Title
Regulation of the rabbit lung mixed-function oxidases by membrane lipid

Author
Hakkinen, Pertti Juhani

Publication Date
1979

Peer reviewed|Thesis/dissertation

REGULATION OF THE RABBIT LUNG MIXED-FUNCTION OXIDASES BY MEMBRANE LIPID

by

Pertti Juhani Hakkinen
B.A., University of California Santa Barbara 1974

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

COMPARATIVE PHARMACOLOGY AND TOXICOLOGY

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA

San Francisco

[redacted]

Date University Librarian

SEP 26 1979

Degree Conferred: . . . . . .

REGULATION OF THE RABBIT LUNG MIXED-FUNCTION OXIDASES BY MEMBRANE LIPID

BY

Pertti Juhani Hakkinen

ACKNOWLEDGEMENTS

The author wishes to acknowledge the following persons:

The Late Dr. Samuel Jacobs - for introducing me to pharmacology and toxicology during my undergraduate days. His death was a great loss and this dissertation is dedicated in his memory.

Dr. Mary Vore (Research Advisor) - for having me as a graduate student, for being a good friend, and with apologies to the Beatles ("Let It Be"), for whispering words of wisdom in my ears during times of trouble.

Dr. Walter Piper - for his assistance in helping me write my dissertation and providing me access to his laboratory facilities.

Dr. John Clements - for his assistance in helping me write my dissertation and providing expertise in matters concerning the physical chemical nature and "good manners" of my research.

Dr. Harold Hodge - for his considerable assistance in all areas of my UCSF experience.

Eva Soliven and Dr.'s Daniel Goodman and Michel Aufrere - for their help, advice, and companionship in the laboratory.

Dr. Violette Sutherland - for her friendly assistance in getting me through the first three years of graduate school.

Diana Schooley, Paula Halverson and Dr. Janet Boyles - for their helpful advice and assistance in the electron microscopy studies.

Dr. Steven Baker, Darline Kalinoski and Dr. Ping Law - for their helpful advice in the binding studies.

Dr. Almira Correia - for her helpful advice, and allowing me to use the equipment in her laboratory.

Beverly Hunter and Kathleen McNamara - for their hard work in typing my dissertation.

Chuck Lee and Leo Davidson - for the many hours spent preparing my tables, figures, and slides.

Special acknowledgement is made for my parents, and my brother, and all of my many other friends at UCSF and ALZA Corporation.

This investigation was supported by Public Health Service Training Grants No. 5T01 GM-00475 and 5T01 GM-07175 (DHEW), NIH Grant HL19605 (DHEW), and Patent Funds from the Graduate Division of the University of California, San Francisco.

Abstract

The objectives of this research were to determine if lipid is an essential component of the lung mixed-function oxidase activity. Two criteria were used to establish the lipid requirement: 1) a decrease in enzyme activity upon removal of lipid and 2) restoration of enzyme activity by the addition of lipid.

Lyophilized rabbit lung microsomes were extracted twice with 1-butanol and twice with acetone at -70°, using a modification of a procedure described previously (Mol. Pharmacol. 10, 963-974, 1974). Extraction removed all of the neutral lipid and 88% of the total phospholipid phosphorus. Recoveries of NADPH-cytochrome c reductase activity and cytochrome P-450 in extracted microsomes were both 70%. Addition of dilauroylglyceryl-3-phosphorylcholine (di-12 GPC) to extracted microsomes increased NADPH-cytochrome c reductase activity to 90% of NADPH-cytochrome c reductase activity in unextracted microsomes but did not effect NADPH-cytochrome c reductase activity in unextracted microsomes. Extraction decreased benzphetamine N-demethylase activity per nmole of P-450 to 65% of activity in unextracted microsomes and addition of di-12 GPC to extracted microsomes increased benzphetamine N-demethylase to that in unextracted microsomes. In contrast, extraction increased 7-ethoxycoumarin 0-deethylase activity to approximately 140% of that in unextracted microsomes; addition of di-12 GPC resulted in no further increase in activity. Extraction increased benzo(a)pyrene hydroxylase activity to approximately 150% and addition of di-12 GPC

increased activity up to 200% of that in unextracted microsomes. The effect of extraction on the apparent K_m ($\mu M \pm SE$) and V_{max} ((nmoles 3-OH benzo(a)pyrene formed/nmole P-450/min) $\pm$ SE) for benzo(a)pyrene hydroxylase activity were as follows: unextracted microsomes--K_m = 10.00 $\pm$ 1.97, V_{max} = 0.31 $\pm$ 0.03; extracted microsomes--K_m = 1.89 $\pm$ 0.37, V_{max} = 0.46 $\pm$ 0.03; extracted microsomes plus di-12 GPC--K_m = 8.23 $\pm$ 1.69, V_{max} = 0.74 $\pm$ 0.08. Addition of di-12 GPC to unextracted microsomes inhibited benzphetamine N-demethylase, 7-ethoxycoumarin 0-deethylase and benzo(a)pyrene hydroxylase activities. These data indicate that lipid is essential for benzphetamine N-demethylase but do not establish a lipid requirement for 7-ethoxycoumarin 0-deethylase and benzo(a)pyrene hydroxylase activities. However, lipid is important in modulating benzo(a)pyrene hydroxylase activity in lung microsomes.

The increased benzo(a)pyrene hydroxylase activity caused by lipid extraction may be due to a change in the partitioning characteristics of benzo(a)pyrene between the aqueous and membrane phase of the microsomal suspension. The association of benzo(a)pyrene to microsomes was determined using a filtration method and was found to be essentially complete (over 85%) within ten seconds after addition of extracted microsomes or unextracted microsomes.

The dissociation of benzo(a)pyrene from lung microsomes was studied by diluting benzo(a)pyrene-preloaded microsomes 80-fold before filtering. Virtually no release of benzo(a)pyrene occurred in the first 15 minutes after dilution. Extracted microsomes bound as much benzo(a)pyrene per

mg of microsomal protein as did unextracted microsomes. It is therefore apparent that extracted microsomes contai a higher concentration of benzo(a)pyrene per unit of membrane which could lead to the increased benzo(a)pyrene hydroxylase activity observed.

The A_{max} of the benzphetamine-induced Type I, the ethanol-induced reverse Type I and the aniline-induced Type II binding spectra was increased 2-6 fold in extracted microsomes when compared to unextracted microsomes. There were no significant change in the spectral dissociation constants. These data indicate that an intact lipid membrane is not required for substrate binding. The increased A_{max} suggests that either more substrate binding sites are available in extracted microsomes or alternatively, more substrate is available for binding in the immediate vicinity of rabbit lung cytochrome P-450 as a result of the lipid extraction procedure.

Table of Contents

VI. RESULTS

List of Tables

List of Figures

List of Plates

VI. RESULTS

LIST OF ABBREVIATIONS

BP	Benzo(a)pyrene
Ci	Curie
DI-12 GPC	dilauroylglyceryl-3-phosphorylcholine
DNA	deoxyribonucleic acid
DPM	disintegrations per minute
EDTA	ethylenediamine tetraacetate
g	gram
K_m	Michaelis constant
K_s	spectral binding constant
l	liter
m	milli-
M	moles per liter
NADP	nicotinamide adenine dinucleotide phosphate
NADPH	reduced NADP
n	nano-
nm	nanometer(s)
R_f	the ratio of the distance traveled by a compound to that traveled by the solvent front
TCA	trichloroacetic acid
TLC	thin-layer chromatography
Tris	Tris-(hydroxymethyl)-aminomethane
UDP	uridine 5'-diphosphate

V_{max} maximum velocity

A_{max} absolute magnitude of the spectra change

3-OH BP 3-hydroxy metabolite of benzo(a)pyrene

I. Introduction and Statement of the Problem

Lipid soluble compounds have to be biotransformed to more water soluble metabolites before they can be excreted from the body. The primary means of accomplishing this is via the mixed-function oxidase system which is located in the liver, kidney, intestines, skin, adrenals and other tissues and organs. The liver is the main site of this biotransformation process. The liver NADPH-dependent mixed-function oxidase system has been resolved into three components essential for enzymatic activity (Figure I-1): 1) a hemoprotein known as cytochrome P-450, 2) a flavoprotein known as NADPH-cytochrome-c reductase, (NADPH-cytochrome P-450 reductase) and 3) a lipid fraction identified as phosphatidylcholine (Lu et al., 1969; Lu et al., 1970; Lu et al., 1972, and Strobel et al., 1970). Lipid was shown to be essential for the transfer of electrons from NADPH-cytochrome-c reductase to cytochrome P-450 (Lu et al., 1969). In addition, the binding of substrates to highly purified cytochrome P-450 has been shown to be dependent on phosphatidylcholine (Guengerich and Coon, 1975).

Many environmental pollutants enter the body via inhalation, with the lung therefore capable of playing a significant role in the biotransformation of many xenobiotics. This role can be either beneficial or detrimental, since similar reactions can decrease or incrase toxicity depending on the substrate and the metabolities formed.

Evidence of oxidative metabolism of foreign compounds by the lung has been obtained in a number of laboratories (Uehleke, 1968; Ichibara et al., 1969; Oppelt et al., 1970; Bend et al., 1972 and Klinger, 1973). The evidence accumulated thus far by Hook, Bend and others indicates that the pulmonary mixed-function oxidase system is similar to the hepatic microsomal mixed-function oxidase system. The similarities include electron transport chains containing cytochrome P-450 and NADPH-cytochrome-c reductase, requirements for oxygen and NADPH for activity, and the ability to metabolize a large number of substrates (Hook et al., 1972; Bend et al., 1972; Hook et al., 1972 and Bend et al., 1973).

The metabolism of most substrates by pulmonary microsomes is slower than that by hepatic microsomes and this may be partially accounted for by a cytochrome P-450 concentration in the lung which is only approximately 25 percent of that in the liver (Hook et al., 1972; Bend et al., 1972; Hook et al., 1972; Bend et al., 1973 and Matsubara and Tochino, 1971). However, Hook and Bend and co-workers (Hook et al., 1972; Bend et al., 1972 and Bend et al., 1973) have shown that the rate of metabolism of some substrates, including benzphetamine, proceeds at a significantly faster rate when calculated per nmole of cytochrome P-450 in rabbit pulmonary microsomes than in rabbit hepatic microsomes.

One approach used in an attempt to understand drug metabolism in the lung and to explain the differences between lung and liver mixed-function oxidase activity involves the detergent solubilization and separation of the system and the study of these components individually

and in combination. Philpot, Fouts and co-workers have used this approach to extensively study the properties of the reconstituted rabbit lung mixed-function oxidase system and have presented evidence for the existence of two forms of pulmonary cytochrome P-450 with marked differences in the ability to metabolize benzo(a)pyrene, ethoxycoumarin and benzphetamine (Philpot et al., 1975; Arinc and Philpot, 1976; Szutowski et al., 1977; Wolf et al., 1978a, and Wolf et al., 1978b). Guengerich (1977) has shown by a similar approach that multiple forms of cytochrome P-450 and possibly NADPH-cytochrome-c reductase are present in both rabbit liver and lung. The reductases of the two organs are apparently identical, but the major pulmonary cytochrome P-450 is immunologically distinct from any of the hepatic cytochrome P-450 forms. However, the proposal of Guengerich that one form accounts for most for the pulmonary cytochrome P-450 appears to be incorrect (Wolf et al., 1978a). Rather, the two forms of cytochrome P-450 appear to be present in the lung in similar amounts (Wolf et al., 1978; Wolf et al., 1978b).

However, despite its apparent power, use of solubilization and reconstitution of mixed-function oxidase systems to determine a possible lipid requirement does have its limitations. Lu and co-workers (Lu et al., 1974) have shown that in the resolved, reconstituted liver microsomal mixed-function oxidase system, several detergents can substitute for the lipid and in higher concentrations, inhibit enzymatic activity. Therefore, in order to establish a lipid requirement, a detergent-free system is essential. Vore and co-workers (Vore et al., 1974a; Vore et al., 1974b) have

developed a detergent-free system which employs acetone and 1-butanol extractions of the hepatic microsomes. Using this techinque, they have removed approximately 80 percent of all phospholipids and all of the microsomal neutral lipids (i.e., cholesterol, mono-, di-, and triglycerides). With this approach, they have established a lipid requirement for the metabolism of benzphetamine and benzo(a)pyrene by the rat hepatic mixed-function oxidase system.

Fleischer and co-workers (Fleischer et al., 1962) have set forth three criteria for establishing the lipid requirement of enzymes: 1) a decrease in enzyme activity upon removal of lipid, 2) a reactivation of the enzyme by the addition of lipid, and 3) evidence that the lipid does in fact bind to the enzyme. The results of Vore et al. (1974a; 1974b) clearly meet the first two criteria. Evidence that the lipid binds to the enyzme requires a preparation of purified cytochrome P-450.

The purpose of this work is to study the lipid requirement of the rabbit lung mixed-function oxidase system using methodology similar to that employed by Vore et al (1974a; 1974b) in their studies of the rat hepatic mixed-function oxidase system.

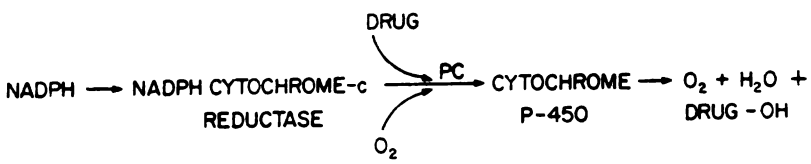
The similarities between liver and lung mixed-function oxidase systems suggest that lipid may also be an essential component of the pulmonary mixed-function oxidase system. Little is thus far known about the lipid requirement of the pulmonary mixed-function oxidase system. Bend et al. (1973) have shown that phospholipase c digestion of lung microsomes decreases activity toward some substrates suggesting that lipid

is required. However, restoration of enzymatic activity to control levels by addition of lipid has not been reported. Reconstitution studies of lung cytochrome P-450 and NADPH-cytochrome c reductase have led to conflicting data. Philpot and co-workers (1975; Arinc and Philpot, 1976) have published dat indicating a lipid requirement for ethoxycoumarin O-deethylation and N-demethylation of benzphetamine using a rabbit lung preparation. In contrast, Orrenius and co-workers (Jernstrom et al., 1975) have shown that using a rat lung partially purified cytochrome P-450 and liver NADPH-cytochrome c reductase preparation, lipid was not required for benzo(a)pyrene hydroxylase activity. The conflicting results may be resolved by use of a detergent-free system.

The molecular ratio of cytochrome P-450 to NADPH-cytochrome c reductase is approximately 20 to 1 in the liver (Estabrook et al., 1969) but closer to 1 to 1 in the lung (Bend et al., 1973; Matsubara et al., 1974). This difference may partially explain the high rate of metabolism of some substrates (i.e., biphenyl and benzphetamine) in the lung. In addition, the different ratios of cytochrome P-450 to NADPH-cytochrome c reductase in lung and liver microsomes suggest that the spatial arrangement of these enzymes in the microsomal membrane is different in the liver and lung and that this difference could be reflected in a unique lipid requirement. Reconstitution experiments eliminate the specific spatial arrangements (electron channels or electron tunnels) of the enzymes involved. This could lead to erroneous conclusions regarding the lipid requirement of the enzymes being studied.

FIGURE I.1

MICROSOMAL ELECTRON TRANSFER SYSTEM



From : Gram, T.E. (1973) DRUG MET. REV. 2, 1-32

II. The Cellular Localization of Pulmonary Mixed-Function Oxidase Activity

The cellular localization of pulmonary mixed-function oxidase activity has been of interest to numerous investigators primarily because of the increasing incidence of lung disease of environmental origin and the realization that many chemical toxins and carcinogens require activation by the mixed-function oxidase system. The precise identification of pulmonary cellular populations possessing mixed-function oxidase activity has been difficult primarily because of the heterogeneity of the lung. Well over 40 distinctive cell types make up the tissues of the lung with no one cell type occupying the major mass of the lung (Sorokin, 1970). Electron microscopy studies show that the major portion of the total lung endoplasmic reticulum (site of the mixed-function oxidase activity) is inside alveolar type II cells and the Clara cells of the bronchial epithelium (Brown, 1974).

Histochemical staining of rat lungs has suggested that hydroxylation of aniline occurs in bronchial epithelium but not in lung parenchyma (Grasso et al., 1971). In contrast, Wattenberg and Leong (Wattenberg and Leong, 1962), using a histochemical method, reported that benzo(a)pyrene hydroxylase activity was found in alveolar walls. However, most lung cancers occur in the bronchi of humans and the trachea of rodents. Histologically, the rodent trachea is similar to the human bronchus since it contains cartilage, compound mucous glands and goblet cells (Cohen and Moore, 1976).

Dirkson and Crocker (1968) produced ultrastructural alterations of columnar respiratory epithelium in suckling rat trachea maintained in organ culture treated with polycyclic aromatic hydro-carbons including benzo(a)prene. Crocker et al (1973), using benzo(a)pyrene and an air pollution composite, produced toxic destruction of all cell types of adult human bronchial mucosa in organ culture. The toxicity of benzo(a)pyrene for adult human respiratory tissue was interpreted in this study as evidence that bronchial microsomal mixed-function oxidases are present and active in the metabolic conversion of benzo(a)pyrene. Pal et al (1975) reported that rat and hamster tracheal rings and human bronchial segments metabolize benz(a)anthracene, 7-methylbenz(a)anthracene and benzo(a)pyrene in a qualitatively similar manner. Grover et al. (1976) showed that human bronchial mucosa in culture appears to activate benzo(a)pyrene.

Benzo(a)pyrene is activated by the microsomal mixed-function oxidase system to epoxides which can then either be converted 1) by epoxide hydrase to the corresponding dihydrodiols or 2) to glutathione conjugates by glutathion S-epoxide transferase or 3) by spontaneous rearrangement to the corresponding phenols. The epoxides can also react with nucleic acids and proteins (Sims and Grover, 1974). Dihydrodiols may be further metabolized by the microsomal mixed-function oxidase system to diol-epoxides which can then bind to DNA (Sims et al., 1974).

Cohen and Moore (1976) studied the metabolism of benzo(a)pyrene by isolated perfused lungs and trachea, and bronchial and lung cultures in the

rat and hamster. They found that benzo(a)pyrene is metabolically activated in the bronchi and trachea and concluded that the high amounts of the dihydrodiol metabolites and their retention in these areas relative to the lungs may partially explain the sensitivity of the trachea and bronchi to hydrocarbon carcinogenesis. Benzo(a)pyrene had also been shown to bind to DNA in cultured human bronchial epithelium (Harris et al., 1974) and in hamster tracheal epithelium (Kaufman et al., 1973). Fisher et al (1977) have conclusively demonstrated the presence of mixed-function oxidase (aniline hydroxylase) activity in rabbit alveolar macrophages. However, the alveolar macrophage was concluded to be a minor site of mixed-function oxidase activity in the lung.

Recently, Boyd (1978) has shown autoradiographically that the reactive metabolite of the pulmonary toxin, 4-ipomeanol is preferentially formed and covalently bound in the pulmonary non-ciliated bronchiolar (Clara) cells of the rat, mouse and hamster. Previous studies by Boyd (1976) have established that the toxic metabolite formed in vivo during cytochrome P-450-dependent oxidative metabolism of 4-ipomeanol is formed in situ in the lung and does not reach the lung by way of the circulation. These results indicate that the Clara cell is a primary locus of mixed-function oxidase activity in the lung. Moreover, since the Clara cell possesses enzymes required for the metabolic activation of certain chemical carcinogens, it must be considered a prime candidate as a cell of origin for at least some bronchogenic cancers.

III. Lipid Requirement of Enzyme Systems

Probably the greatest difference between membrane enzymes and soluble enzymes is the interrelationship of many of the former with lipids (Coleman, 1973). The selective removal and reconstitution of membrane lipids has been widely used for investigating the role of lipids in the biological activities of membrane-bound enzymes. This process also serves as a tool for elucidating the nature of the bonds holding the membrane components together (Razin, 1972).

A membrane-bound enzyme exists in a microenvironment in which its properties may be influenced by: 1) the general chemical and physical nature of the membrane (composition, lipophilic nature, charge, dielectric constant); 2) by specific interactions with individual molecules in its immediate neighborhood (proteins and lipids); 3) by the local effects of its own action and that of its neighbors (Coleman, 1973).

A number of membrane-bound enzymes have been shown to require lipid for their correct functioning (Table III-1). This information has been obtained through the use of detergents, solvents or phospholipases to remove membrane lipid. However, loss of activity following such treatment is not sufficient grounds on its own to establish lipid dependency. This inactivation may also be due to: 1) specific denaturation, unconnected with lipids; 2) inhibitory action of the products of the modification; 3) direct inhibitory actions of the reagents or their contaminants (Coleman, 1973).

Reactivation of the inactive enzyme upon addition of lipid is an essential criterion for establishing lipid dependency. Fleischer et al. (1962) have suggested several criteria which should be obeyed in order to establish a lipid requirement: 1) removal of the lipid; 2) a correlation between removal of the lipid and the loss of activity; 3) a correlation between restoration of the activity and rebinding of the lipid.

Lipid removal techniques are usually as mild as possible with attempts made to lessen the degree of protein denaturation. Essentially all organic solvents used for lipid extraction cause some degree of protein denaturation (Razin, 1972). Solvent extractions are usually carried out in the cold. Contact time with the solvent must be kept short and it may be necessary to eliminate the inhibitory action of the solvent itself on enzyme activity (Coleman, 1963).

Once enzymic activity is decreased by lipid depletion its reconstitution can be tested by the addition of various lipids. Recombination of the lipid with the lipid-depleted membranes is usually done by the method of Fleischer et al. (Fleischer et al., 1962). The membranes are incubated with a sonicated suspension of the lipid. The sonicated lipids seem to interact with the membrane not as individual molecules, but as micelles probably containing several hundred molecules each (Martonosi, 1968).

There is no evidence that the phospholipids required for proper enzyme function participate as cofactors or reaction partners in any enzyme process. The amount needed seems to large for catalytic action

and the chemical specificity appears to be too low. The chemical specificity is sometimes so low that detergents may serve as an adequate substitute for phospholipids (Martonosi, 1968; Duttera et al., 1968 and Lu et al., 1974). It seems more likely that the lipids, by electrostatic and/or hydrophobic interactions, keep the enzyme protein in an enzymatically active form by imposing a certain conformation. Another way in which lipids may affect membrane enzymes is by maintaining an orderly arrangement in the membrane that allows the coupling of enzyme reactions and regulates the access of cofactors and substrates (Razin, 1972).

TABLE III-1

ENYMES SHOWING LIPID REQUIREMENTS

Compounds underlined are highly specific. Abbreviations used: CL, cardiolipin; FA, fatty acid; LPC, lysophosphatidylcholine; LPE, lysophosphatidyl-ethanolamine; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; TG, triglyceride; GPC, glycerophosphorylcholine.

Enzymatic activity	Source	Deactivation	Reactivation
Succinate-0_2, cyto chrome c, coenzyme Q	Beef heart mito-chondria	90% acetone	CL PE PC, mixed lipids
Coenzyme Q-cytochrome c	Beef heart mito-chondria	90% acetone	CL PE PC, mixed lipids
Cytochrome oxidase	Beef heart mito-chondria	90% acetone	PE CL PC, mixed lipids
NADH-0_2, cytochrome c, coenzyme Q	Beef heart mito-chondria	Phospholipase A	PC, mixed lipids
β-Hydroxybutyrate dehydrogenase	Beef heart mito-chondria	Phospholipase A; deoxycholate + salt	PC
NADH-0_2, cytochrome c	Rat liver micro-somes	90% acetone	PC + LPC, mixed lipids
NADPH-dependent hydroxylastins	Rat liver micro-somes	Solvents; deoxy-late + DEAE	Mixed lipids
Phosphatidate phosphatase	Pig kidney micro-somes	n-Butanol; solvents	Mixed lipids
Stearoyldesaturase	Hen liver micro-somes	90% acetone	Mixed lipids + TG + FA PC
GTP-dependent acyl-CoA synthetase	Rat liver mito-chondria	90% acetone	PC
Palmitate acyl-CoA synthetase	Rat liver micro-somes	Phospholipase D	Mixed lipids

TABLE III-1. Continued

Enzymatic activity	Source	Deactivation	Reactivation
Alkeynl-GPC hydrolase	Rat liver microsomes	Phospholipase A,C	PC, Sphingomyelin PE
Phospholipid base exchange	Hen brain microsomes	Deoxycholate + ultrafiltration	PS
Glucose-6-phosphatase	Rat liver microsomes	Phospholipase A,C	LPC PC
UDPglucuronyltransferase	Guinea pig liver microsomes	Phospholipase A,C,	Mixed lipids
Ca^{2+}-ATPase	Rat muscle microsomes	Phospholipase A,C,	LPC PC, FA
Adenyl cyclase	Rat liver plasma membrane	Phospholipase A; solvents	PI, mixed lipids
$(Na^{+}-K^{+})$-ATPase	Various	Phospholipase A; deoxycholate + salt	PS, alkyl- PC
$(Na^{+}-K^{+})$-ATPase, K^{+}-nitrophenylphosphatase	Rat liver plasma membrane	Phospholipase C	PC
UDPhexose: lipopolysaccharide hexotransferase	Salmonella typhimurium	Ethanol	PE, PG, CL, PA
FAD-dependent malate dehydorgenase	Mycobacterium avium	Acetone powder extraction	CL
P-Enolpyruvate phosphotransferase (EII)	S. Typhomurium, E. coli	n-Butanol + urea	PG
Isoprenoid alcohol phosphokinase	Staphyloccus aureus	n-Butanol + acid + DEAE	PG, CL
ATPase, pyrophosphatase	Rhodospirillum rubrum	Phospholipase A	PC, PS, PE

TABLE III-1. Continued

Enzymatic activity	Source	Deactivation	Reactivation
Pyruvate oxidase	E. coli	Mechanical solubilization	LPE, PC, FA, TG

*Table from: Coleman, R. (1973)
Biochem. Biophys. ACTA 300, 1-30

IV. RATIONALE

A. Isolation of Rabbit Pulmonary Microsomes

Mammalian lung is rather resistant to disruption or homogenization due to its richness in connective tissue, particularly collagen, and due to its unique propensity to float in aqueous media. For these reasons, controlled homogenization of lung, short of the total destruction of organelles and intracellular structures, is technically challenging (Gram, 1973).

In contrast to the liver in which two major cell types predominate (hepatocytes and Kupffer cells), normal mammalian lungs consist of "well over 40 distinctive cell types." Most of these cell types are found in other tissues of the body although a few types are unique to the lungs (Sorokin, 1970). Undoubtedly, on quantitative grounds, many of the cell types present may be ignored. Nonetheless, in cytologic terms, the lung is an extremely complex and heterogenous system and prepared cell fractions should be expected to reflect this nonuniformity.

B. The Distribution of Various Marker Enzymes in Subcellular Fractions of Rabbit Lung

Tissue homogenization should ideally disrupt a reasonable number and representative proportion of cells in an essentially random fashion with minimal fragmentation of subcellular particles and redistribution of the enzymes of constitutive components. As noted in Section IV-A, due to the richness in connective tissue of the mammalian lung and its propensity to float in aqueous media, controlled homogenization short of the total destruction of organelles and intracellular structures is technically challenging.

Gram and Bend and co-workers (Gram, 1973); Bend et al., 1973) have studied the distribution patterns of various marker enzymes in the subcellular fractions of rabbit lung and liver. They have shown that the general distribution patterns of the mixed-function oxidase components were very similar in liver and lung and that microsomes exhibited the higest specific activites of mixed-function oxidase markers (e.g., NADPH-cytochrome c reductase, cytochrome P-450, benzphetamine N-demethylase) among the cell fractions. These activites were two to four times higher in microsomes than in any other fraction. Unexpectedly high mixed-function oxidase marker activities were found in the nuclear fractions. This activity can be attributed electron micrographically to the presence of endoplasmic reticulum fragments (microsomes) in the large numbers of partially disrupted cells which settle in the nuclear fraction.

The activity of glucose-6-phosphatase was found to be barely detectable and broadly distributed over all fractions except the soluble fraction in rabbit lung (Gram, 1973; Bend et al., 1973). This is in sharp contrast to the rabbit liver in which glucose-6-phosphatase serves as a classical microsomal enzyme marker (de Duve et al., 1962).

The purities of the various subcellular rabbit lung fractions obtained using a modification (Section V-B) of the microsomal isolation method of Matsubara and co-workers (Matsubara et al., 1974) were assessed by the use of cytochrome P-450 and NADPH-cytochrome-c reductase as microsomal enzyme markers, 5'-nucleotidase is a plasma membrane marker, succinc dehydrogenase as a mitochondrial membrane marker, β-glucuronidase as a lysosomal membrane marker and glucose-6-phosphate dehydrogenase as a soluble enzyme marker.

C. Electron Microscopy of Unextracted and Extracted Rabbit Lung Microsomes

The morphological properties of rabbit liver and lung microsomal pellets have been studied by electron microscopy (Gram, 1973; Bend et al., 1973). The predominant features of the liver microsomal pellet were smooth and rough surfaced vesicles, free ribosomes and glycogen rosettes. Mitochrondria, lysosomes and fragments of the Golgi apparatus were also occasionally observed. In general, these microsomal structures were uniformly distributed across the microscope field. Lung microsomes also contained smooth and rough vesicles, although glycogen rosettes appeared to be absent. In distinct contrast to liver, the microsomal vesicles were not uniformly distributed across the microscope field but occurred as aggregates or clusters. Under high magnification, these aggregates appeared to be composed of vesicles and electron-dense particles (which resembled ribosomes) embedded in an apparently amorphous matrix of long filaments. Fibrillar matrices of this sort were not observed in hepatic microsomes.

D. Measurement of Lung Cytochrome P-450

One of the most notable differences between liver and lung microsomes is their affinity for hemoglobin. Adventitious hemoglobin may interfere with the spectrophotometric determination of cytochromes in cell fractions (Gram, 1973). It has been shown that excess hemoglobin may be essentially completely removed from liver microsomes either by perfusion of the organ in situ or by "washing" the microsomal pellet by resuspension in a dilute salt solution such as 0.15 M KCl and resedimentation (Bend et al., 1973). This is not the case for lung microsomes. Based on the appearance of the carboxyhemoglobin spectral peak at about 428 nm in a carbon monixide difference spectrum, Gram, (1973) has shown that washed particulate fractions of lung bound two to four times as much hemoglobin as comparable liver fractions. Perfusion of lungs with cold isotonic KCl prior to homogenization resulted in the isolation of microsomes having smaller amounts of bound hemoglobin, but the microsomes were still not hemoglobin-free. In addition, resuspension and resedimentation of lung microsomes as many as four times still resulted in microsomes grossly contaminated with hemoglobin (Gram, 1973; Bend et al., 1973).

As hemoglobin has been found to be a contaminant of lung microsome preparations, lung cytochrome P-450 quantifications have been made using the dithionite difference technique where the difference in absorption between the oxidized and reduced forms of carbon monoxide-bound

cytochrome P-450 is recorded. Hemoglobin does not interfere with the quantification of cytochrome P-450 when measured by the dithionite difference technique (Gram, 1973; Bend et al., 1973). This was also found to be the case upon the addition of purified hemoglobin or lyzed erythrocytes to lung microsomes (Gram, 1973).

Philpot, Fouts and co-workers have presented evidence for the existence of two forms of cytochrome P-450 which appear to be present in the lung in similar amounts (Wolf et al., 1978a). In the dithionite difference spectrum, cytochrome I was found to have a Soret maximum at 452 nm and cytochrome II a maximum at 450 nm (Wolf et al., 1978a).

E. NADPH-Cytochrome c Reductase Activity in Extracted and Unextracted Lung Microsomes

NADPH-cytochrome c reductase is a microsomal flavoprotein shown to be one of the enzymes involved in microsomal electron transport. The enzyme has been solubilized and purified and shown to catalyze the reduction of a number of compounds including: cytochrome c (1-electron acceptor), menadione (2-electron acceptor), neotetrazolium and azo-dyes (4-electron acceptors). The equations for the reactions are as follows:

$NADPH + H^+ + 2 \text{ cytochrome } c^{3+} \text{-------} \rightarrow NADP^+ + 2 \text{ cytochrome } c^{2+} + 2H^+$

$NADPH + H^+ + \text{dye} \text{----------} \rightarrow \text{reduced dye} + NADP^+$

One of the assays available for the measurement of enzyme activity depends upon measurement of the rate of cytochrome c reduction. Cytochrome c has a definite absorption spectrum in the oxidized state and an equally definite absorbance in the reduced state (Williams and Kamin, 1967). The reduced form has a pronounced absorption maximum at 550 nm which is absent in the oxidized spectrum. Thus, one can follow the rate of reduction of cytochrome c by observing the increase in optical density at 550 nm with time. Using this assay, Philpot and co-workers (Philpot et al., 1975) have reported NADPH-cytochrome c reductase activity of 60.0 units/mg protein for rabbit pulmonary microsomes.

F. Benzphetamine N-Demethylase Activity in Extracted and Unextracted Lung Microsomes

Benzphetamine, along with many other drugs (aminopyrine, ethylmorphine, N-methyl-aniline, N-methyl-p-chloroaniline etc.) is dealkylated by microsomal enzymes according to the general equation (Oppelt et al., 1970; Bend et al., 1972; Vehleke et al., 1972):

$$R - \underset{H}{N} - CH_3 \xrightarrow[NADPH + 0_2]{microsomes} R - NH_2 + HCHO$$

The rate of metabolism may be followed by either measuring the formation of the demethylated product or the quantity of formaldehyde formed.

Philpot and co-workers (Philpot et al., 1975) have reported a benzphetamine N-demethylase activity of 19.3 nmoles HCHO formed/nmole cytochrome P-450/minute in rabbit lung microsomes. In contrast, Hook and co-workers (Hook et al., 1972a) have reported a benzphetamine N-demethylase activity of 37.5 nmoles HCHO formed/nmole cytochrome P-450/minute in rabbit lung microsomes. Guengerich (1977) has reported a benzphetamine N-demethylase activity of 31 nmoles HCHO formed/nmole cytochrome P-450/minute.

Benzphetamine has been extensively used as a model compound to study the activity of the MFO systems.

G. O-dealkylation of 7-ethoxycoumarin by Extracted and Unextracted Lung Microsomes

The dealkylation of 7-ethoxycoumarin to 7-OH-coumarin has been shown to be catalyzed by cytochrome P-450 (Ullrich and Weber, 1972). Because of the high fluoresence of the product 7-OH-coumarin, 7-ethoxycoumarin O-dealkylase activity has been used as a sensitive probe of mixed-function oxidase activity. It was therefore used as a substrate to determine the lipid requirement of the lung mixed-function oxidase system.

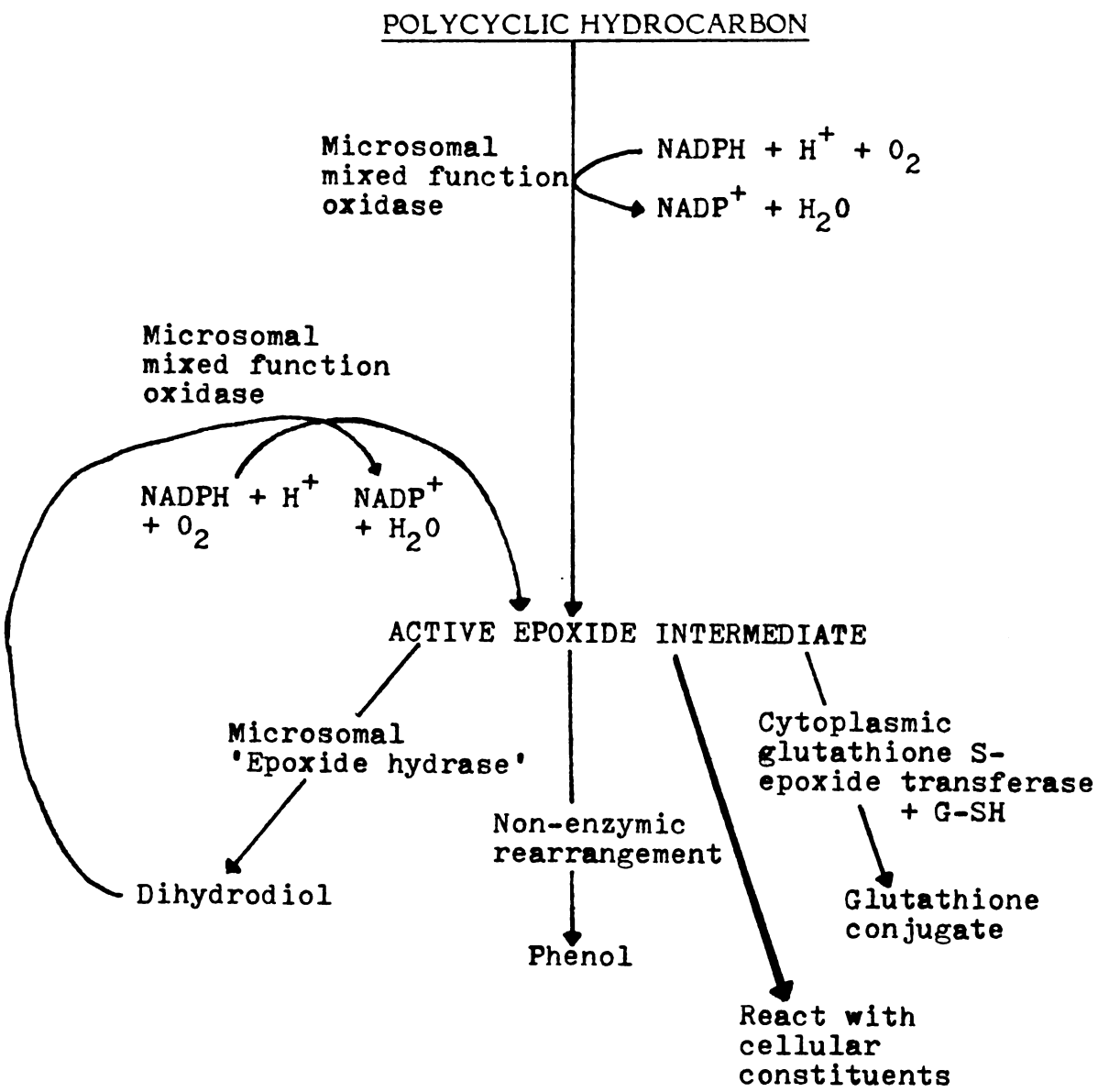
H. Benzo(a)pyrene Hydroxylase Activity in Extracted and Unextracted Lung Microsomes

Polycyclic aromatic hydrocarbons such as benzo(a)pyrene occur widely as atmospheric pollutants resulting from the combustion of fuels and other organic materials, as constitutents of cigarette smoke, in charcoal grilled meats and in the soot of jet planes (National Academy of Sciences, 1972; Shabad, 1971). They are the most widely occurring environmental chemical carcinogens known and promote cancer in many tissues of man and laboratory animals. The respiratory tract is a highly sensitive target organ (Arcos et al., 1968).

Polycyclic aromatic hydrocarbons are metabolized by the microsomal mixed-function oxidase system (aryl hydrocarbon hydroxylase or benzo(a)pyrene hydroxylase) found in many tissues of man and animal species. They are initially metabolized (Figure IV-1) by the microsomal mixed-function oxidase system to epoxides, which can then either be converted 1) by the microsomal epoxide hydrase system to the corresponding dihydrodiols or 2) to glutathione conjugates by the soluble enzyme glutathione S-epoxide transferase or 3) by spontaneous rearrangement to the corresponding phenols (Sims and Grover, 1974). The epoxides, in what is thought to be their toxic reactions, can also react with nucleic acids and proteins. Sims and co-workers have also shown that dihydrodiols may be further metabolized by the microsomal mixed-function oxidase system to diol-epoxides which can then bind to DNA (Sims et al., 1974).

FIGURE IV-1.

METABOLISM OF POLYCYCLIC AROMATIC HYDROCARBONS.



Modified From: Cohen, G.M. in Lung Metabolism ed. by Junod, A.F., and de Haller, R. Academic Press, 1975 p. 190

I. α-Naphthoflavone Studies of Benzo(a)pyrene Hydroxylase Activity

The existence of multiple forms of cytochrome P-450 with different substrate specificities appears to be a major determinant in the metabolic function of mixed-function oxidase systems. Philpot, Fouts and co-workers have presented evidence for the existence of two forms of rabbit pulmonary cytochrome P-450 with marked differences in the ability to metabolize benzphetamine, ethoxycoumarin and in particular, benzo(a)pyrene (Table IV.1) (Philpot et al., 1975; Arinc and Philpot, 1976; Szutowski et al., 1977; Wolf et al., 1978a; Wolf et al., 1978b and Wolf et al., 1979). The two forms of the cytochrome are present in approximately equal amounts (Wolf et al., 1978a).

Knowledge of whether one form of rabbit pulmonary cytochrome P-450 is preferentially destroyed by the lipid extraction procedure is essential for the interpretation of any metabolism data (Table IV.1).

Cytochrome I is highly active in the metabolism of benzphetamine, 7-ethoxycoumarin and benzo(a)pyrene whereas cytochrome II appears to be inactive in the metabolism of either benzphetamine or 7-ethoxycoumarin. Furthermore, the hydroxylated metabolite of benzo(a)pyrene measured fluorometrically (Nebert and Gelboin, 1968) constitutes approximately 72% of the total metabolites formed by cytochrome I and only 45% of the cytochrome II metabolites (Wolf et al., 1978a; 1978b; 1979).

A possible explanation for the increased benzo(a)pyrene hydroxylase (Figure VI-15) and ethoxycoumarin O-deethylase (Figure VI-14) activities

observed per nmole of cytochrome P-450 in extracted microsomes is that the acetone/butanol extraction procedure preferentially denatures cytochrome II in pulmonary microsomes.

To test this hypothesis, the effects of the microsomal enzyme inhibitor α-naphthoflavone on benzo(a)pyrene hydroxylase activity in unextracted and extracted lung microsomes was measured. As seen in Table IV.1, addition of α-naphthoflavone (10^{-4} M, inhibits 99% of cytochrome II and only 6% of cytochrome I-mediated benzo(a)pyrene activity (Wolf et al., 1978b). Thus if, the extraction procedure had preferentially denatured cytochrome II, then α-naphthoflavone should decrease benzo(a)pyrene hydroxylase activity in unextracted microsomes but not in extracted microsomes.

TABLE IV-1

SUBSTRATE METABOLISM IN RABBIT LUNG MIXED-FUNCTION OXIDASE SYSTEMS RECONSTITUTED FROM PURIFIED CYTOCHROME P-450 I AND II

	CYTOCHROME I	CYTOCHROME II
BENZPHETAMINE N-DEMETHYLASE ACTIVITY	+++	—
7-ETHOXYCOUMARIN O-DEETHYLASE ACTIVITY	++	—
BENZO(a)PYRENE METABOLISM	+	+
MAJOR BENZO(a)PYRENE METABOLITE FORMED	3-OH	9,10 DIHYDRODIOL and 3-OH
BINDING OF BENZO(a)PYRENE METABOLITES TO DNA	NO	YES
α-NAPHTHOFLAVONE (10^{-4}M) INHIBITION OF BENZO(a)PYRENE METABOLISM	6% INHIBITION	99% INHIBITION

Modified from Wolf, C.R. et al (1978a; 1978b; 1979)

J. Kinetic Parameters of Benzo(a)pyrene Hydroxylase Activity in Unextracted and Extracted Microsomes

In order to determine if the increased benzo(a)pyrene hydroxylase activity in extracted microsomes was due to an increase in the V_{max} or possibly to a decrease in substrate inhibition or to substrate activation, the kinetics of benzo(a)pyrene hydroxylase activity in unextracted and extracted microsomes was investigated. However, because of the presence of at least two forms of pulmonary cytochrome P-450 with different substrate specificities (Philpot et al., 1975; Arinc and Philpot, 1976; Szutowski et al., 1977; Wolf et al., 1978a; Wolf et al., 1978b; and Guengerich, 1977) and because the enzymes are membrane bound and are impure the K_m values obtained must be interpreted cautiously, and are referred to as K_m apparent.

In order to determine if the increased activity in extracted microsomes was due to an increase in the V_{max} or possibly to decrease in substrate inhibition or to substrate activation, the kinetics of benzo(a)pyrene hydroxylase activity in unextracted and extracted microsomes was investigated.

K. Association and Dissociation of Benzo(a)pyrene with Lung Microsomes

One possible way that lipid extraction increases the apparent affinity of pulmonary cytochrome P-450 for benzo(a)pyrene is by changing the partitioning characteristics of benzo(a)pyrene in the aqueous and/or membrane phase of the microsomes.

The pulmonary mixed-function oxidase system is tightly bound in the membrane of the endoplasmic reticulum. The kinetic properties of many tightly bound membrane enzymes are subject to changes in their lipid environment (Coleman, 1973; Razin, 1972; Zakim and Vessey, 1977). The presence of a lipid matrix may directly affect enzyme activity to produce catalytically active forms of the enzyme, or the lipid matrix may affect catalysis in a manner unrelated to specific lipid-protein interactions.

The relatively high solubility of many nonpolar substrates including benzo(a)pyrene in hydrophobic environments makes the lipid portion of membranes an ideal phase for concentrating such substrates. Sackmann et al. (1973) have proposed a model in which there is rapid diffusion in two dimensions within the plane of a membrane such that substrates in the lipid matrix are able to reach the active sites of membrane-bound enzymes without leaving the plane of the membrane. In this model, the membrane lipids concentrate substrates in the region of the cell containing the metabolizing enzymes and also establish a restricted plane of diffusion between substrate and enzyme. In addition, substrates partitioned within

the lipid matrix of a membrane may not have random orientations. Instead, they may have restricted orientations that are energetically favorable for catalysis.

Schuster and co-workers (Schuster et al., 1975) have studied the interaction of a substituted pleuromutilin with rat liver microsomes using both equilibrium dialysis and spectral techniques. Their results indicate that a large pool of the drug is in the membrane and that its presence is a consequence of the membrane lipids and not the membrane proteins. They suggest that this pool may be in equilibrium with a hydrophobic binding site on cytochrome P-450. Cohen and Mannering (1973) have obtained evidence for such a site on cytochrome P-450 using alcohols to inhibit aniline hydroxylation. The extent of inhibition was found to increase with increasing hydrophobicity of the alcohol. This is consistent with the interaction taking place in a hydrophobic environment.

Additional evidence for both conclusions has been obtained by Ibbetson and Freedman (Ibbetson and Freedman, 1974). Using fluorescence techniques, they have demonstrated that benzo(a)pyrene binding corresponded to a general dissolution into the non-polar matrix of the membrane. Using the data of Ibbetson and Freedman, Parry et al (1976) have calculated a partition coefficient of approximately 4.39×10^3 for benzo(a)pyrene binding between lipid and aqueous phases. However, it is unlikely that the aqueous phase used is completely devoid of protein.

In a study designed to determine whether drug uptake into isolated hepatocytes occurs by passive diffusion or by an energy consuming process,

von Bahr and co-workers (von Bahr et al., 1974) found that drug combination with cellular cytochrome P-450 as measured by formation of the type I spectral change (Section IV-L) was rapid and occurred within a few seconds. The effects of varying temperature and concentration and lipid solubility of the drugs (barbiturates) studied as well as the lack of effect of rotenone preincubation of the cells indicates that drug uptake into the hepatocytes occurs by a non-energy requiring diffusion process. Although not unexpected in view of the number of membranes crossed, the uptake of drug into isolated cells took place within seconds. However, this process is considerably slower than the spectrally observed millisecond binding of substrates to cytochrome P-450 in isolated liver microsomes and liver homogenates (Moldéus et al, 1973; von Bahr et al, 1974).

Blyth and co-workers (Blyth et al, 1971) have found that estradiol and testosterone binding to rat liver microsomes is linear with concentration of the hormone and that the postulated "loose binding site" was not saturable. This data is consistent with the distribution being controlled by a zero-order partition coefficient. Schuster and co-workers (Schuster et al., 1975) also found near linearity of "binding" of pleuromutilin to rat liver microsomes and liposomes, but did eventually saturate the postulated binding site. A likely explanation of this saturation is that with increasing ligand concentration, the ligand itself becomes a significant component of the lipid phase and modifies the solubility properties of the membrane (Parry et al., 1976). This explanation is supported by the fact that changes in lipid composition can vastly modify the bulk properties of a membrane including solubility of substances in it (Parry et al., 1976).

Zakim and Vessey (Zakim and Vessey, 1977) have provided evidence that a membrane-bound hydrophobic substrate (estrone) has direct access to the active site of a membrane-bound enzyme (UDP-glucuronyltransferase) and that membrane-associated estrone, not estrone in solution, is the substrate for UDP-glucuronyltransferase.

No information is available on whether membrane-bound benzo(a)pyrene or benzo(a)pyrene in the aqueous environment serves as the substrate for the pulmonary mixed-function oxidase system. The following experiments were designed to examine: 1)whether lipid extraction changes the partitioning characteristics of benzo(a)pyrene between the aqueous and membrane phase of the microsomal suspension; and 2) whether membrane-bound benzo(a)pyrene or benzo(a)pyrene in the protein-containing aqueous environment serves as the substrate for cytochrome P-450.

L. Substrate Binding to Extracted and Unextracted Lung Microsomal Cytochrome P-450

Cytochrome P-450 interacts with a variety of compounds to give characteristic spectral changes. The addition of a substrate to microsomal suspensions containing cytochrome P-450 induces on of three different types of spectral changes which are known as "Type I spectra", "Type II spectra" and "reverse Type I spectra" or modified Type II (Figure VI-23) (Schenkman et al., 1967; Schenkman et al., 1973). Most substrates of the microsomal mixed-function oxidase system interact with cytochrome P-450 to produce the Type I spectral change.

Early in the study of cytochrome P-450, the observation was made that the addition of substrates to microsomal suspensions containing this cytochrome resulted in a characteristic Soret spectral transition (Narasimhulu et al., 1965) which was suggested, by analogy to other hemin systems, to be a low to high spin conversion of the iron (Type I spectral change) (Hildebrandt et al., 1968; Jefcoate et al., 1969; Estabrook et al., 1968 and Schenkman et al., 1967). This low to high spin transition has been confirmed using electron spin resonance spectroscopy for the cytochromes P-450 from Pseudomonas putida (Tsai et al., 1970; Peterson, 1971) and adrenal cortex mitochrondria (Jefcoate et al., 1973) as well as by magnetic susceptibility determinations on the bacterial system (Peterson, 1971). A direct correlation has been made between changes in the optical

absorbance and electron spin resonance spectral using hepatic microsomal cytochrome P-450 (Waterman et al., 1973). However, in general, no strict correlation exists between the spectral binding constant (K_S), the magnitude of spectral change, and the rate of metabolism of a given substance (Mannering, 1971).

A Type I spectral change in hepatic microsomes is characterized by an increase in absorbance at 387 nm and a decrease in absorbance at 420 nm in the difference spectrum (Ebel et al., 1978). Typical Type I spectra have also been observed upon addition of benzphetamine (a Type I substrate) to rabbit lung microsomes. Although the absolute magnitude of the spectral changes (A_{max}) in liver and lung microsomes differed somewhat, the spectral dissociation constants were very similar (Gram, 1973).

The Type II spectral change is produced by compounds which are basic amines and, in most cases, these compounds are not substrates of the microsomal hydroxylation system (Schenkman, 1967). A Type II spectral change in liver microsomes is characterized by a broad trough centered around 400 nm with an increase in absorbance at about 425 nm and is usually considered to be due to a high to low spin conversion of the heme iron (Ebel et al., 1978). Though not observed invariably, the interaction of aniline with rabbit lung microsomes usually produces a slightly atypical spectrum, qualitatively different from that observed with liver. This atypical spectrum differed from a normal hepatic Type II spectrum in having a broad asymmetrical trough from about 390 to 415 nm and a peak

at about 430 to 435 nm. The A_{MAX} (390-430 nm) for aniline in lung was only about 0.010, and in liver, 0.057 (Gram, 1973).

The reverse Type I spectral change is the mirror image of the Type I spectrum. The addition of methanol, ethanol, acetone, 1-butanol to a microsomal suspension results in the observation of a reverse Type I binding spectrum (Schenkman et al., 1972; Yoshida and Kumaoka, 1975), and it has been assumed to be due to a "competition" between these compounds and the drug substrate for the active site of cytochrome P-450 (Nebert et al., 1977). An alternative explanation has been recently proposed which takes into account the fact that each of these reverse Type I compounds is quite water soluble and is known to effect the dielectric constant of water solutions (Ebel et al., 1978). They will thus have a significant effect on the partition coefficient of extremely hydrophobic compounds by making them more soluble in the aqueous phase while also affecting the solubility in the lipid phase while also affecting the solubility in the lipid phase. Therefore, if the solubility in the aqueous phase is increased more than the solubility in the lipid phase, a larger portion of the compound will be partitioned into the aqueous phase, resulting in a decrease in the lipid phase concentration. In the case of microsomal cytochrome P-450, a spin state change from high to low spin, i.e., a reverse Type I binding spectrum, would occur. However, a more likely hypothesis is that the reverse Type I spectral change is caused by the binding of the sterically accessible oxygen atom of alcohol (e.g., 1-butanol and ethanol) to the sixth ligand of ferric iron thus forming the

G-coordinated low spin cytochrome P-450 (Yoshida and Kumaoka, 1975; Kumaki et al., 1978).

The Type I, Type II and reverse Type I binding spectra in unextracted and extracted lung microsomes were studied as described by Schenkman (Schenkman, 1970) to determine if lipids are required for the binding of substrate to cytochrome P-450. Benzphetamine and aniline were used as Type I and Type II substrates respectively. Ethanol was used as a reverse Type I substrate.

V. METHODS

A. Materials

Acetone and 1-butanol were purchased from Burdick and Jackson (Muskegon, Michigan), Dilauroylglyceryl-3-phosphorylcholine (96-99% pure as judged by chromatographic methods) was purchased from Serdary Research Laboratories Inc. (Ontario, Canada). Benzo(a)pyrene, NADPH, NADP, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, phenolphthalein glucuronic acid, phenolphthalein standard solution, p-iodonitrotetrazolium violet, Type II-S trypsin inhibitor, cytochrome c, glycine, succinic acid, sodium acetate, Trizma^{R}-7.6 and adenosine 5'-monophosphoric acid from Sigma Chemical Company (St. Louis, Missouri). α-Napthoflavone was purchased from Eastman Kodak Company (Rochester, New York) and benzphetamine hydrochloride was a generous gift of Upjohn and Company. 7-hydroxycoumarin was purchased from EGA-Chemie KG (Steinheim, Germany). 7-ethoxycoumarin was synthesized from 7-hydroxycoumarin as described by Ullrich and Weber (1972) and was homogenous as judged by thin-layer chromatography.

B. Isolation of Rabbit Pulmonary Microsomes

Isolation of pulmonary microsomes was achieved by a modification of the procedure used by Matsubara et al (1974) (Figure V-1). Male New Zealand white rabbits (3 kg) fed laboratory chow and tap water ad libitum were sacrificed by a blow to the base of the skull followed by exsanguination. The lungs were removed and placed in a chilled preparation media of 0.154 M KCl and 0.05 M Tris-HCl buffer, pH 7.4. After removal of excess connective tissue, the lungs were homogenized for 30 seconds (Sears Kenmore blender, medium setting) in 3 volumes of buffer containing soybean trypsin inhibitor (2 mg/ml) and then homogenized with a Teflon-glass type Potter-Elvehjem homogenizer (10 passes). The homogenate was centrifuged as described by Matsubara et al. (Matsubara et al., 1974). The final washed pellets were rehomogenized in distilled water and lyophilized and stored at -80^{o} for a maximum of ten days.

Microsomes were prepared using a Lourdes Model-A-2 beta-fuge and Beckman model L ultracentrifuge (Beckman instruments Inc., Palo Alto, California) and lyophilized in a Virtis model 10-145 MR-BA freeze-mobile (The Virtis Company, Gardiner, New York).

FIGURE V-1

PREPARATION OF LYOPHILIZED RABBIT LUNG MICROSOMES

SACRIFICE

↓

- LUNGS INTO 0.154 M KCl AND 0.05 M TRIS-HCl, pH 7.4

↓

- REMOVE EXCESS CONNECTIVE TISSUE AND PLACE LUNGS IN NEW VOLUME OF BUFFER (1:3 w/v) CONTAINING 2 mg/ml SOYBEAN TRYPSIN INHIBITOR

↓

- BLEND FOR 30 SECONDS THEN HOMOGENIZE

↓

- CENTRIFUGE AT 600 g × 10 min

↓

- CENTRIFUGE AT 10,000g × 10 min

↓

- CENTRIFUGE SUPERNATANT AT 16,000g × 10 min

↓

- CENTRIFUGE SUPERNATANT AT 105,000g × 90 min

↓

- RESUSPEND PELLET IN 1mM TRIS-HCl BUFFER, pH 7.4

↓

- CENTRIFUGE AT 105,000g × 90 min

↓

- RESUSPEND PELLET IN DISTILLED WATER(1ml/4g LUNG WT.)

↓

- LYOPHILIZE

Modified from: Matsubara, T. et al (1974) Cancer Research 34, 2196

C. The Distribution of Various Marker Enzymes in Subcellular Fraction of Rabbit Lung

The 600xg (nuclear), 10,000xg (heavy mitochondrial) and 16,000xg (light mitochrondrial) pellets were resuspended in chilled 0.154 M KCl and 0.05 M Tris-HCl buffer, pH 7.4. The 105,000xg pellets (microsomal and washed microsomal) were resuspended in chilled 1 mM Tris-HCl buffer, pH 7.4. All marker enzyme activities except cytochrome P-450 were measured at two protein concentrations to test linearity of enzyme activity with protein concentration.

The levels of cytochrome P-450 were determined by dithionite-reduced difference spectrometry as described by Omura and Sato (1964) (Section V-H). NADPH-cytochrome c reductase activity was measured by the rate of cytochrome c reduction as described by Williams and Kamin (1967) (Section V-I).

The levels of 5'-nucleotidase were determined essentially as described by Ray (1970) except that inorganic phosphate, rather than adenosine, was measured upon termination of the reaction (Waltenbaugh et al., 1978). 5'-AMP was used as the enzyme substrate.

Subcellular fractions were checked for the presence of mitochondrial membranes by measuring the succinic dehydrogenase activity as described by Pennington (1961). The oxidation of succinate was coupled to the reduction of the dye 2-(p-iodophenyl-3(p-nitrophenyl)-5-phenyltetrozolium

(INT) and the formazan produced was extracted with ethyl acetate and measured at 490 nm.

β-glucuronidase levels were measured essentially as described by Fishman et al (1948) using phenolphthalein glucuronidate as the substrate. The number of micromoles of phenolphthalein liberated was determined from a standard curve after measurement of the absorbance at 540 nm.

The levels of glucose-6-phosphate dehydrogenase were determined by the method given in the Worthington 1978 Enzymes Handbook. The reaction velocity was determined by measuring the increase in absorbance at 340 nm resulting from the reduction of NADP. The reaction rate was calculated from the initial linear portion of the curve and was expressed as the change in absorbance at 340 nm/minute/mg protein.

D. Extraction of Lipid from Rabbit Pulmonary Microsomes

Extraction of lipid from pulmonary microsomes was achieved by a modification of the procedure used by Vore and co-workers (Figure V.2) for rat liver microsomes (Vore et al., 1974a; Vore et al., 1974b). In a typical experiment, 40 mg of lyophilized microsomes (14.2 mg protein) were homogenized in 1-butanol (1 ml/10 mg of microsomes), centrifuged, the pellet rinsed (re-homogenized) with 1-butanol and centrifuged followed by two rinsings in acetone (1 ml/10 mg). The final aceton suspension was filtered on a Büchner funnel. The powder was dried under argon and then in a dessicator under vacuum at -20° for 30 minutes. All solvents were kept at -70° in an acetone and dry ice bath and the centrifuge at -15° to -20°. The procedure must be carried out under strictly anhydrous conditions for minimal loss of cytochrome P-450 and NADPH-cytochrome c reductase. The extracted microsomes were then homogenized in 0.1 M potassium phosphate buffer, pH 7.7, and sonicated for 50 seconds. Unextracted microsomes were homogenized and sonicated in a similar fashion.

Sonication was essential in order to keep extracted microsomes in suspension. Without sonication, extracted microsomes accumulated at the bottom of the storage vessel within 30 minutes after homogenization. With sonication, extracted microsomes remained in suspension for at least one hour and could be easily resuspended by vortexing. Before all experiments, extracted and unextracted microsomes were suspended in 0.1 M potassium

phosphate buffer, pH 7.7 and sonicated on ice for 50 seconds with 50% pulsing (25-30 watts) using a Branson sonifier cell disruptor (Branson Ultrasonic Corporation, Danbury, Connecticut).

Extraction of lipid from pulmonary microsomes using one 1-butanol and two acetone extractions was found to result in 15% increase in benzphetamine N-demethylase activity when expressed as nmoles HCOH formed/minute/nmole cytochrome P-450. However, two 1-butanol and two acetone extractions decreased benzphetamine N-demethylase activity to approximately two-thirds of unextracted activity (Section VI-J). Addition of di-12 GPC resulted in a stimulation up to a level approximately equal to that shown by unextracted microsomes. Similar results have been obtained with rat hepatic microsomes (Vore et al., 1974b).

For reasons of uniformity, all other experiments were performed using microsomes which had been extracted twice with 1-butanol and twice with acetone.

FIGURE V-2

EXTRACTION OF LIPID FROM LYOPHILIZED RABBIT LUNG MICROSOMES

- HOMOGENIZE MICROSOMES IN 1-BUTANOL (1 ml/10 mg)
  ↓ CENTRIFUGE
- WASH PELLET WITH 1-BUTANOL
  ↓ CENTRIFUGE
- WASH PELLET IN ACETONE
  ↓ CENTRIFUGE
- WASH PELLET IN ACETONE
  ↓ FILTER
- DRY POWDER WITH N_2 AND THEN IN A DESICCATOR AT −20° FOR 30 MINUTES
  ↓
- HOMOGENIZE MICROSOMES IN 0.1 M POTASSIUM PHOSPHATE BUFFER (1.6 ml/10 mg)
  ↓
- SONICATE FOR 50 SECONDS WITH 50% PULSING

— *Solvents kept at -70° in acetone dry ice bath and centrifuge at -15° to -20°*

— *Unextracted microsomes were homogenized and sonicated similarly*

E. Preparation of Sonicated Synthetic Phosphatidylcholine

Sonicated synthetic phosphatidylcholine (dilauroylglyceryl-3-phosphorylcholine) was prepared by drying a 0.1 ml aliquot of a 20 mg/ml synthetic phosphatidylcholine in chloroform solution at room temperature under a stream of nitrogen. Two ml of 0.02 M Tris-HCL buffer in 1 mM EDTA, pH 7.7 was added and the solution was then sonicated on ice for 45 seconds with 50% pulsing (medium setting) using a Branson sonifier cell disruptor.

The sonicated phosphatidylcholine suspension was clear for the periods required for the assays and yielded reproducible samples. Sonicated lipids probably interact with the microsomal membrane not as inidividual molecules but as micelles containing perhaps several hundred molecules each (Martonosi, 1968).

F. Electron Microscopy of Unextracted and Extracted Rabbit Lung Microsomes

Unextracted and extracted microsomes and a sonicated suspension of di-12 GPC were prepared and di-12 GPC was added to one tube of extracted microsomes in an amount (mg phosphatidylcholine/nmole P-450) equal to that in which maximum reactivation of benzphetamine N-demethylase activity occurred (Section VI-G, Figure VI-13). The cellulose propionate tubes containing the microsomes were spun at room temperature at 100,000 x g for 15 minutes in a Beckman airfuge (Beckman Instruments Inc., Palo Alto, California). The supernatant was decanted and the tips of the tubes were cut off to a level just above the microsomal pellet.

The microsomal pellet was then fixed at 5^{o} for 30 minutes in a combination of 1.5% glutaraldehyde and 1% osmium tetroxide. It should be noted that neither glutaraldehyde nor osmium tetroxide is suitable as a general fixative when used alone, and certain artifacts have been reported in some specimens when glutaraldehyde fixation is followed by fixation with osmium tetroxide. These artifacts arise due to the deleterious effects of glutaraldehyde, such as lipid extraction and cell shrinkage, which occur before the osmium tetroxide is applied (Glauert, 1975). Trump and Bulger (1966) have shown that most of these effects can be minimized or avoided by simultaneous use of glutaraldehyde and osmium tetroxide in a mixed fixative.

After fixation, the pellets were washed with 0.9% saline and left overnight in physiologic saline at 5°. The pellets were next placed in 5% aqueous uranyl acetate for 1 hour. When uranyl acetate is used in this way, it functions not only as a stain but also acts as a fixative, particularly for lipid components (Glauert, 1975). Dehydration was then performed using a standard dehydration series (Glauert, 1965) which consisted of: ethanol 50% for 10 minutes, 70% for 10 minutes, 95% for 10 minutes, 100% twice for 20 minutes each, and propylene oxide twice at 20 minutes each. The pellets were next placed overnight in an embedding media consisting of 1:1 propylene oxice and araldite and then again overnight in 100% araldite. The pellets were then polymerized for 48 hours at 60°.

Sectioning was done with glass knives on Porter-Blum MT1 and MT2 microtomes. The sectioned samples were post-stained for 8 minutes with Reynold's Lead citrate. Micrographs were taken on a Zeiss 952 electron microscope at the magnifications given in the micrographs.

G. Lipid Analysis of Extracted and Unextracted Rabbit Lung Microsomes

The lipid content of the unextracted and extracted microsomes were analyzed by incorporation of labeled precursors, by thin-layer chromatography and by measurement of total phospholipid phosphorus. Additional details of the methodology are described in the legends to the Tables and Figures.

^{3}H-myoinositol (specific activity = 12.5 Ci/mmol, radiochemical purity greater than 99%), ^{14}C-ethanolamine hydrochloride (specific activity = 3.9 mCi/mmol, radiochemical purity greater than 99%) were purchased from New England Nuclear (Boston, Massachusetts).

Silica gel-impregnated glass filter paper was purchased from the Gelman Instrument Company (Ann Arbor, Michigan). Silica gel G pre-coated TLC plates (0.25 mm layer thickness) were purchased from EM Laboratories (Elmsford, New York).

The labeling of phosphatidylcholine, phosphatidylethanolamine and phosphatidylinositol in the microsomal fraction was studied by the method of Jobe and co-workers (Jobe, 1977; Jobe et al., 1978). Each rabbit received either 15 μCi/kg ^{14}choline, 24 μCi/kg ^{14}C-ethanolamine or 63 μCu/kg ^{3}H-inositol as an injection via the marginal ear vein over approximately 45 seconds. Animals were sacrificed 30 minutes after injection and extracted and unextracted microsomes were prepared.

The phospholipid phosphorus content was measured by the method of Bartlett (Bartlett, 1959).

Phospholipid phosphorus in unextracted and extracted microsomes was determined by extracting the phospholipids from aqueous suspensions of the fractions by the chloroform-methanol procedure of Bligh and Dyer (Bligh and Dyer, 1959). Microsomal suspension (0.6-1.0 mg protein in 0.4 ml) was added to 0.5 ml chloroform and 1 ml methanol and vortexed for 20 seconds. An additional 0.5 ml of chloroform and 0.5 ml of water were added followed by mixing and centrifugation at 500 x g. The lower (chloroform) layer was then removed and 50 µl used for the one-dimensional TLC. For the two-dimensional TLC studies, chloroform was removed and evaporated under a stream of nitrogen and the lipids redissolved in 0.3 ml chloroform. Of this solution, 150 µl were spotted for the extracted microsomal lipid extracts and 75 µl were spotted for the unextracted microsomal lipid extracts.

H Measurement of Lung Cytochrome P-450

The concentration of lung cytochrome P-450 was determined by dithionite-reduced difference spectrometry as described by Omura and Sato (Omura and Sato, 1964) using a dual beam spectrophotometer. NADH was also added to reduce interference by cytochrome b_5.

Two milliliters of suspended microsomes to which an excess of NADH was added, were bubbled with carbon monoxide for 30 seconds and placed in two 1 cm path length glass cuvettes. After adjustment of the spectrophotometer, a baseline was run from 410 nm to 500 nm. This was followed by addition of a small amount of sodium dithionite to the sample cuvette followed by a one minute wait to allow all of the cytochrome P-450 to be reduced before running a difference spectrum. Additional small amounts of dithionite were added followed by a spectral scan untl a maximum 450-490 nm spectral change was recorded. An extinction coefficient of 91 mM^{-1} cm^{-1} was used.

I. NADPH-Cytochrome-c-Reductase Activity in Extracted and Unextracted Lung Microsomes

The assay of Williams and Kamin (1967) depends upon the measurement of the rate of cytochrome c reduction by measurement of the absorption at 550 nm. The following were added to a 1 ml cuvette with buffer substituted for NADPH in the blank: microsomes (mg protein = 0.06 mg) in 0.05 M potassium phosphate buffer, pH 7.7 containing 1 x 10^{-4}M EDTA, 36 millimicromoles cytochrome c in the same buffer, and buffer to a volume of 1.0 ml. The reaction was initiated by addition of 0.1 ml of a fresh 1 x 10^{-3}M solution of NADPH in buffer (final volume = 1.1 ml) and a continuous measurement of absorbance at 550 nm was made over 3 minutes. The rate of reduction of cytochrome c was then calculated from the linear portion of the curve. Determinations were made using a Gilford model 2000 multiple sample recorder (Gilford Instrument Laboratories, Oberlin, Ohio).

One unit of NADPH-cytochrome c reductase activity is defined as an absorbance change of 1.0 per minute at 550 nm at 25^{o} in a 1-cm light path. This corresponds to reduction of 0.0476 micromole of cytochrome c per minute per milliliter of reaction mixture (Williams and Kamin, 1967).

J. Benzphetamine N-Demethylase Activity in Extracted and Unextracted Lung Microsomes

Benzphetamine N-demethylase activity was determined by measuring formaldehyde formation by the method of Nash (Nash, 1953) as modified by Cochin and Axelrod (Cochin and Axelrod, 1959). The reaction mixture (2 ml) contained the following: 200 µmoles potassium phosphate buffer (pH 7.4), 7 µmoles $MgCl_2$, 7 µmoles semicarbazide, 2 µmoles EDTA, 2 µmoles NADP, 10 µmoles glucose-6-phosphate, 1.4 units glucose-6-phosphate dehydrogenase, 2 µmoles of benzphetamine, the indicated amounts of di-12 GPC and microsomes.

The incubation mixture was incubated at 37° for 15 minutes. The reaction was stopped with 0.5 ml of cold 20% TCA and the mixture was transferred to plastic centrifuge tubes with Pasteur pipettes. Following centrifugation at 500 x g for 10 minutes, 1.5 ml of the supernatant was transferred to a second set of plastic centrifuge tubes. Nash Reagent (twice the normal concentration) (0.6 ml) was added to each tube, vortexed, and heated in a water bath at 60°C for 15 minutes. Following centrifugation at 500 x g for 10 minutes, the supernatant was transferred to cuvettes using Pasteur pipettes and the absorbance at 412 nm was determined. Known amounts of formaldehyde carried through the incubation and assay procedures served as standards.

K. O-Dealkylation of 7-Ethoxycoumarin by Extracted and Unextracted Lung Microsomes

Microsomal protein and di-12 GPC were added to the incubation vessels in the amounts described in Figure VI-14. The reaction mixture contained 100 μmoles potassium phosphate buffer (pH 7.4), 3 μmoles $MgCl_2$, 1 μmole EDTA, 1 μmole NADP, 5 μmoles glucose-6-phosphate and 0.7 units glucose-6-phospate dehydrogenase and the indicated amounts of microsomal protein and di-12 GPC in a total volume of 1 ml. The reaction was initiated by the addition of 300 nmoles of 7-ethoxycoumarin in 20 μl of acetone. The vessels were incubated at 37^{o} for 20 minutes and the reaction was stopped by transfer of the reaction mixture to tubes containing 5 ml of hexane and vortexing. The reaction mixture was extracted twice with 5 ml of hexane and the hexane discarded to remove unchanged 7-ethoxycoumarin. The reaction mixture was then extracted under 5 ml of ether and 4 ml of the ether layer transferred to a second set of tubes and evaporated to dryness under nitrogen. The residue was dissolved in 2 ml of 0.1 M potassium phosphate buffer (pH 7.4) and extracted again with 5 ml of hexane. The hexane layer was discarded and the fluorescence of the 7-hydroxycoumarin in the buffer phase was determined in an Aminco-Bowman spectrophotofluorometer (excitation wavelength = 338 nm; emission wavelength = 450 nm). Authentic 7-hydroxycoumarin was used as a standard and all results were corrected for the recovery of 7-hydroxycoumarin (97%).

L. Benzo(a)pyrene Hydroxylase Activity in Extracted and Unextracted Lung Microsomes

Benzo(a)pyrene hydroxylase activity was measured using the fluorometric method of Nebert and Gelboin (Nebert and Gelboin, 1968) with slight modification (Lu et al., 1972). The reaction mixture (1 ml) contained the following: 100 µmoles of potassium phosphate buffer, pH 6.8, 3 µmoles $MgCl_2$, 1 µmole EDTA, 1 µmole NADP, 5 µmoles glucose-6-phosphate and 0.7 units glucose-6-phosphate dehydrogenase, benzo(a)pyrene (1.2-39.0 µmoles in up to 20 µl of acetone), the indicated amounts of di-12 GPC and microsomes.

The incubation mixture was incubated a 37^{o} for 15 or 20 minutes. The reaction was stopped with 0.1 ml of acetone. Hexane (3.25 ml) was added and the mixture shaken in an incubator at 37^{o} for 10 mintues. Of the hexane (upper) layer 2.0 ml was transferred into centrifuge tubes, 4.0 ml of 1 N NaOH added and the mixture was shaken for 2 minutes and centrifuged briefly. The fluoroescence of the lower layer was read in an Aminco-Bowman spectrophotofluorometer (excitation wavelength = 394 nm; emission wavelength = 515 nm). Authentic 3-OH-benzo(a)pyrene was used as a standard and all results were corrected for the recovery of 3-OH-benzo(a)pyrene. The activity was expressed as that amount of hydroxylated product which had a fluorescent intensity equivalent to a standard solution of 3-OH-benzo(a)pyrene formed per minute per nanomole

of cytochrome P-450. All assays were performed under linear conditions with respect to time and protein concentrations.

M. Effect of α-Naphthoflavone on Benzo(a)pyrene Hydroxylase Activity in Extracted and Unextraced Lung Microsomes

The indicated amounts (12.5-100 nmoles) of α-Naphthoflavone were added in 5 µl acetone. α-Naphthflavone was added at the same time as the NADPH-generating system.

N. Kinetic Parameters of Benzo(a)pyrene Hydroxylase Activity in Extracted and Unextracted Lung Microsomes

Michaelis constants for benzo(a)pyrene hydroxylase were calculated by the method of Wilkinson (Wilkinson, 1961) with use of a Fortran program of Cleland (Cleland, 1963).

O. Association and Dissociation of Benzo(a)pyrene with Extracted and Unextracted Lung Microsomes

To study the association of benzo(a)pyrene with lung microsomes, we used a modification of the filter method of Williams et al (1976). The filtration assay has been shown to be comparable to equilibrium dialysis with the distinct advantages of being more precise; technically easier and allowing the use of agents that would be degraded during the long incubation period required for dialysis (McDonald et al., 1976).

^{14}C-benzo(a)pyrene (specific activity = 60 mCi/mmol) and ^{3}-H-benzo(a)pyrene (specific activity = 8.3 Ci/mmol) were purchased from Amersham Corporation (Arlington Heights, Illinois). Analysis by 19:1 benzene/ethanol thin-layer chromatography (Sims, 1967) indicated that the ^{14}C-benzo(a)pyrene was greater than 98% pure and that the ^{3}H-benzo(a)pyrene was greater than 96.5% pure.

^{3}H-benzo(a)pyrene was placed in 50 mM Tris buffer, pH 7.6 (see Figure-VI.21). Microsomes were added at zero time and the Siliclad-treated incubation vessels (glass scintillation vials) were incubated at 37^{o} with constant shaking. At timed intervals 100 µl aliquots were removed and rapidly filtered by vacuum through a single Whatman GF/C glass fiber filter to trap the microsomes. The filters were then washed with 6 ml (2 x 3 ml) of ice cold buffer to remove any benzo(a)pyrene not bound to the microsomes. The wash procedure required less than 10 seconds to complete. Duplicate 100 µl aliquots were removed and placed

directly into counting vials to quantitate the total amount of radioactivity present at the end of a given incubation period. To assess binding of benzo(a)pyrene to the filters, duplicate vessels without microsomes were incubated, filtered and washed under identical conditions as those with microsomes and the results used to calculate the background binding. The filters were allowed to dry overnight before counting.

Release or dissociation of benzo(a)pyrene from microsomes was studied in the following manner. Microsomes which had been incubated with benzo(a)pyrene as described above for 15 minutes were diluted 80-fold with buffer at 37° (see Figure VI-20). At timed intervals, 200 ul aliquots of the suspension were removed and filtered, washed and counted as described above. Duplicate 200 ul aliquots were removed and placed directly into counting vials to quantitate the total amount of radioactivity present at the end of a given incubation period.

In a second study, microsomes were incubated with different amounts of ^{14}C-benzo(a)pyrene alone or in the presence of an excess of unlabeled benzo(a)pyrene. The first set of tubes contained the amounts of ^{14}C-benzo(a)pyrene shown in Figure VI-21. 50 mM Tris-HCl buffer, pH 7.6, was added to form a final volume of 2.0 ml. The second set of tubes contained 99 nmoles of unlabeled benzo(a)pyrene and the amounts of ^{14}C-benzo(a)pyrene shown in the figure. 50 mM Tris-HCl buffer, pH 7.6, was added to form a final volume of 2.0 ml. Microsomes (0.1 ml) were added and the mixtures were incubated for 45 mintues at 37°. At the end of the incubation period, the mixtures were filtered under vacuum through

a single Whatman GF/C filter and washed with 4 ml (2 x 2 ml) of ice cold buffer and counted as described above. To assess the binding of benzo(a)pyrene to the filters, duplicate vessels without microsomes were incubated, filtered and washed under identical conditions as those with microsomes and the results used to calculate the background binding.

Initial association and dissociation studies used glass scinitillation vials as the incubation vessels. However, it became apparent that glass had a serious drawback in that the labeled benzo(a)pyrene adsorbed to the incubation vessels. A biphasic loss of label in solution was observed with an initial loss of approximately 15% of the total DPM in solution occurring in the first minute after addition of benzo(a)pyrene followed by a loss of approximately 1% a minute for up to 30 minutes. Identical results were obtained with or without microsomes present in the buffer solution.

Use of plastic or siliclad-treated glass scintillation vials as the incubation vials greatly reduced the loss of total DPM in solution to less than 1% a minute for up to 15 minutes. However, since even the use of these vials resulted in the loss of radioactive benzo(a)pyrene from solution, all experiments were performed as described above with aliquots being removed both for filtration and assessment of the total amount of radioactivity present at the end of a given incubation period.

In an attempt to correlate the release of benzo(a)pyrene with benzo(a)pyrene metabolism, the benzo(a)pyrene hydroxylase activity of microsomes incubated with an equivalent amount of benzo(a)pyrene as those used in the dissociation studies described above was measured using

the fluorometric method of Nebert and Gelboin (Nebert and Gelboin, 1968) with slight modification (Lu et al., 1972).

A radiometric assay was used to assay the total benzo(a)pyrene metabolites formed by lung microsomes (DePierre et al., 1975). The incubation conditions (cytochrome P-450 = 0.028 nmoles/incubation; protein = 0.155 mg/incubation) were the same as described for the fluorometric method described above (except that the substrate used was either labeled ^{3}H-benzo(a)pyrene (0.652 nmoles, specific activity = 8.3 Ci/mmol) or ^{14}C-benzo(a)pyrene (23.34 nmoles, specific activity = 60.7 mCi/mmol) and the reaction was stopped after 15 minutes by the addition of 1 ml or 0.5 n NaOH in 80% ethanol. The sample was then transferred to a centrifuge tube, shaken vigorously with 3 ml hexane for 2 minutes on a Vortex mixer, and spun for 5 minutes at 500 x g. The hexane layer (containing unreacted substrate) was aspirated, 2.5 ml ethyl acetate was added and the sample was shaken vigorously for 30 seconds on a Vortex mixture. Two ml of the upper phase (containing the products) were pipetted into scintillation vials and counted.

The background in this assay was determined by adding the stopping mixture at the same time as the generating system. 0.1-0.4% of the radioactivity added remained in this lower phase. In these experiments, zero-time incubations were routinely carried out and this background has been subtracted from the activities reported.

The percentage of 3-hydroxybenzo(a)pyrene formed was assessed by running the radiometric assay in quadruplicate and determining the

benzo(a)pyrene hydroxylase activity as previously described in two tubes and the total metabolites in the other two tubes.

Benzo(a)pyrene association and dissociation filtrations were performed using a Hoefer model FH-224-OV ten place filter holder (Hoefer Scientific Instruments, San Francisco, California) at a vacuum of approximately 19 mm Hg.

The DPM were determined using a Packard model 3325 Tri-Carb liquid scintillation spectrometer and Scintiverse^{R} aqueous scintillation cocktail (Fisher Scientific Company, Fair Lawn, New Jersey).

P. Substrate Binding to Extracted and Unextracted Lung Microsomal Cytochrome P-450

Extracted and unextracted microsomes were suspended in .1 M phosphate buffer (pH 7.7). Difference spectra were recorded with a dual wavelength recording spectrophotometer.

Unextracted and extracted microsomes were suspended on 1.0 ml buffer to a final protein concentration of 0.96-1.92 mg/ml. When difference spectra were recorded, the same microsomal suspension was divided between two cuvettes. Benzphetamine and aniline were added in water to the sample cuvette and equal volumes of buffer were added to the reference cuvets, unless otherwise stated in figure legends.

Double-reciprocal plots can be derived from spectral titrations at fixed wavelengths (Schenkman, 1970). The double-reciprocal plots are obtained from the kinetic equation as

$$E + S \underset{K_2}{\overset{K_1}{\rightleftharpoons}} C \xrightarrow{K_3} E + P$$

where E is enzyme, C is enzyme-subtrate complex and S is substrate and P the product. Titrations were performed at room temperature in the absence of NADPH or an NADPH-generating system and thus the reaction does not proceed to product P. The reversibility of this first step has been demonstrated in washing experiments by Schenkman et al., (1967).

These workers have defined the "spectral dissociation constant" as being equal to the dissociation constant K_S where

$$K_S = \frac{([E_t] - [C])[S]}{[C]}$$

where E is the total amount of enzyme. By manipulation

$$K_S = \frac{([E_t] - [S])}{[C]} - [S]$$

and

$$[C] = \frac{[E_t][S]}{K_s + S}$$

The reciprocal of this equation

$$\frac{I}{[C]} = \frac{Ks}{[E_t]}\left(\frac{I}{[S]}\right) + \frac{I}{[E_t]}$$

is the equation of a straight line; when the reciprocal of the Type I or Type II spectral change $I/[C]$ is plotted against the reciprocal of substrate level used $1/[S]$, the Y intercept is equal to $I/[E_t]$ or the reciprocal of $[C]_{MAX}$, and the X intercept is equivalent to $-(I/K_S)$.

Q. Other Procedures and General Comments

Cytochrome P-450, NADPH-cytochrome c reductase and glucose-6-phosphate dehydrogenase determinations were measured with an Aminco-Bowman DW-2 spectrophotometer (American Instrument Company, Silver Spring, Maryland) as were the Type I, Type II and reverse Type I binding spectra. Other spectrophotometric determinations (benzphetamine N-demethylase, protein, phosphorus, B-glucuronidase, succinic dehydrogenase and 5'-nucleotidase) were made using a Gilford Stasar II spectrophotometer (Gilford Instrument Laboratories, Oberlin, Ohio). Initial NADPH-cytochrome c reductase assays were determined using a Gilford model 2000 multiple sample absorbance recorder (Gilford Instrument Laboratories, Oberlin, Ohio) and the results did not differ from those obtained with the DW-2 spectrophotometer.

Fluorometric determinations (benzo(a)pyrene hydroxylase and ethoxycoumarin O-deethylase activities) were made using an Aminco-Bowman spectrophotofluorometer (American Instrument Company, Silver Spring, Maryland).

Benzphetamine N-demethylase, ethoxycoumarin O-deethylase, benzo(a)pyrene hydroxylase, benzo(a)pyrene association and dissociation, B-glucuronidase, succinic dehydrogenase and 5'-nucleotidase assay incubations were performed in a Dubnoff metabolic incubator.

Protein determinations were made according to the method of Lowry and co-workers (Lowry et al., 1951), with bovine serum albumin as the standard.

All temperatures given are in degrees centigrade.

VI. RESULTS

A. The Distribution of Various Marker Enzymes in Subcellular Fractions of Rabbit Lung

As seen in Figures VI-1 and VI-2 and Table VI-1, the distribution of cytochrome P-450 and NADPH-cytochrome c reductase was highly localized in the microsomal fractions. Significant activity was also observed in the pre-microsomal pellets which can probably be attributed to the endoplasmic reticulum fragments found in partially disrupted cells (Gram, 1973; Bend et al., 1973). Palade and co-workers have shown that Golgi fractions also contain a very small proportion of the total cellular NADPH-cytochrome c reductase activity (Howell, et al., 1978; Ito, et al., 1978). The NADPH-cytochrome c reductase activity observed in the post-microsomal supernatants is most likely the result of a non-enzymatic reduction of cytochrome c. A number of observations using the postmicrosomal supernatant from rat lung support this conclusion (Johannesen et al., 1977).

The activity of 5'-nucleotidase, a plasma membrane marker, was found to be diffusely distributed in all cell fractions (Figure VI-3 and Table VI-1). The increase in activity of the second microsomal fraction may be the result of the homogenization required to resuspend the first microsomal pellet exposing more of the enzymatically-active surface of the remaining plasma membranes. 5'-Nucleotidase activity was found by

Gram and co-workers to be diffusely distributed in all cell fractions of rabbit liver and lung (Gram, 1973; Bend et al., 1973).

The activity of glucose-6-phosphate dehydrogenase, a cytosol marker, was found to be primarily localized in the first soluble (first post-microsomal supernatant) fraction with activity also observed in the 600 xg, heavy mitochondrial, and light mitochondrial fractions (Figure VI-4 and Table VI-1). This agrees with the liver and lung work of Gram and co-workers (Gram et al., 1973; Bend et al., 1973).

The activity of β-glucuronidase, a lysosomal marker, was found to be diffusely distributed among all cell fractions (Figure VI-5 and Table VI-1). This result agrees with the diffuse distribution of B-glucuronidase in all liver and lung cell fractions found by Gram and co-workers (Gram et al., 1973; Bend et al., 1973).

Lung cell fractions were also checked for possible contamination with mitochondrial membranes by the use of the mitochondrial enzyme succinic dehydrogenase (Figure VI-6 and Table VI-1). Mitochondrial enzyme activity was most highly concentrated in the mitochondrial fractions which agrees with the work of Gram and co-workers (Gram, 1973; Bend et al., 1973). They found the activities of succinate cytochrome c reductase and monoamine oxidase to be most highly concentrated in the mitochondrial fractions of liver and lung.

TABLE VI.1.

THE DISTRIBUTION OF VARIOUS MARKER ENZYMES IN SUBCELLULAR FRACTIONS OF RABBIT LUNG

	600 x g Pellet	Heavy Mitochon	Light Mitochon	Microsomal 1	Soluble 1	Microsomal 2	Soluble 2
Cytochrome P-450 (nmoles/mg protein)	0.045 ± 0.016	0.059 ± 0.002	0.067 ± 0.015	0.248 ±0.030	0.001 ± 0.001	0.197 ± 0.34	0.016 ± 0.005
NADPH Cytochrome c Reductase (nmoles/mg protein/ min)	4.80 + .42	8.33 + 1.24	10.48 ± 0.95	58.12 ± 5.57	1.67 ± 0.80	70.00 ± 4.82	4.32 ± 0.47
Succinic Dehydro-genase (nmoles/mg protein/min)	1140.8 ± 126.0	4379.8 ± 343.1	1792.1 ± 159.9	756.4 ± 53.5	350.1 ± 10.3	618.14 ± 117.5	279.9 ± 120.5
β-Glucuronidase (nmoles/mg protein/ min)	1.28 ± 0.49	3.92 ± 0.78	3.02 ± 0.52	380 ± 0.83	0.44 ± 0.08	3.79 ± 0.80	1.21 ± 0.11
5'-Nucleotidase (nmoles/mg protein/ min)	85.96 ± 14.11	174.20 ± 24.41	246.63 ± 40.33	391.66 ± 44.46	7.18 ± 4.19	556.04 ± 84.77	86.55 ± 11.84
Glucose-6-phosphate Dehydrogenase (ΔA_{340}/mg protein/min)	0.042 ± 0.005	0.018 ± 0.004	0.029 ± 0.006	0.002 ± 0.002	0.100 ± 0.014	0	0.006 ± 0.006

VALUES REPRESENT MEAN ± S.E. OF DUPLICATE DETERMINATIONS FROM THREE SEPARATE EXPERIMENTS

FIGURE VI-1.

SUBCELLULAR DISTRIBUTION OF CYTOCHROME P-450 IN RABBIT LUNG.

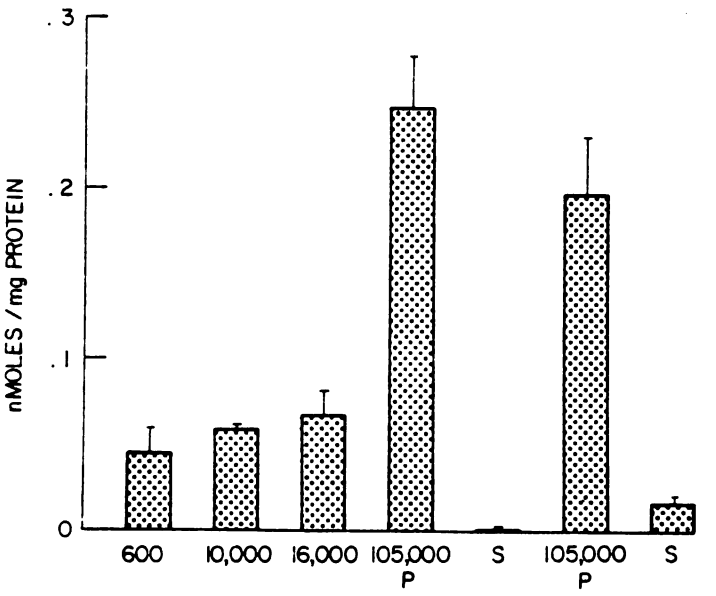


FIGURE VI-2

SUBCELLULAR DISTRIBUTION OF NADPH-CYTOCHROME C REDUCTASE ACTIVITY IN RABBIT LUNG.

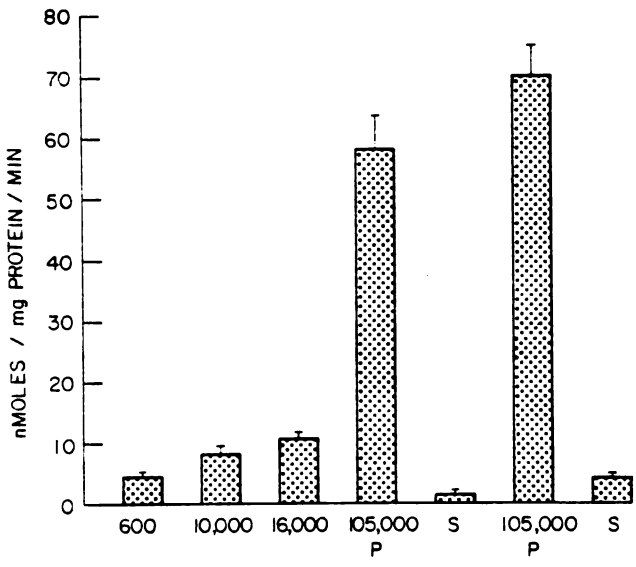


FIGURE VI-3

SUBCELLULAR DISTRIBUTION OF 5'-NUCLEOTIDASE ACTIVITY IN RABBIT LUNG.

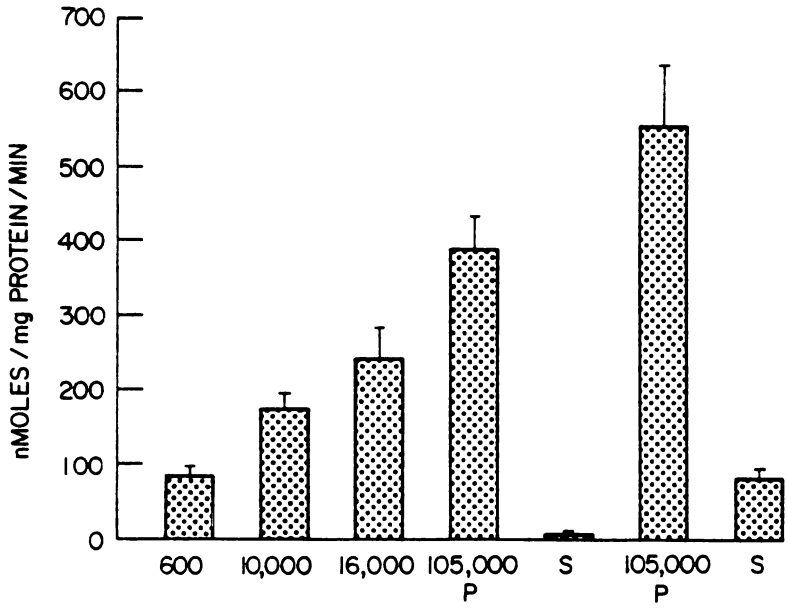


FIGURE VI-4

SUBCELLULAR DISTRIBUTION OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE ACTIVITY IN RABBIT LUNG.

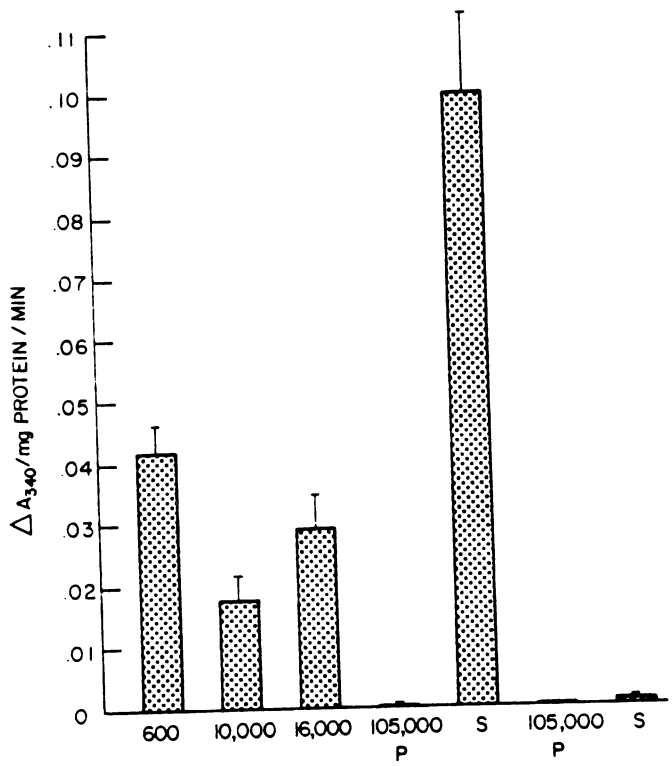


FIGURE VI-5

SUBCELLULAR DISTRIBUTION OF β-GLUCURONIDASE ACTIVITY IN RABBIT LUNG.

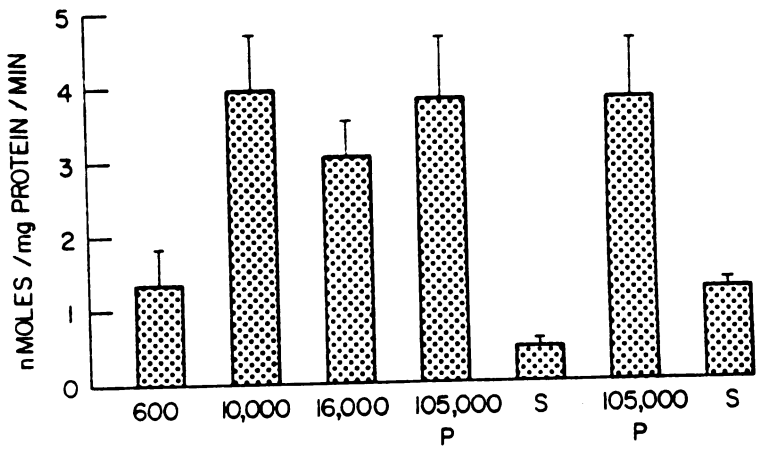
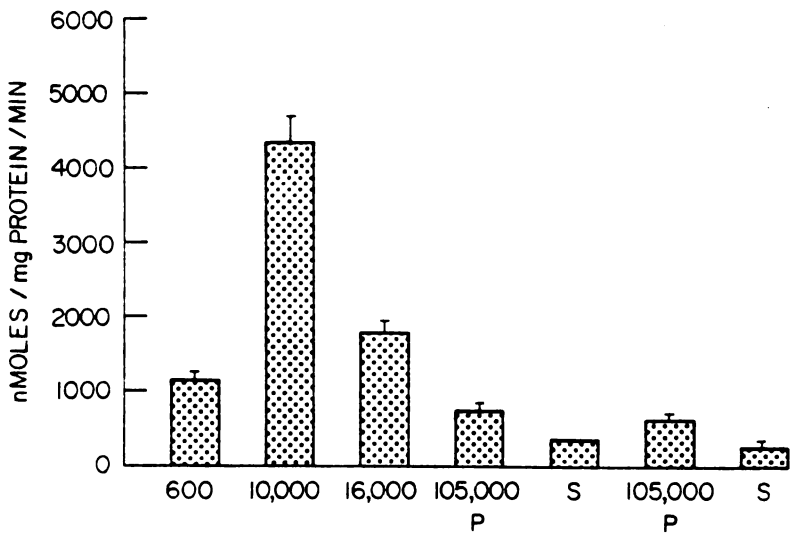


FIGURE VI-6

SUBCELLULAR DISTRIBUTION OF SUCCINIC DEHYDROGENASE ACTIVITY IN RABBIT LUNG.



C. Electron Microscopy of Unextracted and Extracted Rabbit Lung Microsomes

Gram and Bend and co-workers (Gram, 1973; Bend et al., 1973) have observed by electron microscopy that rabbit lung microsomes were composed of aggregates or clusters of vesicles and particles resembling ribosomes embedded in an apparently amorphous matrix of long filaments. They initially fixed suspensions of microsomes overnight in glutaraldehyde followed by an osmium tetroxide postfixation step. however, neither glutaraldehyde nor osmium tetroxide have been shown to be suitable as a general fixative when used alone. Artifacts have been reported which are due to the lipid extracting and cell shrinking effects of glutaraldehyde which occur before postfixation with osmium tetroxide (Glauert, 1975). In addition, osmium tetroxide preserves lipids by forming addition compounds with unsaturated fatty acid chains and also by its solubility in triglycerides. Thus, osmium tetroxide would not be reduced by saturated phospholipids such as di-12 GPC (Meek, 1976). Trump and Bulger (1960) have shown that most of these deleterious effects can be eliminated or minimized by simultaneous use of glutaraldehyde and osmium tetroxide in a mixed fixative.

With the above information in mind, unextracted and extracted lung microsomes were fixed simultaneously in glutaraldehyde and osmium tetroxide and were also placed in aqueous uranyl acetate before

dehydration. Uranyl acetate used this way functions not only as a stain, but also acts as a fixative, particularly for lipid components (Glauert, 1975).

Unextracted lung microsomes were found to be composed of vesicles (with the appearance of smooth endoplasmic reticulum) and electron-dense particles resembling ribosomes (Plates VI-1-V3). However, in distinct contrast to the work of Gram and Bend and co-workers (Gram, 1973; Bend et al., 1973), these vesicles and particles did not appear as clusters or aggregates but were more or less uniformly spread across the field of the micrographs.

In contrast with unextracted microsomes, extracted microsomes contained fewer and in general smaller vescicles and numerous fine electron-dense particles uniformly spread across the field of the micrograph (Plates VI-4-VI-6). The appearance of fewer and smaller vesicles and numerous find electron-dense particles agrees with the results seen with extracted rat liver microsomes (Vore et al., unpublished observations).

Addition of sonicated di-12 GPC to extracted microsomes in a concentration (mg phosphatidylcholine/nmole cytochrome P-450) equal to that used for maximal reactivation of benzphetamine N-demethylase activity (Section VI-G) did not restore the morphology of the microsomes to an unextracted appearance but did result in the appearance of an increased number of vesicles (Plates VI-7 - VI-9). This result agrees with the unpublished observations of Vore and co-workers for extracted rat hepatic microsomes to which sonicated di-12 GPC was added.

The small vesicles and numerous fine electron-dense particles observed in extracted microsomes may consist of globules of (hydrophobic) proteins combined with phospholipids remaining after lipid extraction.

PLATE VI-1

ELECTRON MICROGRAPH OF UNEXTRACTED RABBIT LUNG MICROSOMAL PELLET. SEE "METHODS" (SECTION V-F).

MAGNIFICATION: 16,250x

FIXATION: GLUTARALDEHYDE-OSMIUM TETROXIDE.

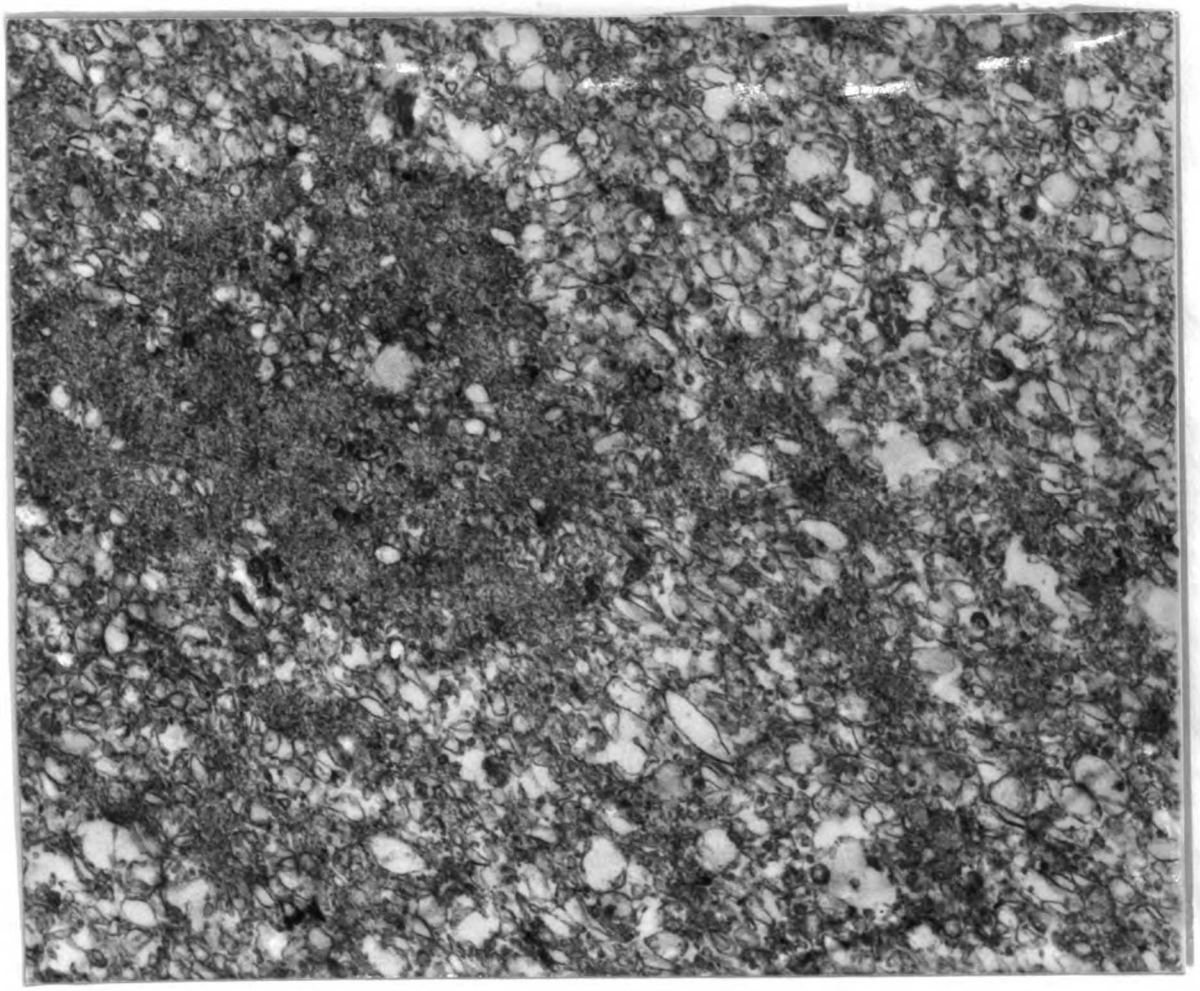


PLATE VI-2

ELECTRON MICROGRAPH OF UNEXTRACTED RABBIT LUNG MICROSOMAL PELLET. SEE "METHODS" (SECTION V-F).

MAGNIFICATION: 34,125x

FIXATION: GLUTARALDEHYDE-OSMIUM TETROXIDE.

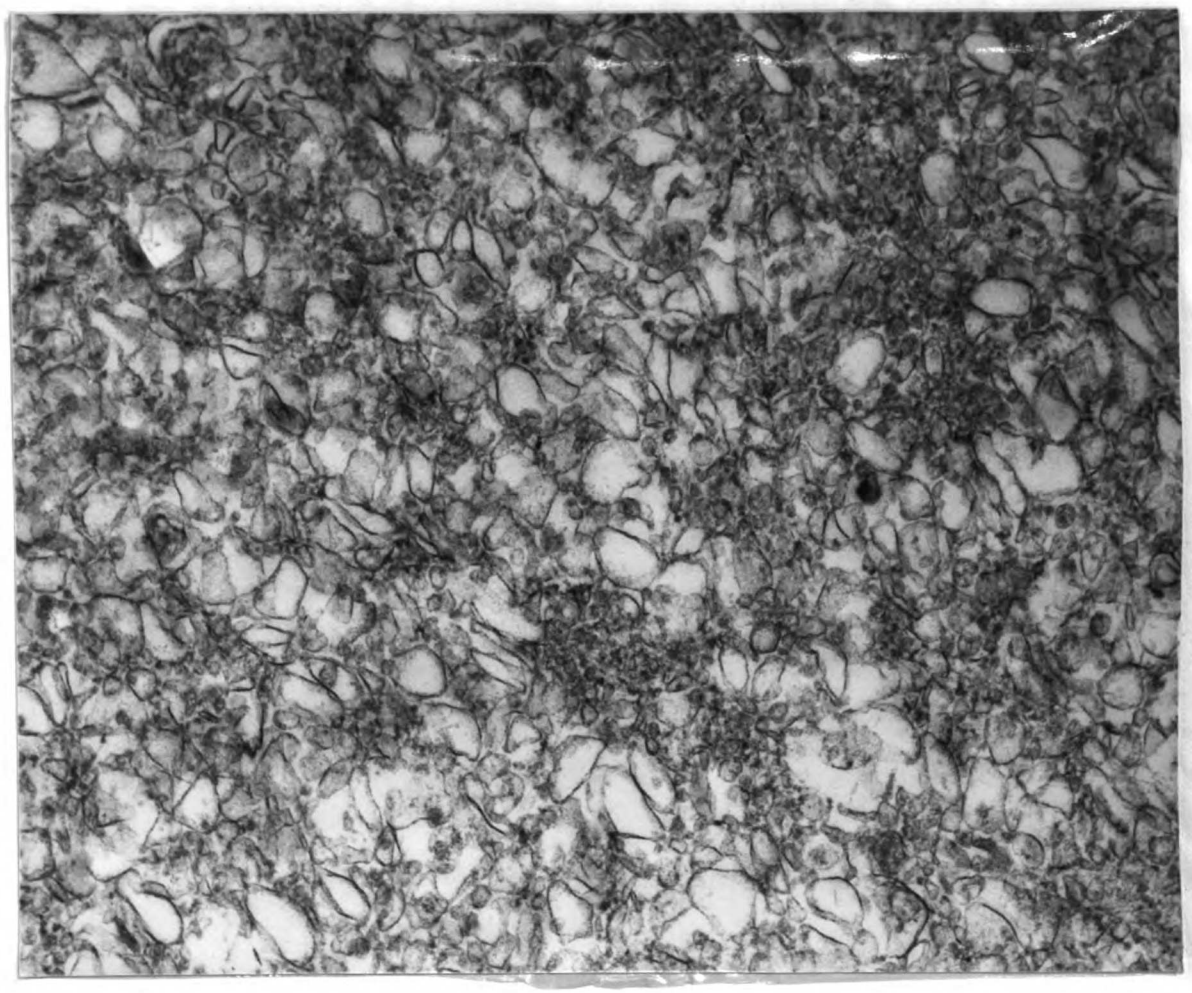


PLATE VI-3

ELECTRON MICROGRAPH OF UNEXTRACTED RABBIT LUNG MICROSOMAL PELLET. SEE "METHODS" (SECTION V-F).

MAGNIFICATION: 94,250x

FIXATION: GLUTARALDEHYDE-OSMIUM TETROXIDE

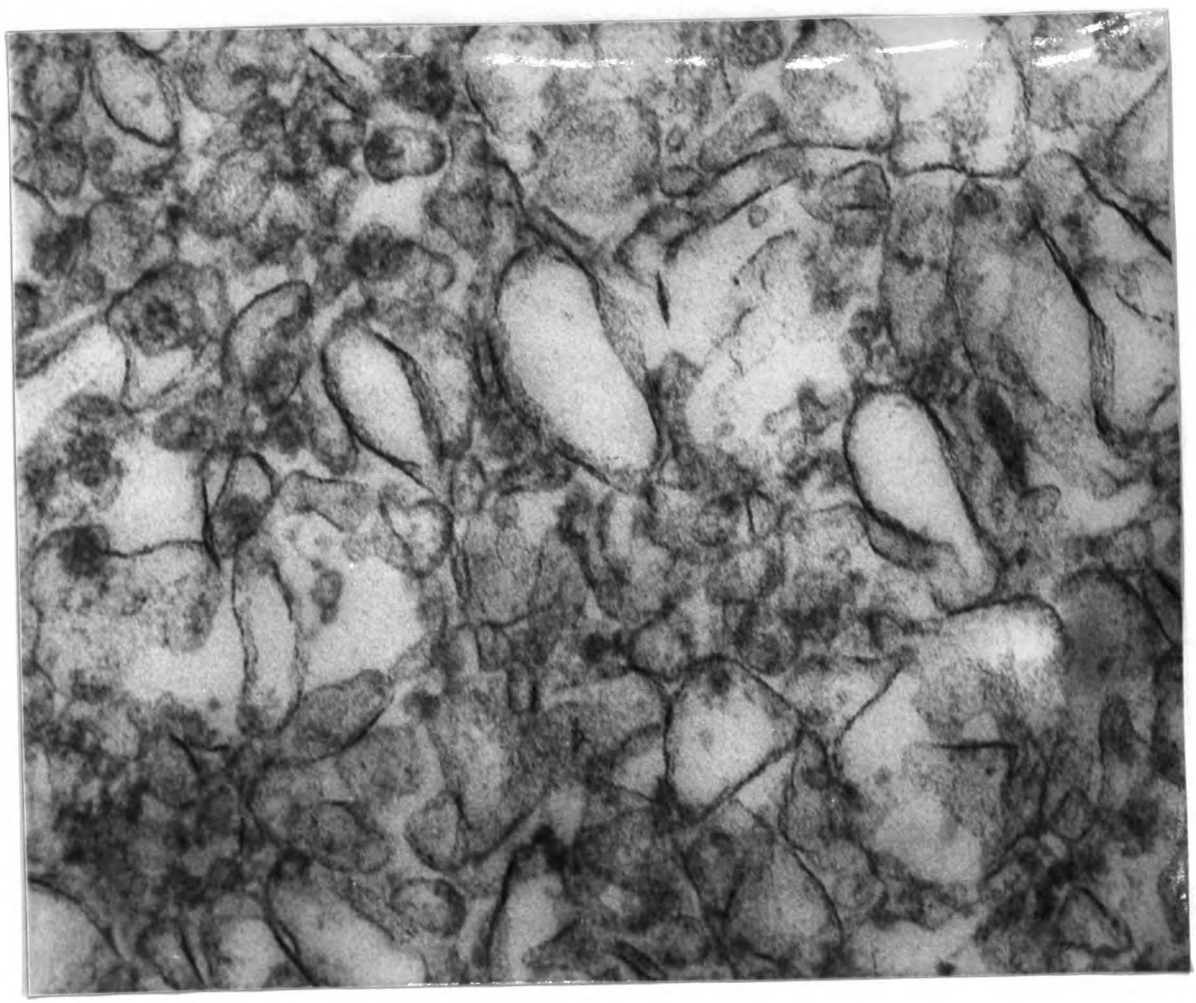


PLATE VI-4

ELECTRON MICROGRAPH OF EXTRACTED RABBIT LUNG MICROSOMAL PELLET. SEE "METHODS" (SECTION V-F).

MAGNIFICATION: 16,250x

FIXATION: GLUTARALDEHYDE-OSMIUM TETROXIDE

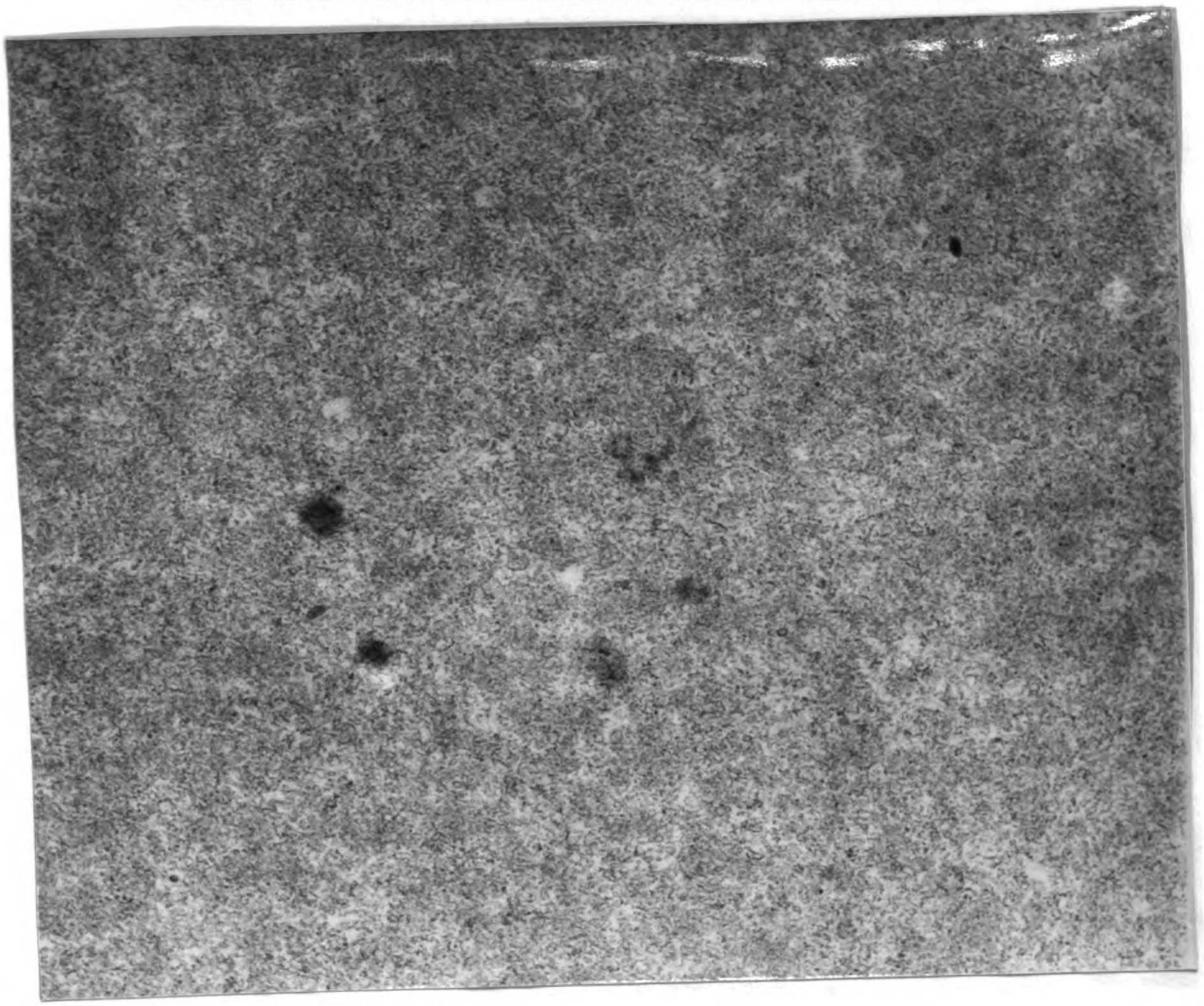


PLATE VI-5

ELECTRON MICROGRAPH OF EXTRACTED RABBIT LUNG MICROSOMAL PELLET. SEE "METHODS" (SECTION V-F).

MAGNIFICATION: 34,125x

FIXATION: GLUTARALDEHYDE-OSMIUM TETROXIDE

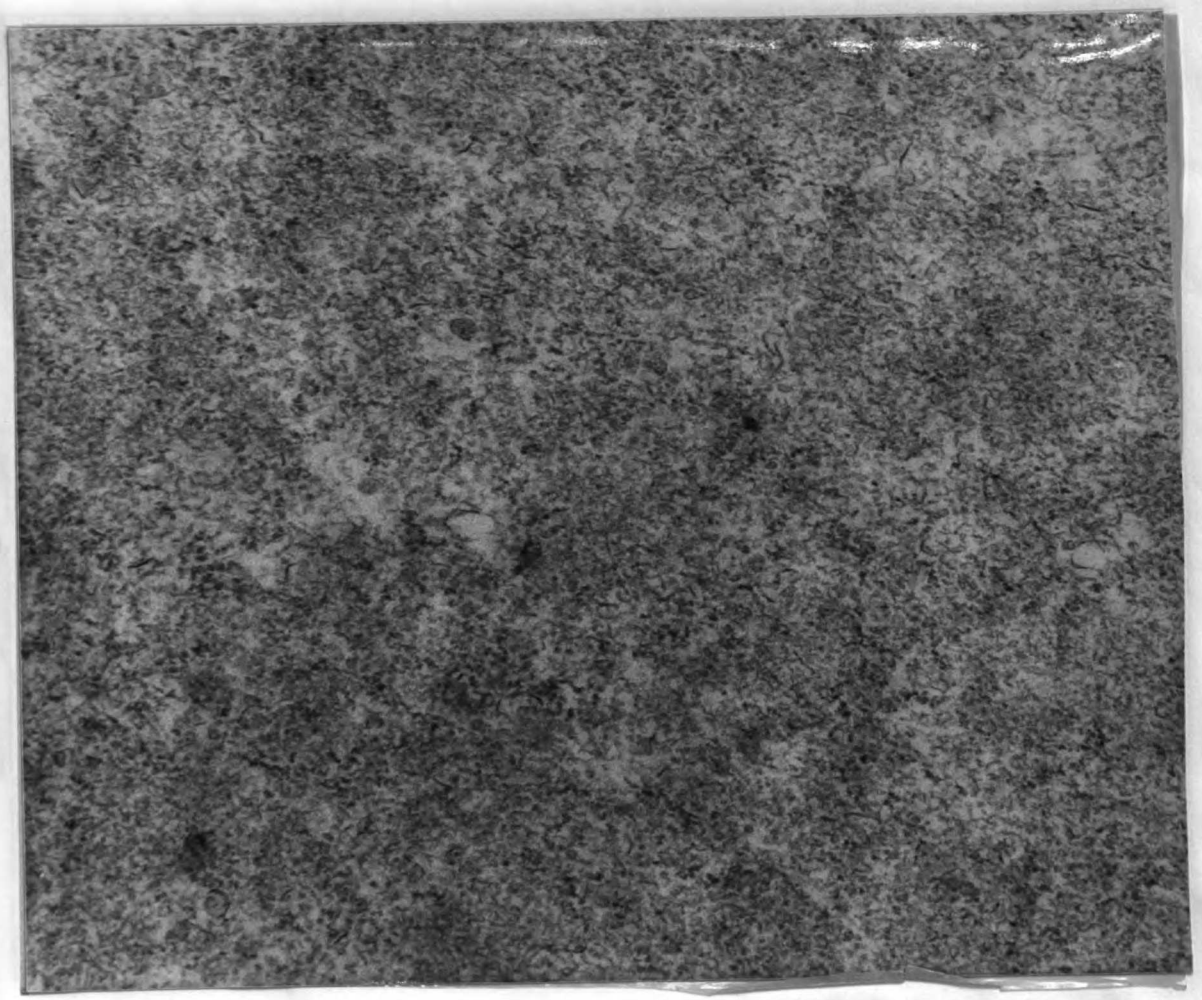


PLATE VI-6

ELECTRON MICROGRAPH OF EXTRACTED RABBIT LUNG MICROSOMAL PELLET. SEE "METHODS" (SECTION V-F).

MAGNIFICATION: 94,250x

FIXATION: GLUTARALDEHYDE-OSMIUM TETROXIDE

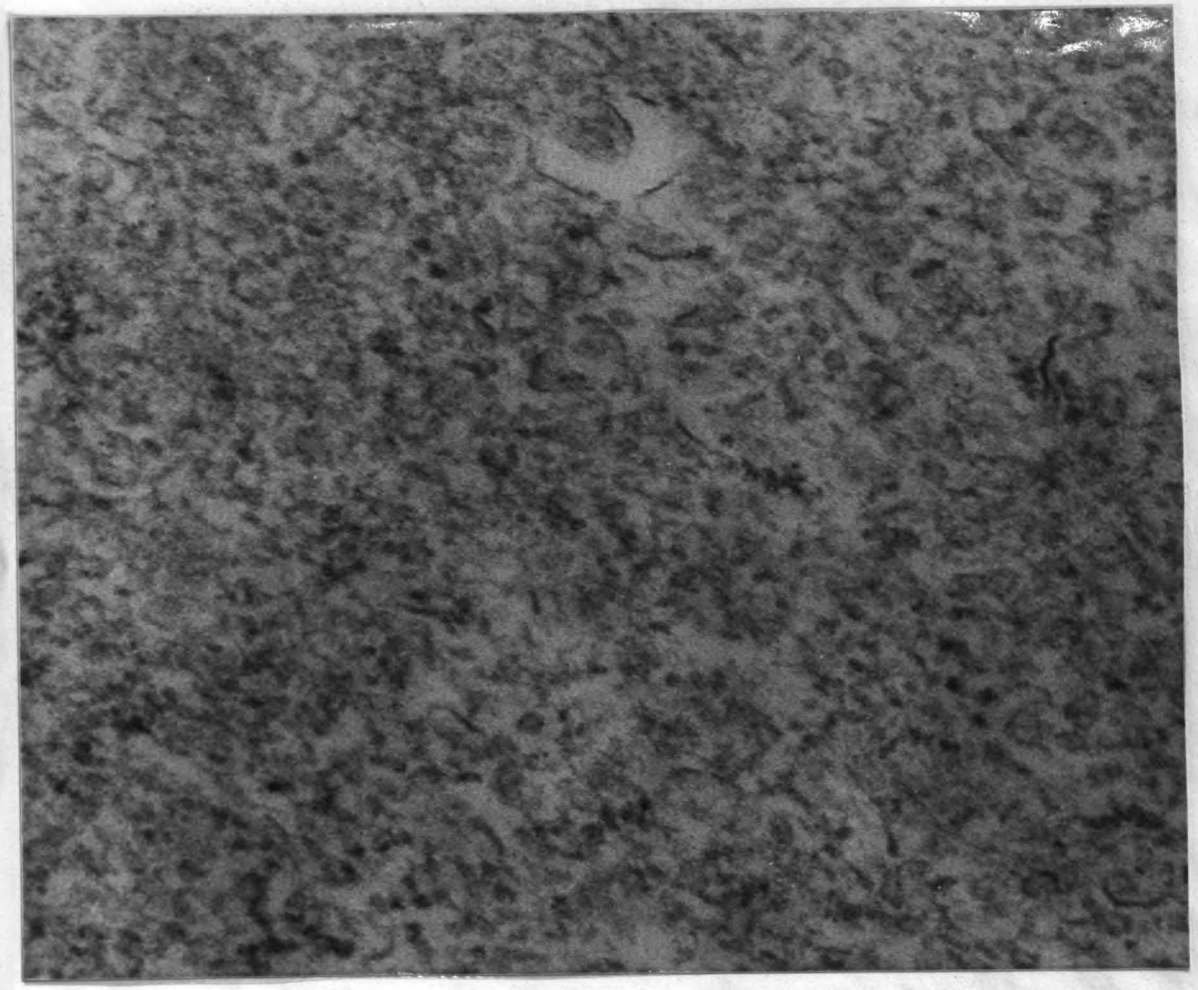


PLATE VI-7

ELECTRON MICROGRAPH OF EXTRACTED RABBIT LUNG MICROSOMAL PELLET AND SONICATED SYNTHETIC PHOSPHATIDYLCHOLINE

SEE "METHODS" (SECTION V-F).

MAGNIFICATION: 16,250x

FIXATION: GLUTARALDEHYDE-OSMIUM TETROXIDE

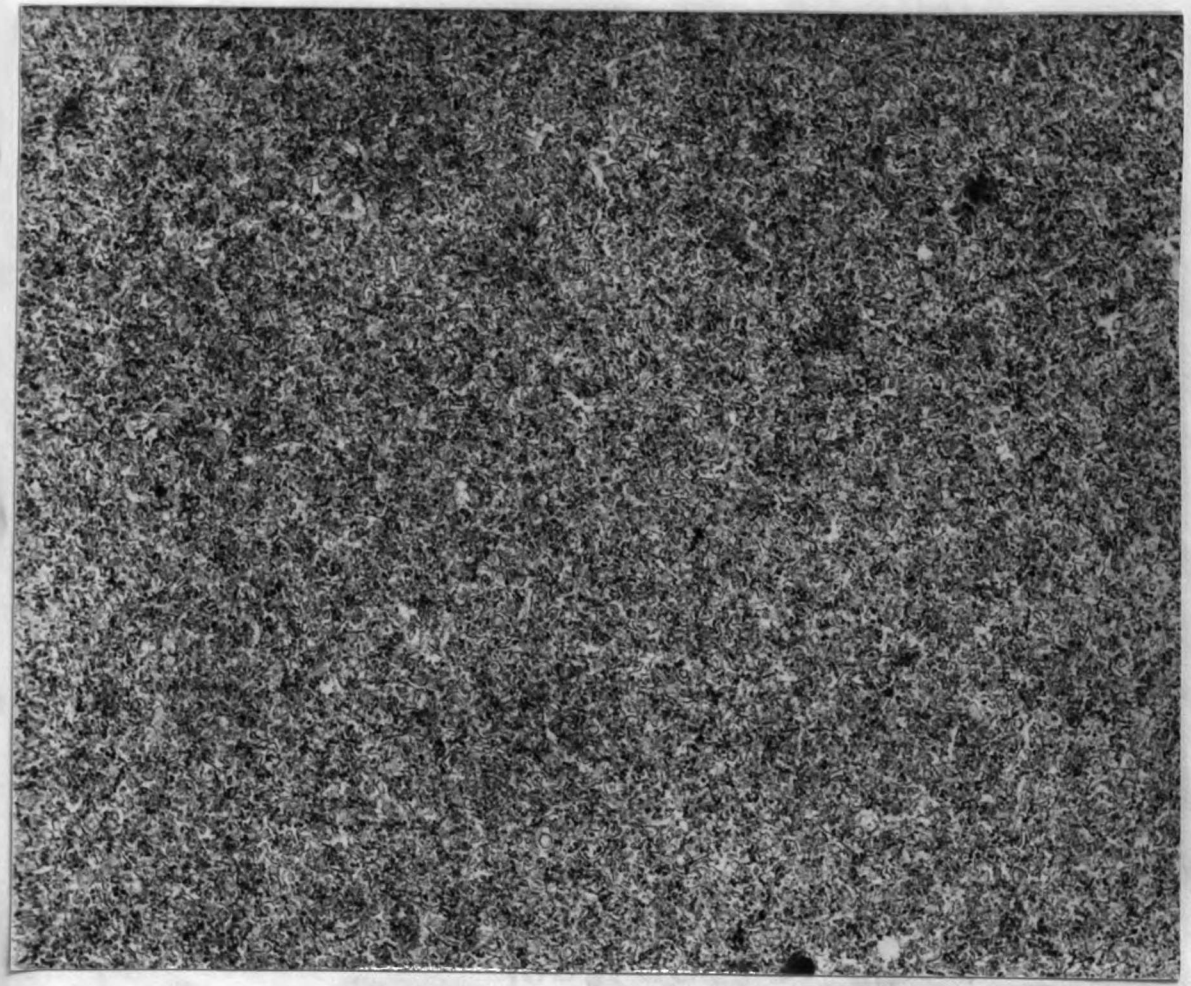


PLATE VI-8

ELECTRON MICROGRAPH OF EXTRACTED RABBIT LUNG MICROSOMAL PELLET AND SONICATED SYNTHETIC PHOSPHATIDYLCHOLINE

SEE "METHODS" (SECTION V-F).

MAGNIFICATION: 34,125x

FIXATION: GLUTARALDEHYDE-OSMIUM TETROXIDE

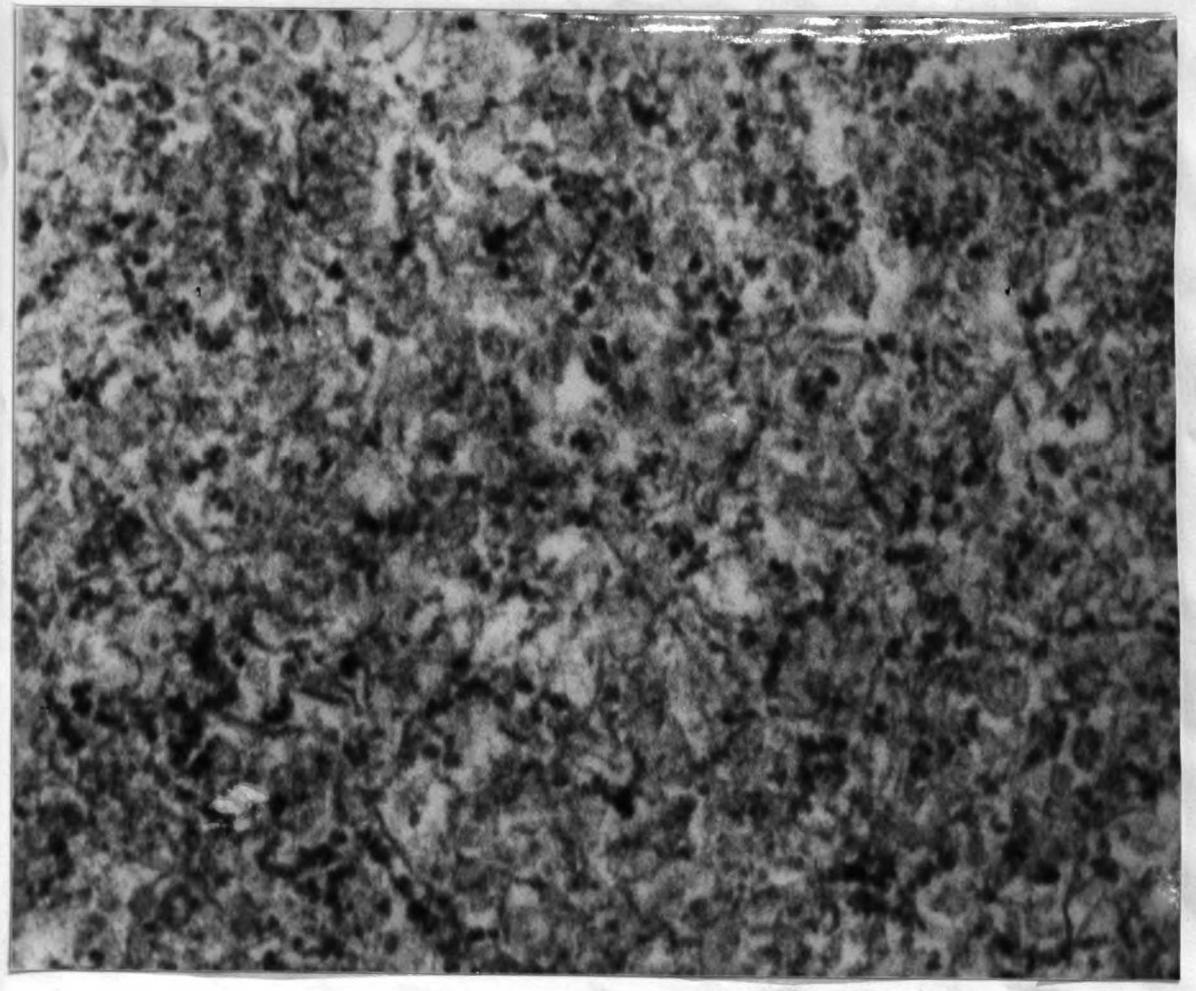
PLATE VI-9

ELECTRON MICROGRAPH OF EXTRACTED RABBIT LUNG MICROSOMAL PELLET AND SONICATED SYNTHETIC PHOSPHATIDYLCHOLINE

SEE "METHODS" (SECTION V-F)

MAGNIFICATION: 94,250x

FIXATION: GLUTARALDEHYDE-OSMIUM TETROXIDE



D. Lipid Analysis of Extracted and Unextracted Microsomes

Jobe and co-workers (Jobe et al., 1978) have studied the phospholipid content in rabbit lung surfactant and subcellular fractions using in vivo pulse labeling with ^{3}H-glycerol and ^{14}C-palmitic acids. Labeled precursors were incorporated into all phospholipid classes in the microsomal fraction simultaneously, and the specific activity of each phospholipid was maximal within 30 minutes following isotope administration. As seen in Table VI-4, approximately 52% of rabbit lung microsomal phospholipid is phosphatidylcholine. Rabbit lung microsomal phosphatidylcholine is 48.5% saturated (Table VI-5). Phosphatidylethanolamine comprises an additional 20% followed by sphingomyelin at 11%. Phosphatidylserine, phosphatidylglycerol and phosphatidylinositol comprise an additional 1-5% each.

In order to obtain consistent results, all studies were done after two 1-butanol and two acetone extractions. As shown in Tables VI-2 and VI-6, approximately 95% of the ^{14}C-phosphatidylcholine, 73% of the ^{14}C-phosphatdiylethanolamine, 14% of the ^{3}H-phosphatidylinositol and 88% of the phospholipid phosphorus content (mg phosphorus/mg protein) were removed by the extraction procedure.

One-dimensional thin-layer chromatography (Figure VI-10) indicates that the extraction procedure used removes all the lung microsomal neutral lipid. This agrees with the results obtained with rat liver microsomes using similar methodology (Vore et al., 1974a; Vore et al., 1974b).

The major phospholipids and glycolipids can be separated, identified and analyzed quantitatively by thin-layer chromatography, particularly in two-dimensional systems (Rouser et al., 1970; Rouser et al., 1969). As shown in Figures VI-7, VI-8 and VI-9, two-dimensional thin-layer chromatograms of egg yolk lipids, polar lipids from extracted microsomes and polar lipids from unextracted microsomes were developed on silica-gel G. Tentative identification of the phospholipids was based on the R_f values of Rouser and co-workers (Rouser et al., 1969; Rouser et al.; 1970) (Table VI-3). As seen in Figures VI-8 and VI-9, the apparent phosphatidylcholine fraction/apparent phosphatidylethanolamine fraction ratio is much smaller in extracted than in unextracted microsomes. This agrees with the data obtained from the use of phosphatidylcholine and phosphatidylethanolamine labelled precursors (Table VI-6).

TABLE VI-2

EFFECT OF BUTANOL AND ACETONE EXTRACTIONS ON VARIOUS COMPONENTS OF RABBIT LUNG MICROSOMES

	UNEXTRACTED MICROSOMES ± S.E	EXTRACTED MICROSOMES ± S.E.	% RECOVERY ± S.E.
CYTOCHROME P-450 (nmoles/mg protein)	0.37 ±0.041 (10)	0.25±0.032 (15)	67.6
CYTOCHROME-C REDUCTASE (units cytochrome-C reduced /min /mg protein)	49.15 ±10 6 (3)	35.53 ±6.8 (3)	72.7 ±2.6
PHOSPHOLIPID PHOSPHORUS CONTENT (mg phosphorus / mg protein)	0.033 ±0.0032(3)	0.004 ±0.0003 (3)	11.9 ± 1.0

FIGURE VI-7

TWO-DIMENSIONAL THIN-LAYER CHROMATOGRAM, ON SILICA-GEL G, OF EGG YOLK LIPIDS

Two-dimensional thin-layer chromatograms on silica gel G developed in solvent system: (1) Chloroform-methanol-28% ammonia (65:25:5, v/v) and (2) Chloroform-acetone-methanol-acetic acid-water (3:4:1:1:0.5, v/v). Chromatogram was visualized by spraying with H_2SO_4 and charring. Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; Sph, sphingomyelin; LPL, less polar lipids-(cholesterol, triglycerides, etc).

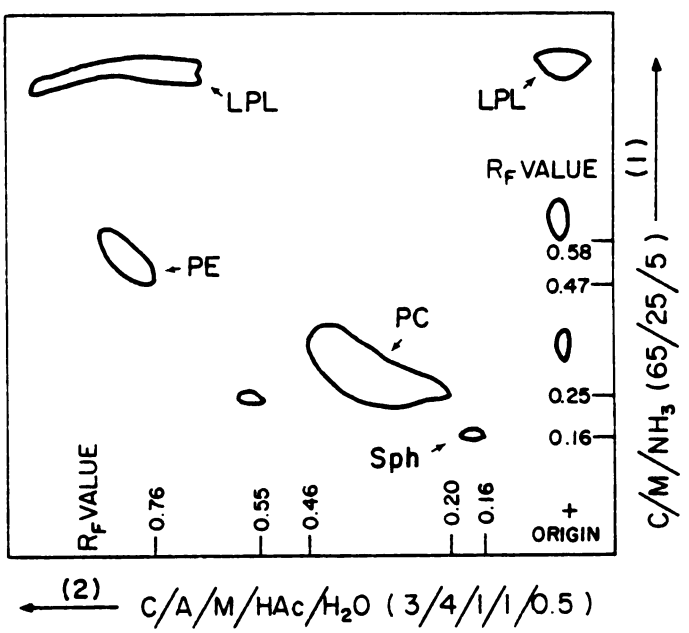


FIGURE VI-8

TWO-DIMENSIONAL THIN-LAYER CHROMATOGRAM, ON SILICA-GEL G, OF POLAR LIPIDS FROM UNEXTRACTED RABBIT LUNG MICROSOMES

Two-dimensional thin-layer chromatograms on silica gel G developed in solvent system: (1) Chloroform-methanol-28% ammonia (65:25:5, v/v) and (2) Chloroform-acetone-methanol-acetic acid-water (3:4:1:1:0.5, v/v). The chromatogram was visualized by spraying with H_2SO_4 and charring. Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; Sph, sphingomyelin; LPL, less polar lipids (cholesterol, triglycerides, etc).

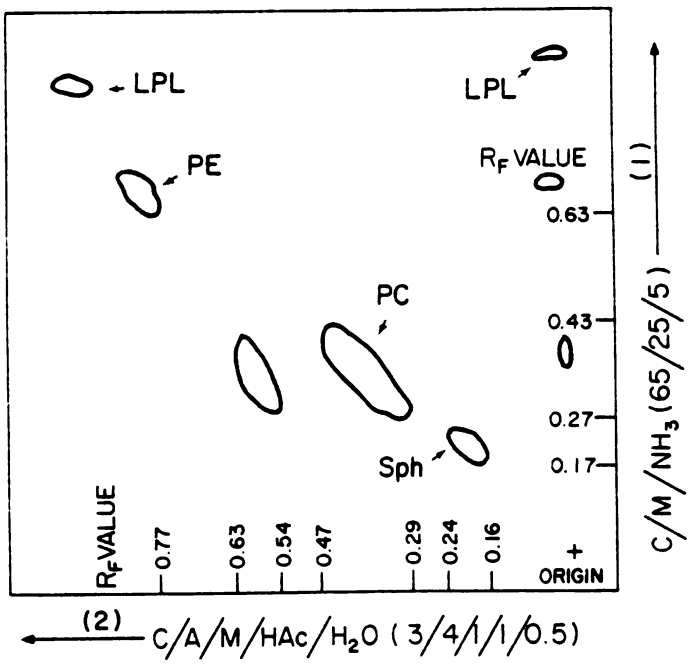

LPL
LPL
PE
R_F VALUE
0.63
PC
0.43
0.27
Sph
0.17
R_F VALUE
0.77
0.63
0.54
0.47
0.29
0.24
0.16
+
ORIGIN
(1)
C/M/NH$_3$ (65/25/5)
(2)
C/A/M/HAc/H$_2$O (3/4/1/1/0.5)

FIGURE VI-9

TWO-DIMENSIONAL THIN-LAYER CHROMATOGRAM, ON SILICA-GEL G, OF POLAR LIPIDS FROM EXTRACTED RABBIT LUNG MICROSOMES

Two-dimensional thin-layer chromatograms on silica gel G developed in solvent system: (1) Chloroform-methanol-28% ammonia (65:25:5, v/v) and (2) Chloroform-acetone-methanol-acetic acid-water (3:4:1:1:0.5, v/v). The chromatogram was visualized by spraying with H_2SO_4 and charring. Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; Sph, sphingomyelin; LPL, less polar lipids (cholesterol, triglycerides, etc.)

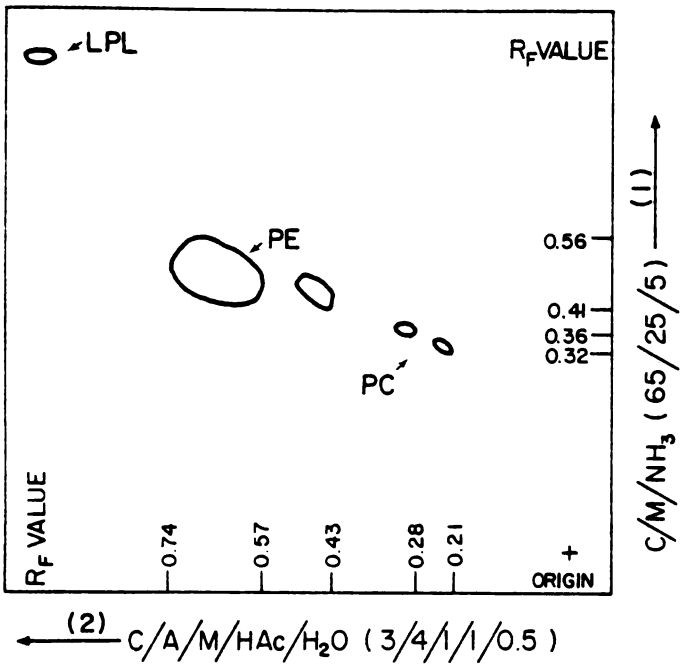

LPL
R_F VALUE
PE
PC
0.56
0.41
0.36
0.32
R_F VALUE
0.74
0.57
0.43
0.28
0.21
ORIGIN
(1) C/M/NH_3 (65/25/5)
(2) C/A/M/HAc/H_2O (3/4/1/1/0.5)

TABLE VI. 3

R_f VALUES OF POLAR LIPIDS ON TLC IN VARIOUS SOLVENTS

Lipid component	R_f values x 100 in solvents**	
	1	2
PHOSPHATIDES		
Lysolecithin	8	15
Phosphatidyl inositol	11	42
Sphingomyelin	22	26
Lecithin	33	40
Lysophosphatidyl ethanolamine	20	40
Phosphatidyl glycerol	37	75
Phosphatidyl ehtanolamine	41	65
Phosphatidyl serine	5	44
Cardiolipin	38	85
Lysophosphatidic acid	5	
Phosphatidicyacid	5	66
GLYCOLIPIDS		
Cerebrosides	45-51	78-84
Cerebroside sulfate	18-20	66-70

** Solvents: 1) Chloroform-methanol-28% ammonia (65:25:5) and 2) chloroform-acetone-methanol-acetic acid-water (3:4:1:1:05)

From: Rouser et al., 1969; 1970.

FIGURE VI-10

CHROMATOGRAM ON SILICA-GEL-IMPREGNATED PAPER OF NEUTRAL LIPIDS FROM UNEXTRACTED RABBIT LUNG MICROSOMES (A), EGG YOLKS (B) AND EXTRACTED RABBIT LUNG MICROSOMES (C).

Egg yolk lipid was used as a standard. Chloroform-methanol (2:1) extracts of both unextracted and extracted microsomes were spotted on silica gel-impregnated glass filter paper (Gelman Instrument Company) and chromatographed in an isooctane-acetic acide (200:3) solvent system. The chromatogram was visualized by spraying with H_2SO_4 and charring. Abbreviations: CE, cholesterol esters; FFA, free fatty acids; TG, triglycerides; C, cholesterol; DG, Diglycerides; MG, monoglycerides; PL, phospholipids.

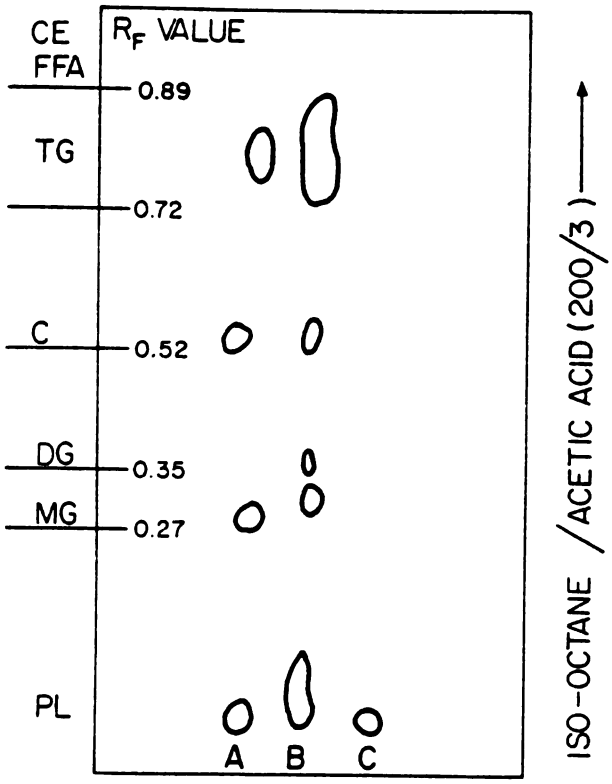

CE
FFA
R_F VALUE
0.89
TG
0.72
C
0.52
DG
0.35
MG
0.27
PL
A
B
C
ISO-OCTANE /ACETIC ACID (200/3)

TABLE VI-4

PHOSPHOLIDPID COMPOSITION OF LUNG FRACTIONS

The results are presented as mean per cent composition ± standard deviation. N, the number of determinations is given in parenthesis

Phospholipid	Parenchyma (7)	Microsomes (8)	Lamellar bodies (10)		Alveolar wash (9)
Phosphatidylcholine	52.5 ± 2.0	51.8 ± 4.0	86.0 ± 5.1	83.8 ± 2.2	
Phosphatidylehtanol-amine	18.4 ± 1.7	20.4 ± 5.3	3.5 ± 0.5	3.6 ± 1.3	
Phosphatidylserine	3.2 ± 0.9	4.7 ± 1.7	0.4 ± 0.1		0.4 ± 0.2
Phosphatidylglycerol	1.6 ± 0.5	1.2 ± 0.6	5.0 ± 1.1		4.7 ± 1.9
Phosphatidylinositol	3.1 ± 0.4	3.6 ± 0.8	4.2 ± 1.9		5.2 ± 1.6
Unknonw phospholipid	6.9 ± 0.7	7.7 ± 1.2	1.2 ± 0.7		0.7 ± 0.2
Sphingomyelin	13.2 ± 2.3	10.8 ± 1.4	0.5 ± 0.4		0.6 ± 0.4
Lysophosphatidyl-choline	1.3 ± 0.6	---	0.7 ± 0.3		---

From Jobe, A. et al. (1978)

TABLE VI-5

FATTY ACID COMPOSITION OF PHOSPHOLIPIDS FROM RABBIT LUNG MICROSOMES

The results are presented as mean per cent composition ± standard deviation of at least four analyses, except for single analyses where no standard error is given. ---, trace amounts or not detected.

	Phosphatidylcholine	Phosphatidylglycerol	Phosphatidylinositol	Phosphatidylethanolamine and phosphatidylserine
14:0	3.2 ± 1.1	4.6 ± 1.5	1.4 ± 0.9	1.7 ± 0.6
16:0	38.4 ± 2.5	38.0 ± 3.7	12.3 ± 3.8	8.8 ± 0.6
16:1	4.5 ± 0.9	5.6 ± 0.6	1.2 ± 1.1	1.5 ± 0.6
18:0	6.9 ± 1.5	6.3 ± 0.2	17.2 ± 2.2	8.5 ± 0.8
18:1	14.6 ± 1.1	23.7 ± 0.7	14.6 ± 1.2	16.7 ± 1.9
18:2	23.4 ± 2.3	19.4 ± 0.2	14.7 ± 2.9	19.2 ± 2.6
18:3 + 20:1	0.6 ± 0.7	0.5 ± 0.7	0.4 ± 0.4	0.3 ± 0.2
20:4	8.4 ± 1.8	1.8 ± 0.2	38.3 ± 6.0	31.3 ± 2.4
Others	----	----	----	11.0 ± 1.0
Saturated	48.5%	48.9%	30.9%	19.0%

From: Jobe, A. et al., (1978)

TABLE VI-6

EFFECT OF BUTANOL AND ACETONE EXTRACTIONS ON LIPID CONTENT OF RABBIT LUNG MICROSOMES.

	UNEXTRACTED MICROSOMES ± S.E.	EXTRACTED MICROSOMES ± S.E.	% RECOVERY ± S.E.
Phosphatidylcholine content * (nmoles choline / mg protein)	0.148 ± 0.013 (3)	0.010 ± 0.002 (3)	5.3 ± 1.3
Phosphatidylethanolamine content * (nmoles ethanolamine / mg protein)	0.060 ± 0.001 (3)	0.016 ± 0.001 (3)	27.1 ± 1.8
Phosphatidylinositol content * (pmoles inositol / mg protein)	0.343 ± 0.020 (3)	0.291 ± 0.011 (3)	85.6 ± 8.1

* Animals sacrificed 30 min after i.v. injection of 15 μCi/kg ^{14}C-choline, 24 μCi/kg ^{14}C-ethanolamine or 63 μCi/kg ^{3}H-inositol

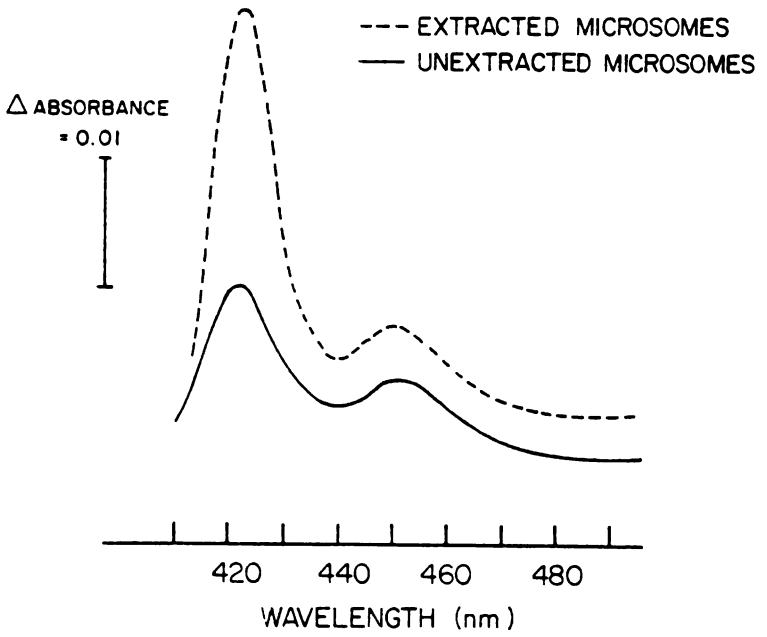
E. Measurement of Lung cytochrome P-450

Typical carbon monoxide difference spectra of dithionite-reduced lung cytochrome P-450 from extracted and unextracted microsomes are shown in Figure VI-11. Extracted microsomes have a large peak at 420 nm which corresponds to the metabolically inactive form of cytochrome P-450. The specific contents (nmoles P-450/mg microsomal protein) of extracted and unextracted microsomes are given in Table VI-2. The extraction procedure results in an approximate one-third loss of specific content which corresponds to the increased amount of cytochrome P-420 present in the difference spectrum of extracted microsomes (Figure VI-11). No change in the position of the Soret maximum was observed which is what would be expected if the extraction procedure did not preferentially destroy one of the two forms of pulmonary cytochrome P-450 which are present in approximately equal amounts (Wolf et al., 1978a).

Arinc and Philpot (1976) have reported that the sum of detectable cytochromes P-450 plus P-420 is increased by the addition of phospholipid to purified rabbit lung microsomes. However, Guengerich (1977) found that the presence of di-12 GPC did not affect the carbon monoxide difference spectrum of purified rabbit pulmonary microsomes. Addition of up to 263 μg of di-12 GPC/nmole cytochrome P-450 to extracted microsomes did not affect the determination of cytochrome P-450 in our studies.

FIGURE VI-11.

CARBON MONOXIDE DIFFERENCE SPECTRUM OF DITHIONITE-REDUCED LUNG CYTOCHROME P-450.



F. NADPH-Cytochrome-c Reductase Activity in Extracted and Unextracted Lung Microsomes

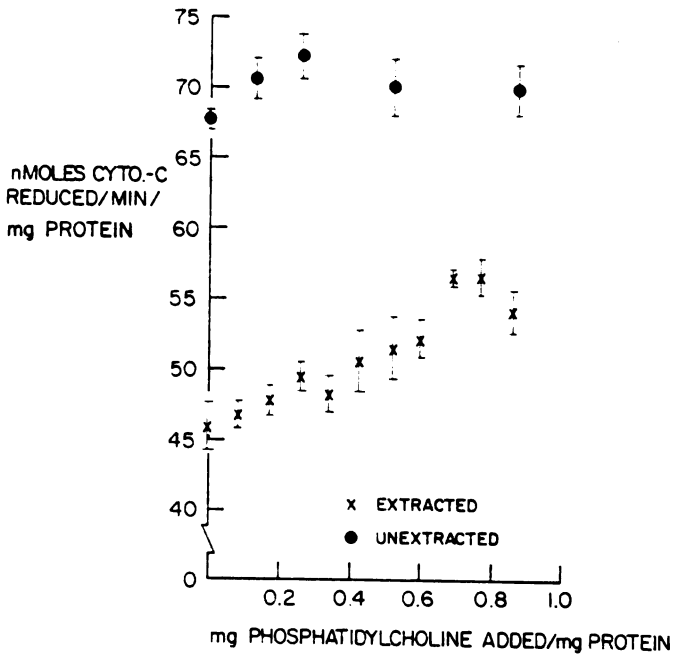
NADPH-cytochrome c reductase activity of rabbit lung microsomes was decreased to approximately two-thirds of unextracted activity after two 1-butanol and two acetone extractions (Figure VI-12). Addition of di-12 GPC resulted in a slight stimulation at low concentrations followed by an apparent leveling off in activity at higher concentrations. However, addition of di-12 GPC did not increase activity to a level similar to that shown by unextracted microsomes. Addition of di-12 GPC to unextracted microsomes did not affect activity.

The observation of a decrease in NADPH-cytochrome c reductase activity upon removal of lipid satisfies one of the three criteria set forth by Fleischer and co-workers (Fleischer et al., 1962) for establishing the lipid requirement of an enzyme. However, full reactivation of the enzyme by the addition of lipid was not accomplished.

FIGURE VI-12

NADPH-CYTOCHROME C REDUCTASE ACTIVITY IN EXTRACTED AND UNEXTRACTED LUNG MICROSOMES. SEE "METHODS" (SECTION V-I).

Protein concentration: extracted = 0.0582 mg/incubation, unextracted = 0.0574 mg/incubation. Points are the mean $\pm$ STD error of three to six determinations.


75
70
65
60
55
50
45
40
0
nMOLES CYTO.-C
REDUCED/MIN/
mg PROTEIN
x EXTRACTED
● UNEXTRACTED
0.2
0.4
0.6
0.8
1.0
mg PHOSPHATIDYLCHOLINE ADDED/mg PROTEIN

G. Benzphetamine N-demethylase Activity in Extracted and Unextracted Lung Microsomes

Benzphetamine N-demethylase activity of rabbit lung microsomes was decreased to approximately two-thirds of unextracted activity after two 1-butanol and two acetone extractions (Figure VI-13). Addition of di-12 GPC resulted in a stimulation up to a level of activity approximately (97%) equal to that shown by unextracted microsomes followed by an inhibition of activity at higher concentrations of added lipid in every experiment, regardless of the amoung of di-12 GPC added to unextracted microsomes, its addition inhibited benzphetamine N-demethylase activity.

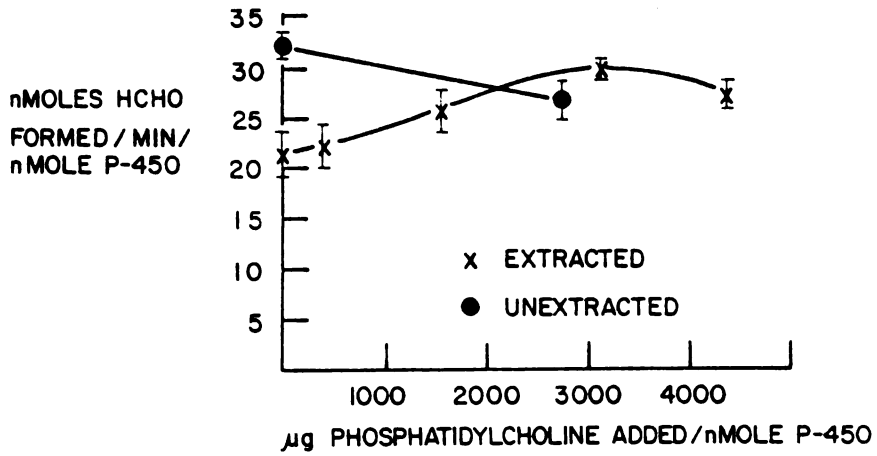
The observations of a decrease in benzphetamine N-demethylase activity upon removal of lipid followed by essentially full reactivation (over 97%) of the enzyme by addition of lipid satisfy two of the three criteria set forth by Fleischer and co-workers (Fleischer et al., 1962) for establishing the lipid requirement of an enzyme. Evidence that the lipid binds to the enzyme is the third criterion set forth by Fleischer et al., and requires the preparation of purified cytochrome P-450.

These results agree with the results of Vore and co-workers (Vore et al., 1974a; Vore et al, 1974b) which established a lipid requirement for the metabolism of benzphetamine by the rat hepatic mixed-function oxidase system. Using reconstituted rabbit lung preparations, Philpot and co-workers (Philpot et al., 1975; Arinc and Philpot, 1976; and Guengerich 1977) have also published data indicating a lipid requirement for the N-demethylation of benzphetamine.

FIGURE VI-13

BENZPHETAMINE N-DEMETHYLASE ACTIVITY IN EXTRACTED AND UNEXTRACTED LUNG MICROSOMES. SEE "METHODS" (SECTION V-J).

Cytochrome P-450 concentrations: extracted = 0.0272-0.036 nmoles/2 ml incubation, unextracted = 0.0321-0.046 nmoles/2 ml incubation. These data represent four separate experiments. The points are the mean $\pm$ STD error of four to eight determinations.


nMOLES HCHO
FORMED / MIN/
nMOLE P-450
35
30
25
20
15
10
5
x EXTRACTED
● UNEXTRACTED
1000
2000
3000
4000
µg PHOSPHATIDYLCHOLINE ADDED/nMOLE P-450

H. O-Dealkylation of 7-Ethoxycoumarin by Extracted and Unextracted Lung Microsomes

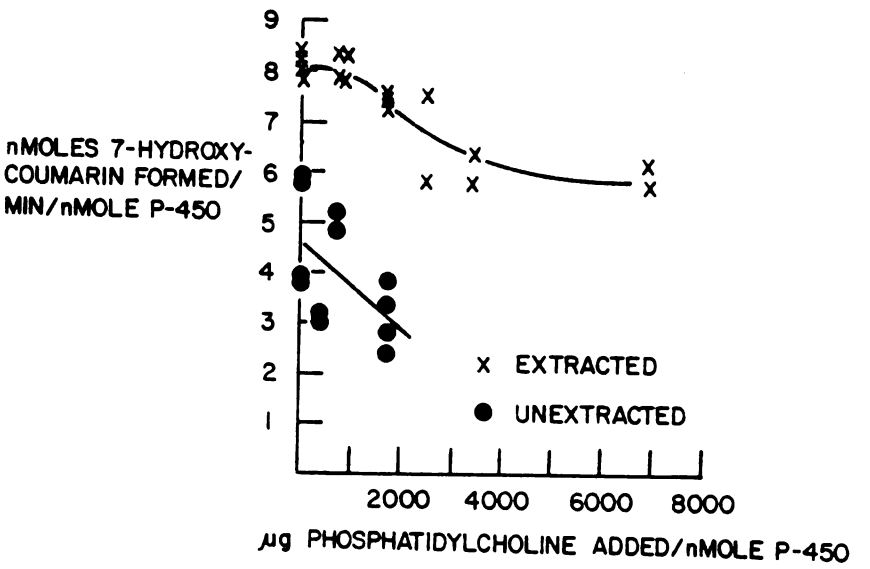
Ethoxycoumarin 0-deethylase activity of rabbit lung microsomes was increase 38%-111% after two 1-butanol and two aceton extractions (Figure VI-14). Addition of di-12 GPC had no effect at low phosphatidylcholine concentrations followed at higher lipid concentrations by a decrease in activity to a level similar to that shown by unextracted microsomes. In every experiment, regardless of the amount of di-12 GPC added, its addition to unextracted microsomes inhibited ethoxycoumarin 0-deethylase activity.

These data do not establish a lipid requirement for ethyoxycoumarin 0-deethylase activity in rabbit lung microsomes but rather suggest that the microsomal membrane suppresses or inhibits activity. In contrast, Philpot and co-workers (Philpot et al., 1975; Arinc and Philpot, 1976) have published data indicating an absolute lipid requirement for ethyoxycoumarin 0-deethylase using a reconstituted rabbit lung cytochrome P-450 and NADPH-cytochrome c reductase preparation.

FIGURE VI-14

O-DEALKYLATION OF 7-ETHOXYCOUMARIN BY EXTRACTED AND UNEXTRACTED LUNG MICROSOMES. SEE "METHODS" (SECTION V.K).

Cytochrome P-450 concentration: extracted = 0.0119-0.0145 nmoles/incubation, unextracted = 0.0119-0.0145 nmoles/incubation. These data points represent individual determinations from two separate experiments. The lines are drawn to best fit by eye.


nMOLES 7-HYDROXY-
COUMARIN FORMED/
MIN/nMOLE P-450
9
8
7
6
5
4
3
2
1
X EXTRACTED
UNEXTRACTED
2000
4000
6000
8000
µg PHOSPHATIDYLCHOLINE ADDED/nMOLE P-450

I. Benzo(a)pyrene Hydroxylase Activity in Extracted and Unextracted Lung Microsomes

Benzo(a)pyrene hydroxylase activity of rabbit lung microsomes was substantially increased after two 1-butanol and two acetone extractions (Figure VI-15). Addition of di-12 GPC resulted in stimulation at low phosphatidylcholine concentrations followed at higher lipid concentrations by a decrease in activity to a level similar to that shown by unextracted microsomes. In every experiment, regardless of the amount of di-12 GPC added, its addition to unextracted microsomes inhibited benzo(a)pyrene hydroxylase activity.

These data do not establish a lipid requirement for benzo(a)pyrene hydroxylase activity in rabbit lung microsomes and contrast with the lipid requirement demonstrated by this method for the metabolism of benzo(a)pyrene by the rat hepatic mixed-function oxidase system (Vore et al 1975a, 1974b) Jernstrom et al., (1975) were also unable to demonstrate a lipid requirement for benzo(a)pyrene hydroxylase activity when a partially purified rat lung cytochrome P-450 and rat liver NADPH-cytochrome c reductase preparation was used.

One possible explanation for the failure to demonstrate a lipid requirement for benzo(a)pyrene hydroxylase is that not enough of the microsomal lipid was removed by the extraction procedure. To test this possibility, microsomes were extracted three times with 1-butanol and twice with acetone. Benzo(a)pyrene hydroxylase activity was decreased to

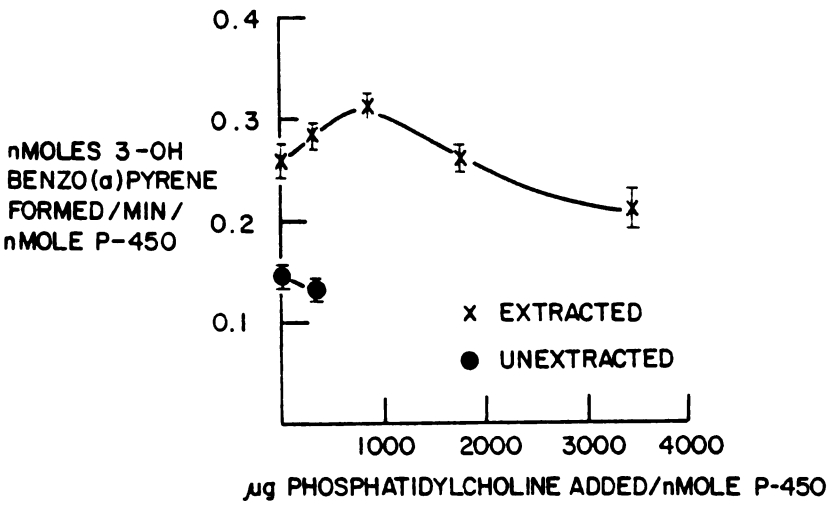
11.6% of unextracted levels in these microsomes. However, addition of di-12 GPC resulted in a further decrease in activity.

Thus, it was not possible to demonstrate a lipid requirement as defined by Fleischer et al., (1962) for benzo(a)pyrene hydroxylase activity regardless of the extent of lipid extraction.

FIGURE VI-15

BENZO(A)PYRENE HYDROXYLASE ACTIVITY IN EXTRACTED AND UNEXTRACTED LUNG MICROSOMES. SEE "METHODS" (SECTION V.L).

Cytochrome P-450 concentration: extracted = 0.0247-0.310 nmoles/incubation, unextracted = 0.0210-0.0590 nmoles/incubation. These data represent four separate experiments. The points are the mean $\pm$ STD error of six to eight determinations.


0.4
0.3
0.2
0.1
nMOLES 3-OH
BENZO(a)PYRENE
FORMED/MIN/
nMOLE P-450
X EXTRACTED
● UNEXTRACTED
1000
2000
3000
4000
µg PHOSPHATIDYLCHOLINE ADDED/nMOLE P-450

J. Effect of α-Naphthoflavone on Benzo(a)pyrene Hydroxylase Activity in Extracted and Unextracted Lung Microsomes

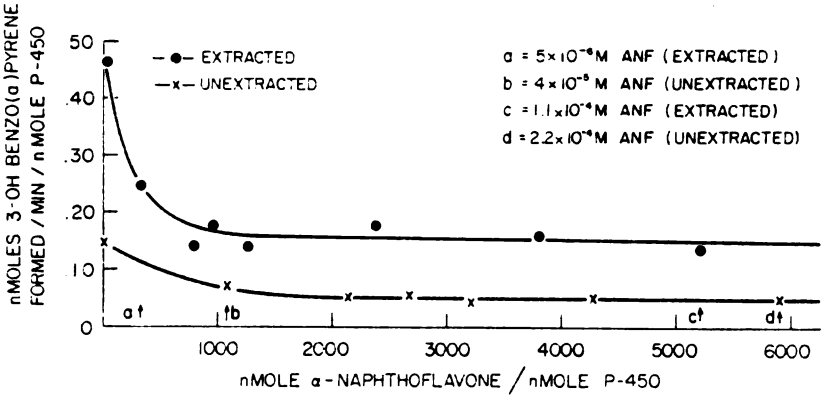
As seen in Figures VI-16 and VI-17, the maximum amount of α-naphthoflavone (ANF) inhibition (expressed as percent activity in the absence of α-naphthoflavone) of benzo(a)pyrene hydroxylase activity was 69% in both extracted and unextracted lung microsomes. α-Naphthoflavone (10^{-4}M) inhibits 99% of cytochrome II-and only 6% of cytochrome I-mediated benzo(a)pyrene metabolism (Wolf et al., 1978b). These results strongly suggest that the lipid extraction procedure employed does not preferentially denature one of the two forms of cytochrome P-450 present in rabbit pulmonary microsomes and that the increased benzo(a)pyrene hydroxylase activity observed in extracted microsomes (Figure VI-15) represents more than enhancement of the cytochrome I concentration.

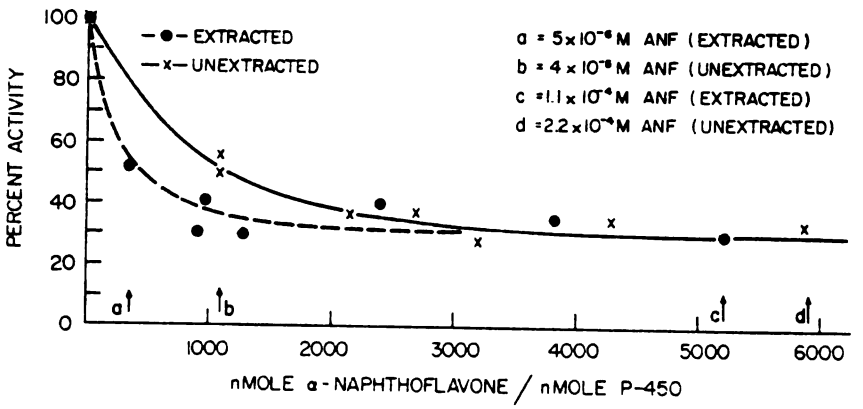
It is important to also note the difference in the concentration required to inhibit activity 50% (unextracted = 4×10^{-5} M ANF; extracted = 5×10^{-6} M ANF) (Figures VI-16 and VI-17). This difference may be due to the removal of 88% of the phospholipid (Table VI-2) which could lead to a greater net concentration of α-naphthoflavone being exposed to the cytochrome P-450 in extracted microsomes.

FIGURES VI-16 AND VI-17

EFFECT OF α-NAPHTHOFLAVONE ON BENZO(A)PYRENE HYDROXYLASE ACTIVITY IN EXTRACTED AND UNEXTRACTED RABBIT LUNG MICROSOMES. SEE "METHODS" (SECTION V.M)

Cytochrome P-450 concentration: extracted = 0.0159-0.0271 nmoles/incubation, unextracted = 0.374 nmoles/incubation. Points are the mean values of duplicate determinations and represent four separate experiments.


nMOLES 3-OH BENZO(a)PYRENE FORMED / MIN / nMOLE P-450
50
.40
.30
.20
.10
0
— ●— EXTRACTED
—x— UNEXTRACTED
a = 5x10⁻⁶M ANF (EXTRACTED)
b = 4x10⁻⁵ M ANF (UNEXTRACTED)
c = 1.1x10⁻⁴M ANF (EXTRACTED)
d = 2.2x10⁻⁴M ANF (UNEXTRACTED)
a
b
c
d
1000
2000
3000
4000
5000
6000
nMOLE α-NAPHTHOFLAVONE / nMOLE P-450


100
80
60
40
20
0
PERCENT ACTIVITY
—●— EXTRACTED
—x— UNEXTRACTED
a = 5x10⁻⁶M ANF (EXTRACTED)
b = 4x10⁻⁵M ANF (UNEXTRACTED)
c = 1.1x10⁻⁴M ANF (EXTRACTED)
d = 2.2x10⁻⁴M ANF (UNEXTRACTED)
a
b
c
d
1000
2000
3000
4000
5000
6000
nMOLE α-NAPHTHOFLAVONE / nMOLE P-450

K. Kinetic Parameters of Benzo(a)pyrene Hydroxylase Activity in Extracted and Unextracted Lung Microsomes

The Lineweaver-Burk plots of benzo(a)pyrene hydroxylase activity in unextracted and extracted lung microsomes are shown in Figure VI-18. Extraction of microsomes caused nearly a five-fold decrease in the apparent K_m and approximately a 50% increase in the V_{max} (Figure VI-18, and Table VI-7). Addition of di-12 GPC (40 μM) to extracted microsomes increased the apparent K_m to near unextracted levels and increased the extracted V_{max} by approximately 50%. These results indicate that although lipid is not required for benzo(a)pyrene hydroxylase activity in lung microsomes (Figure VI-15), lipid is important in the regulation of lung microsomal benzo(a)pyrene hydroxylase.

FIGURE VI-18

LINEWEAVER-BURK PLOTS FOR BENZO(A)PYRENE HYDROXYLASE ACTIVITY IN EXTRACTED AND UNEXTRACTED LUNG MICROSOMES. SEE "METHODS" (SECTION V.N).

These data are representative of three separate experiments. Points are the mean values of duplicate determinations. Cytochrome P-450 concentration: extracted = 0.012 nmoles/incubation, unextracted = 0.018 nmoles/incubation. Protein concentration: extracted = 0.093 mg/incubation, unextracted = 0.1285 mg/incubation.

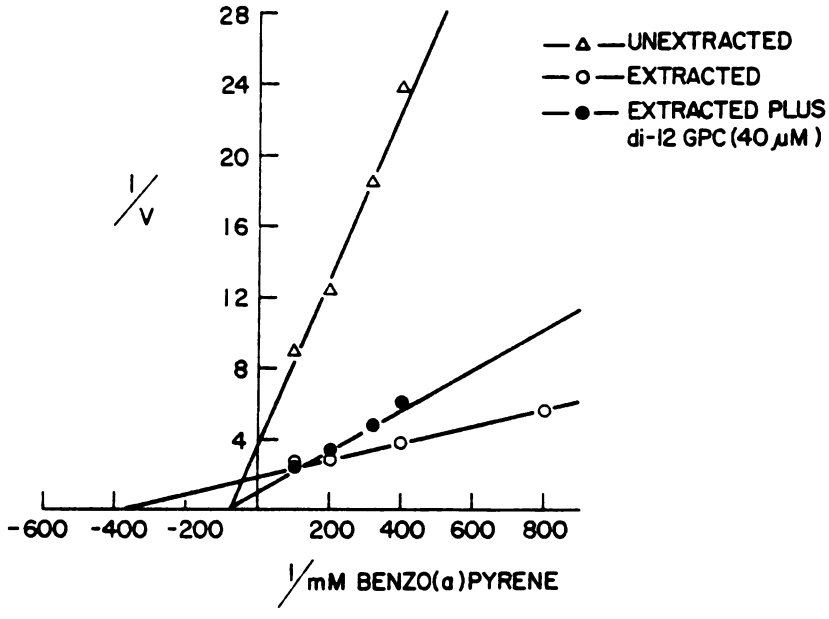

28
24
20
16
12
8
4
1/V
-600 -400 -200 200 400 600 800
1/mM BENZO(a)PYRENE
—△—UNEXTRACTED
—○—EXTRACTED
—●— EXTRACTED PLUS
di-12 GPC(40 μM)

TABLE VI-7

KINETIC PARAMETERS OF BENZO(A)PYRENE HYDROXYLASE ACTIVITY IN EXTRACTED AND UEXTRACTED LUNG MICROSOMES.

	K_m (μM)	V_{max}
UNEXTRACTED	10.00 ± 1.97	0.31 ± 0.03
EXTRACTED (EM)	1.89 ± 0.37	0.46 ± 0.03
EM + DI-12GPC (40 μM)	8.23 ± 1.69	0.74 ± 0.08

The points in table represent the mean value of duplicate determinations from three separate experiments. DI-12 GPC = dilauroylglycerol-3-phosphorycholine. V_{max} = nmmoles 3-hydroxybenzo(a)pyrene formed/min/nmole cytochrome P-450.

L. Association and Dissociation of Benzo(a)pyrene with Extracted and Unextracted Lung Microsomes

One possible way that lipid extraction changes the benzo(a)pyrene hydroxylase activity in extracted lung microsomes is by changing the partitioning characteristics of benzo(a)pyrene in the aqueous and/or membrane phase of the microsomal suspension. Association of benzo(a)pyrene was found to be essentially complete (above 85%) (Figure VI-9) within ten seconds after addition of extracted or unextracted microsomes.

Extracted and unextracted microsomes were suspended in equal protein concentrations prior to the experiment. Within the concentration range used, extracted microsomes bound as much benzo(a)pyrene per mg of microsomal protein as did unextracted microsomes. It is therefore apparent that extracted microsomes contain a higher concentration (i.e. not diluted by lipid) of benzo(a)pyrene per mg of protein which could lead to the increased benzo(a)pyrene hydroxylase activity observed (Section VI-I, Figure VI-15, Table VI-7).

Using fluorescence techniques, Ibbetson and Freedman (1974) have demonstrated that benzo(a)pyrene binding to hepatic microsomal membranes corresponded to a general dissolution into the non-polar matrix of the membrane. When a first order dissociation constant was assumed for this process an apparent K_m of 1 μM was determined. This value is not different from the determined K_m values for benzo(a)pyrene hydroxylation

(Alvares, et al., 1972; Lu, et al., 1972b). That the affinity of benzo(a)pyrene for the membrane is at least as high as that for the specific binding or the enzyme, can be best explained if the enzyme active site is in the lipid environment. Using Ibbetson and Freedman's (1974) data, Parry et al. (1976) have shown that the concentration of a substrate in the microsomal membrane is influenced by the partition coefficient of the substrate between lipid and water, and that the substrate concentration in the membrane influences the apparent K_m. These authors calculated a partition coefficient of approximately 4.39×10^3 for benzo(a)pyrene partitioning between microsomal and aqueous phases. Calculation of an apparent K_m value of 0.23 mM for the hydroxylation of benzo(a)pyrene led to the conclusion that the substrate (benzo(a)pyrene) has of the order of a thousand fold greater affinity (1 μM vs. 0.23 mM) for the enzyme (cytochrome P-450) than it does for the membrane lipids.

In the second association study performed, association of benzo(a)pyrene to the microsomes was not saturable within the concentration range (1.01 nmoles benzo(a)pyrene/nmole cytochrome P-450-1665.40 nmoles benzo(a)pyrene/nmole cytochrome P-450) studied (Figure VI-21). Since the binding of benzo(a)pyrene to the microsomes was not saturable and was linear with respect to the concentration of benzo(a)pyrene added, this data is consistent with the distribution being controlled by a zero-order partition coefficient (Parry et al., 1976).

In studying the dissociation of benzo(a)pyrene from lung microsomes, virtually no release was found to occur in the first 15 minutes following

80-fold dilution of benzo(a)pyrene-loaded microsomes (Figure VI-20). Since the binding is not reversible under the conditions studied, it is more appropriate to speak of a distribution or division of material between the phases rather than a partition coefficient.

In an attempt to correlate the release of benzo(a)pyrene with benzo(a)pyrene metabolism, the benzo(a)pyrene hydroxylase activity of microsomes incubated with an equivalent amount of benzo(a)pyrene was measured. As seen in figure VI-22, in seven minutes only approximately 0.16% of the benzo(a)pyrene in lung microsomes is metabolized to the 3-OH metabolite (fluorometric assay). Use of a radiometric assay to measure total benzo(a)pyrene metabolites (DePierre et al., 1975) led to the estimation that the 3-OH metabolite represents approximately 25% of the total metabolites formed. Thus, only approximately 0.64% of the total benzo(a)pyrene in lung microsomes was metabolized. However, the sensitivity of the binding studies performed is limited by background binding of free benzo(a)pyrene to the filter (approximately 7%). It should be noted that no microsomal protein could be measured in the filtrates whereas the remainder of free benzo(a)pyrene (Figure VI-19) can be accounted for in the filtrates (approximately 5-11% in the association studies).

From these metabolic studies and the release study, we cannot conclude whether or not membrane bound or benzo(a)pyrene in the aqueous environment serves as the substrate for the pulmonary mixed-function oxidase system. However, calculations of the concentrations of

benzo(a)pyrene present in extracted microsomes (Figure VI-20) and in the aqueous phase showed that the concentration of benzo(a)pyrene in the microsomal phase is 0.247 μM. It is doubtful that the concentration in the aqueous phase (0.005 μM) is adequate to support benzo(a)pyrene hydroxylation since the apparent K_m for unextracted and extracted microsomes was in the range of 1.9-10.00 μM (Table VI-7).

FIGURE VI-19

ASSOCIATION OF ^{3}H BENZO(A)PYRENE WITH EXTRACTED AND UNEXTRACTED RABBIT LUNG MICROSOMES. SEE "METHODS" (SECTION V-0).

Data is representative of three separate experiments. Cytochrome P-450 concentration: extracted = 0.021 nmoles/incubation, unextracted = 0.046 nmoles/incubation; protein concentration: extracted = 0.489 mg/incubation, unextracted = 0.489 mg/incubation; final incubation volume = 3.0 ml which consisted of: 2.64 ml Tris-HCl buffer, pH 7.6, 60 nmoles of unlabeled benzo(a)pyrene in 60 µl of acetone, 0.60 nmoles of ^{3}H-benzo(a)pyrene (specific activity = 8.3 Ci/mmol) in 10 µl of acetone and 0.3 ml microsomal suspension.

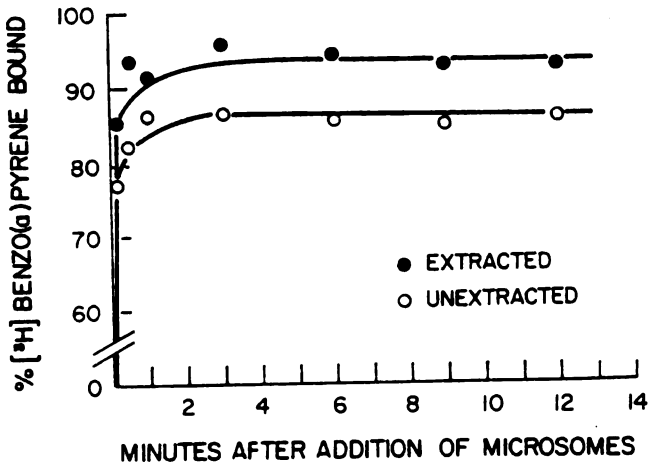

% [³H] BENZO(a)PYRENE BOUND
100
90
80
70
60
0
2
4
6
8
10
12
14
EXTRACTED
UNEXTRACTED
MINUTES AFTER ADDITION OF MICROSOMES

FIGURE VI-20

DISSOCIATION OF ^{3}H BENZO(A)PYRENE FROM EXTRACTED AND UNEXTRACTED RABBIT LUNG MICROSOMES. SEE "METHODS" (SECTION V-0)

Data is representative of three separate experiments. Cytochrome P-450 and protein concentrations are 80x-dilutions of values given in legend to Figure VI-19. Final incubation volume = 8.0 ml which consisted of 7.9 ml 50 mM Tris-HCl buffer, pH 7.6 and 100 µl of the mixture used in Figure VI-19 to measure the association of ^{3}H-benzo(a)pyrene to microsomes.

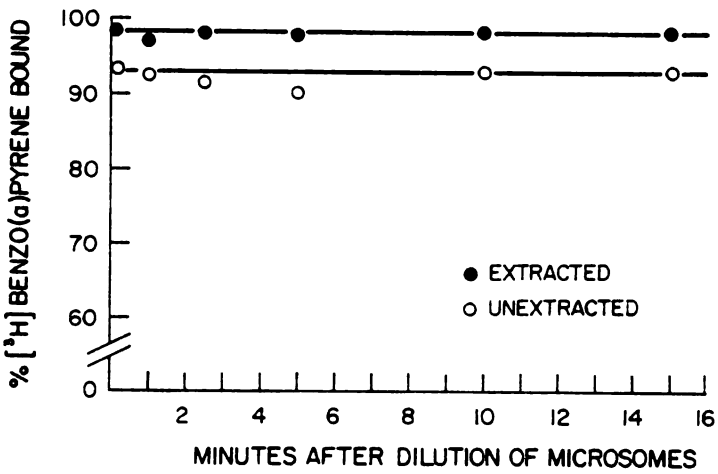

% [³H] BENZO(a)PYRENE BOUND
100
90
80
70
60
0
2
4
6
8
10
12
14
16
MINUTES AFTER DILUTION OF MICROSOMES
EXTRACTED
UNEXTRACTED

FIGURE VI-21

ASSOCIATION OF ^{14}C -BENZO(A)PYRENE ALONE OR IN THE PRESENCE OF AN EXCESS OF UNLABELED BENZO(A)PYRENE WITH RABBIT LUNG MICROSOMES. SEE "METHODS" (SECTION V-0)

These data are representative of three separate experiments. Cytochrome P-450 concentration = 0.0604 nmole/incubation. Benzo(a)pyrene specific activity = 60.7 mCi/mmol).

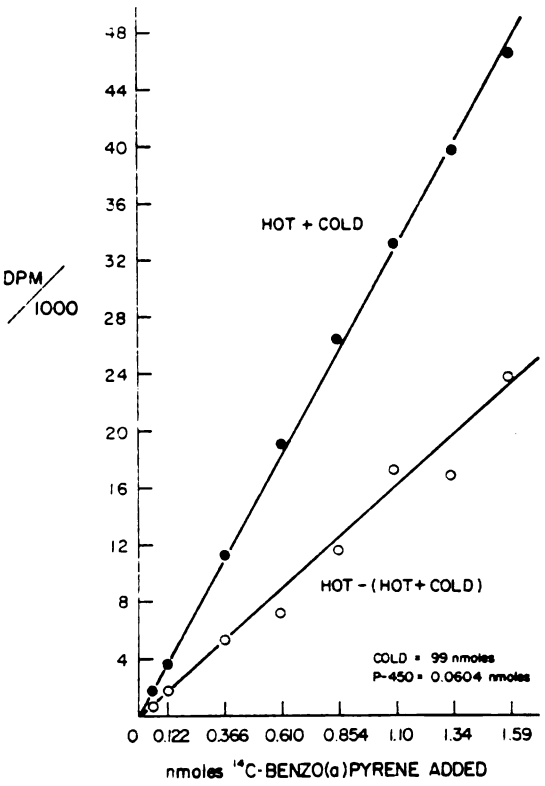
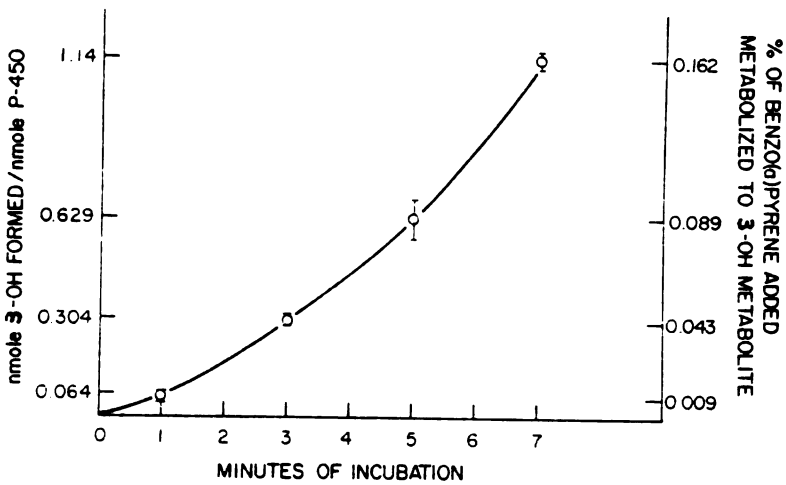

DPM / 1000
48
44
40
36
32
28
24
20
16
12
8
4
HOT + COLD
HOT - (HOT + COLD)
COLD = 99 nmoles
P-450 = 0.0604 nmoles
0
0.122
0.366
0.610
0.854
1.10
1.34
1.59
nmoles ^{14}C-BENZO(a)PYRENE ADDED

FIGURE VI-22

BENZO(A)PYRENE HYDROXYLASE ACTIVITY OF RABBIT LUNG MICROSOMES (% ADDED THAT IS METABOLIZED/MINUTE). SEE "METHODS" (SECTION V-0).

P-450 concentration = 0.0280 nmoles/incubation; protein concentration = 0.155 mg/incubation; points represent the mean $\pm$ STD-error of four determinations.


nmole 3-OH FORMED/nmole P-450
1.14
0.629
0.304
0.064
0
1
2
3
4
5
6
7
MINUTES OF INCUBATION
0.162
0.089
0.043
0.009
% OF BENZO(a)PYRENE ADDED METABOLIZED TO 3-OH METABOLITE

M. Substrate Binding to Extracted and Unextracted Lung Microsomal Cytochrome P-450

An increased magnitude of the spectral changes and slight, if any, changes in the spectral dissociation constants were observed in the Type I (benzphetamine), Type II (aniline) and reverse Type I (ethanol) binding spectra (Figure IV-23-VI-26) of lipid-extracted lung microsomes when compared to unextracted microsomes. These data indicate that the intact lipid membrane is not required for substrate binding. However, even though 95% of the phosphatidylcholine has been removed, the ratio of phosphatidylcholine to cytochrome P-450 is still 680 nmoles phosphatidylcholine/nmole cytochrome P-450 (Jobe, 1977). This it cannot be stated definitively that lipid is not required for substrate binding. The increased absorption maxima in extracted microsomes could be due to an increased substrate concentration at the cytochrome P-450 binding site since removal of the lipid decreases the nonspecific binding sites in the lipid.

It should also be noted that of the two forms of rabbit pulmonary cytochrome, only cytochrome I forms a typical Type I spectrum with benzphetamine while both cytochromes form typical Type I spectra with carbon tetrachloride (Wolf et al., 1978a). If the interaction between benzphetamine and cytochrome I represents an enzyme-substrate complex, it is understandable why benzphetamine is not metabolized by cytochrome II (Wolf et al., 1978a; Wolf et al., 1978b).

FIGURE VI-23

DIFFERENCE SPECTRA OBTAINED BY THE ADDITION OF ETHANOL TO LUNG MICROSOMES. SEE LEGEND TO FIGURE VI-25 FOR METHODS.

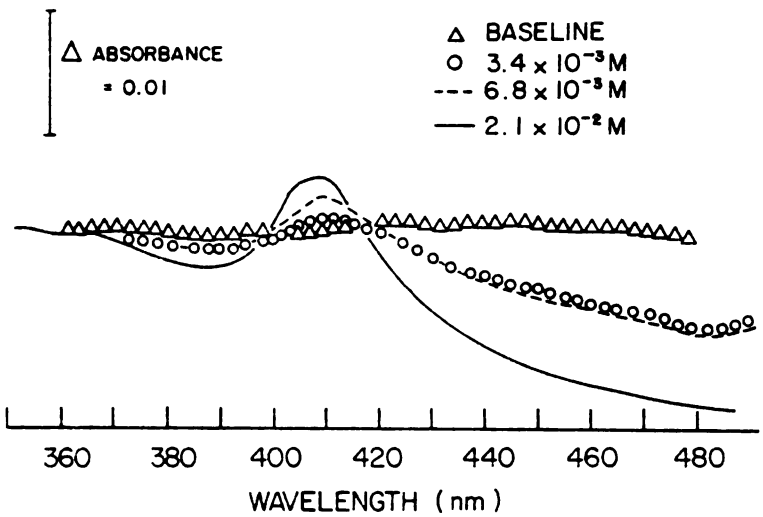

Δ ABSORBANCE
= 0.01
Δ BASELINE
o 3.4 x 10⁻³M
--- 6.8 x 10⁻³M
— 2.1 x 10⁻²M
360
380
400
420
440
460
480
WAVELENGTH (nm)

FIGURE VI-24

DOUBLE-RECIPROCAL PLOTS OF BENZPHETAMINE-INDUCED TYPE I SPECTRAL CHANGE IN EXTRACTED AND UNEXTRACTED LUNG MICROSOMES.

Lung microsomes from control rabbits were lyophilized and suspended at 0.082 nmoles of cytochrome P-450 per milliliter (0.31 mg of protein per milliliter) in 0.1 M potassium phosphate buffer, pH 7.7 ("unextracted"), or were extracted twice with 1-butanol and twice with acetone and suspended to 0.055 nmoles of cytochrome P-450 per milliliter (0.96 mg of protein per milliliter) in the buffer ("extracted"). Benzphetamine was added to the sample cuvette, and an equal volume of buffer to the reference cuvette. $\Delta A_{380-420}$ was calculated per nanomole of cytochrome P-450 to correct for the loss of cytochrome P-450 due to the extraction procedure. The total volume of benzphetamine solution added to extracted and unextracted microsomes did not exceed 1.3% and 7.0% of the volume in the cuvettes respectively.

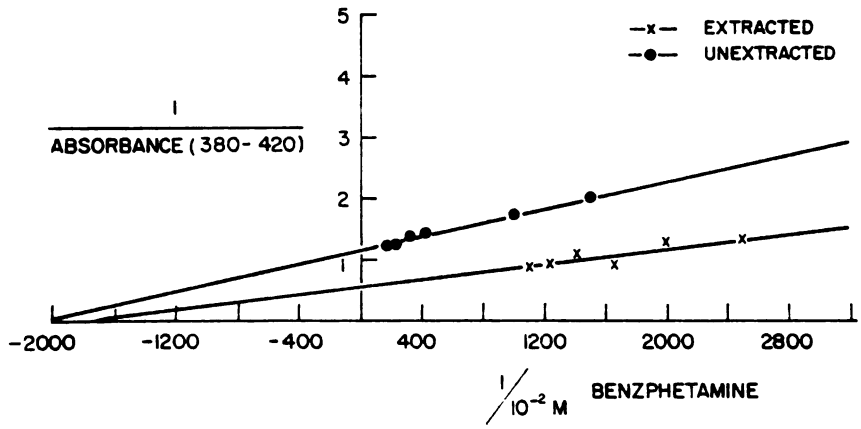

5
4
3
2
1
—x— EXTRACTED
—●— UNEXTRACTED
1
ABSORBANCE (380- 420)
-2000
-1200
-400
400
1200
2000
2800
1/10⁻² M BENZPHETAMINE

FIGURE VI-25

DOUBLE-RECIPROCAL PLOTS OF ETHANOL-INDUCED REVERSE TYPE I SPECTRAL CHANGE IN EXTRACTED AND UNEXTRACTED LUNG MICROSOMES.

Lung microsomes from control rabbits were lyophilized and suspended to 0.103 nmoles of cytochrome P-450 per milliliter (.38 mg of protein per milliliter) in 0.1 M potassium phosphate buffer, pH 7.7 ("unextracted"), or were extracted twice with 1-butanol and twice with acetone and suspended to 0.110 nmoles of cytochrome P-450 per milliliter (1.92 mg of protein per milliliter) in the buffer ("extracted"). Ethanol (100%) was added to the sample cuvette, and an equal volume of buffer to the reference cuvette. $\Delta A_{380-410}$ was calculated per nanomole of cytochrome P-450 to correct for the loss of cytochrome P-450 due to the extraction procedure. The total volume of ethanol added to extracted and unextracted microsomes did not exceed 1.6% and 0.8% or the volume in the cuvettes respectively.

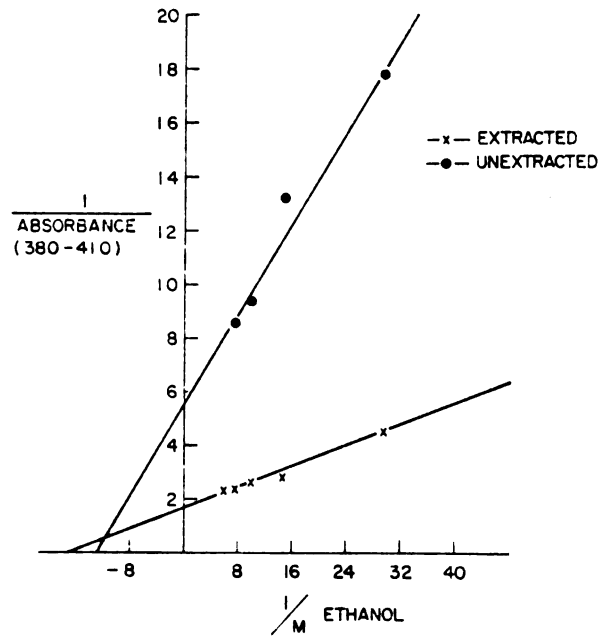
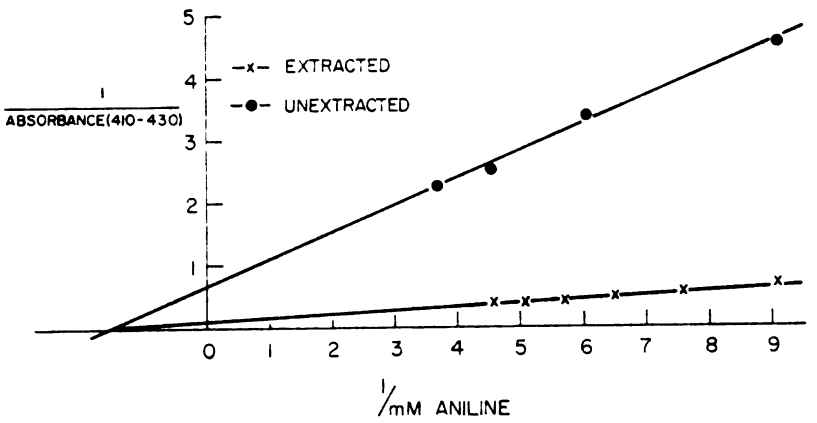

20
18
16
14
12
10
8
6
4
2
1
ABSORBANCE
(380-410)
-x- EXTRACTED
-●- UNEXTRACTED
-8
8
16
24
32
40
1/M ETHANOL

FIGURE VI-26

DOUBLE-RECIPROCAL PLOTS OF ANILINE-INDUCED TYPE II SPECTRAL CHANGE IN EXTRACTED AND UNEXTRACTED LUNG MICROSOMES.

Lung microsomes from control rabbits were lyophilized and suspended to 0.3 nmoles of cytochrome P-450 per milliliter (1.31 mg of protein per milliliter) in 0.1 M potassium phosphate buffer, pH 7.7 ("unextracted"), or were extracted twice with 1-butanol and twice with acetone and suspended to 0.15 nmoles of cytochrome P-450 per milliliter (1.0 mg of protein per milliliter) in the buffer ("extracted"). Aniline was added to the sample cuvette, and an equal volume of buffer to the reference cuvette. $\Delta A_{410-430}$ was calculated per nanomole of cytochrome P-450 to correct for the loss of cytochrome P-450 due to the extraction procedure. The total volume of aniline added to the extracted and unextracted microsomes did not exceed 2.2% and 3.4% of the volume in the cuvettes respectively.


5
4
3
2
1
0
1
2
3
4
5
6
7
8
9
—x— EXTRACTED
—●— UNEXTRACTED
1
ABSORBANCE(410-430)
1/mM ANILINE

DISCUSSION

The objectives of this research were to determine if lipid is an essential component of the rabbit lung mixed-function oxidase system and to characterize its role in mixed-function oxidase activity. Two criteria were used to establish the lipid requirement: 1) a decrease in enzyme activity upon removal of lipid and 2) restoration of enzyme activity by the addition of lipid.

Isolation of rabbit pulmonary microsomes by the procedure used resulted in microsomes with a similar marker enzyme profile as observed previously by Gram (1973) and Bend et al., (Bend et al., 1973). Lyophilization did not affect the NADPH-cytochrome c reductase and mixed-function oxidase activities of the microsomes and the lyophilized microsomes could be stored at -80^{o} for up to ten days without appreciable loss of cytochrome P-450.

The precise identification of pulmonary cellular populations possessing mixed-function oxidase activity has been difficult primarily because of the heterogeneity of the lung. In contrast to the liver in which two major cell types predominate, normal mammalian lungs consist of about 40 cell types with no one cell type occupyng the major mass of the lung (Sorokin, 1970). Thus, in interpreting any results from experiments with lung microsomes, it must be kept in mind that the lung is an extremely complex and heterogeneous system and prepared cell fractions should be expected to reflect this nonuniformity.

Extraction of lyophilized rabbit lung microsomes twice with 1-butanol and twice with acetone removed all of the neutral lipid and 88% of the phospholipid. Using radiolabeled precursors, it was determined that approximately 95% of the ^{14}C-phosphatidylcholine, 73% of the ^{14}C-phosphatidylethanolamine and 14% of the ^{3}H-phosphatidylinositol were removed by the extraction procedure. Approximately 52% of rabbit lung microsomal phospholipid is phosphatidylcholine. Phosphatidylethanolamine comprises an additional 20% and phosphatidylinositol 4% (Jobe et al., 1978). Although Jobe and co-workers (Jobe et al., 1978) have shown that the maximal specific activity of all phospholipids occurred within 30 minutes following isotope administration, it remains to be determined whether these newly formed labeled-phospholipids represent an accurate depiction of all microsomal phospholipid pools.

Observation of unextracted lung microsomes under the electron microscope revealed a matrix composed of vesicles resembling smooth endoplasmic reticulum and electron-dense particles resembling ribosomes. In distinct contrast to the work of Gram and Bend and co-workers (Gram, 1973; Bend et al., 1973), these vesicle particles did not appear as clusters or aggregates but were more or less uniformly spread across the field of the micrographs.

In contrast with unextracted microsomes, extracted lung microsomes contained fewer and in general smaller vesicles and numerous fine electron-dense particles uniformly spread across the field of the micrographs. Addition of sonicated di-12 GPC in a concentration with

respect to cytochrome P-450 equal to that used for maximal reactivation of benzphetamine N-demethylase activity did not restore the morphology of the microsomes to that in unextracted microsomes.

To better understand drug metabolism in the lung and to explain the differences between lung and liver mixed-function oxidase activity detergent it is useful to solubilize the systems and separate components and study them individually and in combination. Philpot, Fouts and co-workers have used this approach to extensively study the properties of the reconstituted rabbit lung mixed-function oxidase system and have presented evidence for the existence of two forms of pulmonary cytochrome P-450 with marked differences in the ability to metabolize benzo(a)pyrene, ethoxycoumarin and benzphetamine (Philpot et al., 1975; Arinc and Philpot, 1976; Szutowski et al., 1977; Wolf et al., 1978a and Wolf et al., 1978b). Guengerich (Guengerich, 1978) has shown by a similar approach that multiple forms of cytochrome P-450 and possibly NADPH-cytochrome-c reductase are present in both rabbit liver and lung. The reductases of the two organs are apparently identical, but the major pulmonary cytochrome P-450 is immunologically distinct from any of the hepatic cytochrome P-450 forms.

However, despite its apparent power, use of solubilization and reconstitution of mixed-function oxidase systems to determine a possible lipid requirement does have limitations. Lu and co-workers (Lu et al., 1974) have shown that in the resolved, reconstituted liver microsomal mixed-function oxidase system, several detergents can substitute for the

lipid and in higher concentrations, inhibit enzymatic activity. Therefore, in order to establish a lipid requirement, a detergent-free system is essential. Vore and co-workers (Vore et al., 1974a; Vore et al., 1974b) have developed a detergent-free system which employs 1-butanol and acetone extractions of the hepatic microsomes. Using this technique, they have removed approximately 80 percent of all phospholipids and all of the microsomal neutral lipids (i.e., cholesterol, mono-, di-, and triglycerides). Using this approach, they have established a lipid requirement for the metabolism of benzphetamine and benzo(a)pyrene by the rat hepatic mixed-function oxidase system.

Fleischer and co-workers (Fleischer et al., 1962) have set forth three criteria for establishing a lipid requirement for an enzyme: 1) a decrease in enzyme activity upon removal of lipid, 2) a reactivation of the enzyme by the addition of lipid, and 3) evidence that the lipid does in fact bind to the enzyme. The results of Vore and co-workers (Vore et al., 1974a; Vore et al., 1974b) clearly meet the first two criteria. Evidence that the lipid binds to the enzyme requires a preparation of purified cytochome P-450.

The similarities between liver and lung mixed-function oxidase systems suggest that lipid may also be an essential component of the pulmonary mixed-function oxidase system. Little is thus far known about the lipid requirement of the pulmonary mixed-function oxidase system. Bend and co-workers (Bend et al., 1973) have shown that the phospholipase c digestion of lung microsomes decreases activity toward some substrates suggesting that lipid is required. However, restoration of enzymatic

activity to control levels by addition of lipid has not been reported. Reconstitution studies of lung cytochrome P-450 and NADPH-cytochrome c reductase have led to conflicting data. Philpot and co-workers (Philpot et al., 1975; Arinc and Philpot, 1976) have published data indicating a lipid requirement for ethoxycoumarin O-deethylation and N-demethylation of benzphetamine using a rabbit lung preparation. In contrast, Orrenius and co-workers (Jernstrom et al., 1975) have shown that, using a rat lung partially purified cytochrome P-450 and liver NADPH-cytochrome c reductase preparation, lipid was not required for benzo(a)pyrene hydroxylase activity. These conflicting results may be resolved by use of a detergent-free system. It should also be noted that reconstitution experiments eliminate the specific spatial arrangement (electron channels or electron tunnels) of the enzymes involved. This could lead to erroneous conclusions regarding the lipid requirement of the enzymes being studied.

In this research, extraction of lung microsomes decreased NADPH-cytochrome c reductase and benzphetamine N-demethylase activities to approximately 65% of unextracted activity. Addition of sonicated synthetic phosphatidylcholine to extracted microsomes increased NADPH-cytochrome c reductase activity to 90% of unextracted activity and increased benzphetamine N-demethylase activity to unextracted levels. In contrast, extraction increased 7-ethoxycoumarin O-deethylase and benzo(a)pyrene hydroxylase activities. Addition of sonicated di-12 GPC to extracted microsomes resulted in no further increase in 7-ethoxycoumarin O-deethylase activity but further stimulated benzo(a)pyrene hydroxylase activity.

Addition of sonicated di-12 GPC to unextracted microsomes did not affect NADPH-cytochrome c reductase activity and inhibited benzphetamine N-demethylase, ethoxycoumarin O-deethylase and benzo(a)pyrene hydroxylase activities.

The observations of a decrease in benzphetamine N-demethylase activity upon removal of lipid followed by essentially full reactivation (over 97%) of the enzyme by addition of lipid satisfy two of the three criteria set forth by Fleischer and co-workers (Fleischer et al., 1962) for establishing the lipid requirement of an enzyme.

The effect of extraction on the kinetic parameters of benzo(a)pyrene hydroxylase activity were examined and it was found that the extracted microsomes had an apparent K_m approximately five-fold less than unextracted microsomes and that addition of di-12 GPC (40 μM) increased the apparent K_m of extracted microsomes to approximately that of unextracted microsomes. These data could be explained by the presence of nonspecific binding sites for benzo(a)pyrene in the microsomal membrane which compete with cytochrome P-450 for the substrate, thus mimicking the kinetics of a competitive inhibitor. Extraction may therefore decrease the apparent K_m by removing the nonspecific binding sites in the lipid. The increase in the apparent K_m in extracted lung microsomes seen after addition of di-12 GPC suggests that benzo(a)pyrene is partitioning into the lipid and thus decreasing the amount of substrate available for binding to cytochrome P-450.

Alternatively, extraction could remove endogenous ligands of cytochrome P-450 which are competitive inhibitors of benzo(a)pyrene. Whether or not these proposed inhibitors are in fact endogenous substrates of cytochrome P-450, it is unlikely that the inhibitors are free fatty acids or cholesterol since extraction of rat hepatic microsomes with acetone alone removes neutral lipids (including free fatty acids and cholesterol) and only trace amounts of phospholipids with no effect on the apparent K_m or V_{max} for benzo(a)pyrene hydroxylase activity (Vore, et al., 1974b). However, postulation that a competitive inhibitor of benzo(a)pyrene is removed by extraction does not agree with the increase in V_{max} observed in extracted microsomes. From these results, lipid can be concluded to be important in the regulation of benzo(a)pyrene hydroxylase activity in lung microsomes.

That a lipid requirement was observed for benzphetamine N-demethylase activity but not for benzo(a)pyrene hydroxylase activity contrasts with the results of Vore and co-workers (Vore et al., 1974a; Vore et al., 1974b) in which lipid requirements were shown for the metabolism of benzphetamine and benzo(a)pyrene by the rat hepatic mixed-function oxidase system.

A difference in lipid requirements between liver and lung microsomes is not surprising when one considers the known differences between the respective enzyme systems. While the reductases of the two organs are apparently identical, the major pulmonary cytochrome P-450 is immunologically distinct from any of the hepatic cytochrome P-450 forms

(Guengerich, 1977). The molecular ratio of cytochrome P-450 to NADPH-cytochrome c reductase is approximately 20 to 1 in the liver (Estabrook et al., 1969) but closer to 1 to 1 in the lung (Bend et al., 1973; Matsubara et al., 1974). This difference in ratios may partially explain the high rate of metabolism of some substrates (i.e., benzphetamine and biphenyl) in the lung (Hook et al., 1972; Bend et al., 1972; Bend et al., 1973). In addition, the different ratios of cytochrome P-450 to NADPH-cytochrome c reductase in lung and liver microsomes suggest that the spatial arrangement of these enzymes in the microsomal membrane is different in the liver and lung and that this difference could be reflected in an unique lipid requirement.

It should be noted that lipid extraction may affect mixed-function oxidase activity of lung microsomes by changingin the ability of protein (e.g., cytochrome P-450 and NADPH-cytochrome c reductase) to move laterally on the plane of the membrane. Two possible mechanisms are conceivable for functional interactions among component proteins fo membrane-bound multienzyme systems. One possibility is that the interactions are effected by the lateral motion and subsequent collision of the proteins on the plane of membranes, whereas another possibility is that the proteins exist in membranes as functional clusters in which the interactions take place directly. In an effort cytochrome to elucidate the actual mechanism, cytochrome P-450 and NADPH-cytochrome c reductase, both purified from rabbit liver microsomes, have been incorporated into the membrane of phosphatidylcholine vesicles by the cholate dialysis method. Results of studies of the reduction of

cytochrome P-450 by NADPH at 25° in which the molar ratio of the reductase to the cytochrome was kept constant but the amount of purified egg-yolk phostphatidylcholine, relative to the two proteins, was varied, suggest that the interaction between the two proteins is effected by their random collision caused by their lateral mobilities on the plane of the membrane of phoshatidylcholine vesicles (Taniguchi, et al., 1979). The extraction procedure employed in this study may thus alter the ability of cytochrome P-450 and NADPH-cytochrome c reductase to move laterally on the plane of the membrane and this altered mobility may at least partially explain the results obtained.

As discussed previously, one possible way that lipid extraction changes the benzo(a)pyrene hydroxylase activity in extracted microsomes is by changing the partitioning characteristics of benzo(a)pyrene between the aqueous and the membrane phase of the microsomal suspension. Within the concentration range used, extracted microsomes were found to bind as much benzo(a)pyrene per mg of microsomal protein as did unextracted microsomes. Since, there are 480 nmoles phospholipid/mg protein (1297.3 nmoles phospholipid/nmole cytochrome P-450) present in unextracted rabbit lung microsomes (Jobe, 1977), it is conceivable that nonspecific binding sites in the phospholipid present can act as a sink for substrates such as benzo(a)pyrene and thereby decrease the amount of substrate at the active site of cytochrome P-450. The increased benzo(a)pyrene hydroxylase activity observed in extracted microsomes can thus be explained by the removal of nonspecific bindign sites in the lipid phase.

Due to the low level of benzo(a)pyrene hydroxylase activity in the lung, no conclusions could be drawn from the studies performed as to whether benzo(a)pyrene in the membrane or aqueous phase serves as the substrate for benzo(a)pyrene hydroxylase. However, association of benzo(a)pyrene with extracted and unextracted microsomes was essentially complete within 10 seconds after addition of benzo(a)pyrene to the microsomal suspension and no significant release of benzo(a)pyrene from the microsomes was observed even 15 minutes after an 80-fold dilution of the microsomes. Thus, it appears likely that the pool of benzo(a)pyrene within the membrane is the substrate for pulmonary cytochrome P-450. In a similar approach, Zakim and Vessey (Zakim and Vessey, 1977) have shown that membrane-bound estrone serves as the substrate for hepatic microsomal UDP-glucuronyl transferase.

Cytochrome P-450 interacts with a variety of compounds to give characteristic spectral changes. The addition of a substrate to microsomal suspensions induces the formation of one of three different types of spectral changes. An increased magnitude of the spectral changes and slight, if any, changes in the spectral dissociation constants were observed in the Type I (benzphetamine), Type II (aniline) and reverse Type I (ethanol) binding spectra of extracted lung microsomes when compared to unextracted microsomes. These data indicate that the intact lipid membrane is not required for substrate binding. However, even though 95% of the phosphatidylcholine has been removed, there are still 680 nmoles of phosphatidylcholine present per nmole cytochrome P-450. Thus,

it cannot be stated definitively that lipid is not required for substrate binding. The increased absorption maxima in extracted microsomes could be due to an increased substrate concentration at the cytochrome P-450 binding site since removal of the lipid decreases the nonspecific binding sites in the lipid.

Finally, it has been suggested that 1-butanol forms a reverse Type I binding spectrum by interacting directly with the heme-iron of cytochrome P-450 (Yoshida and Kumaoka, 1975; Nebert et al., 1976), due to the nucleophilic hydroxyl group. Since the lipid extraction procedure employed in this study utilizes 1-butanol, 1-butanol could affect the binding spectra observed with aniline, ethanol and benzphetamine. However, Van Den Berg and co-workers (Van Den Berg et al., 1979) found that 1-butanol interfered with both Type II and Type I binding in mouse liver microsomes and that the magnitude of the spectral changes produced by Type I and Type II substrates was diminished. Thus, the results suggest that the increased magnitude of the spectral changes observed in the Type I, Type II and reverse Type I binding spectra of extracted lung microsomes when compared to unextracted microsomes cannot be explained by any residual 1-butanol present in the microsomes following the extraction procedure.

REFERENCES

Arcos, J.C., Argus, M.D. and Wolf, G. (1968). In Chemical Induction of Cancer, Vol. 1, Part 2, p. 324. Academic Press, New York.

Arinc, E. and Philpot, R.M. (1976). Preparation and properties of partially purified pulmonary cytochrome P-450 from rabbits. J. Biol. Chem. 251: 3213-3220.

Alvares, A.P., Schilling, G.R. and Kuntzman, R. (1968) Differences in the kinetics of benzpyrene hydroxylation by hepatic drug-metabolizing enzymes from phenobarbital, and 3-methylcholanthrene treated rats. Biochem. Biophys. Res. Commun. 30: 588-595.

Bartlett, G.R. (1959). Phosphorus assay in column chromatography. J. Biol. Chem. 234: 466-468.

Bend, J.R., Hook, G.E.R., Easterling, R.E., Gram, T.E. and Fouts, J.R. (1972). A comparative study of the hepatic and pulmonary microsomal mixed-function oxidase systems in the rabbit. J. Pharmacol. Exp. Ther. 183: 206-217.

Bend , J.R., Hook, G.E.R. and Gram, T.E. (1973). Characterization of lung microsomes as related to drug metabolism. Drug Metab. Dispo. 1: 358-367.

Bligh, E.G. and Dyer, W.J. (1959). A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. 37: 911-917.

Blyth, C.A., Freedman, R.B. and Rabin, B.R. (1972). Sex specific binding of steroid hormones to microsomal membranes of rat liver. Nature New Biol. 230: 136-139.

Boyd, M.R. (1976). Role of metabolic activation in the pathogenesis of chemically induced pulmonary diseases: mechanism of action of the lung-toxic furan, 4-ipomeanol. Env. Hlth. Persp. 16: 127-138.

Boyd, M.R. (1977). Evidence for the Clara cell as a site of cytochrome P-450-dependent mixed function oxidase activity in lung. Nature 269: 713-715.

Brown, E.A.B. (1974). The localization, metabolism, and effects of drugs and toxicants in lung. Drug Metab. Rev. 3: 33-87.

Cleland, W.W. (1963). Computer programmes for processing enzyme kinetic data. Nature 198: 463-465.

Cochin, J. and Axelrod, J. (1959). Biochemical and pharmacological changes in the rat following chronic administration of morphine, nalorphine and normorephine. J. Pharmacol. Exp. Ther. 125: 105-110.

Cohen, G.M. and Mannering, G.J. (1973). Involvement of a hydrophobic site in the inhibition of the microsomal p-hydroxylation of aniline by alcohols. Mol. Pharmacol. 9: 383-397.

Cohen, G.M. and Moore, B.D. (1976). Metabolism of ^{3}H -benzo(a)pyrene by different portions of the respiratory tract. Biochem. Pharmacol. 25: 1623-1629.

Coleman, R. and Hubscher, G. (1963). Metabolism of phospholipids. VII. On the lipid requirement for phosphatidic acid phosphatase activity. Biochim. Biophys. Acta. 73: 257-266.

Coleman, R. (1973). Membrane-bound enzymes and membrane ultrastructure. Biochim. Biophys. Acta 300: 1-30.

Crocker, T.T., O'Donnell, T.V. and Nunes, L.L. (1973). Toxicity of benzo(a)pyrene and air pollution composite for adult bronchial mucosa in organ culture. Cancer Res. 33: 88-93.

de Duve, C., Wattiaux, R. and Bandhuin, P. (1962). In Advances in Enzymology (F.F. Nord, ed.) Vol. 24, pp. 291-358. Interscience Publishers, New York.

DePierre, J.W., Moran, M.S., Johannesen, K.A.M. and Ernster, L. (1975). A reliable, sensitive, and convenient assay for benzpyrene mono-oxygenase. Analyt. Biochem. 63: 470-484.

Dirksen, E.R. and Crocker, T.T. (1968). Ultrastructural alterations produced by polycyclic aromatic hydrocarbons on rat tracheal epithelium in organ culture. Cancer Res. 28: 906-923.

Duttera, S.M., Byrne, W.L. and Ganoza, M.C. (1968). Studies on the phospholipid requirement of glucose 6-phosphatase. J. Biol. Chem. 243: 2216-2228.

Estabrook, R.W., Hildebrandt, A.G., Remmer, H., Schenkman, J.B., Rosenthal, O. and Cooper, D.Y. (1968). In Biochemie des Sauerstoffe, 19. Colloquium der Gesellschaft fur Biologische Chemie (Hess, B. and Staudinger, H.J., eds.) pp. 142-177, Springer-Verlag, Berlin.

Fisher, A.B., Huber, G.A. and Furia, L. (1977). Cytochrome P-450 content and mixed-function oxidation by microsomes from rabbit alveolar macrophages. J. Lab. Clin. Med. 90: 101-108.

Fishman, W.H., Springer, B. and Brunetti, R. (1948). Application of an improved glucuronidase assay method to the study of human blood B-glucuronidase. J. Biol. Chem. 173: 449-456.

Fleischer, S., Brierley, G., Klouwen, H. and Slautterback, D.B. (1962). Studies of the electron transfer system XLVII. The role of phospholipids in electron transer. J. Biol. Chem. 237: 3264-3272.

Glauert, A.M. (1975) In Fixation, Dehydration and Embedding of Biological Sepcimens, pp. 7-113. North-Holland, Amsterdam.

Gram, T.E. (1973). Comparative aspects of mixed function oxidation by lung and liver of rabbits. Drug Metab. Rev. 2: 1-32.

Grasso, P., Williams, M., Hodgson, R., Wright, M.G. and Gangolli, S.D. (1971). The histochemical distribution of aniline hydroxylase in rat tissues. Histochem. J. 3: 117-126.

Grover, P.L., Hewer, A., Pal, K. and Sims, P. (1976). The involvement of diol-epoxide in the metabolic activation of benzo(a)pyrene in human bronchial mucosa and in mouse skin. Int. J. Cancer 18: 1-8.

Guengerich, F.P. and coon, M.J. (1975). RAtes of individual steps in hydroxylation reactions catalyzed by reconstituted liver microsomal system containing cytochrome P-450. Pharmacologist 17: 216.

Guengerich, F.P. (1977) Preparation and properties of highly purified cytocrome P-450 reductase from pulmonary microsomes of untreated rabbits. Mol. Pharmacol. 13: 911-923.

Haris, C.C., Genta, V.M., Frank, A.L., Kaufman, D.G., Barrett, L.A., McDowell, E.M. and Trump, B.F. (1974). Carcinogenic polynuclear hydrocarbons bind to macromolecules in cultured human bronchi. Nature 252: 68-69.

Hildebrandt, A., Remmer, H. and Estabrook, R.W. (1968). Cytochrome P-450 of liver microsomes--one pigment or many. Biochem. Biophys. Res. Commun. 30: 607-612.

Hook, G.E.R., Bend, J.R., Hoel, D., Fouts, J.R. and Gram, T.E. (1972a). Preparation of lung microsomes and a comparison of the distribution of enzymes between subcellular fractions of rabbit lung and liver. J. Pharmacol. Exp. Ther. 182: 474-490.

Hook, G.E.R., Bend, J.R. and Fouts, J.R. (1972b). Mixed-function oxidases and the alveolar macrophage. Biochem. Pharmacol. 21: 3267-3277.

Hook, G.E.R. and Bend, J.R. (1976). Pulmonary metabolism of xenobiotics. Life Sci. 18: 279-290.

Howell, K.E., Ito, A., and Palade, G.E. (1978) Endoplasmic reticulum marker enzymes in golgi fractions. What does this mean? J. Cell Biol. 79: 581-589.

Ibbetson, A.L. and Freedman, R.B. (1974). Fluorescence study of the interaction of 3,4-benzpyrene with microsomal membranes. Biochem. Soc. Trans. 2: 343-345.

Ito, A., and Palade, G.E. (1978) Presence of NADPH-cytochrome P-450 reductase in rat liver golgi membranes. J. Cell Biol. 79: 590-597.

Ichibara, K., Kusunose, E. and Kusunose, M. (1969). Microsomal hydroxylation of decane. Biochem. Biophys. Acta 176: 713-719.

Howell, K.E., Ito, A., and Palade, G.E. (1978) Endoplasmic reticulum marker enzymes in golgi fractions. What does this mean? J. Cell Biol. 79: 581-589.

Jacobson, M.S., Levin, W., Poppers, P.J., Wood, A.W. and Conney, A.H. (1974). Comprison of the 0-dealkylation of 7-ethoxycoumarin and the hydroxylation of benzo(a)pyrene in human placenta: effect of cigarette smoking. Clin. Pharmacol. Ther. 16: 701-710.

Jefcoate, C.R.E., Gaylor, J.L. and Calabrese, R.L. (1969). Ligand interactions with cytochrome P-450. I. Binding of primary amines. Biochemistry 8: 3455-3463.

Jefcoate, C.R., Simpson, E.R., Boyd, G.S., Brownie, A.C. and Orme-Johnson, W.H. (1973). The detection of different states of the P-450 cytochromes in adrenal mitochondria; changes induced by ACTH. Ann N.Y. Acad. Sci. 212: 243-261.

Jernström, B., Capdevila, B., Jakobsson, S. and Orrenius, S. (1975). Solubilization and partial purification of cytochrome P-450 from rat lung microsomes. Biochem. Biophys. Res. Commun. 64: 814-822.

Jobe, A. (1977). The labeling and biological half-life of phosphatidylcholine in subcellular fractions of rabbit lung. Biochim. Biophys. Acta 489: 440-453.

Jobe, A., Kirkpatrick, E. and Gluck, L. (1978). Labeling of phospholipids in the surfactant and subcellular fractions of rabbit lung. J. Biol. Chem. 253: 3810-3816.

Johannesen, K., DePierre, J.W., Bergstrand, A., Dallner, G. and Ernster, L. (1977). Preparation and characterization of total, rough and smooth microsomes from the lung of control and methylcholanthrene-treated rats. Biochim. Biophys. Acta 496: 115-135.

Kaufman, D.G., Genta, V.M., Harris, C.C., Smith, J.M., Sporn, M.B. and Saffiotti, V. (1973). Binding of ^{3}H-labeled benzo(a)pyrene to DNA in hamster tracheal epithelial cells. Cancer Res. 33: 2837-2841.

Kumaki, K., Sato, M., Kon, H. and Nebert, D.W. (1978). Correlation of Type I, Type II, and reverse Type I difference spectra with absolute changes in spin state of hepatic microsomal cytochrome P-450 iron from five mammalian species. J. Biol. Chem. 253: 1048-1058.

Klinger, W. (1973). Amidopyrine-N-demethylation by lung 9000 xg supernatant of newborn and adult rats. Acta Biol. Med. Germ. 31: 467-469.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951). Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193: 265-275.

Lu, A.Y.H., Strobel, H.W. and Coon, M.J. (1969). Hydroxylation of benzphetamine and other drugs by a solubilized form of cytochrome P-450 from liver microsomes: lipid requirement for drug demethylation. Biochem. Biophys. Res. Commun. 36: 545-551.

Lu, A.Y.H., Strobel, H.W. and Coon, M.J. (1970). Properties of a solubilized form of cytochrome P-450-containing mixed-function oxidase of liver microsomes. Mol. Pharmacol. 6: 213-220.

Lu, A.Y.H., Kuntzman, R., West, S., Jacobson, M. and Conney, A.H. (1972). Reconstituted liver microsomal enzyme system that hydroxylates drugs, other foreign compounds, and endogenous substrates. II. Role of the cytochrome P-450 and P-448 fractions in drub and steroid hydroxylations. J. Biol. Chem. 247: 1727-1734.

Lu, A.Y.H. and West, S.B. (1972) Reconstituted liver microsomal enzyme system that hydroxylates drugs, other foreign compounds, and endogenous substrates. III. Properties of the reconstituted 3,4 benzpyrene hydroxylase system. Mol. Pharmacol. 8: 490-500.

Lu, A.Y.H., Levin, W. and Kuntzman, R. (1974). Reconstituted liver microsomal enzyme that hydroxylates drugs, other foreign compounds and endogenous substrates. VII. Stimulation of benzphetamine N-demethylation by lipid and detergent. Biochem. Biophys. Res. Commun. 60: 266-272.

Mannering, G.J. (1971). In Fundamentals of Drug Metabolism and Drug Disposition (Ladu, B.N., Mandel, H.G., Way, E.L. eds.), Williams and Wilkins, Baltimore, pp. 206-252.

Martonosi, A., Donley, J. and Halpin, R.A. (1968). Sarcoplasmic reticulum. III. The role of phospholipids in the adenosine triphosphatase activity and Ca^{++} transport. J. Biol. Chem. 243: 61-70.

Matsubara, T., and Tochino, Y. (1971). Electron transport systems of lung microsomes and their physiological functions. I. Intracellular distribution of oxidative enzymes in lung cells. J. Biochem. (Tokyo) 70: 981-991.

Matsubara, T., Prough, R.A., Burke, M.D. and Estabrook, R.W. (1974). The preparation of microsomal fractions of rodent respiratory tract and their characterization. Cancer Res. 34: 2196-2203.

McDonald, J.M., Bruns, D.E. and Jarett, L. (1976). Characterization of calcium binding to adipocyte plasma membranes. J. Biol. Chem. 251: 5345-5351.

Meek, G.A. (1976). Practical Electron Microscopy for Biologists. John Wiley and Sons, London, pp. 418-419.

Moldeus, P., Grundin, P., von Bahr, C. and Orrenius, S. (1973). Spectral studies on drug-cytochrome P-450 interaction in isolated rat liver cells. Biochem. Biophys. Res. Commun. 55: 937-944.

Narasimhulu, S., Cooper, D.Y. and Rosenthal, O. (1965). Spectrophotometric properties of a triton-clarified steroid 21-hydroyxylase system of adrenocorticol microsomes. Life Sci. 4: 2101-2107.

Nash, T. (1953). The colorimetric estimation of formaldehyde by means of the Hantzsch reaction. Biochem. J. 55: 416-421.

National Academy of Sciences (1972): Particulate Polycyclic Organic Matter. Biologic Effects of Atmospheric Pollutants. Division of Medical Sciences, National Research Council, Washington, 361 pp.

Nebert, D.W. and Gelboin, H.V. (1968). Substrate-inducible microsomal aryl hydroxylase in mammalian cell culture. J. Biol. Chem. 243: 6242-6249.

Nebert, D.W., Considine, N. and Owens, I.S. (1973). Genetic expression of aryl hydrocarbon hydroxylase induction. VI. Control of other aromatic hydrocarbon-inducible monooxygenase activities at or near the same locus. Arch. Biochem. Biophys. 157: 148-159.

Nebert, D.W., Kumaki, K., Sato, M. and Kon, H. (1976). Further characterization of drug interactions with hepatic microsomal cytochrome P-450. Hoppe-Seyer's Z. Physiol. Chem. 357: 1044-1045.

Nebert, D.W., Kumaki, K., Sato, M. and Kon, H. (1977). Microsomes and Drug Oxidations. (Ullrich, V., Hildebrnadt, A., Roots, I., Estabrook, R.W. and Conney, A.H., eds.) Pergamon Press, Oxford, pp. 224-231.

Omura, T. and Sato, R. (1964). The carbon monoxide-binding pigment of liver microsomes. I. Evidence for its hemoprotein nature. J. Biol. Chem. 239: 2370-2378.

Oppelt, W.W., Zange, M., Ross, W.E. and Remmer, H. (1970). Comparison of microsomal drug hydroxylation in lung and liver of various species. Res. Commun. Chem. Path. Pharmacol. 1: 43-56.

Pal, K., Grover, P.L. and Sims, P. (1975). The metabolism of carcinogenic polycyclic hydrocarbons by tissue of the respiratory tract. Biochem. Soc. Trans. 3: 174-175.

Parry, G., Palmer, D.N. and Williams, D.J. (1976). Ligand parititioning into membranes: its significance in determining K_m and K_s values for cytochrome P-450 and other membrane bound receptors and enzymes. Febs Lett. 67: 123-129.

Pennington, R.J. (1961). Biochemistry of dystrophic muscle. Mitochrondrial succinate-tetrazolium reductase and adenosine triphosphatase. Biochem. J. 80: 649-654.

Peterson, J.A. (1971). Camphor binding by Pseudomonas putida cytochrome P-450. Arch. Biochem. Biophys. 144: 678-693.

Philpot, R.M., Arinc, E. and Fouts, J.R. (1975). Reconstitution of the rabbit pulmonary microsomal mixed-function oxidase system for solubilized components. Drug. Metab. Dispos. 3: 118-126.

Ray, T.K. (1970). A modified method for the isolation of the plasma membrane from rat liver. Biochim. Biophys. Acta 196: 1-9.

Razin, S. (1972). Reconstitution of biological membranes. Biochim. Biophys. Acta 265: 241-296.

Rouser, G., Kritchevsky, G., Yamamoto, A., Simon, G., Galli, C. and Bauman, A.J. (1969). Methods in Enzymology, Academic Press, New York, Vol. 14, pp. 272-317.

Rouser, G., Fleischer, S. and Yamamoto, A. (1970). Two dimensional thin layer chromatographic separation of polar lipids and determination of phospholipids by phosphorus analysis of spots. Lipids 5: 494-496.

Sackmann, E., Trauble, H., Galla, H.J. and Overath, P. (1973). Lateral diffusion, protein mobility, and phase transitions in Escherichia coli membranes. A spin label study. Biochemistry 12: 5360-5369.

Schenkman, J.B., Remmer, H. and Estabrook, R.W. (1967). Spectral studies of drug interaction with hepatic microsomal cytochrome. Mol. Pharmacol. 3: 113-123.

Schenkman, J.B. (1970). Studies on the nature of the Type I and Type II spectral changes in liver microsomes. Biochem. 9: 2081-2091.

Schenkman, J.B., Cinti, D.L., Orrenius, S., Moldeus, P. and Kraschnitz, R. (1972). The nature of the reverse Type I (modified Type II) spectral change in liver microsomes. Biochemistry 11: 4243-4351.

Schenkman, J.B., Cinti, D.L., Moldeus, P.W. and Orrenius, S. (1973). Newer aspects fo substrate binding of cytochrome P-450. Drug Metab. Dispo. 1: 111-120.

Schuster, L., Fleschurz, C. and Helm, I. (1975). On the interaction of a lipophilic drug with different sites of rat-liver microsomes. Equilibrium studies with a substituted pleuromutilin. Eur. J. Biochem. 51: 511-519.

Sims, P. (1967). The metabolism of benzo(a)pyrene by rat liver homogenates. Biochem. Pharmacol. 16: 613-618.

Sims, P. and Grover, P.L. (1974). Epoxides in polycyclic aromatic hydrocarbon metabolism and carcinogenesis. Adv. Cancer Res. 20: 165-274.

Sims, P., Grover, P.L., Swaisland, A., Pal, K. and Hewer A. (1974). Metabolic activation of benzo(a)pyrene proceeds by a diol-epoxide. Nature 252: 326-327.

Sorokin, S.P. (1970). In Morphology of Experimental Respiratory Carcinogenesis (Nettesheim, P., Hanna, M.G., Jr. and Deatherage, J.W., eds.) U.S. Atomic Energy Commission, Washington, D.C., pp. 3-41.

Strobel, H.W., Lu, A.Y.H., Heidema, J. and Coon, M.J. (1970). Phosphatidylcholine requirement int he enzymatic reduction of hemoprotein P-450 and in fatty acid, hydrocarbon, and drug hydroxylation. J. Biol. Chem. 245: 4851-4854.

Szutowski, M., Wolf, C.R. and Philpot, R.M. (1977). The rabbit pulmonary mixed-function oxidase system: purification of two forms of cytochrome P-450 and reconstitution of activity. Pharmacologist 19: 161.

Taniguchi, H., Imai, Y., Iyanagi, T. and Sato, R. (1979). Interaction between NADPH-cytochrome P-450 reductase and cytochrome P-450 in the membrane of phosphatidylcholine vesicles. Biochem. Biophys. Acta 250: 341-356.

Trump, B.F. and Bulger, R.E. (1966). New ultrastructural characteristics of cells fixed in a glutaraldehyde-osmium mixture. Lab. Invest. 15: 368-379.

Tsai, R., Yu, C.A., Gunsalus, I.C., Peisach, J., Blumberg, W., Orme-Johnson, W.H. and Beinert, H. (1970). Spin-state changes in cytochrome P-450_{CAM} on binding of specific substrates. Proc. Natl. Acad. Sci. USA 66: 1157-1163.

Uehleke, H. (1968). Extrahepatic microsomal drug metabolism. Excerpta Med. Int. Congr. Ser. No. 181, pp. 94-100.

Ullrich, V. and Weber, P. (1972). The O-dealkylation of 7-ethoxycoumarin by liver microsomes. HoppeSeyler's Z. Physiol. Chem. 353: 1171-1177.

Van Den Berg, A.P., Noordhoek, J. and Koopman-Kool, E. (1979). The relation between the sex-dependency of Type I binding of ethylmorphin and the 1-butanol-induced spectral change in mouse liver microsomes. Biochem. Pharmacol. 28: 31-36.

von Bahr, C., Vadi, H., Grundin, R., Moldeus, P. and Orrenius, S. (1974). Spectral studies on the rapid uptake and subsequent binding of drugs to cytochrome P-450 in isolated rat liver cells. Biochem. Biophys. Res. Commun. 59: 334-339.

Vore, M., Hamilton, J.G. and Lu, A.Y.H. (1974a). Organic solvent extraction of liver microsomal lipid. I. The requirement of lipid for 3,4-benzpyrene hydroxylase. Biochem. Biophys. Res. Commun. 56: 1038-1044.

Vore, M., Lu, A.Y.H., Kuntzman, R. and Conney, A.H. (1974b). Organic solvent extraction of liver microsomal lipid. II. Effect on the metabolism, of substrates and binding spectra of cytochrome P-450. Mol. Pharmacol. 10: 963-974.

Waltenbaugh, A.A. and Friedmann, N. (1978). Hormone sensitive calcium uptake by liver microsomes. Biochem. Biophys. Res. Commun. 82: 603-608.

Wattenberg, L.W. and Leong, J.L. (1962). Histochemical demonstration of reduced pyridine nucleotide dependent polycyclic hydrocarbon metabolizing systems. J. Histochem. Cytochem. 10: 412-420.

Wilkinson, G.N. (1961). Statistical estimatations on enzyme kinetics. Biochem. J. 80: 324-332.

Williams, C.H. Jr. and Kamin, H. (1967). Microsomal triphosphopyridine nucleotide-cytochrome c reductase of liver. J. Biol. Chem. 237: 587-595.

Williams, L.T., Jarett, L. and Lefkowitz, R.J. (1976). Adipocyte B-adrenergic receptors. Identification and subcellular localization by (-)- ^{3}H dihydroalprenolol binding. J. Biol. Chem. 251: 3096-3104.

Wolf, C.R. Szutowski, M.M., Ball, L.M. and Philpot, R.M. (1978a). The rabbit pulmonary monooxygenase system: characteristics and activities of the two forms of pulmonary cyto-P-450. Chem. Biol. Interact. 21: 29-43.

Wolf, C.R., Ball, L., Bend, J.R. and Philpot, R.M. (1978b). Metabolism of benzo(a)pyrene in rabbit pulmonary monooxygenase systems reconstituted from purified components: activities of cytochrome P-450 I and II. Fed. Proceed. 37: 643.

Worthington 1978 Enzymes Handbook (1978). Worthington Biochemical Corporation, Freehold, New Jersey, pp. 84-85.

Yoshida, Y. and Kumaoka, H. (1975). Studies on the substrate-induced spectral change of cytochrome P-450 in liver microsomes. J. Biochem. (Tokyo) 78: 455-468.

Zakim, D. and Vessey, D.A. (1977). Membrane-bound estrone as substrate for microsomal UDP-glucuronyltransferase. J. Biol. Chem. 252: 7534-7537.