PULMONARY SURFACTANT INHIBITION FOLLOWING CARDIOPULMONARY BYPASS

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FOLLOWING

CARDIOPULMONARY BYPASS

BY

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Abstract

Following cardiopulmonary bypass (CPB) patients exhibit varying amounts of pulmonary dysfunction. This study examined the possibility that plasma which leaked into the alveoli could cause a reduction in the ability of pulmonary surfactant to reach very low surface tension (surfactant inhibition). The possibility that CPB could cause an increase in any component of plasma which might inhibit surfactant function was also examined. Plasma from adult and child patients was tested. Surfactant was prepared from pig lungs. Surfactant function was measured as ability to lower surface tension on a Langmuir-Wilhelmy balance. Plasma from normals and CPB patients before and after bypass was mixed with pig surfactant and tested on the balance. The surface load of surfactant was constant while the amount of plasma was varied.

Plasma from normals, adult patients and child patients inhibited the surfactant. Serum, fibrinogen, albumin and globulins also inhibited surfactant in this assay. The amount of inhibition was proportional to the amount of protein added. No increased inhibition was seen in post-bypass vs. pre-bypass samples in adults or children. Inhibitor(s) are present in normal plasma and do not appear

to be increased by the bypass procedure. It is possible that plasma components entering airspaces through leaking lung membranes may inhibit surfactant from proper function, and may contribute to lung collapse and edema seen in the ARDS of any etiology.

The nature of the inhibition was examined by characterization of the inhibitor and by studying models of protein-surfactant interaction on the surface balance.

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List of abbreviations and definitions

ARDS	adult respiratory distress syndrome
RDS	respiratory distress syndrome
CPB	cardiopulmonary bypass
sam	surface active material; surfactant
pigsam	surfactant prepared from pig lungs
BAL	bronchoalveolar lavage
PL	phospholipid
PC	phosphatidylcholine
DPPC	dipalmitoyl phosphatidylcholine
DSPC	distearoyl phosphatidylcholine
PG	phosphatidylglycerol
PI	phosphatidylinositol
mN/m	milliNewtons per metre; SI units of surface tension equivalent to dynes/cm
gamma-min	surface tension at minimum surface area; a measure of surfactant function
delta gamma-min	change in surface tension at minimum surface area from that of surfactant alone; a measure of the effect of the test material on surfactant function
EM	electron microscopy
C	centigrade
SACE	serum angiotensin converting enzyme
MW	molecular weight; units = daltons

g	gram
mg	milligram
ug	microgram
ml	millilitre
ul	microlitre
EDTA	ethylenediamine tetraacetic acid
THAM	tris hydroxymethyl aminomethane
PEG	polyethylene glycol
TLC	thin layer chromatography

Chapter 1

Introduction

1.1 Overview

This thesis addresses the problem of lung surfactant dysfunction following surgery in which cardiopulmonary bypass (CPB) is employed. The literature has been reviewed with consideration of:

1. lung surfactant, under the headings
   surfactant function in health
   alveolar and surfactant structure
   surfactant composition, synthesis and turnover
   surfactant component function and physical properties,
2. acute respiratory failure, under the headings
   respiratory distress syndrome (RDS) of the newborn
   adult respiratory distress syndrome (ARDS)
   respiratory distress following CPB
   surfactant and ARDS, and
3. the hypothesis that lung surfactant may be inactivated by inhibitors from plasma which may leak into the alveoli through lung membranes damaged during acute lung injury, under the heading

surfactant inhibitors including their association with cardiopulmonary bypass.

It is considered that an essential feature of pulmonary surfactant is its ability to lower the surface tension at the alveolar air-water interface to very low values. The phrase "inhibition of surfactant" and related phrases will be used throughout this thesis to mean prevention of the ability of surfactant to reach low surface tension during compression of a surfactant monolayer in a Langmuir-Wilhelmy balance.

1.2 Surfactant Function in Health

Surfactant is found in the acellular lining layer of the alveoli. It has the ability to lower surface tension (Pattle, 1955, 1965; Clements 1956, 1957, 1962; Clements, Hustead, Johnson & Gribetz, 1961). This property counteracts the tendency to alveolar collapse, facilitates opening of fluid-filled alveoli, increases lung compliance, decreases work of breathing, and decreases transalveolar hydrostatic forces forming pulmonary edema (Notter & Morrow, 1975; Notter, Finkelstein & Taubold, 1983; Bredenberg, Paskanik & Nieman, 1983; Beck & Lai-Fook, 1983). Open alveoli facilitate gas exchange by improving oxygenation and CO_2 excretion. It has been postulated that the lining layer of the lung aids the clearance of particulate matter and the suppression of invading microorganisms (Schwartz & Christman, 1979; Schlimmer, Austgen & Ferber, 1983).

There is both a functional and structural basis for putative surfactant activity. Mammalian lungs were found to contain this very surface active material (Pattle, 1955; Clements, 1956) Pattle (1955) observed the highly stable characteristics of lung edema foam. Clements (1956) observed low surface tension and hysteresis of extracts of lung minces tested on the surface tension balance. These low surface tensions corresponded to calculations made from pressure-volume curves of inflated lungs (Brown, 1957; Clements, 1957). Lung surfactant deficiency was implicated as the primary cause of the respiratory distress syndrome of the newborn when extracts of minces from lungs taken from premature infants dying with respiratory distress were shown to have increased surface tensions on the surface balance (Avery & Mead, 1959). Photography demonstrated collapse of terminal airways with inflating pressures less than 10 cm H_2O in excised preterm fetal rabbit lungs with absent surfactant, and the beneficial effects of instilling exogenous surfactant were seen (Enhorning, 1977a, 1977b). Scanning electron microscopy was used to show surface tension effects on lung architecture in air-filled, saline-filled and detergent-washed lungs (Bachofen, Gehr & Weibel, 1979).

Surface tension has been measured in situ (Schurch, Goerke & Clements, 1976, 1978; Schurch, 1982). Microdroplets of fluorocarbon and silicone oils deposited in alveoli formed contact angles which were dependent on the surface tension. In cat lungs the surface tension was 10 mN/m at 70% of total

lung volume and was decreased to less than 1 mN/m at 40% of total lung volume (=functional residual capacity). At 40% of total lung volume the surface tension was stable for 10 minutes then increased steadily to 9 mN/m over 1 hour.

1.3 Alveolar and surfactant structure

The alveoli are the small air-filled sacs at which the airways terminate. Their large surface area and thin walls facilitate the exchange of gases from air to blood and vice-versa. The walls separating the alveoli contain endothelial capillaries and basement membranes, an abbreviated interstitium, an epithelial basement membrane and the continuous alveolar epithelial cell layer (Figure 1-1). Adjacent to the alveolar epithelium is the alveolar lining layer containing surfactant.

The structure of the alveolar lining layer has been studied using the electron microscope. The acellular alveolar lining consists of a uniform 4.2 nm layer at the air interface, the surface film, and a heterogeneous layer between the surface film and alveolar epithelium, the hypophase (Kikkawa, 1970). Freeze-fracture electron microscopy has shown the surface film to be smooth and without large, globular shadows, suggesting a phospholipid layer with no protein (Kikkawa & Manabe, 1978). It has been estimated that the surface film is composed of at least 90% disaturated phosphatidylcholine, based on the behaviour of surface films and lung compliance data (Hildebran, Goerke &

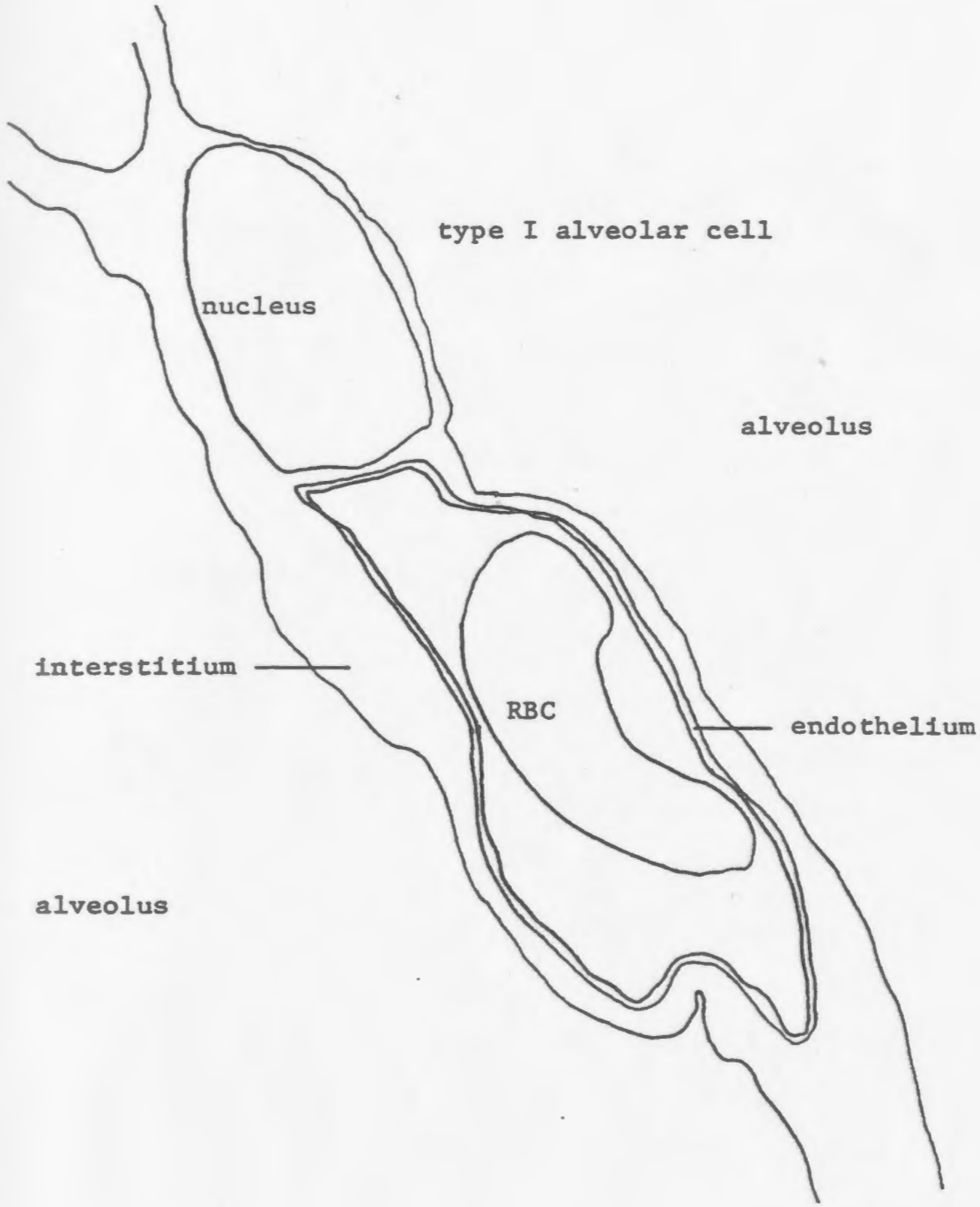


Figure 1-1 The alveolar-capillary membranes

Clements, 1979; Clements, 1977). The hypophase contains structures thought to be lipid aggregates in the forms of vesicles, lamellae and lattice-like arrangements called tubular myelin (Kikkawa, 1970; Manabe, 1979). Structural continuity has been demonstrated on electron microscopy between the hypophase structures and the surface film, and between the hypophase structures and the lamellar bodies being discharged at type II alveolar cell surfaces (Manabe, 1979; Williams & Benson, 1981). Figure 1-2 depicts the putative formation of surfactant in the alveolus.

The alveolar epithelial cells form a continuous cell layer covering the alveolar basement membrane. There are two cell types. The cell type covering the majority of the alveolar surface is the type I cell. These cells are broad and flat. Type II cells are however more numerous (Kuhn, 1982) and are cuboidal in shape. The most distinctive cytologic feature of the type II cells and the most reliable identifying criteria are the secretory organelles called lamellar bodies. They consist of a membrane enclosing concentric or parallel osmiophilic lamellae on electron microscopy.

The lamellar bodies begin as multivesicular bodies of the Golgi apparatus and endoplasmic reticulum. The mature organelle results from accumulation of lipid in the form of lamellae and some protein which is associated with residual vesicular structures within the lamellar bodies and with the outer membrane. No protein-like structures were seen to be associated with the interior lamellae on electron microscopy

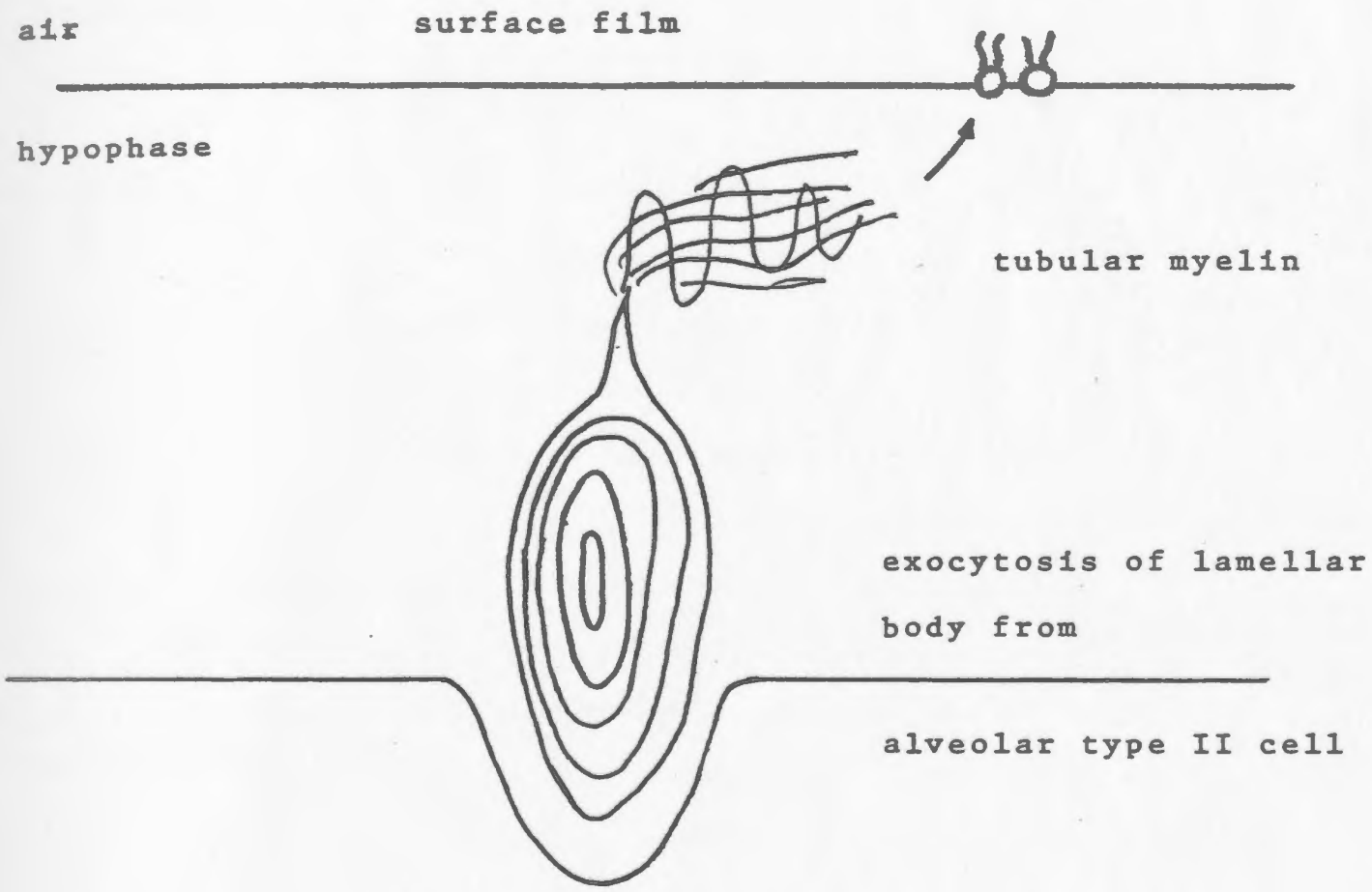


Figure 1-2 Surfactant formation in the alveolus

(Kikkawa & Manabe, 1978). The exact site of combining protein and lipid is uncertain. The lamellar body discharges its contents into the hypophase by exocytosis (Askin & Kuhn, 1971). The use of radiolabelled palmitate confirmed that lamellar body palmitate was a precursor of alveolar lining palmitate (Young, Kremers, Apple, Crapo & Brumley, 1981; Jobe, 1979; Jobe, Kirkpatrick & Gluck, 1978; Kotas, 1982).

The control of secretion involves cholinergic and B-adrenergic stimulation and prostaglandins, and is not fully understood (Dobbs & Mason, 1979; Nicholas & Barr, 1981; Delahunty & Johnston, 1979; Oyarzun & Clements, 1977, 1978). Steroids, thyroid hormones, and insulin affect synthesis and secretion (Avery, Wang & Taeusch, 1973; Liggins, 1969; Liggins & Howie, 1972; Mavis & Vang, 1981; Smith & Bogues, 1980). Steroid effects involve a mediator from adjacent fibroblasts (fibroblast pneumocyte factor) (Smith, 1979).

1.4 Surfactant: Chemical composition, synthesis and turnover

Samples of the alveolar lining layer are best obtained by bronchoalveolar lavage. Extracts of lung minces contain more cellular components (Morgan, Finley & Fialkow, 1965; Tanaka & Takei, 1983). The alveolar lining layer obtained by lavage is diluted many times by the lavage saline, and is contaminated by upper airway secretions and by blood

components (Reifenrath & Zimmermann, 1973). Centrifugation techniques are employed to obtain concentrates which are highly enriched in surface activity. It is this material which is known as surfactant. A standard protocol for preparing surfactant is not yet established.

The composition of lung surfactant differs slightly between mammalian species, and between investigators due to differences in processing techniques. As a rough average, the percentage composition by weight is 10% protein, 10% neutral lipid, and 80% phospholipid. The distinguishing feature of surfactant lipid is the high proportion of dipalmitoyl phosphatidylcholine (DPPC) and phosphatidylglycerol (PG). Such high relative quantities of these two lipids are not found in other body tissues. The ratio of DPPC to PG, approximately 7:1, is similar in type II cells, lamellar bodies and in lavage. This is consistent with the view that surfactant originates in the lamellar bodies of type II cells (Kikkawa & Smith, 1983). For a review see Keough (1984) and Sanders (1982).

The lamellar body of the alveolar type II cell is the major storage site for surfactant lipid. DPPC and PG are most highly concentrated in the lamellar bodies of the cell. Synthesis of the phospholipids occurs primarily in the endoplasmic reticulum of the type II alveolar cell (Chevalier & Collet, 1972). DPPC may be synthesized directly from dipalmitoyl diglyceride and CDP-choline, or by acylation of 1-palmitoyl 2-lysophosphatidylcholine by

palmitoyl-coenzyme A (the de- and re-acylation remodeling pathway) (Smith & Kikkawa, 1978; Frosolono, Slivka & Charms, 1971; Ishidate & Wienhold, 1981). A minor route would be the transacylation of 1-palmitoyl 2-lysophosphatidylcholine. Substrate availability may determine which pathway is used in vivo.

Fatty acids, particularly palmitic acid may be synthesized by the type II cells de novo, or may be taken up from the pulmonary circulation (Smith & Kikkawa, 1978). Cholesterol is derived from the pulmonary circulation.

The mechanisms of lipid turnover are not fully established. Hypophase lipid is somehow removed from the alveolus. Hyperventilation increases the rate of bulk removal (Oyarzun, Clements & Baritussio, 1980). Macrophages and mucociliary mechanisms may be involved (Yoneda, 1976; Eckert, Lux & Lachmann, 1983; Nicholas & Barr, 1981). DPPC may be recycled into the lamellar bodies of type II cells (Magoon, Wright, Baritussio, Williams, Goerke, Benson, Hamilton & Clements, 1983; Oyarzun et al, 1980; Jobe, 1979; Hallman, Epstein & Gluck, 1981). Pinocytic uptake of lipid and apoprotein has been demonstrated (Kikkawa & Kaibara, 1972; Williams & Benson, 1981).

The hypophase also contains several hydrolytic enzymes which may regulate some of the surfactant turnover (Orlowski, Orlowski, Kilburn & Lesser, 1981; Hook, 1978).

1.5 Surfactant: component function, physical properties and structure

Lung surfactant has the essential physical properties of (1) ability to lower surface tension on film compression to less than 9 mN/m, (2) rapid adsorption of the surface active agent(s), (3) rapid spreading of the surface active agent(s) and (4) slow film collapse rates at high surface pressures. These abilities should exist in the physiologic temperature range about 37 degrees C (Notter & Shapiro, 1981; Taeusch, Clements & Benson, 1983; Hawco, Davis & Keough, 1981). Studies of the individual components of surfactant have application to surfactant replacement in surfactant-deficient states and the synthesis of such materials.

Surface film studies on the tension surface balance have shown that a DPPC monolayer has the unique characteristic of lowering surface tension to approximately zero on film compression at 37 degrees C. No other molecular species in lung surfactant will approach this surface tension at 37 degrees C (Clements, 1967, 1973). The effect of temperature on liquid condensed-liquid expanded phase transition is important with regard to fluidity of the lipid acyl groups and ability to achieve a high packing density on film compression. The decreased free energy state of high packing density is seen as low surface tension. Substitution of acyl groups with carbon chains of varying lengths and double bonds affects lipid fluidity and phase transition temperatures, and hence ability to attain high

packing density and low surface tension on film compression (Keough, 1984). Slow film collapse rates at high surface pressures are also characteristic of pure DPPC monolayers and not of other molecular species at 37 degrees C (Keough, 1984; Goerke & Gonzales, 1981; Hildebran et al, 1979).

It is generally felt that some selective mechanism allows DPPC to form the surface film to account for the low surface tensions achievable only by pure DPPC monolayers. This mechanism may involve the process of selective lipid insertion into the monolayer, selective exclusion (squeeze-out) from the surface film on compression, or some protein-mediated modification of the surface film, analogous to the phospholipid exchange proteins of cellular biochemistry.

The function of the other components of lung surfactant are not so easily defined. Because DPPC does not have the properties of rapid adsorption and spreading, the function of the other components may relate to assisting adsorption and spreading, and investigations have been performed with this idea in mind. The literature presents conflicting evidence about the function of these components so that no definite statement can be made at this time.

The negatively-charged lipids, eg PG, were found to increase adsorption of lipid to the surface and enhance formation of tubular myelin in studies by King & MacBeth (1981). In contrast, Beppu, Clements & Goerke (1983) and Obladen, Brendlein & Kempien (1979) did not find that the

negatively-charged lipids increased the adsorption of lipid to the surface. PG is known to be characteristic of mature lung surfactant. It increases during the late stages of gestation to replace phosphatidylinositol (PI) as the predominant negatively-charged phospholipid. The lavage fluid of adult respiratory distress patients contains an abnormally high ratio of PI/PG. Lung surfactant deficient states have abnormalities in the negatively-charged lipid component (Hallman, Spragg, Harrell, Moser & Gluck, 1982).

Surfactant lipids other than DPPC generally have phase transition temperatures less than 37 degrees C and would be expected to enhance fluidization and spreadability at 37 degrees C (Villalonga, 1968; Phillips & Hauser, 1974). This would aid the formation of the monolayer.

Cholesterol may also contribute to surface film formation by enhancing bilayer instability (Keough, 1984).

Surfactant apoproteins increase with gestational age in the amniotic fluid along with PG. Human lung apoproteins have been described as two bands on polyacrylamide gels under reducing conditions at 30,000-40,000 daltons and at 60,000-70,000 daltons (Shelley, Balis, Paciga, Espinoza & Richman, 1982; Taeusch et al, 1983). Perhaps the larger protein is a dimer of the smaller apoprotein. These apoproteins are specific to the alveoli. They have been found to be associated with the lamellar bodies of the alveolar type II cells, but the mechanism of association is unknown. The apoprotein may be essential to the formation

of tubular myelin in the alveolar hypophase and aid adsorption of lipid to the surface film (Taeusch et al, 1983; Benson, Williams, Sueishi, Goerke & Sargeant, 1984; King & MacBeth, 1981; Tanaka, Takei & Kanazawa, 1983; Notter, Finkelstein & Taubold, 1983). However, removal of 90% of the proteins from an animal lung surfactant did not affect the surface properties of the resulting lipid when tested in a pulsating bubble surfactometer (Metcalfe, Enhorning & Possmayer, 1980; Yu, Harding, Smith & Possmayer, 1983).

Divalent cations, eg calcium, magnesium, may be needed by surfactant to achieve low surface tensions (King & Macbeth, 1981; Benson et al 1984; Kobayashi & Robertson, 1983; Weber, Yu & Possmayer, 1983). Again, it is postulated that the divalent cation may aid the formation of tubular myelin.

The formation of the surface monolayer of lipid involves some transformation of lipid present in bilayers present in the alveolar hypophase. Theoretical considerations of monolayer formation come from studies of spontaneous lipid conformations in an aqueous environment. Such studies have suggested that the lipid would exist as some form of lipid bilayer. Bilayer instability induced by calcium, PG, or apoprotein could lead to the presence of non-bilayer lipid conformations such as inverted micelles and inverted hexagonal phase from which a surface monolayer might form. Tubular myelin, the lipid aggregate structures in the hypophase, may contain lipid in bilayer and unstable bilayer

forms from which the surface monolayer film is formed. Alternatively, the tubular myelin may be produced concomitantly with the lipid monolayer as another nonbilayer conformation (Keough, 1984).

1.6 Respiratory distress syndrome of the newborn

Respiratory distress syndrome of the newborn, or hyaline membrane disease is associated with surfactant deficiency. Extracts of lung minces from infants with this syndrome fail to reach low surface tensions (Avery & Mead, 1959). The surfactant deficiency is associated with immaturity of the lung and type II surfactant-producing cells.

Increased incidence of infant respiratory distress syndrome is seen in premature newborns (Avery & Mead, 1959; Avery et al , 1973; Usher, Allen & McLean, 1971), perinatal asphyxia (Rokos, Vaeusorn, Nachman & Avery, 1968), familial disposition (Graven & Misenheimer, 1965), male sex (Naeye, Burt, Wright, Blanc & Tatter, 1971), maternal diabetes mellitus (Hubbell & Drorbaugh, 1965), maternal obesity (Naeye, Freeman & Blanc, 1974), and hypothyroidism (Gibson, Blackmore, Wijeratne & Wrathall, 1976). These conditions may be speculated to retard development of the lung surfactant system during fetal maturation. Corticosteroids are used to stimulate maturation of the surfactant system in cases of premature labour (Avery et al, 1973; Liggins, 1969; Liggins & Howie, 1972). They accelerate epithelial maturation in the developing lung (Kikkawa, Kaibara,

Motoyama, Orzalesi & Cook, 1971; Motoyama, Orzalesi, Kikkawa, Cook, Zigas, Kaibara & Wu, 1971). A peptide factor from fibroblasts which are apposed to type II cells may be important in regulating the response of the alveolar epithelium to steroids (Smith, 1979; Marin, Dameron & Relier, 1982). Stress and the B-adrenergic agonists can stimulate secretion of DPPC from immature type II cells.

The lung matures rapidly during the last quarter of gestation. DPPC production is markedly increased, as is PG production. The ratio of lecithin to sphingomyelin is used as a maturity index of fetal lung (Gluck, Kulovich, Borer, Brenner, Anderson & Spellacy, 1971). Ratios of 2:1 or greater reliably indicate maturity, whereas ratios of 1.5-2:1 are less reliable (Harvey, Parkinson & Campbell, 1975). Infants with RDS have both reduced quantities and qualitative changes in the lecithins of their lungs. The qualitative changes include i) a decreased proportion of disaturated phosphatidylcholine and ii) an increase in lipids which have acyl chains with increased fluidity at 37 degrees C (Shelley, Kovacevic, Paciga & Balis, 1979; Morley, Hill, Brown, Barson & Davis, 1982; Keough, 1984). Both the reduction in DPPC and the increase in unsaturated fluid lipids would contribute to surfactant dysfunction.

The increase of PG in amniotic fluid is also an index of fetal lung maturity (Kulovich, Hallman & Gluck, 1979). PG may function to increase DPPC production as it activates cholinephosphate cytidylyltransferase, an enzyme in the

synthetic pathway of DPPC (Stern, Kovac & Weinhold, 1978; Feldman, Kovac, Drunginis & Weinhold, 1978).

Recently, a specific surfactant inhibitor has been described in the airways of premature lambs and infants with RDS (Ikegami, Jacobs & Jobe, 1983; Jobe, Ikegami, Jacobs, Jones & Conaway, 1983). This inhibitor is associated with leaking lung membranes. It is a protein but has been only partially characterized.

Studies of exogenous surfactant for infant RDS show great promise. Synthetic surfactants, surfactants made from bovine lung lavage lipids alone, and enriched with DPPC, and surfactants from human amniotic fluid are being tested. Theoretical considerations of DPPC content and the functions of fluidizing lipids, calcium and proteins to perhaps aid adsorption and spreading are relevant. Recent trials of surfactant replacement have claimed improved ventilation and survival in neonates with RDS (Fujiwara, Chida, Watabe, Maeta, Morita & Abe; 1980; Hallman, Merritt, Schneider, Epstein, Mannino, Edwards & Gluck, 1983; Smyth, Metcalfe, Duffy, Possmayer, Bryan & Enhorning, 1983).

1.7 Adult Respiratory Distress Syndrome

The adult respiratory distress syndrome (ARDS) describes acute respiratory failure in the setting of shock, sepsis, trauma and other illnesses. ARDS with sepsis and multiorgan failure is the principal cause of death in patients surviving initial hemorrhage or head injury. It is

estimated to affect 150,000 persons per year in the United States, and has a 50% mortality despite modern intensive care techniques (Connors, McCaffree & Rogers, 1981). Many victims are young and previously healthy.

The respiratory failure involves an acute lung injury, a noncardiogenic pulmonary edema with leaking lung membranes, surfactant deficiency, and pulmonary fibrosis. If not lethal, resolution takes weeks with only partial reversibility in structural derangements. Permanent reduction in vital capacity or air flow obstruction is seen in one-third of survivors, but subtle abnormalities in exercise tolerance and gas exchange may persist in all cases (Elliot, Morris & Cengiz, 1981).

The acute lung injury is usually secondary to a systemic illness, commonly shock, sepsis and trauma. Some systemic inflammatory components, eg. complement, leukocyte products, platelet products or fibrin degradation products injure the pulmonary capillary endothelium (Rinaldo & Rogers, 1982). The alveolar capillary endothelium is also damaged and a permeability type of pulmonary edema ensues. Concurrent damage to the surfactant system results in alveolar collapse and increased pulmonary edema. Sepsis and oxygen toxicity augment lung damage and likely influence the fibrotic response (Fisher, 1980).

In the intensive care unit, life-support technology has improved greatly. The Swan-Ganz pulmonary artery catheter allows some distinction between hydrostatic and permeability

edema, and provides for monitoring of cardiopulmonary physiologic indices. The use of positive end-expiratory pressure (PEEP) ventilation has been claimed to improve survival in ARDS (Ashbaugh, Petty, Bigelow & Harris, 1969). PEEP may prevent surfactant aggregation (Wyszogrodski, Kyei-Aboagyek, Taeusch & Avery, 1975) and reduce oxygen toxicity by lowering inspired oxygen requirements (Petty & Ashbaugh, 1971). Steroids may modify both the inflammatory injury and fibrotic response (Hammerschmidt, White, Craddock & Jacob, 1979; Skubitz, Craddock, Hammerschmidt & August, 1981; Hesterberg & Last, 1981; Brigham, Bowers & McKeen, 1981), but the role of steroids in the management of RDS is not yet defined. Clinical trials testing the efficacy of steroids have not been conclusive. Sibbald, Anderson, Reid, Holliday & Driedger (1981) suggested that high-dose steroids may reduce alveolar-capillary permeability in human septic ARDS if used early in the course of the illness. However, Thompson, Gurley, Lutz, Jackson, Kvols & Morris (1976) did not find that high-dose steroids affected outcome or physiologic responses in a double-blinded study of 60 patients with septic shock. Extracorporeal membrane oxygenation did not improve the mortality in a multicentre study (NIH, 1979).

Clinical management of ARDS has not been very successful in reducing the mortality of the disease. Treatment to date may be considered as aggressively supportive. Therapeutic considerations now necessarily will come from the study of pathogenic mechanisms.

The pathology of ARDS lungs shows capillary microthrombi, endothelial damage, an interstitium filled with mesenchymal and inflammatory cells, interstitial edema and hemorrhage, loss of alveolar epithelial type I cells and proliferation of type II cells. Destruction of normal lung architecture is followed by pulmonary vascular obliteration, lung parenchymal fibrosis and alveolar loss (Lamy, Fallat, Koeniger, Dietrich, Ratliff, Eberhart, Tucker & Hill, 1976; Katzenstein, Bloor & Leibow, 1976).

Investigation of the acute lung injury suggests that complement activation could be a factor common to several etiologic illnesses, eg. sepsis, shock, trauma, pancreatitis, burns, endotoxemia, soft tissue injury, and cardiopulmonary bypass. Complement activation recruits neutrophils (Craddock, Hammerschmidt, White, Dalmasso & Jacob, 1977). Neutrophils can damage the pulmonary endothelium, interstitium and alveolar epithelium through lysosomal superoxide radicals (Sacks, Moldow, Craddock, Bowers & Jacob, 1978) and proteases (Janoff, White, Carp, Harel, Dearing & Lee, 1979). Neutrophil proteases can cause intravascular coagulation and platelet aggregation. Platelet-fibrin thrombi have been observed in ARDS lungs (Saldeen, 1976). Fibrin degradation products and prostaglandins and leukotrienes have both neutrophil chemotactic and microvascular permeability altering properties (Manwaring, Thorning & Curreri, 1978; Piper, 1983). Perhaps all of these systems - complement,

neutrophils, platelets, coagulation - are involved in the acute lung injury of ARDS. They may potentiate and amplify the effects of each other. It may not be that one system is solely responsible for the lung injury, although for therapeutic approaches this would be ideal.

Normal defense mechanisms may be deficient in the states predisposing to ARDS. Fibronectin is a glycoprotein that exists in tissue as an intercellular adhesive and in the plasma as an opsonizing factor. Fibronectin depletion in sepsis has been observed (Saba & DiLuzio, 1969; Schumacker & Saba, 1980). This would both increase alveolar-capillary membrane permeability and decrease the effectiveness of the reticuloendothelial system to clear particulate microthrombi and bacterial toxins. Antiprotease defense against leucocyte proteases may be impaired. Alpha-1-antitrypsin is an antiprotease that is found to be inactivated in the lung lavage of ARDS patients (Carp & Janoff, 1980; Bruce, Boat, Martin, Dearborn & Fanaroff, 1981).

For reviews of ARDS see Moore, Lyons, Pierce, Morgan, Drinker, MacArthur & Dammin (1969), Connors et al (1981), and Rinaldo & Rogers (1982).

1.8 Respiratory distress following cardiopulmonary bypass

Following cardiopulmonary bypass operations (CPB), variable degrees of lung dysfunction are seen. Most commonly today there are minor atelectasis and slight decreases in lung volume. Respiratory distress syndrome

(RDS), which was quite common before hemodilution techniques were instituted, occurs infrequently in the whole patient patient population, but RDS still remains a significant complication of CPB especially in cases of prolonged bypass and in children (Kirklin, Westaby, Blackstone, Kirklin, Chenoweth & Pacifico, 1983).

The RDS which follows CPB operations exhibits clinical and pathophysiologic characteristics seen in other forms of ARDS. There is a permeability type of pulmonary edema, atelectasis, loss of lung volumes, increased shunting, impaired gas exchange, and the tendency to infection and multiple organ failure (Ratliff, Young, Hackel, Mikat & Wilson, 1973; Wilson, 1974). In such states, the mortality is high. Pathophysiologically, exposure of the blood to the extracorporeal circulation - the foreign surfaces of plastic (polyvinyl chloride) tubing, the stress and shear forces of roller pumps and the suction apparatus - causes damage to blood components. Microaggregates of damaged blood components and a complement-initiated inflammatory response are seen (Kirklin et al, 1983; Craddock, Fehr, Brigham, Kronenberg & Jacob, 1977). The lung vascular endothelium is damaged (Ionescu, 1981). The interstitium shows hemorrhage, edema and infiltrates of polymorphs. The alveolar epithelial cells are damaged. The pathogenic mechanisms of complement, leukocyte superoxide radicals and proteases, platelets, prostaglandins and leukotrienes, and fibrin degradation products may be invoked in this acute injury to

the lung. As well, some investigators have associated the onset of RDS following CPB with possible anaphylactic reactions to fresh frozen plasma (Hashim, Kay, Hammond, Kopf & Geha, 1984; O'Connor, Erskine & Pringle, 1981) and to heparin-protamine interaction (Best, Sinosich, Teisner, Grudzinskas & Fisher, 1984; Olinger, Becker & Bonchek, 1980).

Surfactant dysfunction has been investigated as part of the lung injury following CPB. Extracts of minces of lung specimens from patients who died following CPB showed increased surface tensions (18 mN/m for ARDS lungs vs 7 mN/m for normals) (Gardner, Finley & Tooley, 1962). Extracts of minces of lungs from dogs subjected to CPB also showed increased surface tensions (Gardner et al, 1962; Hepps, Roe, Wright & Gardner, 1963; Mandelbaum & Giammona, 1964; Panossian, Hagstrom, Nehlsen & Veith, 1969; Camishion, Fraimow, Kelsey, Tokunaga, Davies, Joshi, Cathcart & Pierucci, 1968).

The surfactant dysfunction may result from decreased production of surfactant or production of dysfunctional surfactant, increased breakdown of surfactant components, or surfactant inactivation. Damage to the alveolar epithelial type II cells may cause decreased production of surfactant. The injury would seem more likely to result from the inflammatory response than from ischemia due to pulmonary artery occlusion during the CPB. In isolated dog lung perfusions, periarterial hemorrhage and edema preceded any

increase in minimum surface tensions of lung extracts (Panossian et al, 1969). Occlusion of the pulmonary artery in dogs produced surfactant dysfunction only after 16 hours of ligation (Gardner et al, 1962; Mandelbaum & Giammona, 1964; Morgan & Edmunds, 1967). When one lung was kept ischemic by clamping of the pulmonary artery during CPB, it did not develop microscopic lung injury, but the lung whose pulmonary artery was left open did develop such changes (Lesage, Tsuchioka, Young & Sealy, 1966).

Evidence for decreased production or increased turnover of surfactant is that a reduction in the lamellar bodies of alveolar type II cells was seen post-CPB in dogs (Baritussio & Clements, 1981; Sobonya, Kleinerman, Primiano & Chester, 1972). Evidence for increased turnover of surfactant is that interruption of pulmonary blood flow during CPB may result in increased surfactant removal from the lung (Morgan, 1971).

The effects of hemodilution have been tested by examination of extracts of minces of lungs from dogs on CPB (Camishion et al, 1968). With whole blood prime, increased minimum surface tensions were observed at the end of the CPB of one hour duration. However with hemodilution pump priming techniques, there was no increase in minimum surface tensions of the extracts until 14 hours after CPB. It was suggested that hemodilution protected by diluting a plasma inhibitor of surfactant activity produced during CPB.

In another study, extracts of minces of lungs from dogs

placed on CPB were tested 24 hours after bypass (Hepps et al, 1963). The extracts showed a mean minimum surface tension on the surface balance of 22 mN/m when whole blood prime was used in the pump, compared with 11 mN/m when hemodiluted blood was used.

The timing of the lung injury in relation to the CPB and the timing of surfactant dysfunction may not be the same. With respect to the timing of surfactant dysfunction, decreased surface activity was seen immediately after 1-2 hours of CPB with whole blood prime (Camishion et al, 1968), and after 15-24 hours with hemodilution techniques (Hepps et al, 1963). With respect to the timing of the lung injury and the timing of the leaking lung membranes, tracer studies with labelled albumin demonstrated maximum alveolar-capillary leak at 2 hours following CPB (hemodilution technique) of 1-2 hours duration in patients (Royston, Catley, Higenbottam, Wallwork & Minty, 1983). Maximum lung damage as indicated by decreased levels of serum angiotensin converting enzyme (SACE), a lung endothelial enzyme, also occured at 2 hours following CPB. Improvement of the leak and SACE levels was seen by 48 hours after CPB.

Our studies address the possibility that surfactant dysfunction may result from inhibitors which leak into the alveoli from the plasma during the acute lung injury. The timing of production of such postulated inhibitors may concur with timing of the lung injury immediately following CPB, or sometime later with the observed timing of

surfactant dysfunction (eg. at 15-24 hours with hemodilution techniques).

1.9 Surfactant and ARDS

The abnormalities of lung mechanics in ARDS, namely atelectasis, decreased lung volumes and increased compliance, fit well with surfactant dysfunction. As well, surfactant deficiency would increase pulmonary edema, and decrease normal particulate and bacterial clearance mechanisms (Hallman et al, 1982; Notter & Morrow, 1975; Notter & Shapiro, 1981; Schwartz & Christman, 1979; Schlimmer et al, 1983). Surfactant abnormalities are likely part of the consequences of the primary lung injury rather than a mechanism of primary injury.

Investigation of surfactant in ARDS has shown decreased function and abnormal compostion. Abnormal lipid-protein aggregates were observed in surfactant from patients with ARDS (Petty, Reiss, Paul, Silvers & Elkins, 1977; Petty, Silvers, Paul & Stanford, 1979). The surfactant had minimum surface tensions on film compression comparable to that of normals (18 mN/m) but the surfactant from the ARDS lungs had a higher compressibility than normals, ie. compression produced hysteresis loops more like those of pure proteins, than of DPPC. In surfactant prepared from extracts of minces of lungs from patients who died with ARDS, the DPPC content was decreased relative to other lipids (von Wichert & Kohl, 1977). It was concluded that the ineffective

surface activity seen in ARDS surfactant was the result of decreased DPPC content.

Bronchoalveolar lavage (BAL) from patients with ARDS, and other respiratory diseases, eg. pneumonia, malignancy, and chronic obstructive lung disease, was compared with that from normal volunteers (Hallman et al, 1982). The total phospholipid of ARDS BAL was not much different from that of normals or from other respiratory diseases, but the lecithin/sphingomyelin ratio, PG and disaturated lecithin were low in ARDS BAL. Moreover, these indices normalized with recovery of lung function. BAL preparations from ARDS patients were not surface active compared with BAL from normals (minimum surface tensions of 14-20 mN/m and 3-5 mN/m, respectively). Phospholipase A_2 activity was low in BAL from ARDS patients compared with that from normals and the other lung diseases, suggesting no increase in the catabolism of phospholipids in ARDS. Plasma myoinositol in ARDS patients was low unlike newborns with RDS where high plasma myoinositol levels may decrease synthesis of PG.

In another study, BAL from patients with ARDS was found to contain a reduced proportion of palmitic acid in the total fatty acid compared with that from a control group of patients undergoing bronchoscopy for local lung lesions (Baughman, Stein, MacGee, Rashkin & Sahebjami, 1984).

Animal models of acute lung injury also show surfactant abnormalities. Pulmonary edema has been induced in dogs with rapid intravenous dextran infusion (Said, Avery, Davis,

Banerjee & El-Gohary, 1965). The surface activity of extracts from damaged lung were reduced when compared with that from normal lung. This surfactant dysfunction was associated with abnormal pressure-volume curves and morphologic and histologic damage. Oleic acid has been injected intravenously into cats (Hamilton, Hustead, Peltier, Kuenzig, Strandmark & Rosenbaum, 1964). Extracts of lung minces from treated animals showed increased minimum surface tension (22 mN/m) when compared with that of control lungs (<10 mN/m). The lungs were grossly hemorrhagic, the alveoli filled with blood and exudate, and there was disorganization of the alveolar architecture. In dogs exposed to high oxygen tensions respiratory distress and pulmonary edema was seen (Morgan, Finley, Huber & Failkow, 1965). The BAL of dogs with pulmonary edema showed high surface tensions and a decrease in the proportion of palmitic acid and disaturated lecithin in the total lipids of the BAL when compared with BAL from normals. Electron microscopy showed alterations in the structure of the lamellar bodies of the type II alveolar epithelial cells. Another investigation of high oxygen tensions in dogs showed decreased surface activity of lung lavage fluid obtained after 5 hours of exposure (Trapp, Patrick & Oforsagd, 1971). The total quantity of phospholipid and palmitate was decreased in the BAL of treated dogs in comparison to that from control dogs. N-nitroso-N-methylurethane has been injected subcutaneously into dogs (Ryan, Liau, Bell, Hashim

& Barrett, 1981). The lungs showed microatelectasis, interstitial and alveolar edema, and hyaline membranes. BAL and lung tissue were analysed for disaturated phosphatidylcholine (PC). Both alveolar and type II cell disaturated PC levels were decreased compared to a control group, suggesting that surfactant changes resulted from injury to the alveolar type II cells. Lung injury has been induced in mice with butylated hydroxytoluene (Smith, 1983). Lamellar body volume density was decreased at the time of alveolar type II cell mitosis. This implied that the metabolism of proliferating type II alveolar cells at the time of injury was not directed to surfactant production.

Surfactant dysfunction may result from decreased production, increased breakdown, or inactivation. In acute lung injury there is evidence for decreased production of DPPC. Not much investigation has addressed the breakdown and turnover of surfactant in acute lung injury. The possibility of surfactant inactivation by plasma inhibitors reaching the alveoli through damaged lung blood-air membranes has been investigated and will be reviewed in the next section.

1.10 Surfactant inhibitors including their association with cardiopulmonary bypass

Gardner et al (1962) tested extracts of minces from normal dog lungs on a surface tension balance. The compression cycle was timed at 30 minutes. Heparinized whole blood was

pumped through a bubble oxygenator in vitro for several hours, then 5 ml of this blood was mixed with the extract of normal dog lung. The minimum surface tensions were 18-22 mN/m on compression (n=6). When the same extracts were mixed with unperfused blood, the minimum surface tensions were 6-8 mN/m. It was concluded that blood exposed to CPB contained surfactant inhibitors whereas normal blood did not prevent surfactant from reaching low surface tensions on the surface balance. These inhibitors were suggested to be free lipids released following the denaturation of protein. Protein is known to be denatured by CPB.

Mandelbaum & Giammona (1964) added dog blood to extracts of minces from normal dog lungs. Normal blood, blood aged for 2-4 hours at room temperature, and blood pumped through an oxygenator circuit with no tissue contact for 2-4 hours increased the minimum surface tension from 10 mN/m to 28-32 mN/m. However blood taken from dogs during 5 hours of CPB did not increase the minimum surface tension significantly. The quantity of blood added was unstated. It was concluded that a circulating surfactant inhibitor had been removed from the blood during CPB in the dogs. Neither Gardner or Mandelbaum described how the blood and lung extracts were mixed or in what quantities they were mixed prior to testing on the surface balance.

Tierney & Johnson (1965) tested extracts of minces of rabbit lungs on the surface balance. Three grams of lung were minced, then stirred for 5 minutes in 50 ml of 0.9%

saline. The extracts were poured into the surface balance trough through cotton gauze to remove tissue pieces. If 1 to 6 ml of normal rabbit blood or serum was added to the saline during extraction, the minimum surface tension was usually but not always elevated. The minimum surface tension was also increased if i) the temperature was raised to 42 degrees C, ii) the extract was prepared with distilled water, iii) phospholipase C was incubated with the extract and iv) cholesterol or oleic acid was added to the surface. If 10 ml of blood was added beneath the surface film, no significant effect upon the minimum surface tension was seen. The method of adding the blood beneath the surface was not stated. They concluded that adequate mixing of blood and a sufficient quantity of blood were essential before significant change in minimum surface tension would occur. They concurred with Gardner et al (1962) that free lipid could inhibit surfactant. Alterations in the pressure-volume characteristics of the lung could result when the blood-air barrier was damaged and the composition of the surface changed.

Taylor & Abrams (1966) extracted surfactant from rabbit, lamb and human lungs. Extracts were mixed with human plasma protein fractions, incubated for 5 minutes then tested on a subphase of saline on the surface balance. Plasminogen, plasmin, albumin, and gamma-globulins when mixed in equimolar or greater amounts with surfactant did not affect the minimum surface tension of the surfactant. The

molecular weight of the surfactant was not specified. However an equimolar amount of fibrinogen did increase the minimum surface tension, and the minimum surface tension was increased with increasing amounts of fibrinogen. The protein-surfactant mixture was placed on the surface using the glass rod method of Trurnit (1960).

Balis, Shelley, McCue & Rappaport (1971) mixed washings from rabbit lungs with serum and citrated plasma in a ratio of 5:1. The minimum surface tension of the mixtures placed on a subphase of Ringer's solution was elevated to 18-20 mN/m. Centrifugation of the mixtures produced a pellet which had high surface activity (minimum surface tensions of 0-5 mN/m). This was a reversible form of surfactant inactivation. When lung washing was mixed with heparinized or recalcified citrated plasma, a clot developed in the mixture. The clot contained myeloid figures in the fibrinous deposits. Eluate of the washing-plasma mixture yielded a pellet after centrifugation which had high minimum surface tensions (20-25 mN/m). It was suggested that this type of surfactant inactivation depleted surfactant by coagulation and might represent a mechanism of surfactant inactivation in hyaline membrane disease.

Miyahara (1969) tested mixtures of fibrinogen, fibrin and fibrinogen breakdown products, serum, plasma, and whole blood with lung washings, and found that all had inhibitory effects on the surface activity of the surfactant.

Tabak & Notter (1977) added proteins to a preformed

surface monolayer of DPPC using the glass rod method of Trurnit (1960). Bovine fibrinogen and albumin did not affect the minimum surface tension in ratios of 1-2 molecules of protein per molecule of phospholipid at the surface.

Rufer & Stolz (1969) found that on the surface balance, pure protein hypophases (albumin, globulin, fibrinogen) were surface active. Monolayers of rat lung surfactant and pure DPPC placed on the protein hypophases showed increased minimum surface tensions and decreased hysteresis. The rat lung surfactant was added to the surface of the hypophase as a dry powder. The DPPC was added to the surface using a 50% propandiol solution as the spreading agent.

In summary, the literature presented a confusing picture of the effects of blood or blood components on lung surfactant activity (Table 1-1). Surfactant inhibition was tested as the ability to prevent low surface tension on compression using the Langmuir-Wilhelmy surface balance. Possible explanations for the variability in the results are the varied methods of i) preparing the lung surfactant, ii) mixing the surfactant and the blood or blood component, and iii) testing the mixture on the surface balance.

1.11 Statement of the problem

The literature relevant to the hypothesis of surfactant dysfunction due to inactivation by plasma inhibitors has been reviewed. It was proposed to seek answers to the

Table 1-1: Literature findings on surfactant inhibition

Does normal blood inhibit surfactant?

no	yes
Gardner et al (1962)	Mandelbaum et al (1964)
Tierney et al (1965) (blood beneath the surface)	Tierney et al (1965) (usually, when blood mixed with lung extract)

Do plasma proteins inhibit surfactant?

no	yes
Taylor et al (1966) (albumin, globulins)	Taylor et al (1966) (fibrinogen only)
Tabak et al (1977)	Balis et al (1971)
	Miyahara (1969)
	Rufer et al (1969)

Does CPB blood inhibit surfactant?

no	yes
Mandelbaum et al (1964) (in vivo)	Mandelbaum et al (1964) (in vitro)
	Gardner et al (1962) (in vitro)

following questions as the answers were not clear from the literature:

1. Do normal plasma and serum inhibit surfactant?
2. Does CPB produce or increase surfactant inhibitors in plasma, and if so, what time are these inhibitors produced in relation to the CPB?
3. Is CPB in children different from in adults with respect to the production of possible surfactant inhibitors?
4. What is the nature of any observed inhibition?

Information gained from this study should allow a clearer understanding of lung dysfunction involving leaking membranes with entrance of plasma or plasma fractions into the alveolar spaces.

1.12 Research plan

This study of surfactant inhibition involved tests of the ability of lung surfactant to reach low surface tensions on a surface tension balance. Inhibition was defined as the prevention of the surfactant from reaching low surface tension. As previously stated, there are differences in the methods used to test surfactant-potential inhibitor mixtures. A precise definition of methods of testing was required.

The preparations for testing surfactant-potential inhibitor mixtures involved the preparation of lung

surfactant and definition of the test conditions. The test conditions considered were the surface balance conditions (treatment of the trough, the hypophase solution, the layering of the test sample on the hypophase solution, the time of equilibration on the surface before testing, the solution used for spreading the test material), the method of plasma preparation, the method of mixing the surfactant and potential inhibitor, and the time of interaction of the mixture before testing.

Tests of normals and patients undergoing CPB surgery were examined to investigate the possible presence of inhibitors in the blood of normal adults and in adult and child patients.

The nature of the inhibition was investigated by comparing the effects of plasma, serum and various plasma protein fractions on surfactant. As well, an attempt was made to define the chemical nature of the inhibitor. Also, an effort was made to determine if the specific surfactant inhibitor of premature infants and lambs with RDS (Ikegami et al, 1983; Jobe et al, 1983) was present in the plasma from normal individuals and CPB patients.

Chapter 2

Preparations

2.1 Preparation of Lung Surfactant

Surfactant used for most of the tests was prepared from pig lungs. Temporal and fiscal reasons of economy were: freshly removed lungs from pigs (killed by electrocution) were readily obtained from Newfoundland Farm Products, and the yield of surfactant per man-hour of preparation was higher using pig lungs than with smaller, more expensive laboratory animals. One batch of surfactant from rabbits was also used.

Surfactant can be prepared from extracts of lung minces or from lung lavage. Lung lavage yields a "cleaner" surfactant which is less contaminated by cellular constituents (Morgan et al, 1965b; Tanaka & Takei, 1983). Lung lavage was used for the preparations in this study.

Pig lungs were obtained from the local abbatoir and transported to the laboratory on ice. The trachea was intubated with a plastic Tygon tube and tied in place with household string. Whole lung lavage was performed with a cold saline solution, solution #1, containing NaCl .15M, $MgCl_2$ .003M, $CaCl_2$ .003M, and THAM .005M, adjusted to pH 7.4 (see appendix A.1) (King & Clements, 1972). The lungs were

filled with the saline (approximately 2 litres) then drained by turning them upside-down and gently compressing them manually. This was repeated three times. The recovery volumes were not recorded as it was not intended to quanitate the yield of the surfactant. The lungs were received with some sections collapsed and hemorrhaged. Foamy, blood-tinged fluid was seen in the cut tracheae. This may have been partly the result of rough handling at the abbatoir, and partly may have been associated with the pigs' reaction to slaughter.

The initial procedure for purification of surfactant was a modification of the technique described by Yu, Harding, Smith & Possmayer (1983) for cow lungs (see appendix A.2). This involved 3 centrifugation steps using a saline solution. With dialysis and lyophilization the total time of processing the preparation was 4 days. The resuspended pig lung surfactant had good surface active properties when tested on the surface balance (minimum surface tension < 10 mN/m), but contained a relatively low phospholipid content as determined by phosphorus assay (appendix A.6) and as compared to that reported in the literature, which is 80-90% by weight (Table 2-1).

The low phospholipid content was thought to reflect contaminating materials, mainly proteins. Since contaminating proteins and other blood constituents could be confounding to subsequent tests of mixtures of surfactant and plasma, other processing techniques were used to improve the phospholipid content.

Table 2-1:
Pigsam preparations:
object to produce surfactant
with low surface tension (gamma-min)
of less than 10 mN/m and containing
80-90% phospholipid (PL) by weight

date of harvest	colour of lavage	processing technique	gamma-min on surface (10 ug load)	% PL by wt
13/7	bloody	Yu et al (1983)	5 & 7	50%
	bloody	Yu et al (1983) and NaBr	0 & 0	86%
18/7	bloody	King & Clements (1972)	2 & 3	85%
	bloody	Yu et al (1983) and NaBr	5,7 & 0	85%
	clean	Yu et al (1983)	2,0 & 0	74%
1/8	bloody	Yu et al (1983) and NaBr	0,5 & 0	85%
	bloody	Yu et al (1983) Na Br and Shelley et al (1977)	4 & 5	83%
8/8	bloody	Yu et al (1983) and NaBr	2 & 5	68%
	bloody	Yu et al (1983), Na Br and Shelley et al (1977)	5 & 5	86%

The procedure of King & Clements (1972) and the procedure of Shelley, Paciga & Balis (1977) were tried. These techniques involved separating surfactant with a high phospholipid content from contaminating proteins. NaBr was used to create density gradients, and centrifugation separated the surfactant from the proteins as they settled at their isopycnic densities in the gradient. It was noted that the NaBr floatation step removed the blood-staining of the material and the material became white.

The King & Clements (1972) protocol was followed for the production of their "W1" fraction containing the surface active material. The modification of the protocol of Shelley et al (1977) used in this study, involved using material prepared from the protocol of Yu et al (1983) and which had been centrifuged in one NaBr floatation step, then centrifuging this material once in a discontinuous NaBr density gradient. The method of this study differed from that of Shelley et al (1977) only in that two similar discontinuous NaBr density gradient centrifugations were used in their protocol.

It was found that once the phospholipid content was about 85% by weight, additional centrifugations did not increase the phospholipid content higher than 85%. For the test preparation used in the study (date of harvest 1/8, Table 2-1), one density gradient centrifugation improved the phospholipid content to 85% by weight.

The protocol from which our stock supply of surfactant was

produced included one centrifugation to remove cells, a centrifugation to pellet the surfactant, one centrifugation with NaBr to float the surfactant, a second pelleting centrifugation, dialysis and lyophilization (appendix A.2). The phospholipid fraction was 85% by weight as determined by phosphorus assay (lipid weight taken as 25 times the phosphorus weight), and had gamma-min less than 10 mN/m. Gamma-min is the surface tension obtained on compression of a surfactant monolayer at minimum surface area of the surface tension balance. The methods of testing surface tension on the surface tension balance are described in the following section 2.2. In Table 2-1, this surfactant is listed as pigsam harvested on 1/8, processed through Yu et al (1977) and NaBr. Seven pig lungs were used, yielding about 200 mg of lyophilized material. For testing, the lyophilized material was resuspended in solution #1 using a glass stirring rod, vortexing and sonication (appendix A.6).

The criteria for satisfactory surfactant used in this study, employed % phospholipid (PL) by weight and gamma-min < 10 mN/m. Other characteristics for surfactant have been stated in the literature (Notter & Shapiro, 1981; Taeusch et al, 1983) but were not tested at this time.

Once the processed material was 85% PL by weight further centrifugations did not increase the % PL composition. This may indicate association of the specific lung apoprotein to the PL in the surfactant. It is interesting to note that the surfactant preparations with low % PL composition, which

implied a high protein content, also had low minimum surface tensions (Table 2-1).

2.2 Test conditions for the surface balance

Surface tension measurements were performed on a Kimray-Greenfield surfactometer (a Langmuir-Wilhelmy balance, Figure 2-1) housed in a box to maintain temperature and humidity. The air temperature was 37 $\pm$ 2 degrees C and the subphase temperature was 33 $\pm$ 2 degrees C. An open beaker of H_2O was kept within the box. The trough had an available surface area of 53.4 cm^2 which could be compressed to 7.5 cm^2. A cycle speed of compression and relaxation took 34 seconds.

The balance was zeroed after the platinum flag had been roughened with fine emory paper, rinsed and heated to an orange colour over a bunsen burner. A subphase of 25 ml of solution #1, which was prepared with doubly distilled water (the second distillation using $KMnO_4$), was poured into the trough, and the surface cleaned by repetitive compression and aspiration until the surface tension was 70 mN/m.

The sample to be tested was gently laid onto the surface of the subphase by touching a droplet formed at the end of a Hamilton syringe needle to the surface (Figure 2-2).

An example of the surface tension hysteresis loop of a surfactant monolayer (Figure 2-3) compressed and expanded on the surface balance is found in Figure 2-4. Surfactant function was measured as the surface tension at the minimum surface area during the first compression.

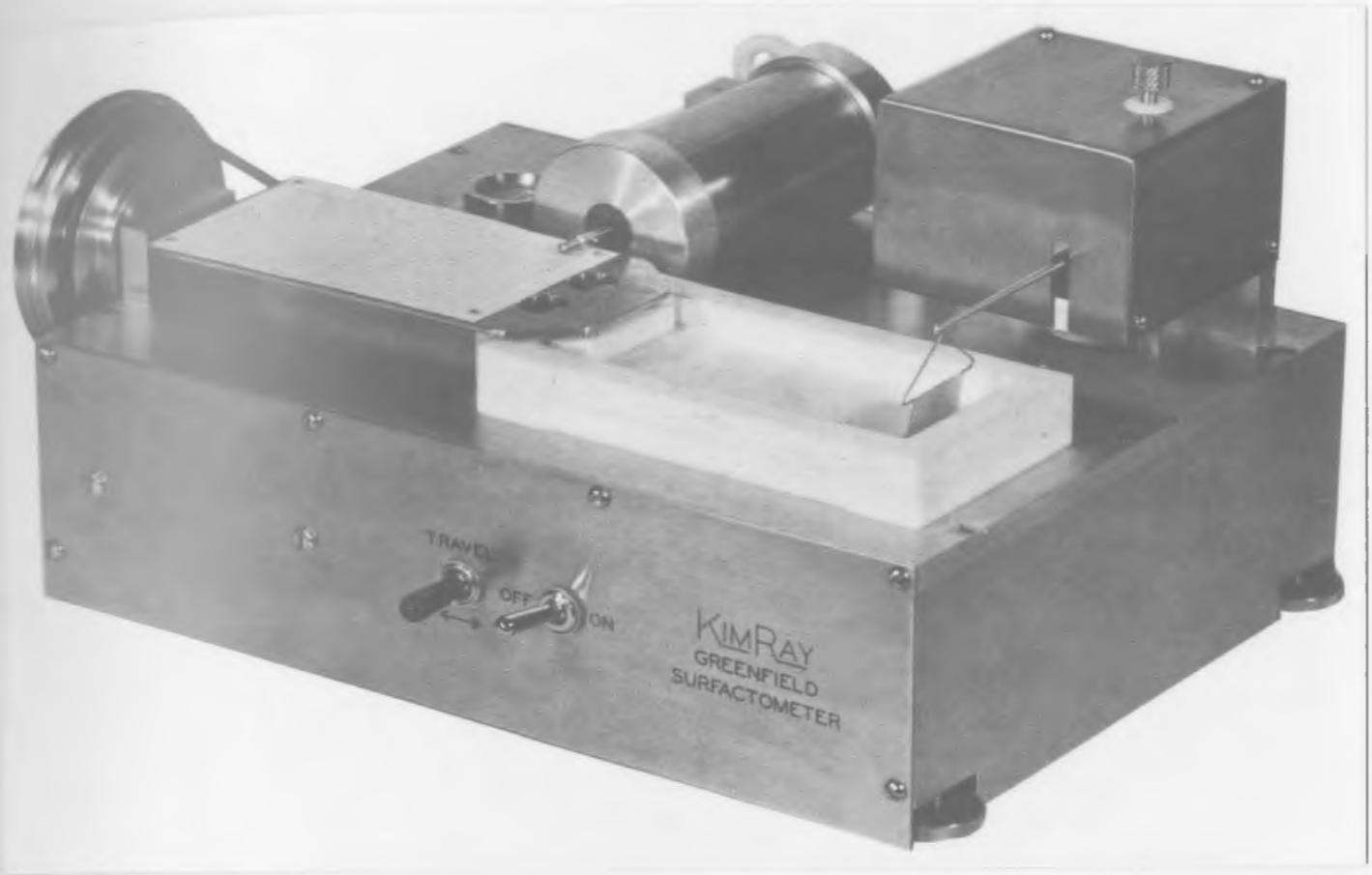


Figure 2-1: A Langmuir-Wilhelmy surface tension balance

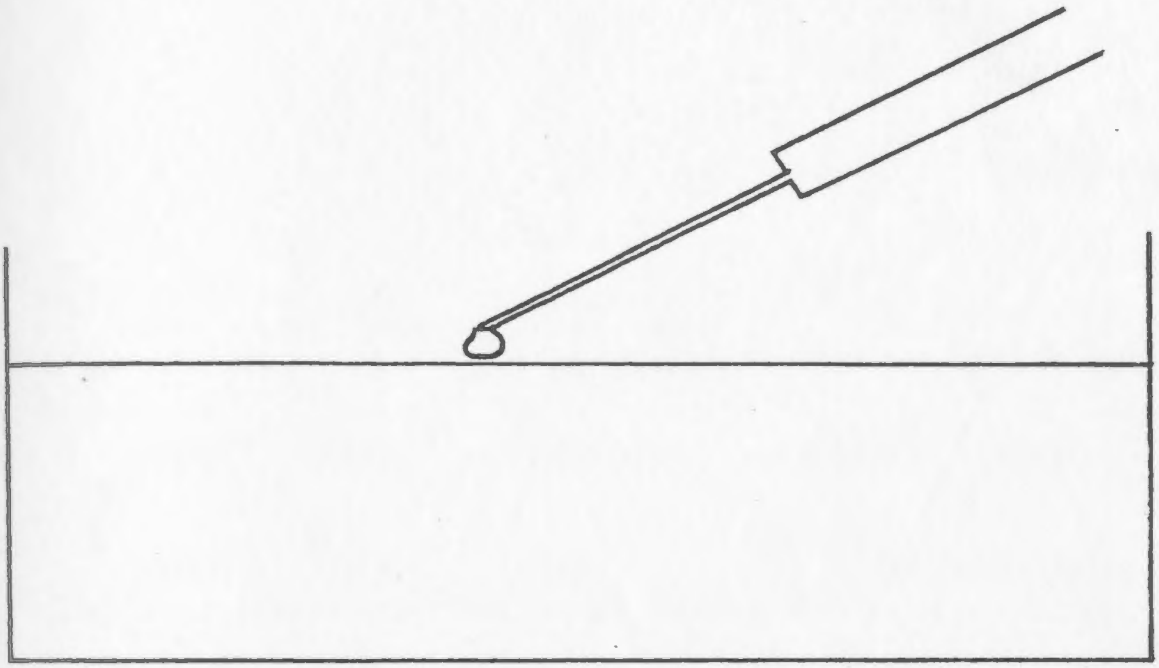


Figure 2-2 Droplet technique for monolayer formation

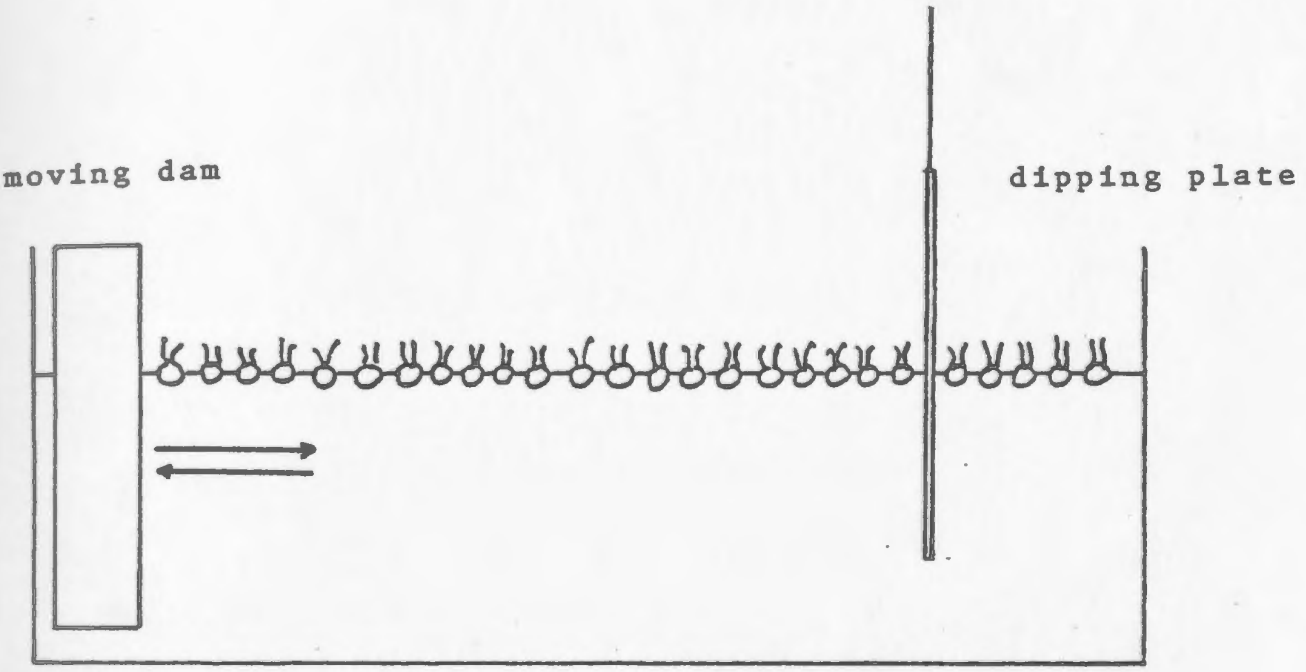


Figure 2-3 Surfactant monolayer

$LaCl_3$-DSPC-DPPC treatment

The teflon trough and moving barrier were treated with solution of a saturated lecithin and lanthanum chloride (Hildebran et al, 1979; Goerke & Gonzales, 1981). Before treatment the teflon trough was rinsed with distilled water then methanol:chloroform, 1:1 v:v, and air-dried. The side walls of the trough and barrier dam were swabbed with a cotton-tipped applicator dipped in a solution of 0.1M $LaCl_3$ and 0.125mM DSPC in methanol. This was allowed to dry for at least 5 minutes. 25 ml of solution #1 was placed in the trough. 5 ul of a 2 mg/ml DPPC solution in hexane:ethanol, 9:1 v:v, was placed on the surface of the hypophase. The surface was then compressed for 3 cycles. A second 5 ul of the DPPC solution was placed on the surface and compressed for 3 cycles.

The $LaCl_3$-DSPC-DPPC treatment of the trough seemed to decrease the amount of leak of surface active material around the barrier dam and up the side walls of the trough. The compression shoulder approaching 5 mN/m was sharper and occured earlier during the compression phase for the treated troughs as compared with the untreated troughs (Figure 2-5).

Test for surface leak

An objective test of leak of surface active molecules from the surface and the performance of the balance was a test of area/molecule for DPPC. DPPC, 3 ul of a 2 mg/ml solution, was compressed on the surface. The area at which 20 mN/m

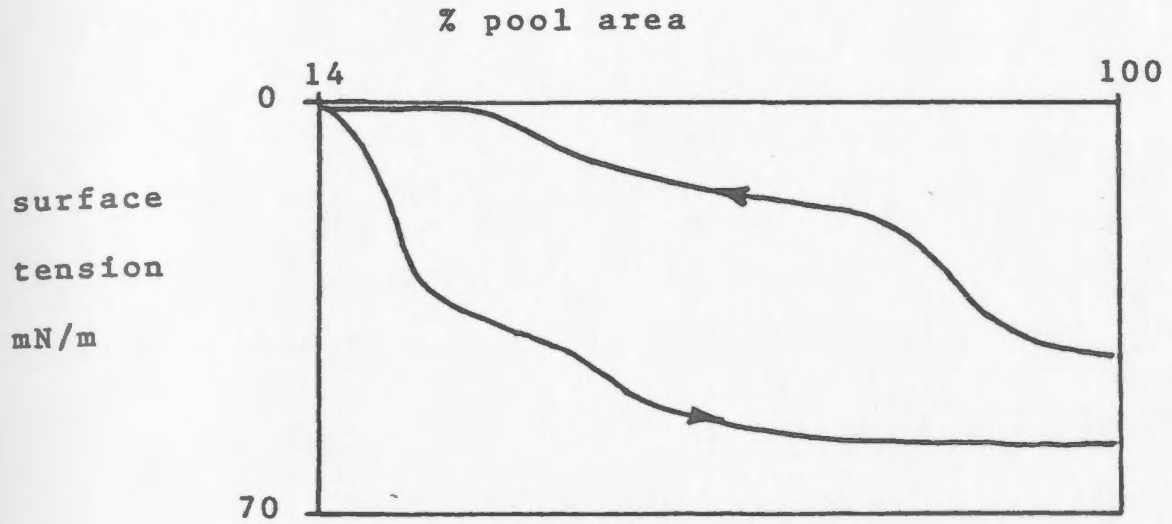


Figure 2-4 Example of surfactant hysteresis loop of surface tension

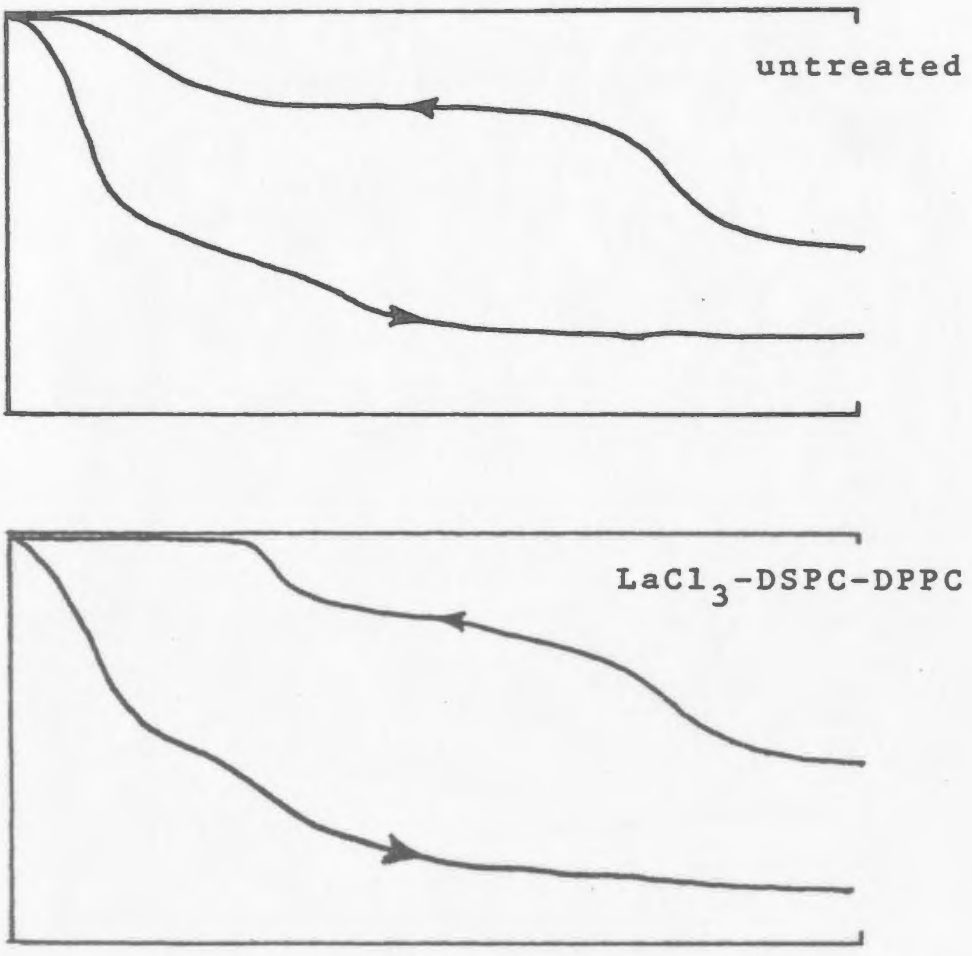


Figure 2-5 The effect of $LaCl_3$-DSPC-DPPC

was reached was used to calculate the area/molecule ratio. This value was checked against the value of 45 angstroms^2/molecule for DPPC at 20 mN/m (Trauble, Eibl & Sawada, 1974). Calculated values less than 45 angstroms^2/molecule indicated that surface active molecules were being lost from the surface and that corrective measures were needed (eg. change of trough and/or barrier, or retreatment with $LaCl_3$-DSPC-DPPC).

Effect of the hypophase solution

The effect of the hypophase solution was tested using a surface load of 10 ug of surfactant. The solutions tested were solution #1 (with 0.15M NaCl, 0.003M $CaCl_2$, 0.003M $MgCl_2$, 0.005M THAM, pH 7.4) and a 0.6M sucrose solution (with $CaCl_2$ 0.003M, THAM 0.005M, pH 7.4) (Baritussio, Magoon, Goerke & Clements, 1981). Compression was started 5 minutes after the surfactant had been placed on the surface.

The minimum surface tensions (gamma-min) were:

solution #1 - 2 & 5 mN/m

sucrose - 2 & 3 mN/m.

Effect of layering time

The effect of the length of time from placing the surfactant on the surface to beginning compression (layering time) was tested using 10 ug of surfactant on a hypophase of solution #1.

The minimum surface tensions were:

1 min - 4 & 4 mN/m

3 min - 3 & 4 mN/m

5 min - 2,5,0 & 7 mN/m

10 min - 3 & 3 mN/m

Effect of surfactant surface load

The surface load of surfactant was tested using a hypophase of solution #1 and a layering time of 5 minutes.

The minimum surface tensions were:

10 ug - 2 & 5 mN/m

20 ug - 3 & 4 mN/m

Effect of spreading solution

The spreading solution, which is the solution used to resuspend lyophilized surfactant, was tested with 10 ug of surfactant on a hypophase of solution #1. Recall that DPPC has poor spontaneous spreading characteristics. Two solutions were tested: solution #1 and an isopropanol:water, 2:1 v:v, solution. The effect of layering time was also considered since time for the isopropanol to evaporate and residual isopropanol on the surface might affect the ability of the surfactant to reach low surface tensions.

The minimum surface tensions were:

isopropanol:water - 6 & 6 mN/m (5 min layering)
- 4 & 6 mN/m (10 min layering)

solution #1 - 2 & 5 mN/m (5 min layering)
- 3 & 3 mN/m (10 min layering)

Summary

There was no obvious difference between the hypophase solutions of sucrose and solution #1. There was no obvious difference between the layering times from 1 to 10 minutes. There was no obvious difference between the surfactant surface loads of 10 and 20 ug. There may be a difference in the spreading agents solution #1 and isopropanol:water. The isopropanol:water did not improve the gamma-min, and may have even moved it away from zero. The isopropanol may denature the protein of surfactant and plasma in subsequent testing and influence the results.

2.3 Test conditions for surfactant-plasma interactions and methods of plasma preparation

The effects of plasma on surfactant function have been tested with various methods on the surface balance. The method used in this study involved placing a premixed suspension of plasma and surfactant on the surface of a hypophase solution of saline. This model of surfactant and protein interaction tested the situation where surfactant and protein both exist at the alveolar air-water interface.

The initial collection of plasma from human subjects was performed with Vacutainer tubes containing heparin. Heparin was used as an anticoagulant instead of EDTA for two reasons: i) EDTA would chelate calcium which may be important for surfactant function, and ii) heparin is used as the anti-coagulant during CPB. The blood was centrifuged in a bench-top centrifuge (IEC clinical centrifuge; setting = 6) for 5 minutes and the plasma removed with a pasteur pipette. The total protein concentration of the plasma was determined using the Biuret method (Gornall, Bardawill & David, 1949) (appendix A.8).

The teflon trough and barrier dam had been prepared with $LaCl_3$-DSPC-DPPC. The hypophase solution used was solution #1, and the layering time 4 minutes.

Surfactant and plasma were mixed in a glass vial by vortexing and by passing the mixture into and out of a 50 ul Hamilton syringe seven times. The mixtures tested were surfactant-plasma at 2:1, 1:1, 1:2 v:v. The surfactant-plasma mixture was placed on the surface with a constant surface load of 10 ug (5 ul of a 2 mg/ml solution) of surfactant. The time of interaction, ie. the time for mixing in the glass vial before placing on the surface of the hypophase, was tested at 5 and 30 minutes. The effect of dilution was tested by mixing the surfactant with a saline solution (Ringer's lactate) at 2:1, 1:1, 1:2 v:v. Again the surface load of surfactant was kept constant at 10 ug.

The data showed that surfactant alone reached lower surface tensions than mixtures of surfactant and plasma, defined as surfactant inhibition. Figure 2-6 is an example of the surface tension diagrams of a mixture of surfactant (7.5 ug phospholipid) and plasma (10 ul or approximately 0.8 mg total protein) and of surfactant (7.5 ug PL) alone. It was seen that the degree of surfactant inhibition increased with the amount of plasma added. Delta gamma-min, the difference in minimum surface tension of the surfactant-plasma mixture from that of surfactant alone, was plotted on the y-axis vs the volume of plasma added on the x-axis (Figure 2-7). The gamma-min for each aliquot of resuspended pigsam had been tested before and after each series of tests of the mixtures of plasma and surfactant. It was determined that the line of best fit was linear for both the 5 min and 30 min times of interaction, ie. delta gamma-min, a measure of the degree of inhibition, varied directly with the volume of plasma. There was no significant difference between the data for the 5 vs 30 minutes of interaction time, $p>.25$. The statistical methods are discussed in section 2.4.

There was no effect of dilution of surfactant with the saline solution on the gamma-min at 2:1, 1:1, 1:2 v:v, which corresponds to 2.5, 5.0, 7.5 ul of diluting solution, respectively. Further consideration of dilutional effects is considered in a later section.

A question of the effect on plasma proteins by vacutainer rubber was raised. The literature suggested that the serum

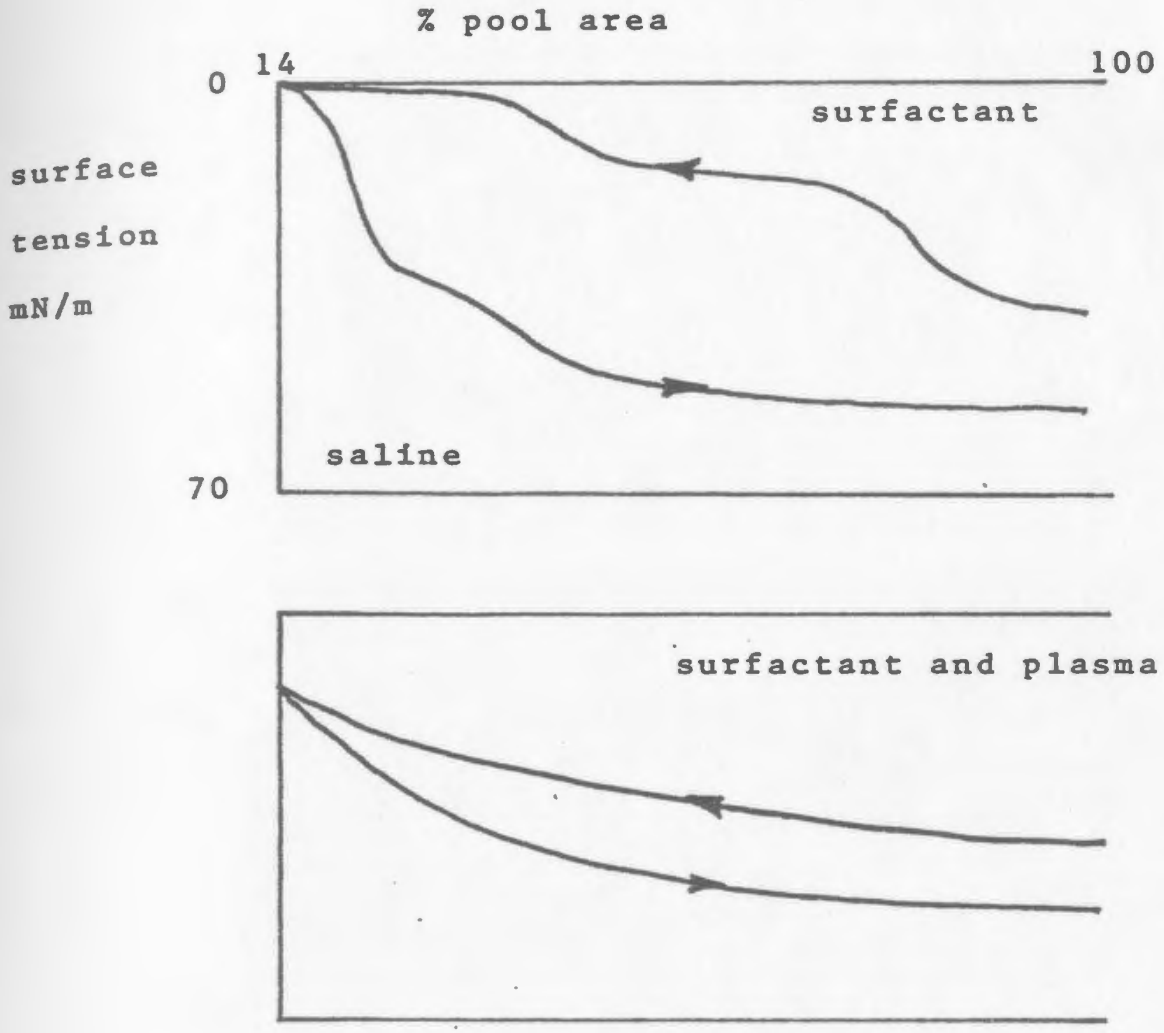


Figure 2-6 The effect of plasma on surfactant

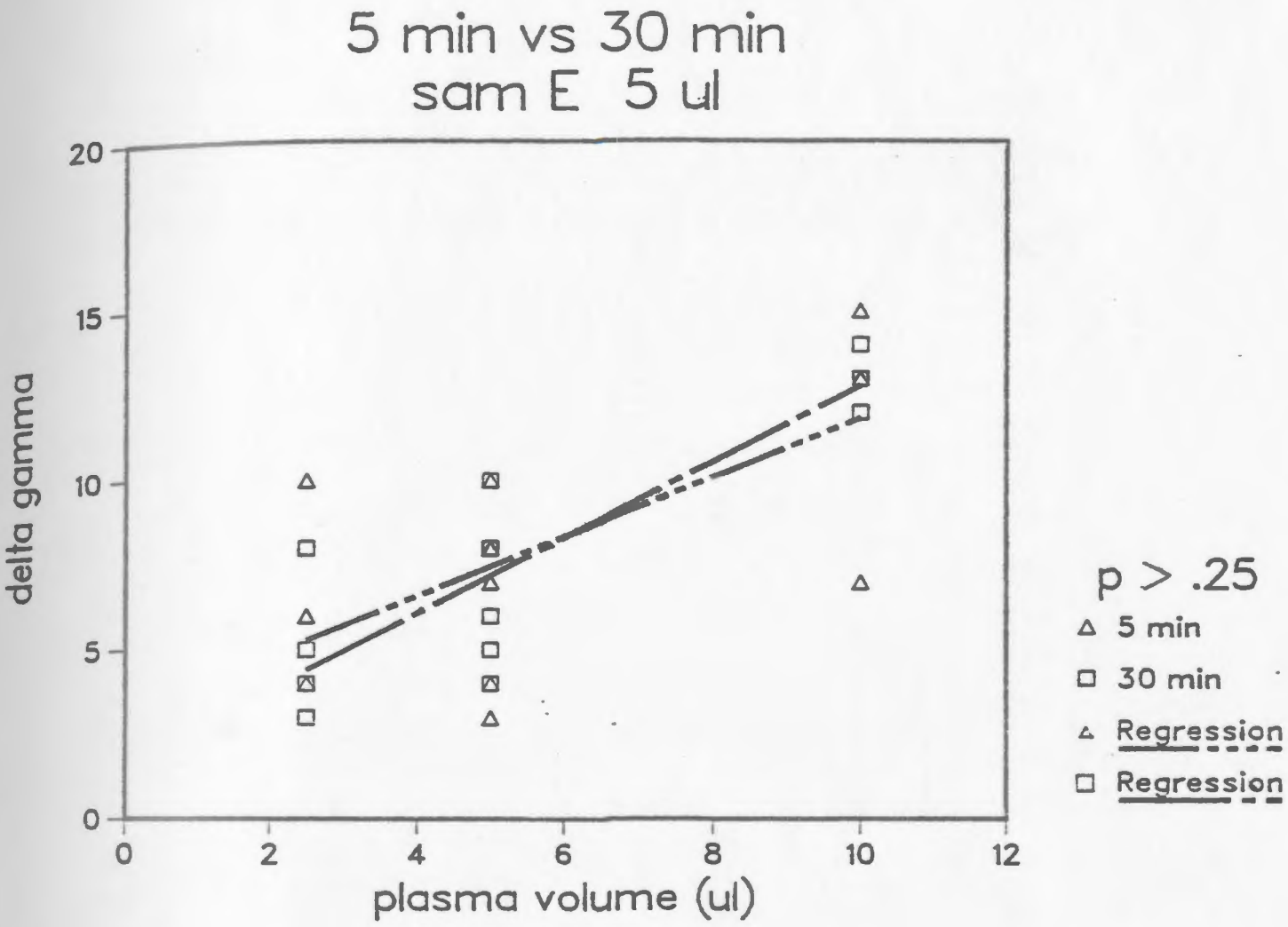


Figure 2-7 The effect of interaction time:

5 min vs 30 min

levels of certain drugs may be lowered because the rubber may displace the drug from alpha-1-glycoprotein binding sites (Ooi, Pozanski & Smith, 1980). In any event we chose to collect the blood subsequently using plain glass syringes and plain glass tubes with parafilm for covering.

2.4 Statistical methods

These methods were developed with the expert advice of Dr.A.Cornish, Department of Statistics, MUN.

The data was plotted with (x,y) coordinates. The method for determining the line of best fit for the data used quadratic regression analysis. By definition of delta gamma-min, the line of best fit should go through the origin.

If the line of best fit was linear and went through the origin, then for the regression line y=a+bx, the t-value, ie. the value of the t-statistic given by the regression, for the "a" term would not be significant. If "a" was significant, then the linear regression line of best fit for the data did not go through the origin. Within the range of x tested the linear regression line of best fit with the significant y-intercept ("a"), would better describe the data than a regression line forced through the origin. Between the origin and the lowest x value tested, the line may have a different slope.

If the line of best fit was non-linear, then the quadratic term in the regression would have a significant t-value.

Other lines of best fit may then be tested (eg. power, exponential, log) as these may be written as a polynomial to the n-th degree. If the quadratic term was not significant, then the linear regression line was truly the line of best fit. One need not apply other lines (eg. power, exponential, log) to the data.

As determined, all groups were best described by linear regression, except one, the adult CPB-2, sam F group (a result of the next section on the study of the effect of CPB on surfactant inhibition). Comparisons between the groups were tested using the overall F-test method for coincident linear regressions (Zar, 1974). The lines of best fit were used for comparisons, not the forced lines through the origin.

2.5 Rabbit surfactant control

To check that the inhibitory effects of human plasma were not peculiar to pig lung surfactant, a small quantity of rabbit lung surfactant was prepared using the same protocol used for the BAL for pig lungs. The rabbit surfactant had a phospholipid content of 80% by weight as determined by phosphorus assay.

Mixtures of normal adult human plasma and rabbit lung surfactant were tested under the same test conditions on the surface balance. It was observed that the plasma prevented the rabbit surfactant from reaching low surface tensions, and that the amount of inhibition varied directly with the surface load of plasma (Figure 2-8).

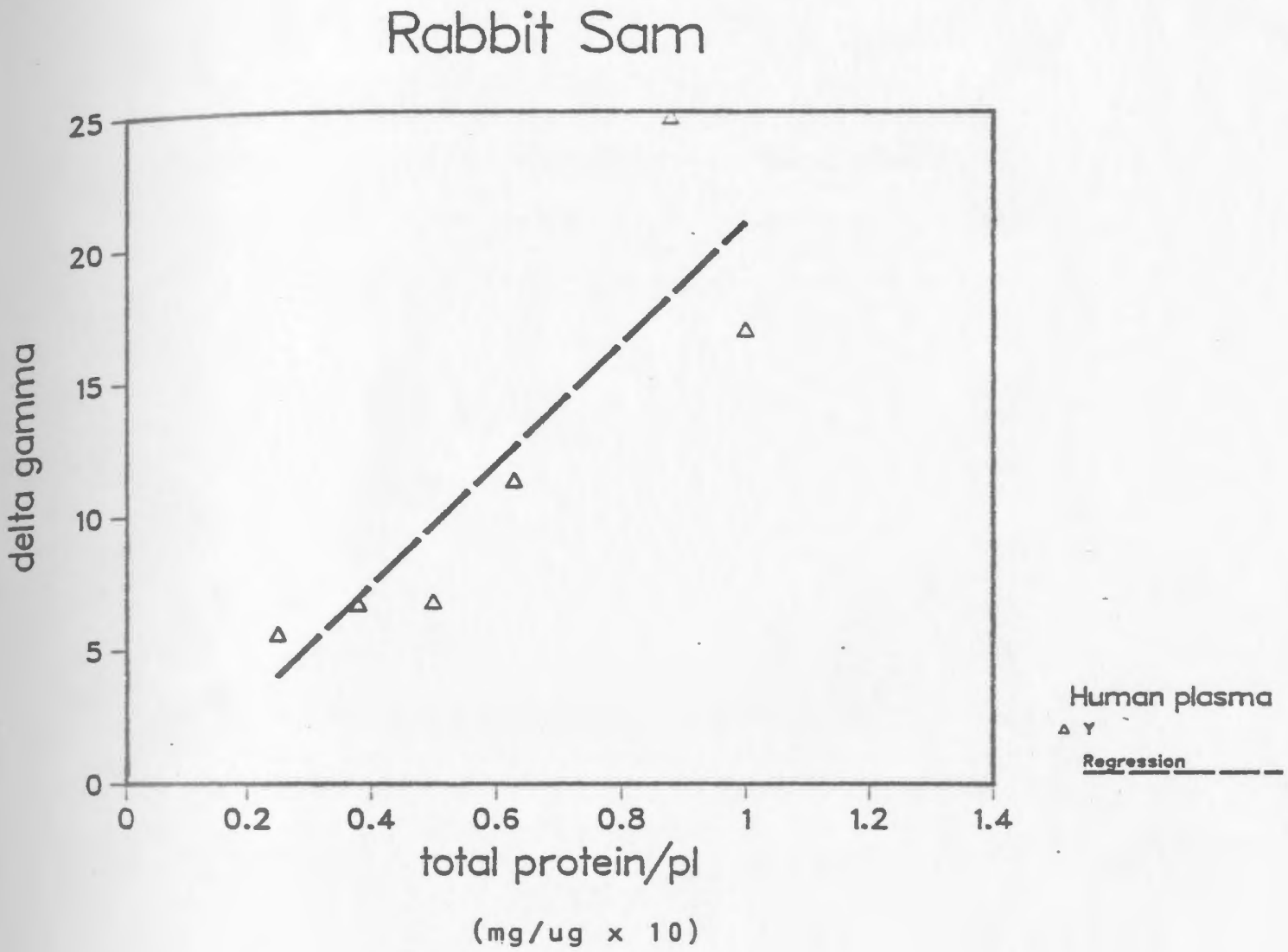


Figure 2-8 The effect of human plasma on rabbit lung surfactant

2.6 Summary

Plasma was shown to prevent surfactant from reaching low surface tensions under the stated conditions of testing. The result was not peculiar to pig lung surfactant. Methods of statistical comparisons between test groups were developed using regression analysis and an overall F-test.

Chapter 3

A study of the effect of cardiopulmonary bypass on surfactant inhibitors

3.1 Introduction

It was observed in the last section that plasma inhibited surfactant. In this chapter the effect of CPB on plasma was investigated with regard to the production or elevation of levels of circulating surfactant inhibitors in the plasma.

There is lung dysfunction following CPB which ranges in severity from minor atelectasis to RDS. RDS is a rare complication of elective adult cardiac surgery employing CPB, but is still seen with significant frequency in children and in cases with prolonged bypass. The CPB is thought to invoke an inflammatory response in the blood which injures the lung blood-air membranes. The hypothesis was that plasma components which leaked into the alveoli through the damaged membranes could inhibit surfactant from proper function. Studies on the effect of CPB on potential inhibitors in plasma had not been previously investigated in humans.

The test groups consisted of normal adults, adult patients undergoing elective CPB surgery and child patients

undergoing CPB surgery. Protocols were approved by the Human Investigation Committee of the Faculty of Medicine, Memorial University of Newfoundland. Permission to collect blood was obtained from the hospitals, physicians and subjects involved.

Samples were taken at several times perioperatively in the adult patients to examine the timing of surfactant dysfunction in relation to the timing of the lung injury. Hemodilution was used in all cases. Bubble oxygenators (Cobe) were used for most of the CPB but membrane oxygenators (Cobe) were also used occasionally.

3.2 Methods

The blood was collected using plain glass syringes and plain glass tubes with parafilm covering. Heparin was added to the tubes when plasma was required, at a dose of 30 units/ml blood.

Blood from the adult patients was collected at four times, all from an indwelling arterial line. The first sample, CPB-1, was taken on the morning of the operation before the patient was taken into the operating room. The second sample, CPB-2, was taken once the patient was asleep but before CPB was started, to account for possible effects of the anesthetic drugs. The third sample, CPB-3, was taken from the CPB circuit immediately on completion of CPB. The fourth sample, CPB-4, was taken 20-24 hours post-operatively in the intensive care unit.

Blood from the child patients was taken from an indwelling arterial line while the patient was asleep and before CPB was started. A second sample was taken immediately on completion of CPB. (see page 62a)

The blood from the adult normals and the adult CPB groups, CPB-1 and CPB-2, was also used to prepare serum.

Plasma prepared from the blood was kept at room temperature until testing. The tests of surfactant inhibition were done within ten hours of blood collection. The plasma-surfactant mixtures were mixed in a glass vial by vortexing and by passing the mixture into and out of a 50 ul Hamilton glass syringe. The mixture was allowed to interact for at least 5 minutes before testing. A hypophase of solution #1 was used in the teflon trough prepared with $LaCl_3$-DSPC-DPPC. The mixture was added to the surface with the droplet technique and allowed to spread and equilibrate for 4 minutes before compression of the surface was started. The minimum surface tension, gamma-min, was the surface tension at minimum surface area on the first compression cycle. The total protein concentration of the plasma was determined by the Biuret method. The amount of plasma was varied while the surface load of surfactant was constant at 10 ug. The ability of the pigsam to reach low surface tension was checked by testing for the gamma-min of each aliquot of pigsam before and after each of the series of tests of the mixtures of plasma and surfactant.

The effect of hemodilution on the possible inhibitory components was considered. From the literature it seemed

In the pediatric patients, blood was taken once before CPB and once following CPB. Additional samples as in the adults, were not taken for reasons of patient comfort and the small blood volume in these pediatric patients. Also, the adult data on preliminary examination had not showed a significant effect between groups.

The adults ranged in ages from 49 to 70, and the male to female ratio was 4:1. All of the adult patients underwent elective operations for coronary artery bypass grafting.

The child patients ranged in ages from 6 months to 6 years. The male to female ratio was 1:2. The children underwent repairs of congenital heart defects.

likely that the inhibitor was a protein. For this reason, and the fact that any non-proteinaceous inhibitors would be diluted equally with the plasma proteins, the tests of surfactant inhibition were performed with respect to the total protein mixed with the surfactant. ie. Total protein was used as a simple measure to standardize for inhomogeneous concentrations or dilutions among individual samples. Also, the phospholipid content varied by approximately 12% between surfactant suspensions. The variation in the amount of plasma mixed with the constant surface load of surfactant was expressed in terms of mg of total protein/ug of phospholipid. For convenience of statistical calculations, the ratio, mg total protein/ug PL, was multiplied by a factor of 10 to bring the numbers in the range 0 to 1.25. ie. The values of mg total protein/ug PL were in the range 0 to 0.125, were multiplied by 10, and expressed at 0 to 1.25 with the units, mg protein/ug PL x 10.

During the study two suspensions of surfactant were used. Both came from the same batch of lyophilized pig lung surfactant, harvested on day 1/8/83, and processed in our protocol. The resuspensions were performed on different days and are denoted sam E and sam F. The method of resuspending the lyophilized material using solution #1, a glass stirring rod, vortexing and sonication, was the same. Aliquots of both were stored at -20 degrees C. Although the concentration of surfactant was 2 mg/ml the phospholipid

concentrations (taken as 25 times the phosphorus concentration) were different. The concentrations were: sam E, 1.5 mg/ml PL and, sam F, 1.3 mg/ml PL. A likely reason for the different phospholipid concentrations was the hygroscopic nature of the lyophilized surfactant, and differences in water content of the material at the different times of resuspension. Surfactant surface loads of sam E, 7.4 ug PL, and sam F, 6.5 ug PL (equivalent to 10 ug surfactant) were used in tests on the surface balance.

Delta gamma-min, the difference in the surface tensions at minimum surface area of the mixture of plasma and surfactant and of surfactant alone, was plotted against the total protein/phospholipid load on the surface. Regression analysis was used to determine if the line of best fit was linear. Analysis of variance and an overall F-test of the lines of best fit was used to compare the data between groups.

The groups consisted of the different patient populations (normal adults, adult patients and child patients) and the times when the blood was collected (eg. CPB-1 to -4, pre- and postpump). The subjects from whom blood was collected and tested with sam E were separate from those tested with sam F. ie. Sam E and sam F tests were not performed on the test samples from the same patients.

A significance level of 0.05 was used.

Control tests of pump priming solution and dilution

Solution from the pump priming circuit was tested to investigate possible effects of heparin and plasticizers. This solution contained human albumin, heparin, $NaHCO_3$ and Ringer's lactate. Samples were taken once the solution had been circulated in the pump circuit. Tests compared the effect on surfactant function of priming solution from the pump to that of human albumin alone. There was no significant difference between the effects of the two solutions, $p>.25$ (Figure 3-1).

Tests on the effects of dilution were repeated. Surfactant was mixed with varying quantities of solution #1 which approximated the range of volumes used when adding the plasma. These volumes were greater than those used in the previous tests of 2:1, 1:1, 1:2 v:v, because hemodilution techniques were used in the CPB procedures. ie. The plasma protein concentrations were decreased by dilution and in order to test the same range of protein loads on the surface more plasma volume was required. Only one test of dilution with sam E was performed because supply was depleted. Sam G, 1.5 ug PL/ul, surface load, 7.5 ug PL, was used in additional tests of the effect of dilution.

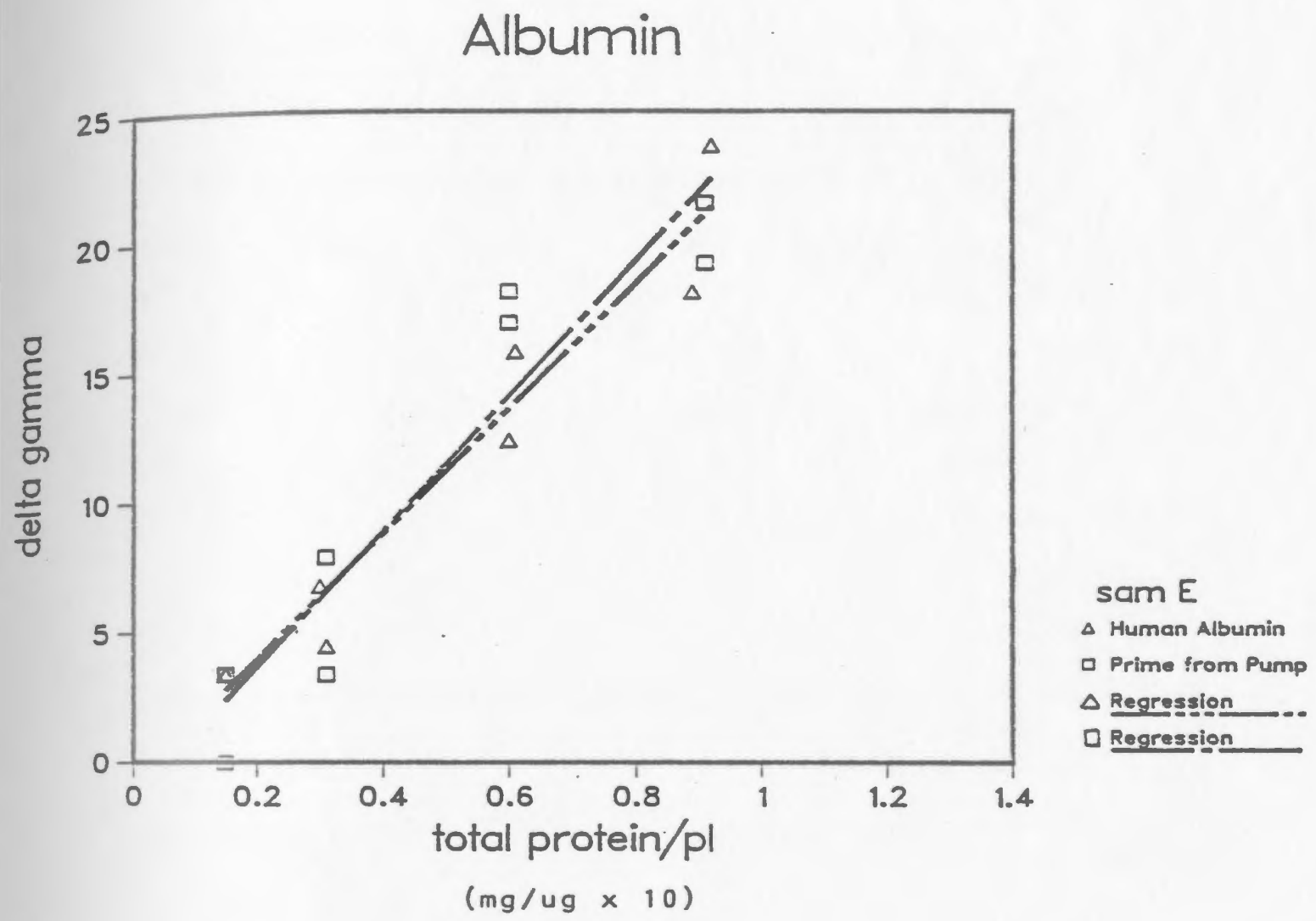


Figure 3-1 Comparison of albumin alone vs priming solution from the pump

3.3 Results

The data from the plots of all groups were best described by linear regression except for one group, the adult CPB-2, sam F group. The t-value for the quadratic term in the quadratic regression was significant (p=.002), implying that the line of best fit was not linear. The finding of one non-linear line of best fit seemed out of context when all the other lines of best fit were linear. A sound practical, chemical or physiologic reason for such an event was not apparent. As well the linear regression described the data for this group with a correlation coefficient which compared well with those of the other groups (Table 3-1). Thus, the linear regression line of best fit was used in comparisons involving the group adult CPB-2, sam F. Table 3-1 gives the parameters of the lines of best fit of the test groups.

Of the adult patients entered in the study, none developed ARDS. Of the child patients, one developed RDS following CPB and subsequently died.

The effect of CPB on potential surfactant inhibitors in plasma was determined by three sets of comparisons between pre- and postpump groups in adults and children. There was internal control in each comparison, ie. the pre- and postpump groups used blood samples from the same group of patients. The first compared the adult CPB plasma groups 1 to 4, using sam E (Figure 3-2). The second compared the adult CPB plasma groups 1 to 4, using sam F (Figure 3-3). Again, the patients from whom blood was collected and tested

with sam E were not the same patients from whom blood was collected and tested with sam F. The third test compared the child CPB groups pre- and postpump plasma, using sam F (Figure 3-4). There was no significant difference between the groups, $p>.25$, for all three comparisons. Increased inhibition was not seen in the plasma of adults taken 20-24 hours after CPB, a time when postoperative pulmonary complications are usually apparent. Thus no significant increase in inhibition by plasma as a consequence of CPB was seen in either children or adults.

To investigate possible differences between adult patients and child patients, the effects of surfactant inhibition by the plasma sampled from these groups were compared in three tests. Comparisons were made i) between the adult CPB-2 and the child prepump groups, ii) between the adult CPB-3 and the child postpump groups, and iii) between the pooled data of the adult CPB groups 1 to 4, and the pooled data of the child pre- and postpump groups. Previous comparisons had shown that there was no difference among the adult CPB groups 1 to 4, or between the child pre- and postpump groups. The validity of comparing the adult and child patients using the pooled data was checked using a test of variance. The variance of the data was estimated to be the same for all of the groups tested.

There was a significant difference between the adult CPB-2 plasma, sam F, $n=4$, group and the child prepump plasma, sam F, group, $n=5$, $.025>p>.01$. The data for the adult CPB-2

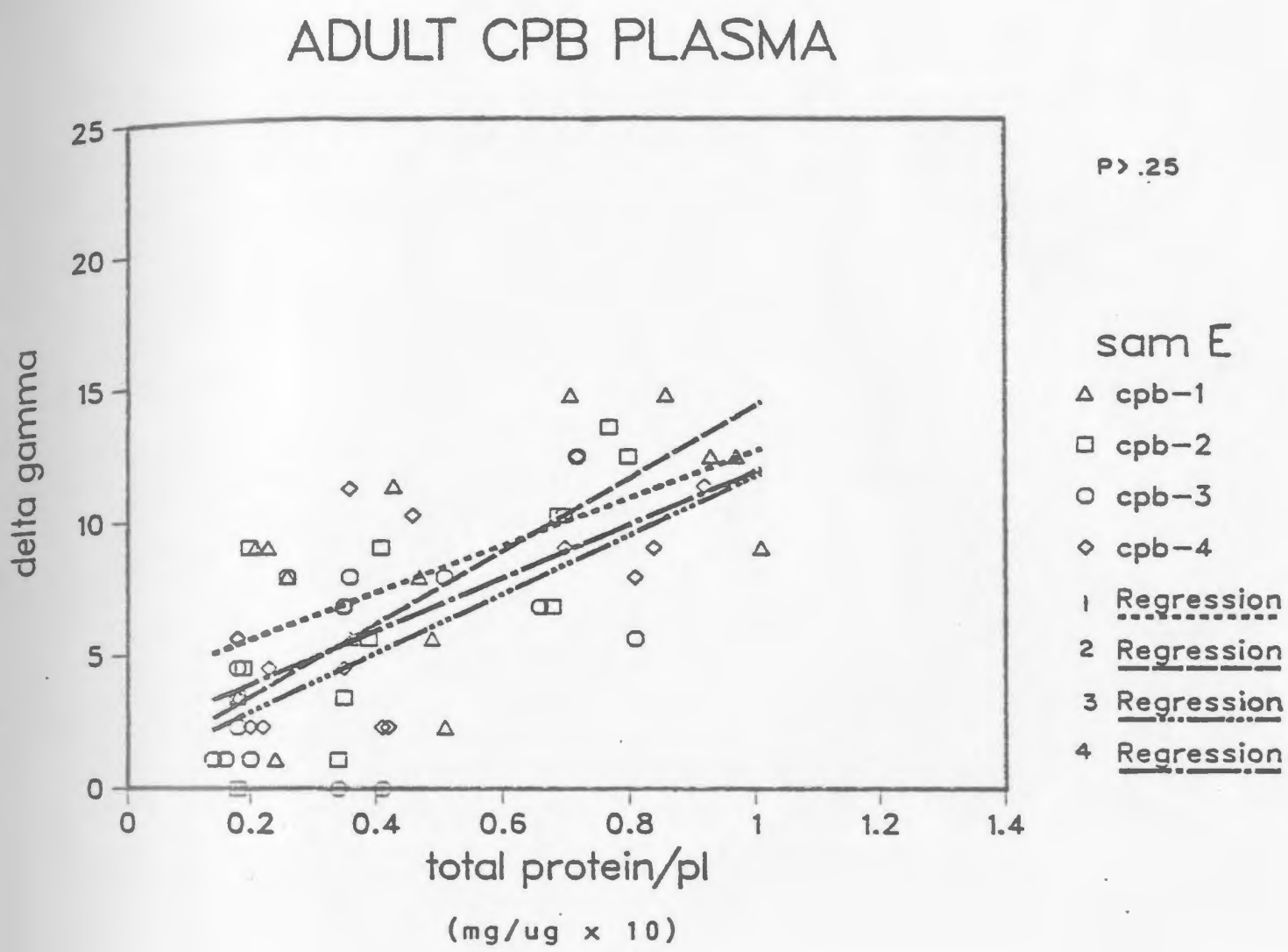


Figure 3-2 Comparison of adult CPB plasma, groups 1 to 4, sam E

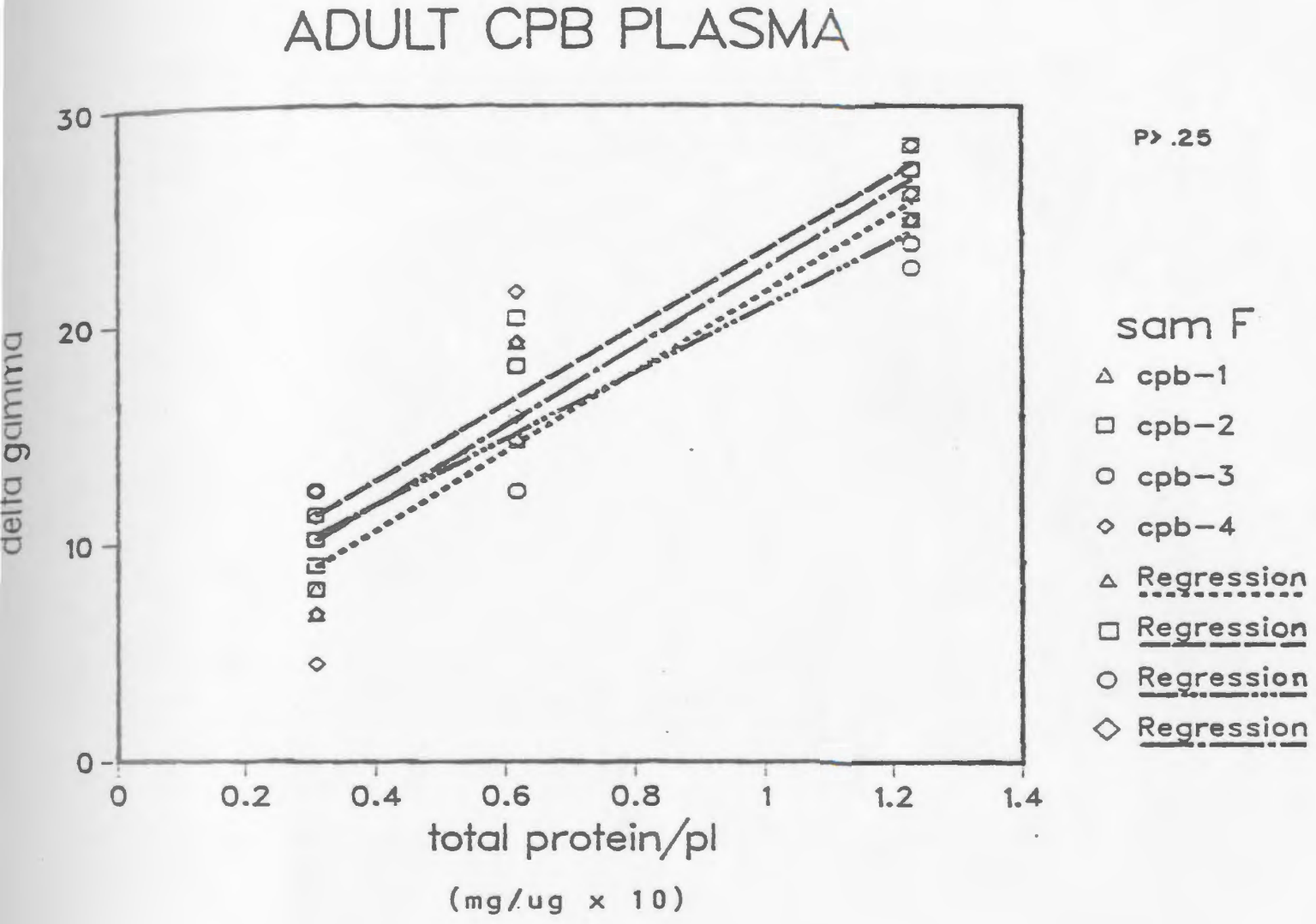


Figure 3-3 Comparison of adult CPB plasma, groups 1 to 4, sam F

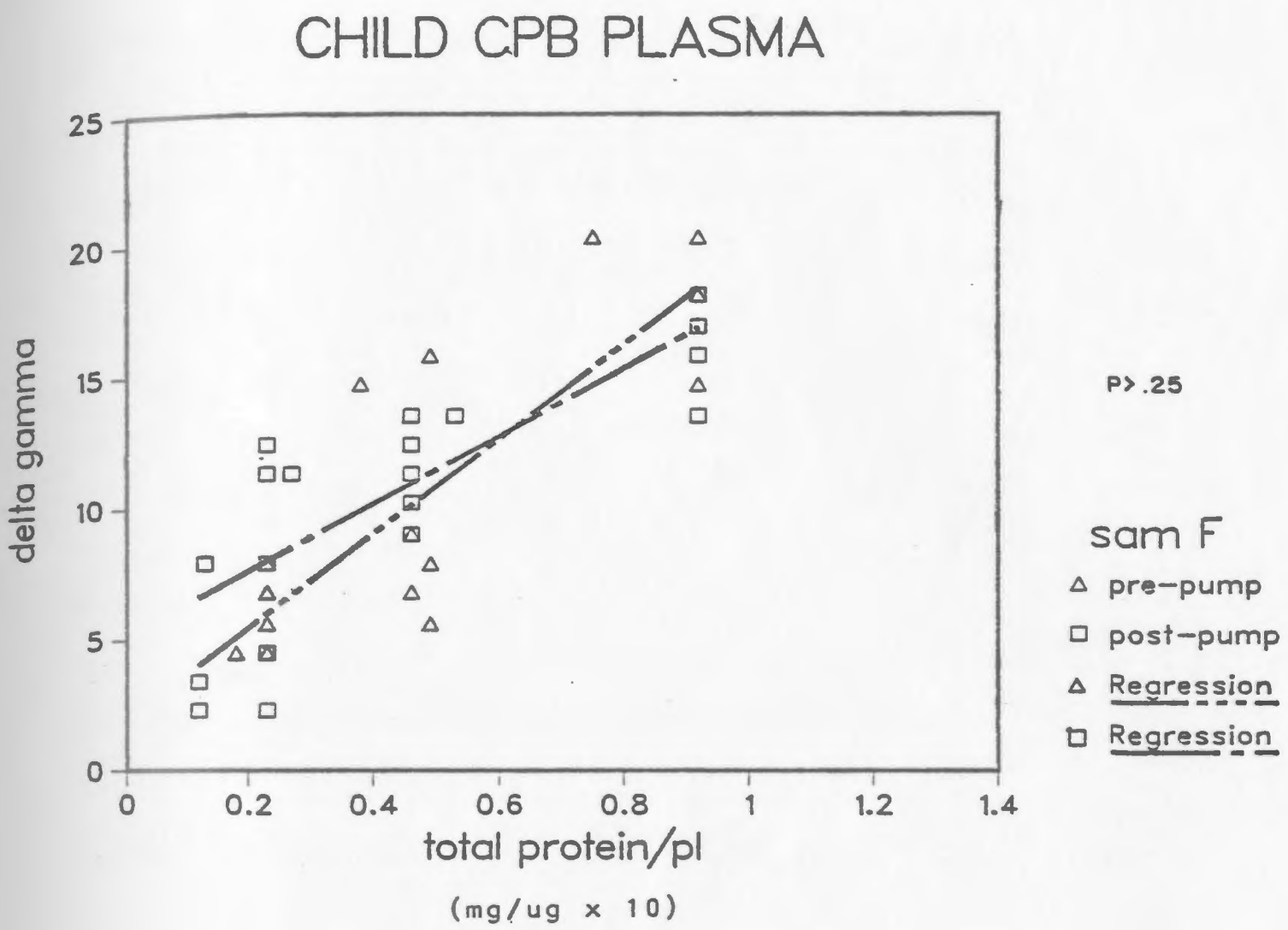


Figure 3-4 Comparison of child CPB plasma, prepump vs postpump, sam F

group showed slightly greater inhibition than that for the child prepump group. The difference was due mainly to a difference in elevation (y-intercept) and not to a difference in slope (Table 3-1). There was no significant difference between the adult CPB-3 plasma, sam F, n=4, group and the child postpump plasma, sam F, group, n=5, $.10>p>.05$. There was a significant difference between the pooled data from the adult CPB groups 1 to 4, plasma, sam F, n=14, and the pooled data from the child pre- and postpump groups, plasma, sam F, n=10, $p<.01$. Again slightly more inhibition was seen with the adult patients than with the child patients (Figure 3-5).

Possible differences in the effects of plasma and sera on surfactant inhibitors in plasma was investigated by comparing plasma and sera in the normal adult groups for sam E and sam F. In both comparisons (plasma vs sera, sam E; plasma vs sera, sam F) there was a significant difference, $p<.0005$. Plasma was more inhibitory than sera in both comparisons (Figures 3-6 and 3-7). The differences were in the slopes of the regression lines for both comparisons.

To investigate the possibility of differences between patients and normals, comparisons were made using an individual CPB group, CPB-1, for the comparison of adult normal and adult CPB patient plasma. The CPB-1 group was chosen because the patients at this time would not have been subjected to anesthetic or surgery. Comparisons were also made using the pooled data from the adult CPB-1 to -4

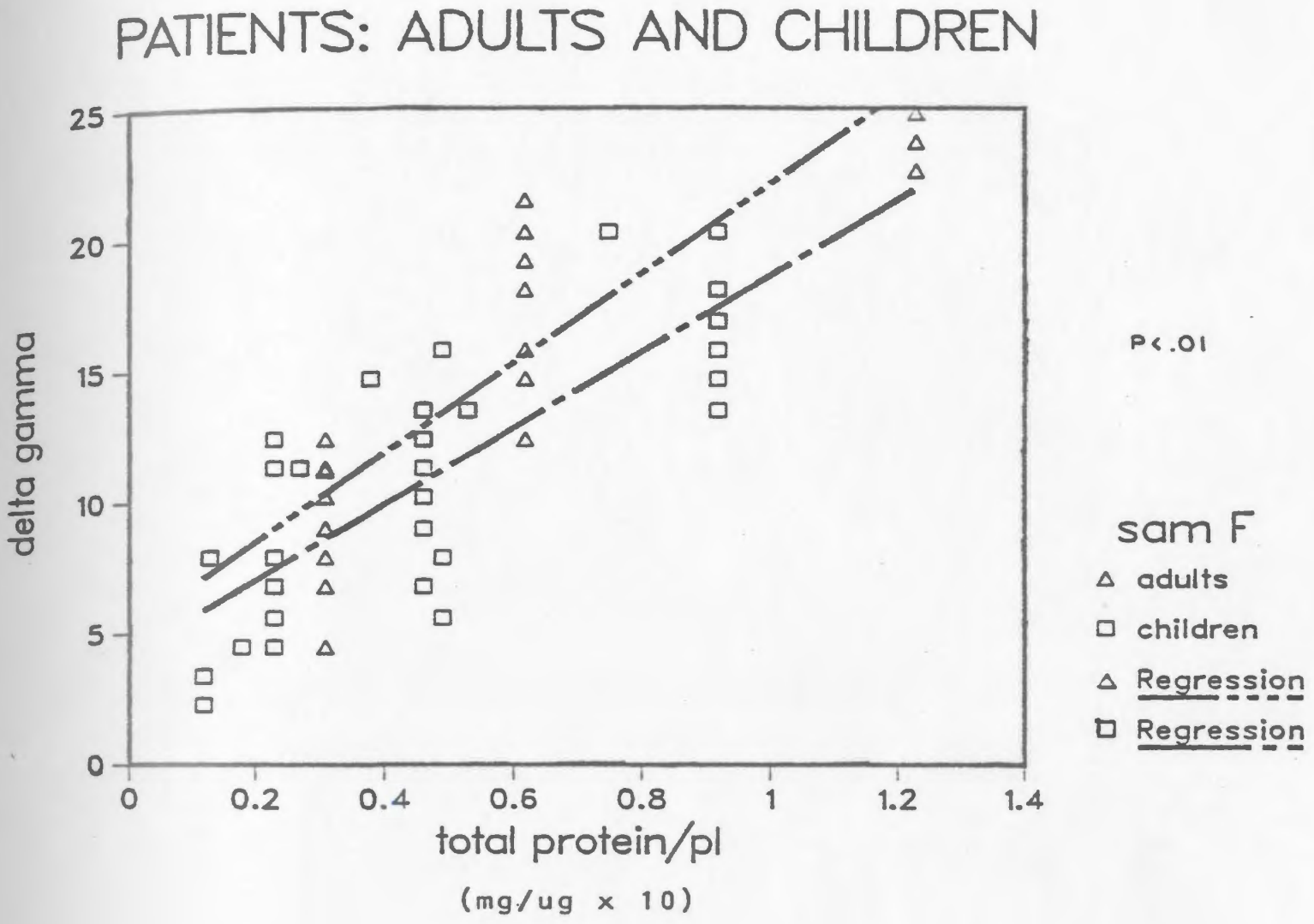


Figure 3-5 Comparison of adult CPB vs child CPB plasma, sam F

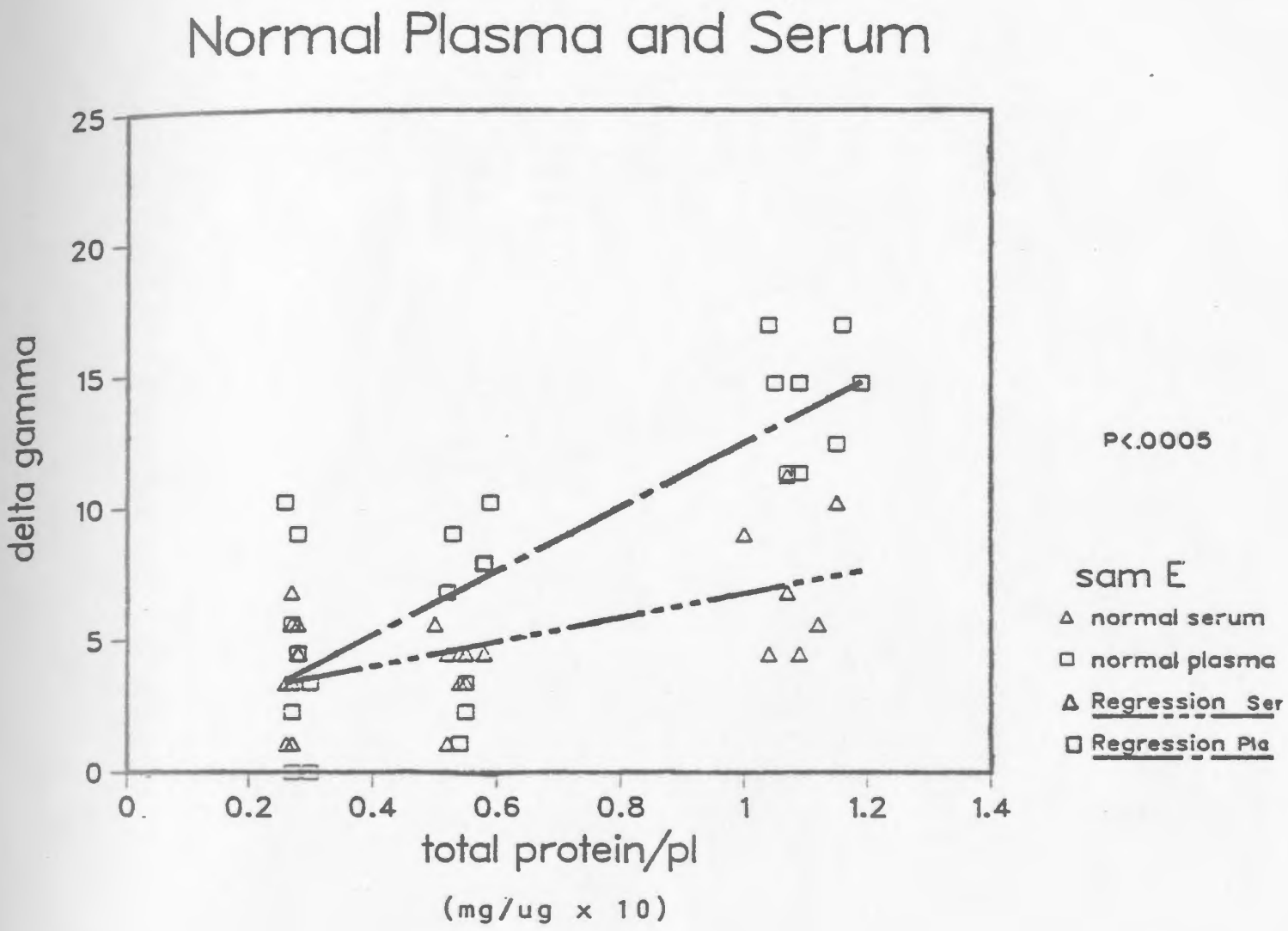


Figure 3-6 Comparison of plasma vs serum, normal adults, sam E

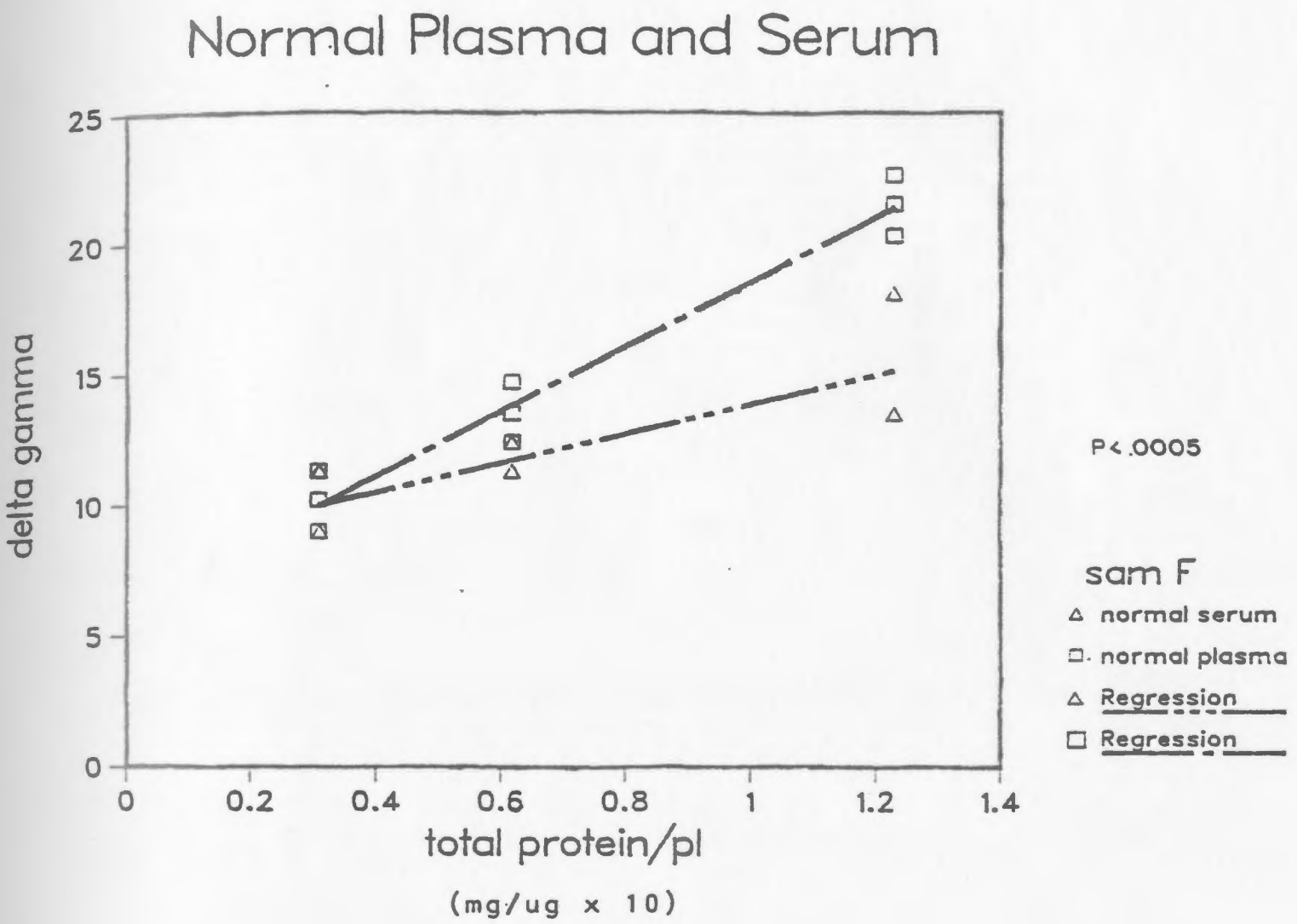


Figure 3-7 Comparison of plasma vs serum, normal adults, sam F

groups, justified by a test of variance for the data of the groups.

There was no difference between the group normal adult plasma, sam E, n=8, and the group adult CPB-1 plasma, sam E, n=5, $.25>p>.10$. There was also no difference between the group normal adult plasma, sam E, n=8, and the pooled data from the adult CPB-1 to -4 plasma, sam E, n=19, $p>.25$ (Figure 3-8). In the respective tests using sam F, there was no difference between the group normal adult plasma, sam F, n=3, and the group adult CPB-1, plasma, sam F, n=2, $.10>p>.05$. However, there was a difference between the group normal adult plasma, sam F, n=3, and the collective adult CPB 1 to 4 plasma, sam F, n=14, $p<.005$ (Figure 3-9). Here, the plasma from the patients showed more inhibition than that from the normal adults, and the difference was in the slopes of the regression lines.

Possible differences between normal adults and adult patients might exist in the sera. Sera was prepared from the adult patients only in the CPB-1 and CPB-2 groups. The pooled "n" for the data of tests with sera collected from the adult patients was five for the sam E group, and four for the sam F group. Here, the pooled "n" equaled the actual number of patients.

There was a significant difference between sera from the group normal adults, sam E, n=8, and the group adult patients CPB 1 and 2, sam E, n=5, $p<.01$. In these sam E - sera tests, the patients showed more inhibition than did

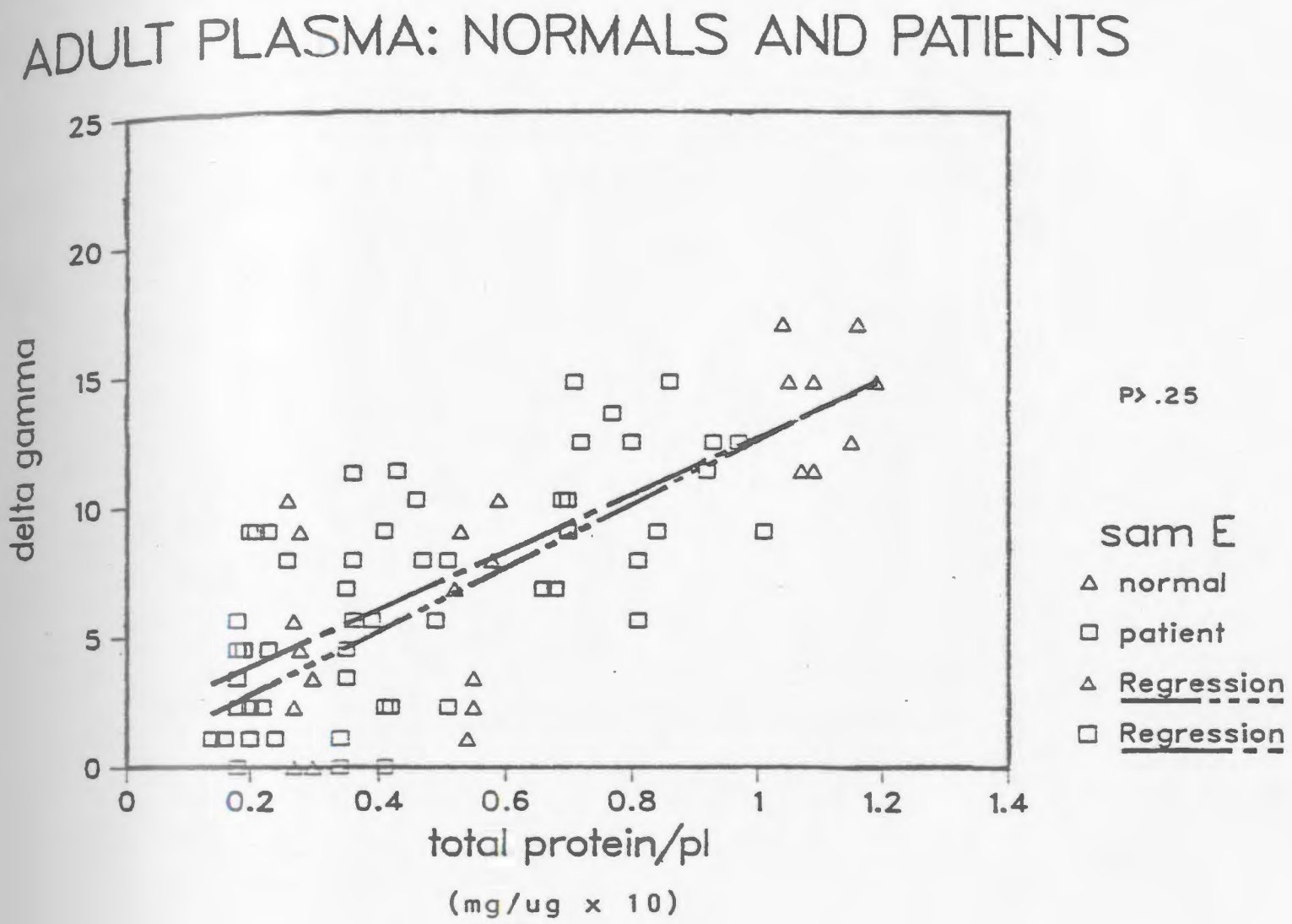


Figure 3-8 Comparison of normal adults vs adult CPB patients, sam E , plasma

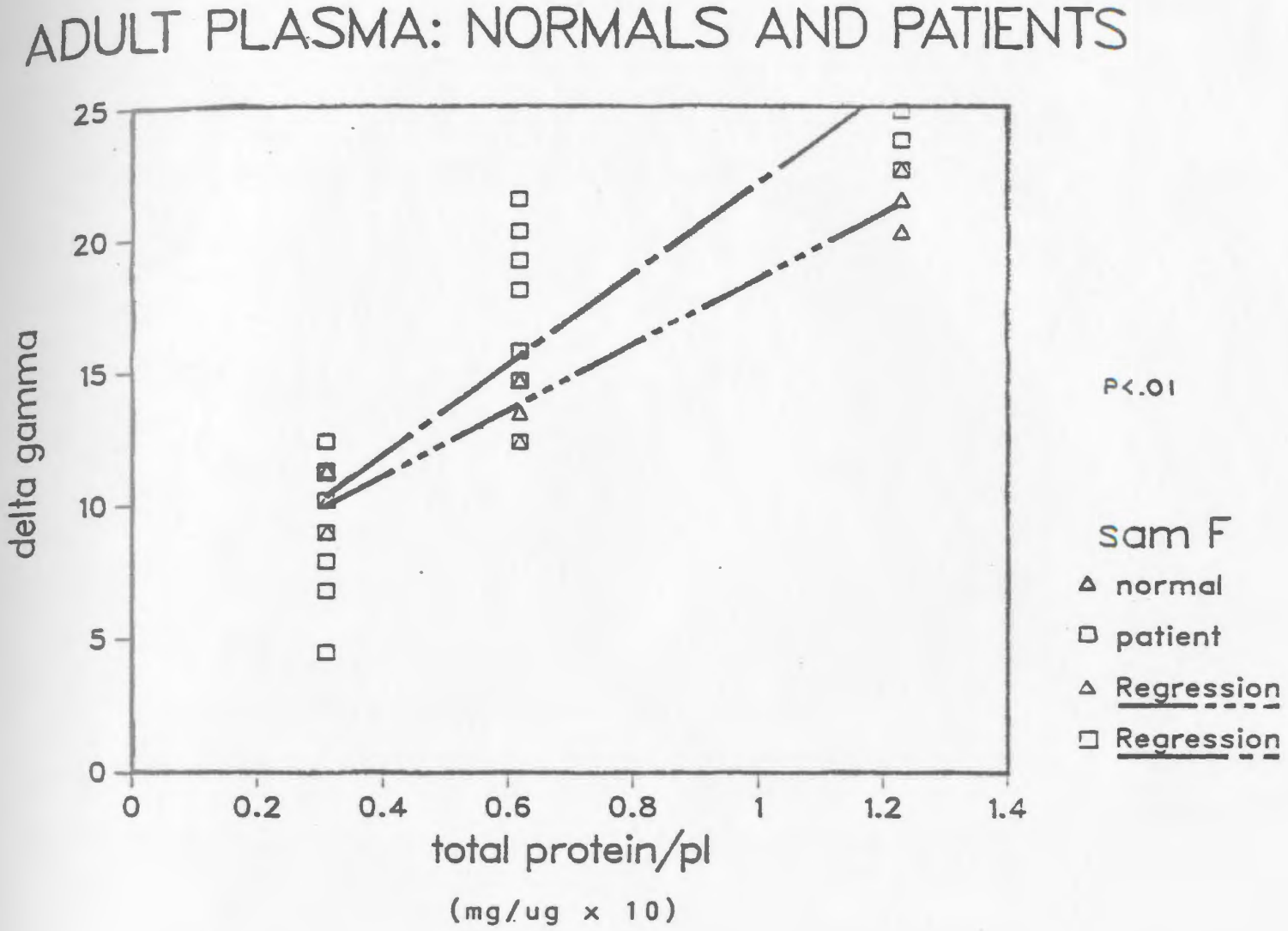


Figure 3-9 Comparison of normal adults vs adult CPB patients, plasma, sam F

normals (Figure 3-10). The difference was in the slopes of the linear regression lines. There was also a significant difference between sera from the group normal adults, sam F, n=3, and the group adult patients CPB 1 and 2, sam F, n=4, $p<.005$. However, in these sam F - sera tests, the normals showed more inhibition than did the patients (Figure 3-11). Here, the difference was in the elevations of the linear regressions.

In the above comparisons, the sam E tests were conducted separately from the sam F tests. There was a 12% difference in the surface load of phospholipid (sam E, 7.4 ug PL; sam F, 6.5 ug PL). To test for differences in the effects of plasma and sera on the two surfactant suspensions, the data from the adult normal groups were used. There was a significant difference between the normal adult plasma group using sam E and that using sam F, $p<.0005$ (Figure 3-12). There was also a significant difference between the normal adult sera group using sam E and that using sam F, $p<.0005$ (Figure 3-13). In both comparisons the sam F tests showed more inhibition than did the sam E tests, with the differences accounted for by differences in elevation of the regression lines.

Table 3-1 gives the parameters of the lines of best fit of the test groups, summarizing the data found in the preceding plots of delta gamma-min vs total protein/PL (mg/ug x 10). The number of patients involved is given by "n". The y-intercept of the lines of best fit is given by "a" and the

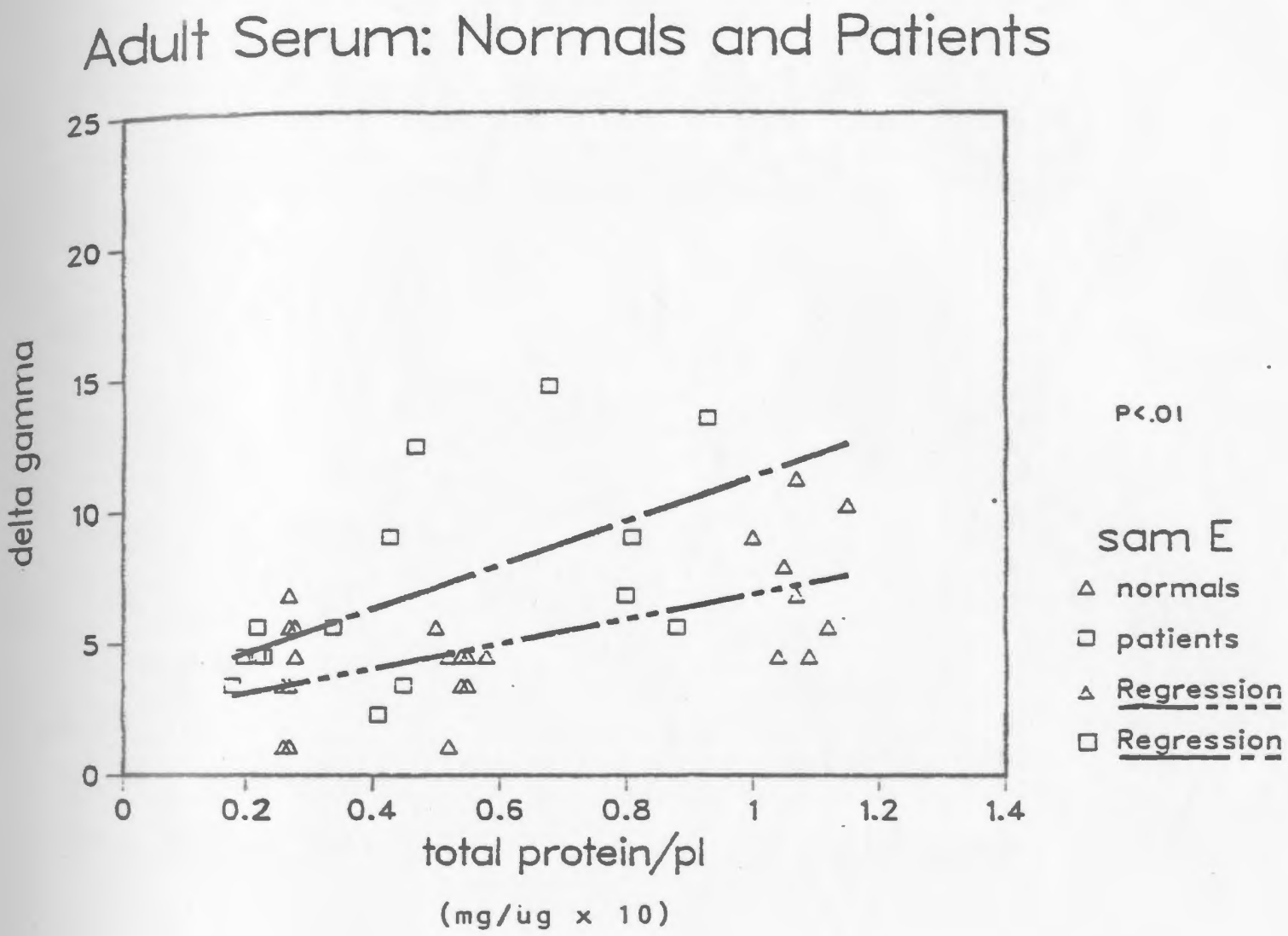


Figure 3-10 Comparison of normal adults vs adult CPB patients, sera, sam E

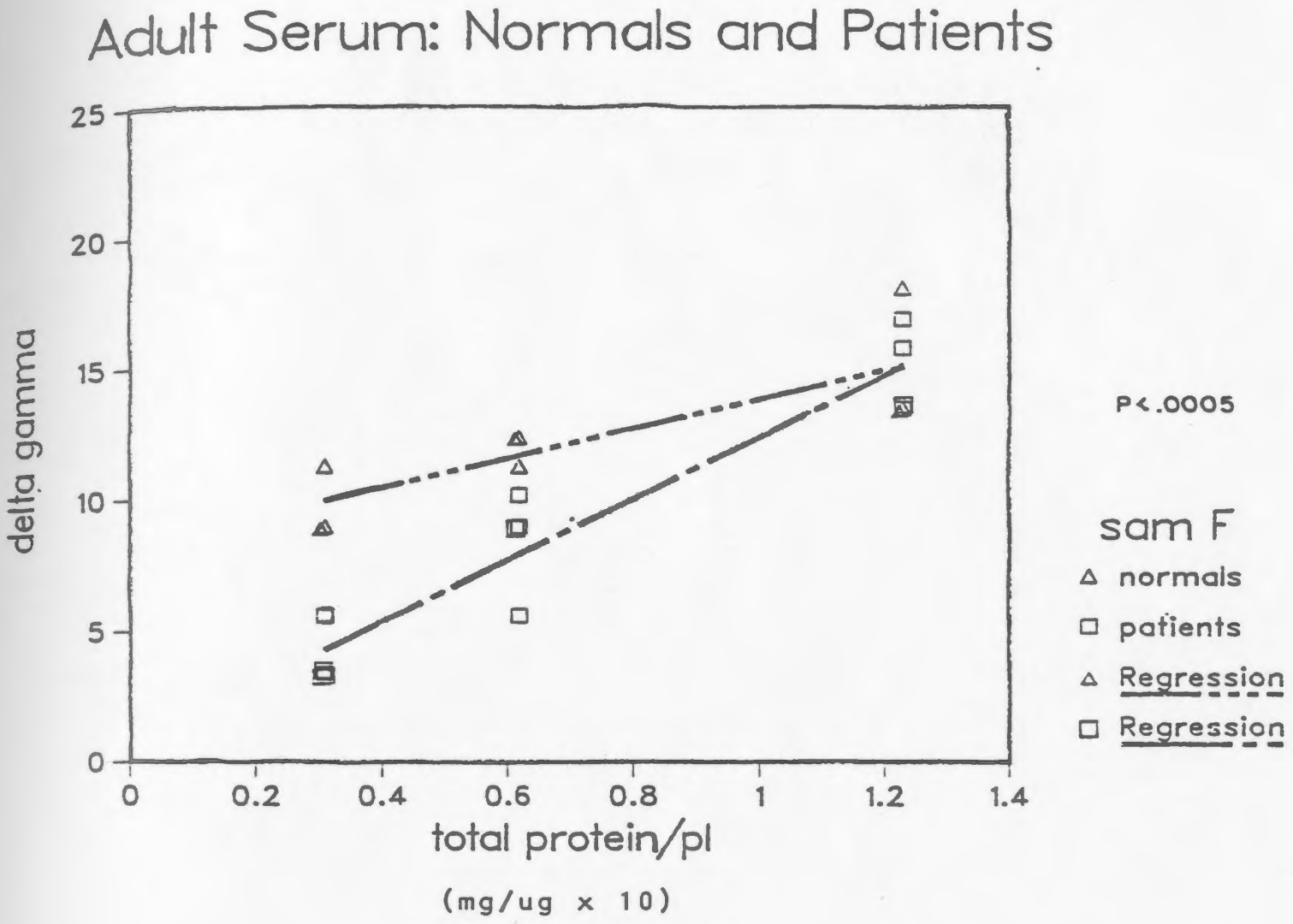


Figure 3-11 Comparison of normal adults vs adult CPB patients, sera, sam F

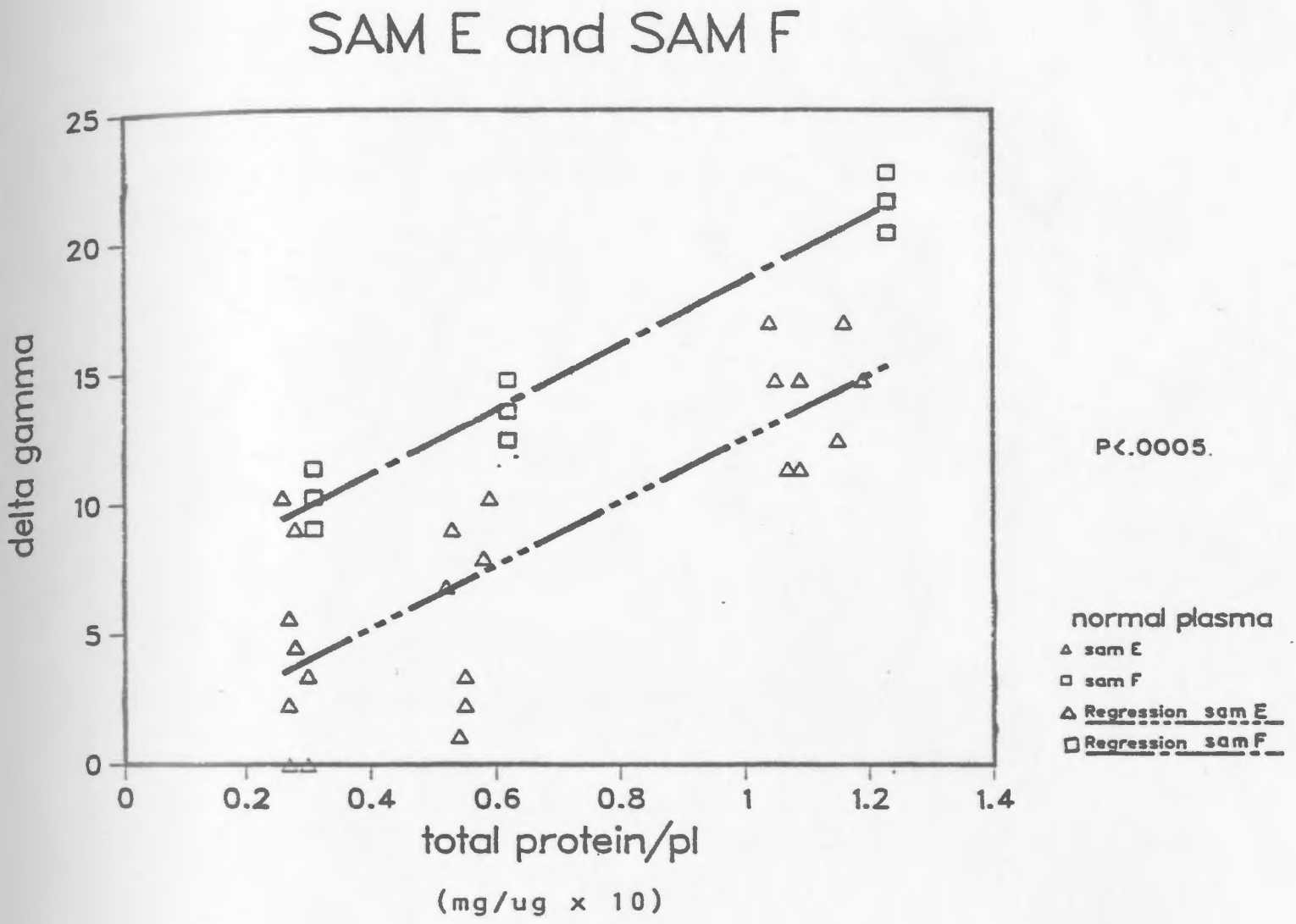


Figure 3-12 Comparison of sam E vs sam F, normal adult plasma

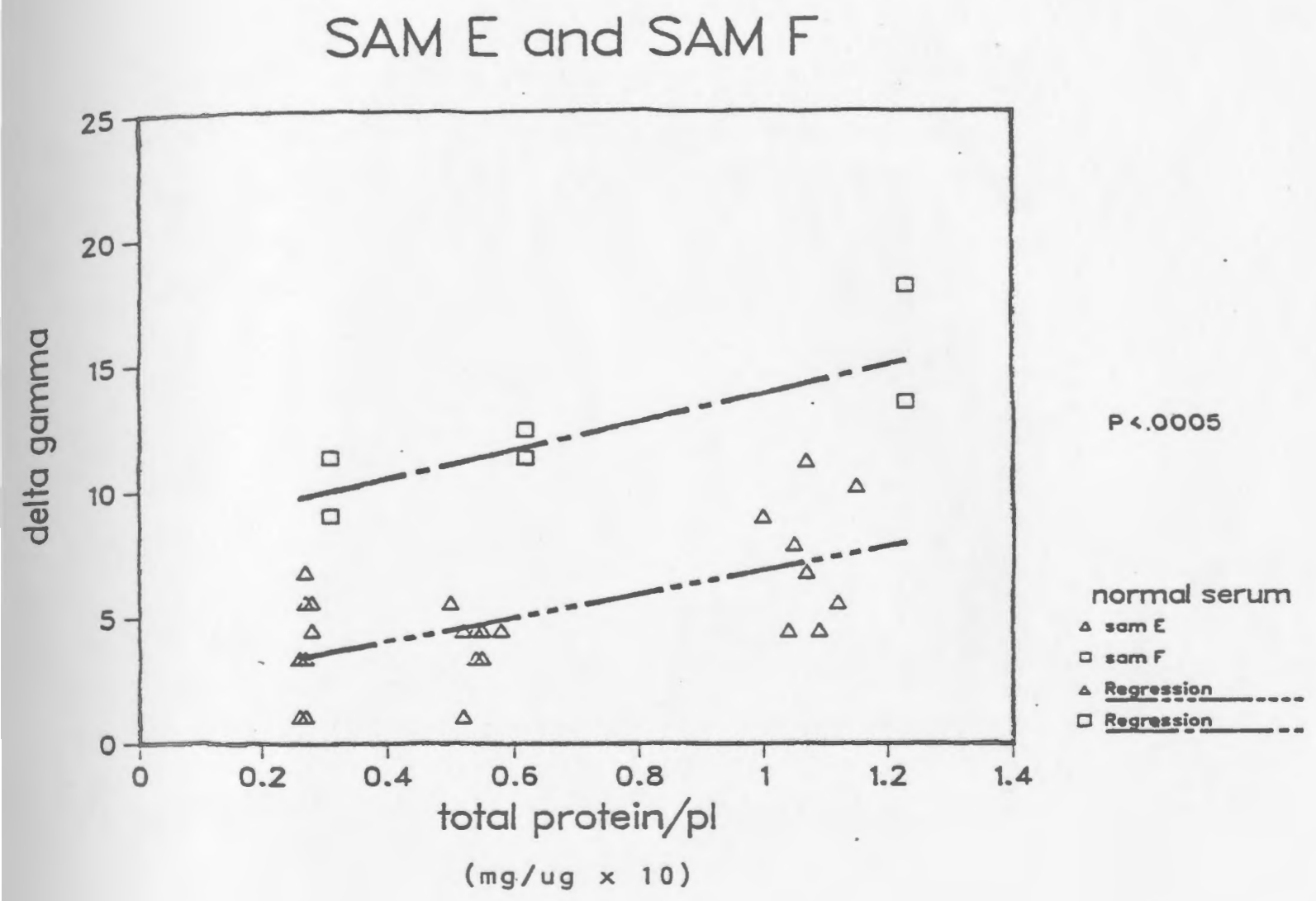


Figure 3-13 Comparison of sam E vs sam F, normal adult sera

slope of the lines of best fit is given by "b". The correlation coefficient of the lines of best fit is given by "r^2", and is an indicator of the fit of the line to the data. Rough comparisons of the groups may be made on the basis of the "a", "b" and "n" values. Actual statistical probabilities for comparisons have been given above in the text, and also are summarized in Table 3-2.

Table 3-1: Parameters of the lines of best fit of the test groups

	n	a	b	r^2
Normals, sam E				
plasma	8	0.4	12.1	.67
serum	8	2.2	4.7	.38
Normals, sam F				
plasma	3	6.1	12.4	.96
serum	3	8.2	5.6	.69
Adult CPB plasma, sam E				
CPB-1	5	3.8	8.8	.38
CPB-2	4	0.7	13.6	.57
CPB-3	5	0.7	11.0	.41
CPB-4	5	1.9	9.9	.48
CPB-all		1.7	10.9	.46
Adult CPB plasma, sam F				
CPB-1	2	3.3	18.1	.91
CPB-2	4	5.8	17.5	.90
CPB-3	4	5.7	15.1	.91
CPB-4	4	4.5	18.0	.84
CPB-all		5.1	17.0	.87
Adult CPB-1 or -2 serum				
sam E	5	3.0	8.3	.33
sam F	4	0.7	11.7	.90
Child CPB plasma, sam F				
prepump	5	1.9	18.0	.74
postpump	5	5.0	12.8	.67
overall		3.8	14.9	.69

Table 3-2: Summary of Comparisons

Comparison	significant difference	p-value
adult CPB plasma, sam E, groups 1 to 4	no	>.25
adult CPB plasma, sam F, groups 1 to 4	no	>.25
child CPB plasma, sam F, pre- vs postpump	no	>.25
adult CPB-2 vs child prepump, plasma, sam F	yes	<.01
adult CPB-3 vs child postpump, plasma, sam F	no	>.05
adult patients (CPB-1 to -4) vs child patients (pre- and postpump), plasma, sam F	yes	<.01
adult normals vs patients (CPB-1 to -4),plasma, sam E	no	>.25
adult normals vs CPB-1, plasma, sam E	no	>.10
adult normals vs patients (CPB-1 to -4), sam F	yes	<.01
adult normals vs CPB-1, plasma, sam F	no	>.05
adult normals vs patients (CPB-1 or -2), sera, sam E	yes	<.01
adult normals vs patients (CPB-1 or-2), sera, sam F	yes	<.01

Table 3-2 (continued)

Comparison	significant difference	p-value
plasma vs sera, normal adults, sam E	yes	<.01
plasma vs sera, normal adults, sam F	yes	<.01
sam E vs sam F, normal adults, plasma	yes	<.01
sam E vs sam F, normal adults, sera	yes	<.01

The effect of prolonged bypass was examined in one child patient, who subsequently died of cardiac and pulmonary complications. RDS was evident in this patient postoperatively. There was no obvious difference between samples taken before bypass, at pump time one and one-half hours and at pump time 3 hours (Figure 3-14).

There was no effect of dilution when sam E was diluted with 20 ul of solution #1. This is a ratio of surfactant:solution #1, 1:4 v:v. There was a slight effect of dilution on surfactant ability to reach low surface tensions with sam G (Figure 3-15). Inhibition was seen in the higher ranges of dilution at volumes greater than 10 ul. Delta gamma-min increased linearly with increasing dilution. Note that sam G was not used in the tests of surfactant inhibition by plasma or sera.

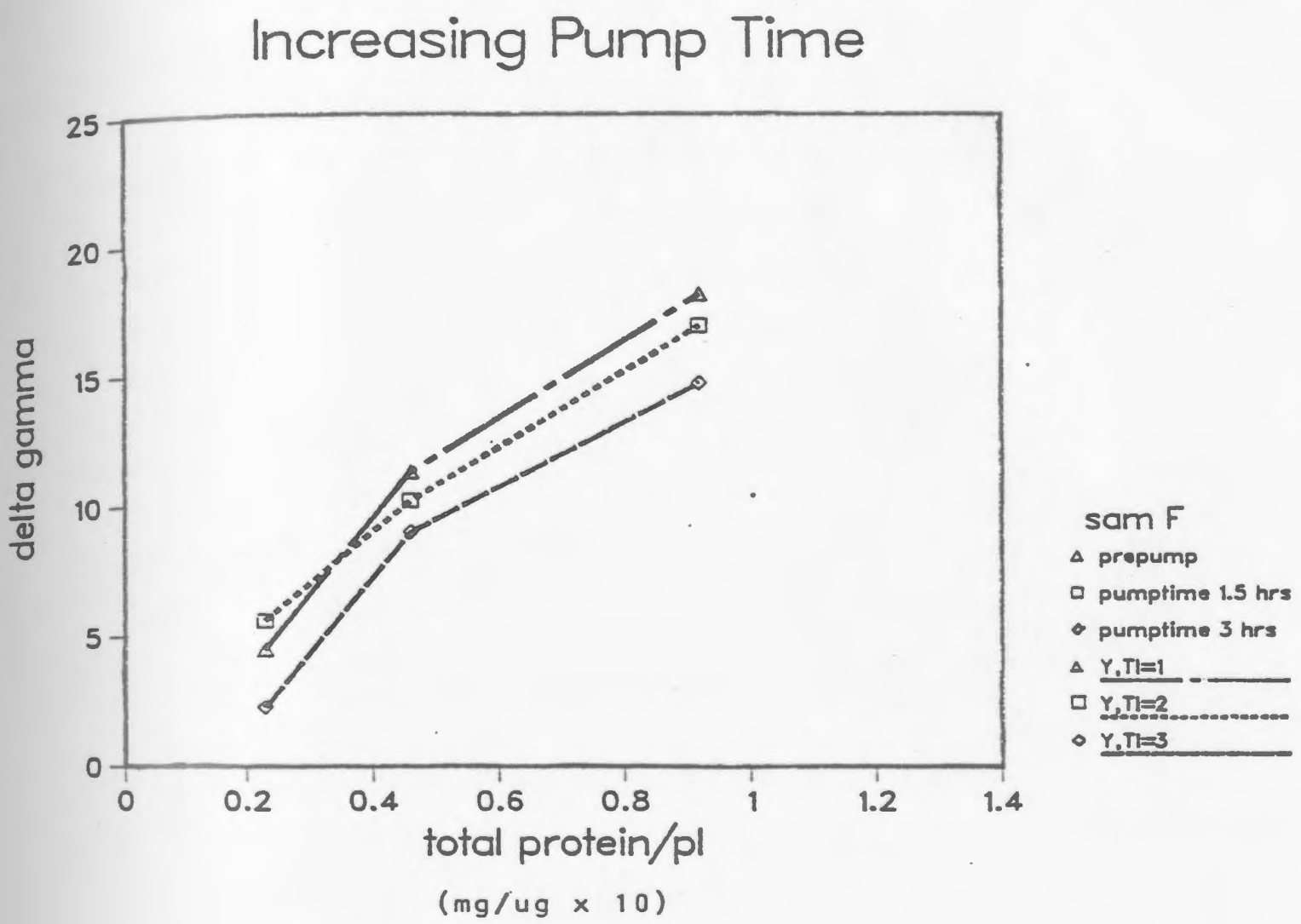


Figure 3-14 The effect of prolonged bypass in one child patient

3.4 Discussion

Cardiopulmonary bypass is a known etiologic agent of acute lung injury, and although the incidence of respiratory distress syndrome following operations involving CPB is small in elective adult cardiac surgery today, RDS remains a significant complication in pediatric cases and cases involving prolonged bypass. CPB provided a setting where an inflammatory response induced in the blood might produce or increase levels of potential circulating inhibitors. The inflammatory response is a mechanism whereby blood-air membranes could be injured, and plasma constituents could enter into the alveoli. The pathophysiologic characteristics of acute lung injury and the clinical characteristics of RDS following CPB are similar to those of ARDS. CPB is included in the long list of etiologic factors of ARDS.

The literature did not present a clear view of the effect of normal blood or plasma proteins on surfactant function, or of CPB on surfactant inhibitors in plasma. Normal blood was found to inhibit surfactant on the surface balance (Mandelbaum & Giammona, 1964; Tierney & Johnson, 1965). In contrast, Gardner et al (1962) found that surfactant was not inhibited by blood from normal individuals. And, Tierney & Johnson (1965) found that blood from normals injected into a hypophase solution of surfactant on the surface balance did not inhibit surfactant. Test conditions were noted to vary between these investigations. Blood pumped in vitro through

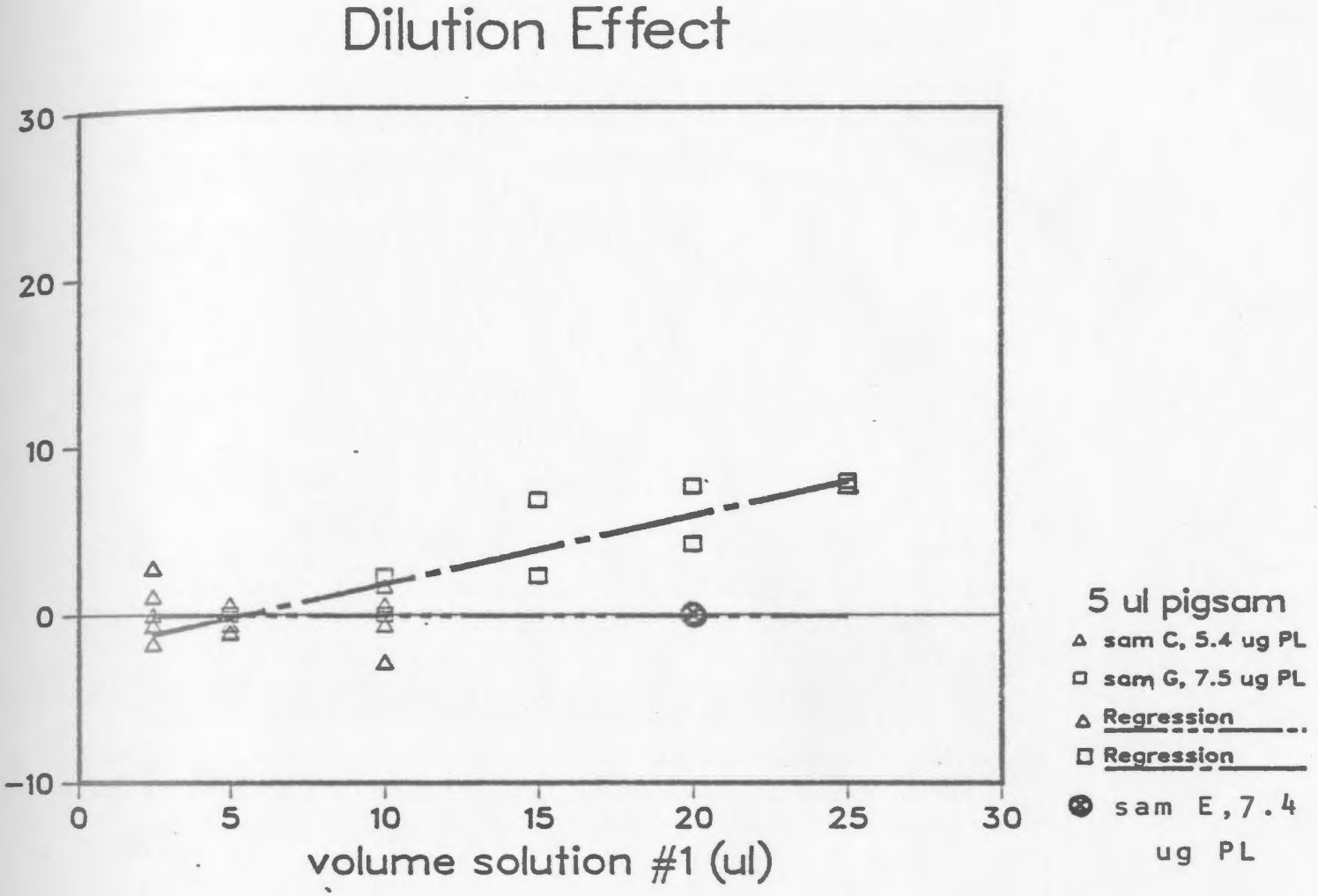


Figure 3-15 The effect of dilution on the surfactant

oxygenator circuits with no tissue contact was observed to inhibit surfactant in tests on the surface balance (Gardner et al, 1962; Mandelbaum & Giammona, 1964). However, blood taken from dogs after five hours of CPB did not inhibit surfactant when tested under the same conditions (Mandelbaum & Giammona, 1964).

It was suggested that the timing of surfactant dysfunction with hemodilution techniques was delayed by 15-24 hours following CPB (Camishion et al, 1968; Hepps et al 1963), whereas the maximum injury to the lung occured at 2 hours following CPB (Royston et al, 1983). No previous investigation had studied surfactant inhibition in human subjects undergoing CPB.

In this study, conditions for testing plasma-surfactant mixtures were defined. The model of plasma-surfactant interaction used in the study placed both plasma and surfactant on the surface of a hypophase solution on the surface balance. Tests using this assay would correspond to the situation in the alveolus where both plasma and surfactant would exist at the air-water interface.

In plasma-surfactant tests on the surface balance under these test conditions, plasma from the adult normal groups, all the adult patient groups and the child patient groups inhibited surfactant in a direct relationship between delta gamma-min and total protein load (except the adult group CPB-2, sam F).

The effect of CPB on the production of plasma inhibitors

or the increase in levels of inhibitors was studied. In consideration of a possible timing effect, postpump samples were taken in the adult patients at two times. The first was immediately after CPB, just before lung injury may be maximum, and the second at 20-24 hours after CPB, when postpump pulmonary complications would be apparent. Hemodilution was used in all cases of CPB in the study.

No difference was seen comparing prepump and postpump groups in adults or children. There was no increased inhibition in samples taken at either of the postpump times in the adult patients. The tests were internally controlled, ie. pre- and postpump blood was taken from the same patients. There was no additional effect of heparin or possible plasticizers.

Increased inhibitors may have been produced by the CPB but they may not have been detected in this study. Possible reasons include decay of the inhibitor between the time of sampling and the time of testing, and the presence of inhibitors in the airways but not in plasma. For example, prostaglandins and related compounds have half-lives of minutes and phospholipases may be present locally in the airways.

CPB patient plasma was significantly different between child and adult plasma groups, sam F, in two comparisons. These were the comparisons of i) the adult CPB-2 group and the child prepump group, and ii) the pooled data from the adult CPB groups 1 to 4 and the pooled data from the child

CPB pre- and postpump groups. In both of these comparisons the adult patients showed slightly more inhibition than did the child patients. However in another comparison of adult patients and child patients, adult CPB-3 plasma, sam F, vs child postpump plasma, sam F, a significant difference was not seen. This result certainly does not explain the observation that children have more lung dysfunction following CPB than do adults.

One case of prolonged bypass was examined. No obvious increase in inhibition was seen in the prolonged pump time of 3 hours. This may reflect the lack of increase in inhibition seen with blood exposed to CPB in general.

Comparisons were made between adult normals and adult CPB patients. No conclusive difference between the effects of normal and patient blood was seen. There was no difference between normal adults and adult patients in tests using plasma in three of four tests. One comparison, however, of plasma taken from normal adults and adult patients showed more inhibition with patients than with normals. There were differences between normal adults and adult patients using sera, but the differences were in the opposite direction when tests were done with the two surfactant preparations. If patients did have some increased surfactant inhibitor in their blood, it might be speculated that the elevated levels may be a result of stress. The increased catecholamines might elevate fibrinogen levels and other acute phase proteins or increase levels of free fatty acids or glucose.

The stress state might then predispose the patient to surfactant inhibition if there was damage to the lung blood-air membranes.

Results of tests for plasma and sera from normals using sam E were different from those of the respective tests using sam F. There are two possible explanations. The first is that the phospholipid/protein ratio may be critical to the ability of the surfactant to reach low surface tensions. With the lower surfactant phospholipid surface load used in tests with sam F, 6.5 ug PL, more inhibition was seen for the range of protein surface load tested. With the higher surfactant surface load used in tests with sam E, 7.4 ug PL, less inhibition was seen. Note again that the surface loads of sam E and sam F differed only by 12%. The second possibility is that some unknown factor may have been present in the sam F suspension to inhibit its activity, even though the method of suspending the lyophilized pig lung surfactant was the same for both, both originated from the same batch of processed lung washings, and the minimum surface tensions of both sam E and sam F suspensions were the same (< 5 mN/m). The possibility that dilutional effects may account for differences did not seem applicable for this range of dilution. ie. To dilute sam E to the concentration of sam F would involve adding less than one ul of diluting saline to the surface load of surfactant.

Scatter in the data occured even though the experiments were performed under well-defined conditions. The scatter

perhaps is a reflection of the varied findings in the literature. Variability may be found in human blood samples, in the preparation of surfactant from lung lavage and the efficacy of the surfactant preparation to reach low surface tensions, in methods of blood collection, in test conditions of mixing the surfactant and potential inhibitor (blood, plasma or protein), and in conditions of testing the mixtures on the surface balance.

Trurnit (1960) studied proteins at the air-water interface. A glass rod of specified length and diameter was used to place the protein solution onto the surface of the hypophase solution. It was suggested that with this method more protein adsorbed to the air-water interface than with a droplet technique. It was stated that as much as 60% of the protein was lost into the hypophase using a droplet technique. However, when Tabak & Notter (1977) tested the interaction of proteins and DPPC at the air-water interface using the glass rod technique, albumin and fibrinogen did not inhibit DPPC from reaching low surface tension on compression. It was noted that the DPPC was added to the surface before the protein. Taylor & Abrams (1966) and Miyahara (1969) used the glass rod to spread mixtures of the surfactant and protein onto the surface. However while Miyahara reported that fibrinogen, albumin and the globulins all inhibited surfactant, Taylor & Abrams (1966) found that fibrinogen prevented surfactant from reaching low surface tensions, but that albumin and the globulins did not. Thus

the glass rod technique did not eliminate inconsistencies in the literature data. In the experiments of this thesis, some variability may have arisen from variable amounts of protein adsorbing to the surface using the droplet technique, and variable loss of protein onto the side walls of the trough.

Other authors tested surfactant extracts and blood on the surface balance (Gardner et al, 1962; Mandelbaum & Giammona, 1964; Tierney & Johnson, 1965), but used the extracts of lung minces as the hypophase solution. Surfactant would be needed in much larger quantities using this method compared with the method using a hypophase of saline which was employed in the experiments of this study. Rufer & Stolz (1969) used a protein solution as the hypophase and layered the surfactant onto the surface with an alcohol spreading solution. These different methods may account for some of the variability in the results of the literature.

The effects of dilution were variable. In the one test using sam E there was no effect of dilution to 1:4 v:v (20 ul of solution #1). In the previous tests of dilution with sam C, there was no effect of dilution to 1:2 v:v. With sam G however, inhibition was seen at dilutions greater than 1:2 v:v, 10 ul. The lipid aggregate structures in surfactant would be preserved in this range of concentration. This degree of dilution would not disaggregate these structures because the concentration of lipid, at least 10^{-6} M, would still be much greater than the critical micellar

concentration, approximately 10^{-10} M. It was noted that sam G had been stored in lyophilized form at -20 degrees C for a longer period of time (approximately 8 months) compared with sam E (3 months) and sam F (4 months). The effect of prolonged storage time on the surfactant was not studied.

If dilution did increase the degree of inhibition in the tests using sam E and sam F, this would be important particularly for the postpump hemodiluted samples. The corrected values for delta gamma-min would show less inhibition than the indicated values, implying that the postpump samples were less inhibitory than indicated. In any event this would not deter from the conclusion that CPB did not increase inhibition.

In conclusion, the data are consistent with the possibility that plasma components entering the airspaces through leaking lung membranes may inhibit surfactant from proper function, and may contribute to the collapse and edema of the lungs seen in the ARDS of any etiology. CPB did not increase the levels of plasma inhibitors in the assay used in this study.

Chapter 4

Nature of the inhibition

4.1 Characterization of the inhibitor

4.1.1 Introduction

The literature has suggested that components in the plasma may affect surfactant function. These include proteins, free fatty acids and cholesterol. Most of the literature articles reviewed have investigated the protein fractions of plasma as the inhibitory component (Taylor & Abrams, 1966; Balis et al, 1971; Miyahara, 1969; Tabak & Notter, 1977; Rufer & Stolz, 1969; Tierney & Johnson, 1965). Tierney & Johnson (1965) also tested the effects of cholesterol and oleic acid on surface films of rabbit lung surfactant prepared from extracts and found that the minimum surface tension was increased. Gardner et al (1962) suggested that blood which had been circulated through a CPB pump inhibited surfactant by the release of free lipids following the protein denaturation caused by the pump. Hallman et al (1982) found that the lung surfactant-deficient states of RDS in newborns and in adults were associated with abnormalities in the negatively-charged lipids with a high ratio of PI/PG.

In the introductory section, "Surfactant: component function, physical properties and structure", it was presented that the surface film must contain a high proportion of DPPC because low minimum surface tension was not achievable by other compounds at 37 degrees C. The addition of any substance to the surfactant surface layer might prevent the achievement of low surface tension by displacing DPPC from the surface or by interfering with the phospholipid monolayer packing on compression of the surface.

Recently Ikegami et al (1983) and Jobe et al (1983) described a specific surfactant inhibitor in the airways of premature infants and lambs with RDS. This was associated with leaking lung membranes as shown by a high rate of appearance in the airways of radioactively-labelled albumin which had been given intravenously. The specific inhibitor was tested using the pulsating bubble apparatus (the dynamic alveolar model) after Nozaki (1970). The inhibitor was isolated from airway washings. The washings were centrifuged on 0.7M sucrose. The material floating on the sucrose was pelleted with a second centrifugation. The specific surfactant inhibitor was isolated from the supernatant from this second centrifugation by successive polyethylene glycol (PEG) precipitations. The specific inhibitor was found in the fraction precipitating between 25 and 50% PEG, the PEG 50% fraction. It was found to increase minimum surface tension to large degrees with only small

amounts of protein relative to surfactant. It was indicated that the protein isolated by PEG fractionation was greater than 20 times more inhibitory than the proteins of the airway washings as a whole. The specific inhibitor was partially characterized as a substance with MW > 12,000. It was not inactivated by boiling or lipid solvent extraction techniques. It was completely inactivated by an unspecified protease.

In the studies of this thesis plasma did inhibit surfactant from reaching low minimum surface tension. The following questions were asked: Was the inhibitor in plasma a protein? and, Was the specific surfactant inhibitor described by Ikegami et al (1983) and Jobe et al (1983) present in plasma?

Experiments were performed i) to test if the inhibitor was a protein, ii) to look for the Ikegami-Jobe specific surfactant inhibitor in plasma, and iii) to compare plasma from normals with plasma from patients using the fractionated components of plasma.

4.1.2 Plasma treatments

Methods

Plasma, which was anticoagulated with heparin, was taken from normals and patients (hemodiluted post-pump blood). The plasma was treated with a general protease, proteinase K (Sigma P-0390 lot 14F-0142). This enzyme, prepared from the fungus, Tritirachium album, is reported to have wide

spectrum activity, cleaving the peptide bonds adjacent to the carboxyl groups of aliphatic and aromatic amino acids with blocked alpha-amino groups. The proteinase was added to the plasma in a ratio of 50 ug enzyme:50 mg plasma protein (1:1000 by weight; personal communication from Dr. W. Davidson, Department of Biochemistry, Memorial University of Newfoundland), and incubated overnight at 37 degrees C. A clot was formed. The supernatant (Brinkmann Instruments centrifuge 3200 for Eppendorf centrifuge tubes; 10 min at 12,000 gav) was tested for surfactant inhibition and was analysed using polyacrylamide gel electrophoresis.

0.5 ml portions of the plasma from the same normals and patients were diluted with solution #1, 1:10 v:v, and placed in a bath of boiling water. The supernatant (IEC bench-top centrifuge; setting = 6; 10 min) was used to test for surfactant inhibition and was analysed by polyacrylamide gel electrophoresis. It was noted that whole plasma when boiled yielded a coagulated solid with no supernatant. This was not suitable for testing.

0.5 ml portions of the plasma from the same normals and patients were dialysed with a 12,000 MW exclusion membrane against solution #1 in the Eppendorf centrifuge tubes (appendix Figure A-2). The volume of the bath was 500 ml and was changed two times. The dialysis proceeded overnight at 4 degrees C and with constant stirring. The dialysed plasma was used to test for surfactant inhibition and was analysed using polyacrylamide gel electrophoresis.

Surface studies were performed on mixtures of surfactant (the sam F suspension) and treated plasma or plasma fractions as described previously. Polyacrylamide gel electrophoresis was run with the aid of Dr. W.Davidson, Department of Biochemistry, MUN (appendix A.9).

Results

It was observed that all of the treated plasma samples inhibited the surfactant from reaching low surface tensions (Table 4-1). Figures 4-1 and 4-1(a) show examples of the polyacrylamide gel electrophoresis patterns of the treated plasma samples. Comparing the values of delta gamma-min at protein/PL = 0.77 (mg/ug x 10) for normals and patients, there were no large differences between the untreated, proteinase treated and dialysed samples. The values of delta gamma-min were in the range 12 to 17 mN/m, which most likely is within experimental error under these test conditions.

The effects of dilution were not tested with sam F because of lack of supply. Inferences from the previous data on dilutional effects indicated that surfactant might be inhibited by dilution if the volumes of the test sample added to the surfactant was greater than 10 ul. No conclusions about dilution effects were made from comparisons of the hemodiluted patient samples and the plasma from normals.

The effects of dilution were particularly relevant to the

supernatants of the boiled plasma samples. The protein content of these supernatants were low relative to untreated plasma. Tests at the protein/PL range of 0.77 (mg/ug x 10) were not performed because very large volumes would be needed. The observed values of delta gamma-min for the supernatants of the boiled plasma samples tested at 0.1 (mg/ug x 10) were likely appropriate for the low amount of protein and the volumes employed (15-20 ul). That no higher values of delta gamma-min were observed might suggest that no other inhibitors were present after boiling, ie. free fatty acids, cholesterol, etc. Protein staining was not seen following polyacrylamide gel electrophoresis of these boiled samples, likely because the concentration of protein was too low (Figures 4-1, 4-1 (a)). The dialysed samples showed inhibition comparable to the untreated plasma. This indicated that the inhibitors had MW > 12,000 daltons. Polyacrylamide gel electrophoretic patterns were similar to those of untreated plasma (Figures 4-1, 4-1(a)).

The samples treated with the proteinase also showed inhibition similar to untreated plasma. This was unexpected in our view that the inhibitors were proteins. The possibilities were: i) the inhibitory effect seen was due to smaller peptides, or ii) the enzyme did not work. In fact, the polyacrylamide gel electrophoretic patterns did not show any smearing of bands in the protease-treated samples which might be expected after general proteolytic activity (Figures 4-1, 4-1(a)). Possible explanations for

Table 4-1: Tests of treated plasma using sam F 7.8 ug PL

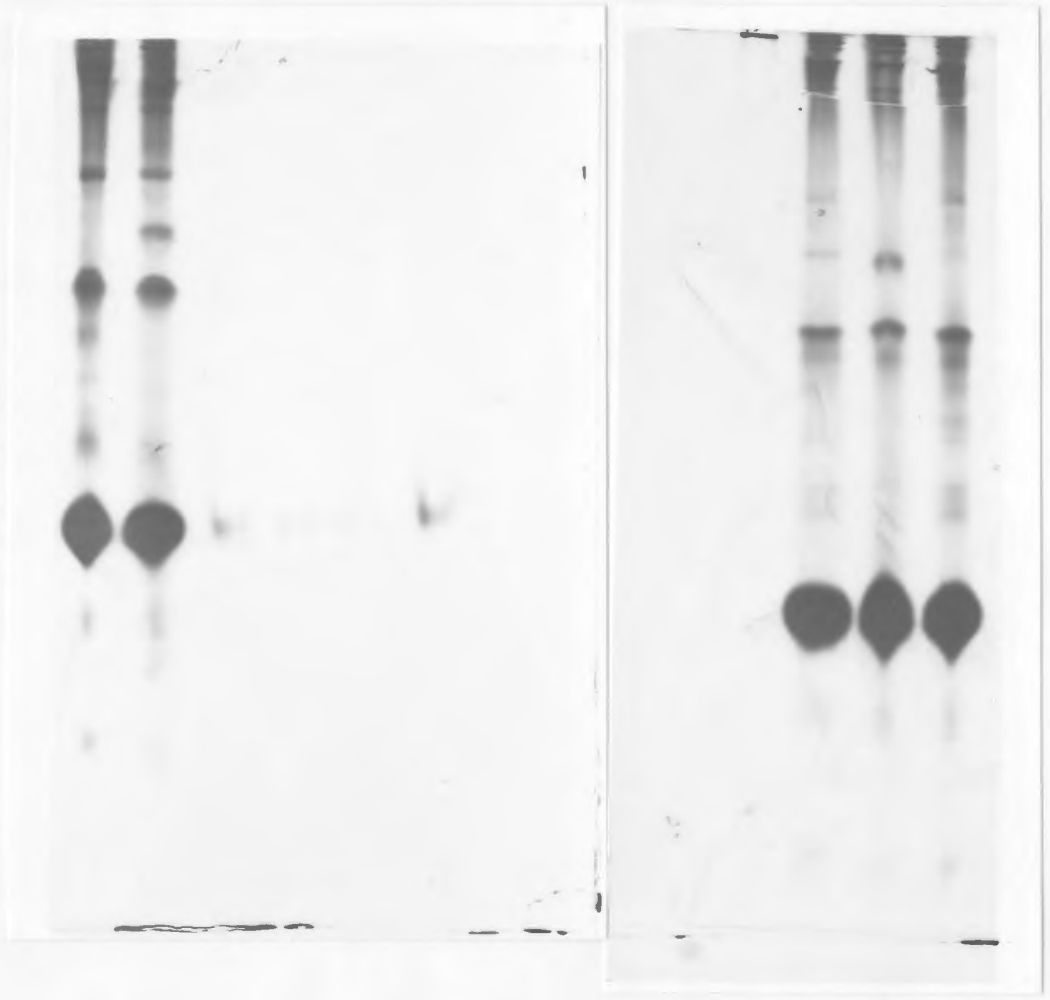
	T.Protein/PL (mg/ug x 10)	Delta gamma-min, mN/m normals (n=2)	patients (n=2)
untreated	0.77	13.5 ±0.5 (7 & 8 ul)*	15 ±2 (13 & 14 ul)
proteinase	0.77	12 ±0 (7 & 8 ul)	13 ±1 (13 & 14 ul)
dialysed	0.77	16.5 ±2.5 (9 & 10 ul)	16.5 ±.5 (15 & 17 ul)
boiled	0.1	3 ±0 (15 & 15 ul)	2.5 ±1.5 (15 & 20 ul)

*Volumes of plasma sample applied to the surface

lack of proteinase activity are i) proteinase autolysis and autodigestion, and ii) the presence of natural antiproteases in plasma. We did not further pursue investigation of the proteinase due to expense and time restrictions.

There was no appreciable difference between the treated plasma taken from normals and patients in the small number of samples tested.

In summary, the data were consistent with the interpretation that the inhibitors present in plasma were



1 2 3 4 5 6 7 8 9 10

Figure 4-1: Polyacrylamide gel electrophoresis of the treated plasma and PEG fractions

Lanes 1,10 - untreated plasma
Lanes 2,9 - proteinase-treated plasma
Lanes 3-6 - PEG 50 fraction
Lane 7 - supernatant of boiled (diluted) plasma
Lane 8 - dialysed plasma

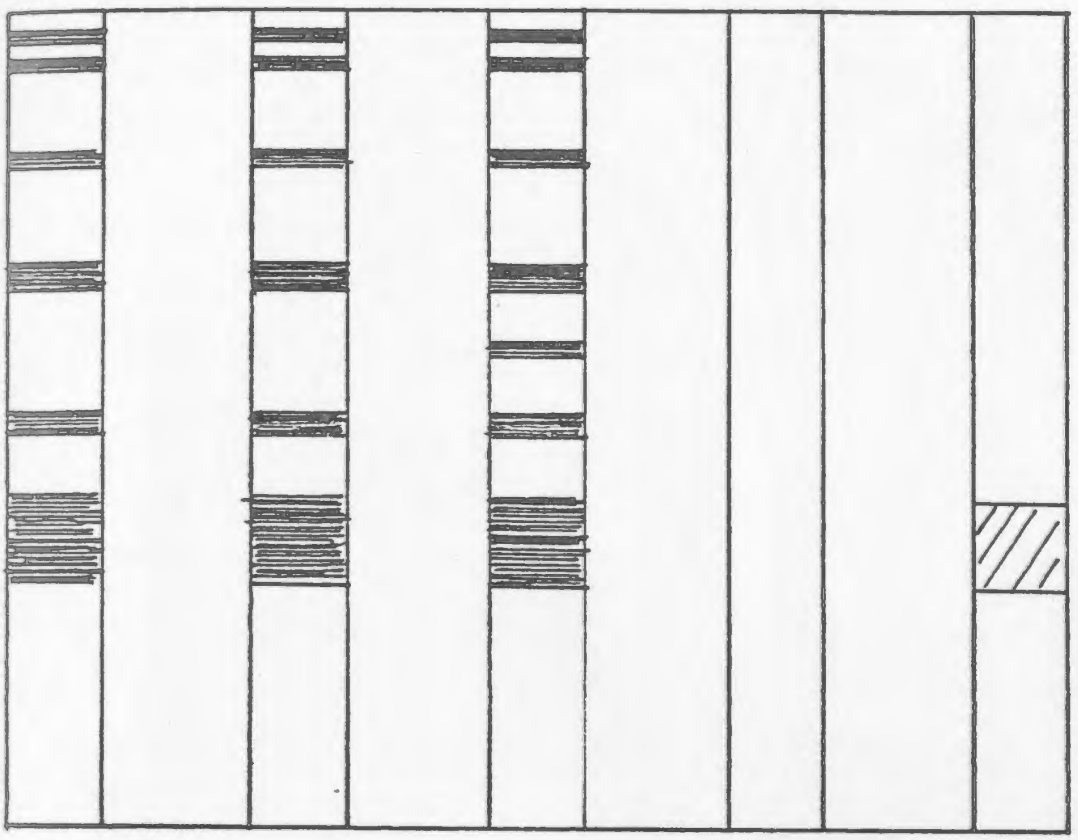


Figure 4-1a Polyacrylamide gel electrophoresis of the treated plasma samples and the 50% PEG fraction

proteins. Inhibition by other plasma components seemed unlikely.

4.1.3 Polyethylene glycol fractionation

Polyethylene glycol (PEG) fractionation of plasma proteins was used to investigate two potential effects. First, it was used to determine if any possible differences between the plasma of normals and patients could be localized to different plasma protein fractions, eg. fractions containing globulins, fibrinogen, albumin. Second, an attempt was made to find the specific surfactant inhibitor of Ikegami and Jobe (Ikegami et al, 1983; Jobe et al, 1983) in the plasma of normal adults and adult patients.

PEG is a high molecular weight polyalcohol which chaotropically alters the activity of the water surrounding proteins, hence causing precipitation. PEG with an average MW of 3350 was used (after Ikegami et al, 1983). Solutions with the 3350 MW polymer have relatively low viscosity, and are reported to cause no denaturation of plasma proteins at room temperature. Manipulation at room temperature is advantageous in comparison to the manipulations at freezing temperatures required by fractionation with ethanol and ammonium-sulfate (Cohn, McMeekin, Oncley, Newell & Hughes, 1940; Cohn, Gurd, Surgenor, Barnes, Brown, Derouaux, Gillespie, Kahnt, Lever, Liu, Mittelman, Mouton, Schmid & Uroma, 1950; Edsall & Anson, 1947). The PEG concentrations and pH changes used were outlined by Ikegami et al (1983).

The first fraction, the precipitate obtained at a PEG concentration of 17%, pH 7.0 (PEG 17%), should contain as major components fibrinogen and the globulins. The second fraction, the material precipitated between 17 and 25% PEG, pH 5.4 (PEG 25%), was expected to contain albumin as the major component. The last fraction, the material precipitated between 25 and 50% PEG, pH 5.4 (PEG 50%), was expected to contain the specific surfactant inhibitor (Ikegami et al, 1983; Polson, Potgieter, Largier, Mears & Joubert, 1964).

An important consideration was the likely inhibition of surfactant by the PEG. There would be residual PEG with the precipitated protein. It was asked: Could the inhibitory component of the PEG be accounted for and subtracted out of the total inhibition, leaving only the inhibition due to the protein? Or, must the residual PEG be removed from the proteins?

The PEG fractionation procedure is described in appendix A.10. Tests of surfactant inhibition were conducted on the surface balance with the same methods used previously.

PEG was found to inhibit surfactant from reaching low minimum surface tensions. The degree of inhibition varied directly with the amount of PEG. The relationship, delta gamma-min = 1.91 + 0.27 [ug PEG/ug PL] describes the data given in appendix A.11 for the line of best fit for a plot of delta gamma-min vs PEG/PL. The concentration of PEG could be estimated using the refractive index of the

solution. From the data given in tables in appendix A.11 it was found that the refractive index of a solution of PEG and bovine serum albumin (BSA) was the sum of the refractive indices of the BSA and PEG components in solution:

$$RI(BSA) = -.36 + 0.1\ [mg\ BSA/ml]$$

$$RI(PEG) = 0.8\ [g\ PEG/dl]$$

$$RI(total) = RI(BSA) + RI(PEG)$$

Once the concentration of PEG in the protein fraction was determined by refractive index the contribution to the inhibition caused by that amount of PEG could be calculated. The effect of PEG on the total inhibition was then subtracted from the total leaving the inhibition due to the protein. In this way, the inhibition due to the protein alone was determined. Addition of the delta gamma-min due to protein and that due to PEG was assumed to give the total delta gamma-min.

In Table 4-2, "NF" denotes plasma taken from a normal individual and "TM", that from one patient. The PEG fractions were the precipitates obtained at the PEG concentrations 17%, 17-25% and 25-50%. The determination of the concentration of PEG was based on the concentration of protein determined by the Biuret method, and observations of the refractive indices of solutions of BSA and PEG as outlined previously. The total delta gamma-min were observed data. The determination of the contribution to the total delta gamma-min by the PEG was based on the observed

delta gamma-min for the PEG solution. The determination of the contribution to the total delta gamma-min of the protein was based on the assumed relationship, total delta gamma-min = delta gamma-min due to PEG + delta gamma-min due to protein.

It was found that the calculated inhibition due to the residual PEG was much greater than the observed total inhibition of the protein-PEG solution in some cases (Table 4-2). Attempts were made to remove the PEG from the protein since the inhibition due to the residual PEG could not be accounted for by the above methods and could not be ignored. Attempts to remove the PEG by dialysis with an 8,000 MW exclusion membrane failed. It was postulated that the PEG, MW 3350, would form micelles which would not be able to penetrate the membrane. Next, ethanol was used to precipitate the protein, leaving the PEG still dissolved in the ethanol. The method is described in the appendix A.12. Unfortunately a low yield of protein was recovered following the ethanol precipitation, eg. 59% of the PEG 17% fraction, and 9% of the PEG 25% fraction. The precipitated protein also contained residual PEG. The presence of PEG was tested by adding one drop of the protein solution to 5 ml of Nessler's reagent (Polson et al, 1964). The formation of a white precipitate indicated the presence of at least 0.01% PEG.

The PEG 50% fraction was analysed using polyacrylamide gel electrophoresis. Only small quantities of protein had been

Table 4-2: Polyethylene glycol (PEG) fractions

	total protein/PL (mg/ug x 10)	delta gamma-min, mN/m total	PEG	protein
NF 17%	.05	9.0	4.0	5.0
[PEG] =	.12	12.4	7.0	5.4
29 mg/ml	.23	13.5	12.1	1.4
	.46	14.7	22.3	- 7.6
	.69	15.8	32.5	-16.7
TM 17%	.03	2.3	3.8	- 1.5
[PEG] =	.05	13.5	5.7	7.8
55 mg/ml	.13	13.5	11.4	2.1
	.51	13.5	40.0	-26.5
NF 25%	.09	13.3	6.6	6.7
[PEG] =	.19	15.6	11.3	4.3
68 mg/ml	.39	24.4	20.7	3.7
	.76	25.6	39.5	-13.9
TM 25%	.08	10.2	7.7	2.5
[PEG] =	.15	13.5	13.5	0
167 mg/ml	.39	20.3	30.8	-10.5
	.77	21.5	59.7	-38.2
NF 50%	.002	7.0	6.7	0.3
[PEG] =	.003	11.3	11.5	- 0.2
138 mg/ml	.008	12.2	25.8	-13.6
	.017	14.8	49.7	-34.9
	.033	17.4	97.4	-80.0
TM 50%	.004	2.3	5.4	- 3.1
[PEG] =	.008	7.9	9.0	- 1.1
102 mg/ml	.013	13.5	12.5	1.0
	.021	16.9	19.6	- 2.7
	.042	24.8	37.2	-12.4

recovered. Only one faint band was seen, and this band had comparable electrophoretic mobility to albumin. The possibilities are that i) there was no Ikegami-Jobe specific surfactant inhibitor present in adult plasma or that quantities were insufficient to be detected by this assay, and ii) the specific inhibitor protein was associated with albumin. In a personal communication with Dr. Ikegami it was suggested that the specific inhibitor protein was present in plasma in small quantities but that it was very hard to isolate from albumin.

PEG fractionation was not pursued further at this time. It was felt that time constraints should limit the development of methods to separate residual PEG from the protein fraction, and the separation of albumin from the specific inhibitor.

4.2 Mechanisms of surfactant inhibition by proteins

4.2.1 Plasma proteins

Introduction

Others have commented on possible mechanisms of plasma protein inhibition of surfactant. Balis et al (1971) suggested that fibrinogen inactivated surfactant by incorporating the surfactant into a clot and so prevented access of surfactant to the air-water interface. Serum inhibited surfactant from reaching low surface tension but this inhibition was reversed by centrifugation to separate

the surfactant from the serum proteins. In contrast centrifugation of surfactant-fibrinogen mixtures did not yield disinhibited surfactant.

Inhibitory effects of plasma proteins on surfactant were found to be variable in the reports of the literature. Taylor & Abrams (1966) found that albumin and the globulins did not inhibit surfactant, and Tabak & Notter (1977) found that fibrinogen and albumin did not inhibit DPPC. In contrast, Taylor & Abrams (1966) found that fibrinogen did inhibit surfactant, and Miyahara (1969) and Rufer & Stolz (1969) found that fibrinogen, albumin and the globulins did inhibit surfactant.

In this section tests investigating the effects of the individual plasma protein fractions on surfactant were performed with the assay used in previous experiments of this thesis.

Methods

The human plasma proteins, fibrinogen, albumin, and the globulins were obtained from a commercial supplier. Mixtures of protein and surfactant were placed on the surface of a saline hypophase by the droplet technique in the same manner as used in previous tests. Because the fibrinogen was prepared with citrate which could chelate the calcium necessary for surfactant function, the effect of a citrate solution was tested on the surfactant (Figure 4-2). The citrate content was calculated for each total protein/PL

load tested for fibrinogen. Citrate was listed by the manufacturers as 15% of the weight of the fibrinogen compound. A value for inhibition due to citrate content was determined from the line of best fit for citrate effect (Figure 4-2). The effect of the citrate contained in each total protein/PL load tested for fibrinogen was subtracted from the total delta gamma-min of the fibrinogen data. The delta gamma-min for the fibrinogen data were corrected for the effect of citrate in this manner.

Results

Fibrinogen was found to be a potent inhibitor of the surfactant (Figure 4-3). High values of delta gamma-min were observed at low protein loads. The line of best fit was not linear but seemed to asymptotically approach a value of delta gamma-min between 25-30 mN/m. This would seem reasonable since fibrinogen alone (0.6 mg on the surface) was surface active with a minimum surface tension in the range of 25-30 mN/n (Figure 4-4).

Albumin did not have an asymptotic curve in the range of protein tested. Rather, the line of best fit was linear in this range (Figure 4-3). Surface films of albumin alone (1.0 mg on the surface) had minimum surface tensions of 25-30 mN/m (Figure 4-4). Therefore at high ratios of protein to PL, a limiting value of 25-30 mN/m would be expected.

The globulins were found to inhibit surfactant. The

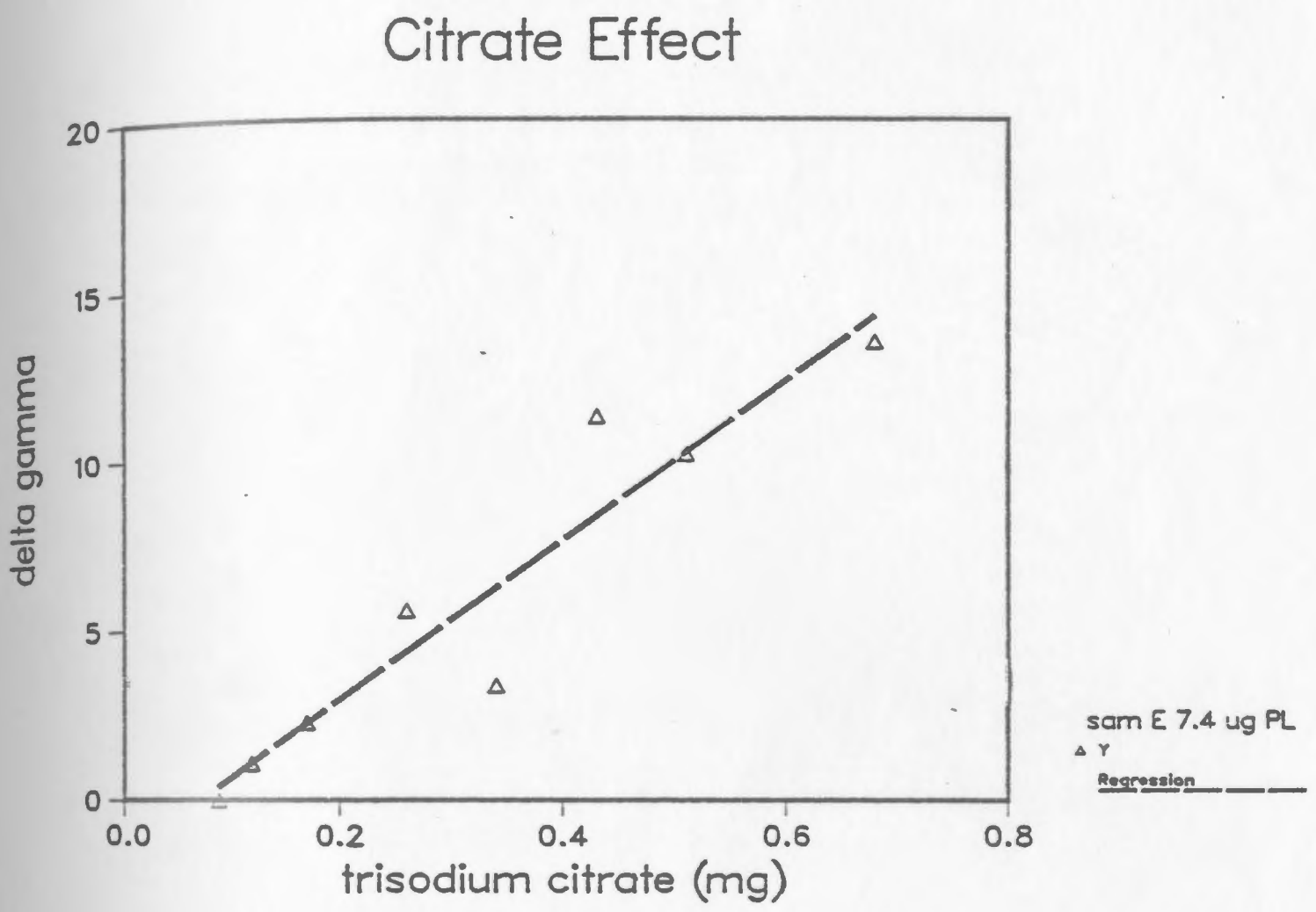


Figure 4-2 The effect of citrate on surfactant

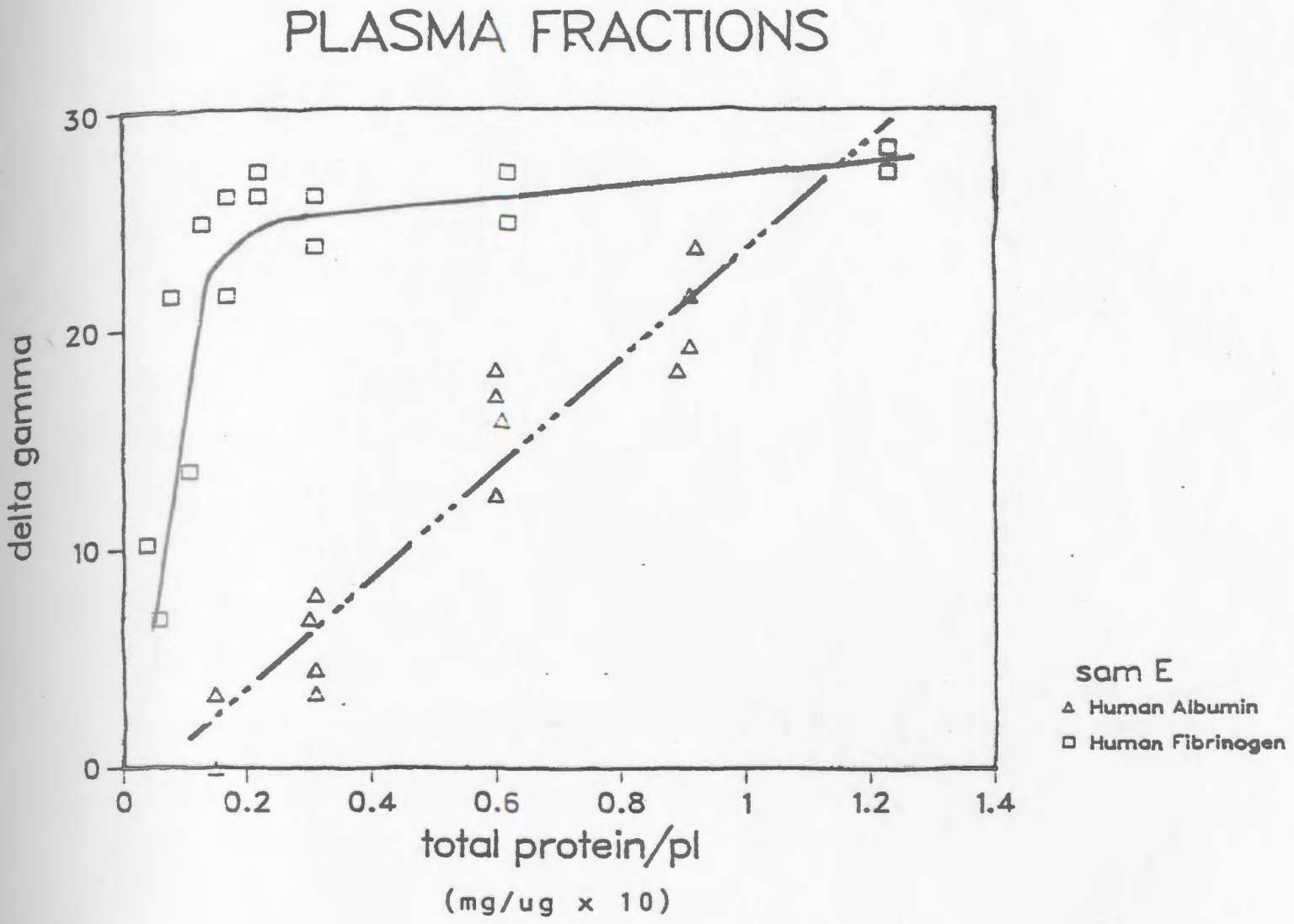


Figure 4-3 The effect of fibrinogen and albumin on surfactant

asymptotic line seemed to approach a value of 15-20 mN/m (Figure 4-5), which is different from those of fibrinogen and albumin. Monolayers of pure globulins (0.9 mg on the surface) had minimum surface tensions in the range of 15-20 mN/m (Figure 4-4).

Plasma and serum were seen previously to inhibit the surfactant in a direct relation between delta gamma-min and the ratio total protein/phospholipid. Plasma was more inhibitory than serum. The increased inhibition of plasma may be attributed to the presence of fibrinogen in plasma and not serum, and to the high degree of inhibition seen with fibrinogen.

In a hypothetical case where albumin was the only inhibitory component of serum, the delta gamma-min of the serum effect could be expressed in terms of albumin diluted with some non-inhibiting protein. About 50% of the protein in serum consists of albumin. If albumin was the only inhibitory component of serum then serum should have about one-half of the inhibitory effect of albumin on a per mg total protein basis. The dashed line of Figure 4-6 represents the inhibitory effect of albumin diluted by one-half with non-inhibiting protein. The line of best fit for the serum showed less inhibition than this dashed line. This would indicate that the other components of serum may be interacting with the albumin to decrease inhibitory activity. Simple summation effects of each of the serum protein components does not seem to be applicable due to some protein-protein interaction (Figure 4-6).

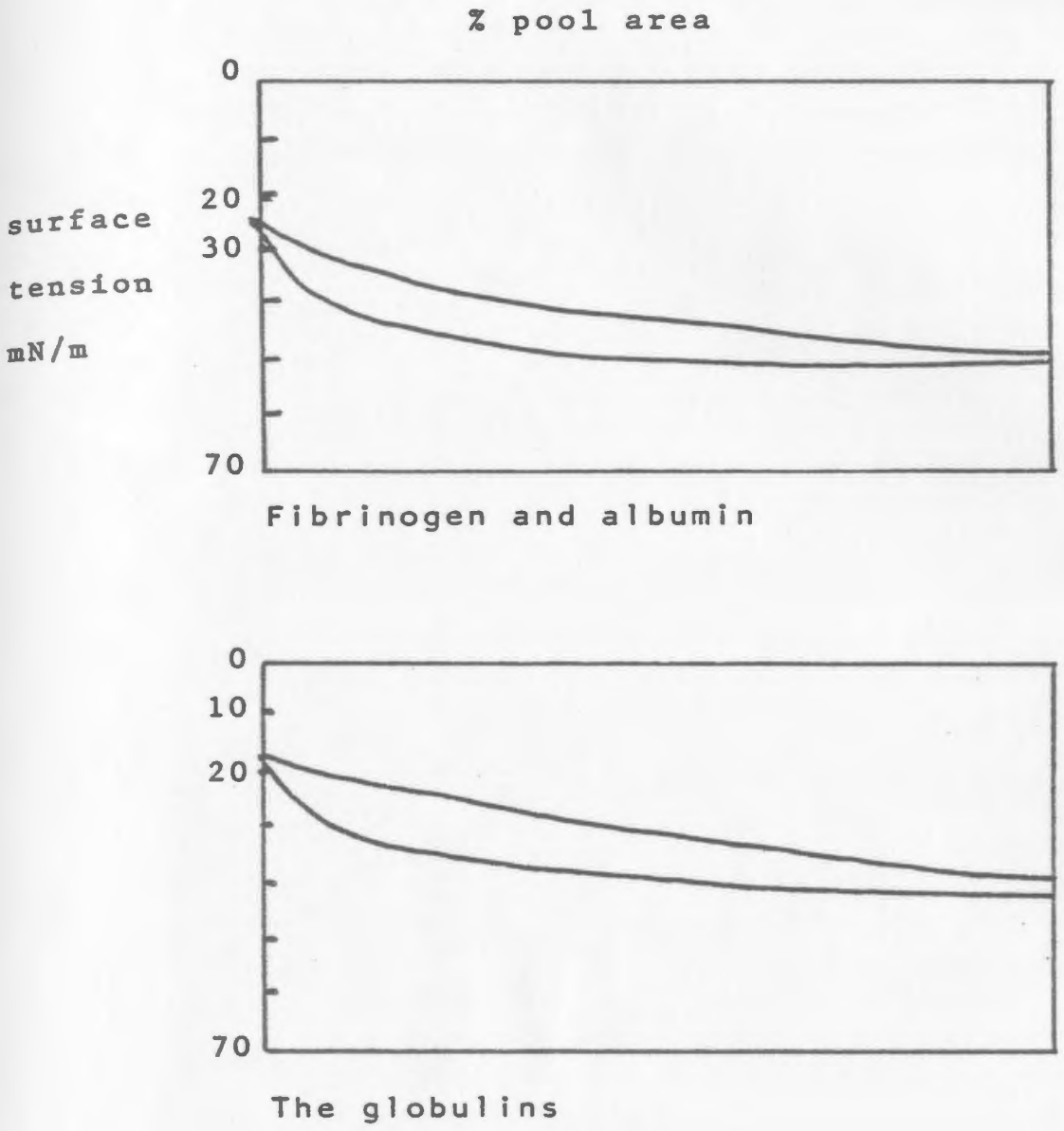


Figure 4-4 Surface tension diagrams for surface films of the plasma proteins

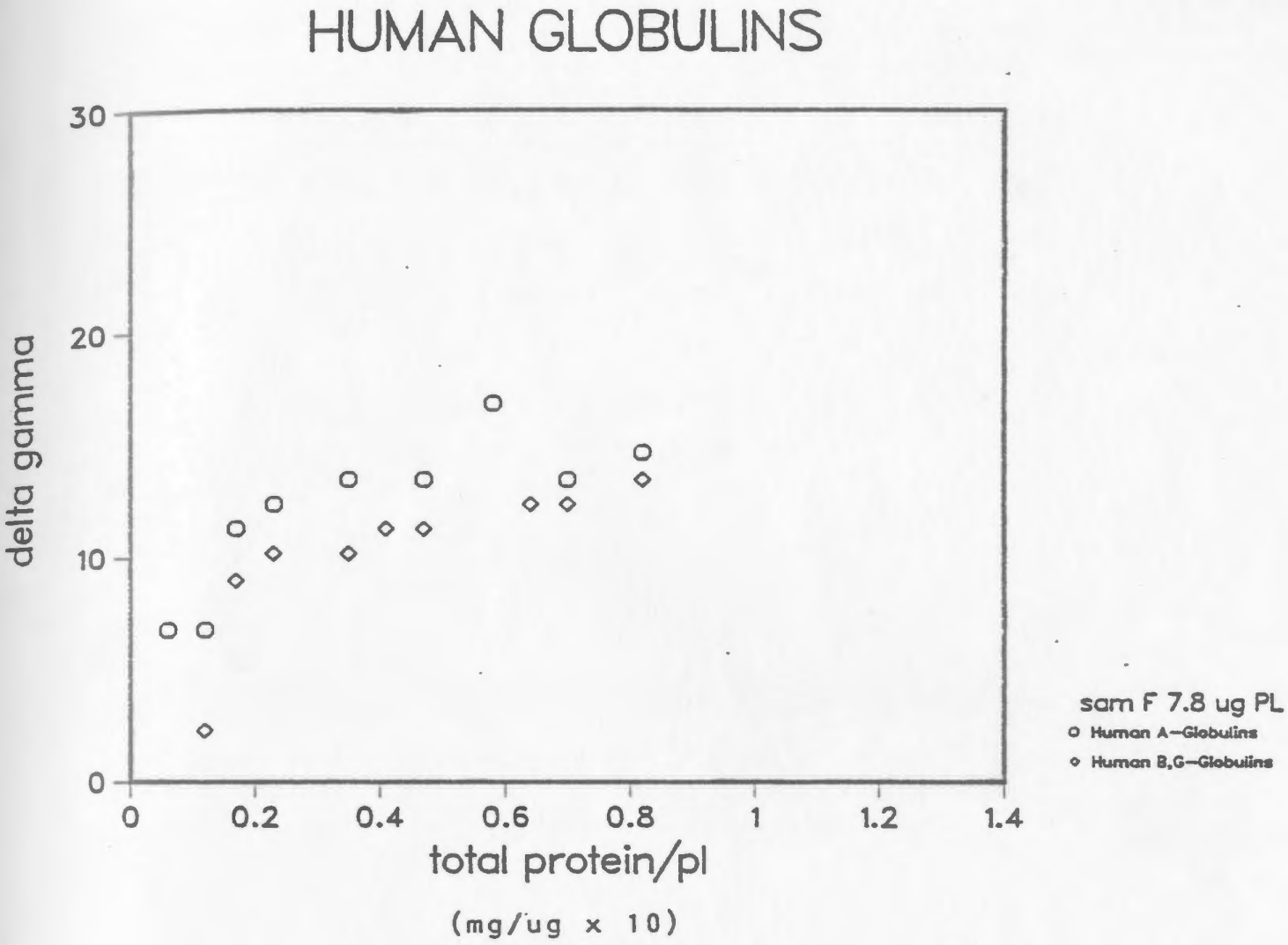


Figure 4-5 The effect of the globulins on surfactant

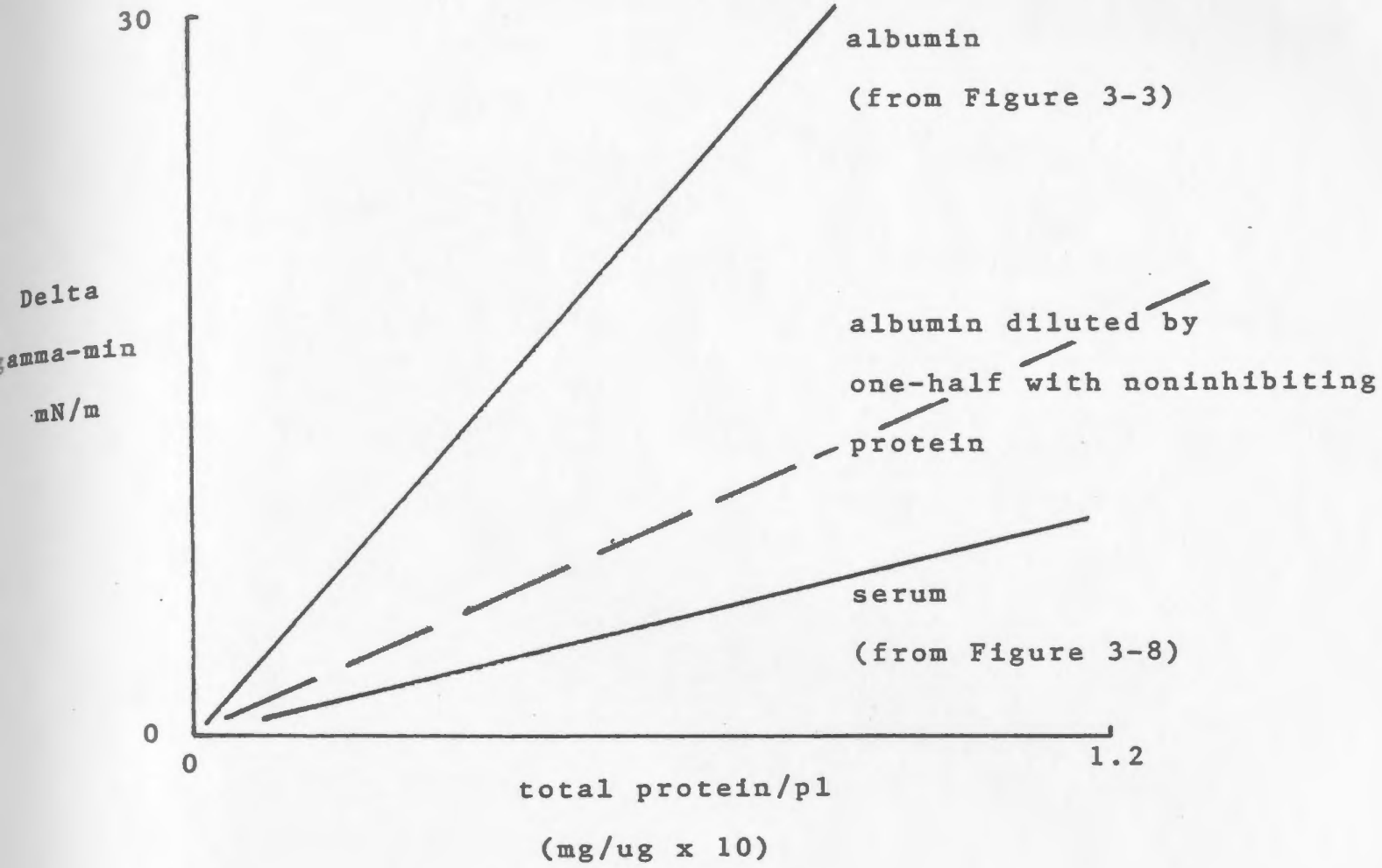


Figure 4-6 Comparison of the effects of serum and albumin on surfactant

4.2.2 Proposed mechanism of inhibition

Surfactant was inhibited from reaching low surface tensions by all of the plasma protein fractions. This inhibition was increased as the amount of protein relative to phospholipid was increased.

The ability to reach low surface tension reflects the relative amount of DPPC on the surface. Other substances do not possess as much ability to lower surface tension at 37 degrees C. The low surface tension results from the ability of DPPC to achieve high packing density and a low free energy state on compression at 37 degrees C. Other substances like proteins which are surface active, if placed in a surface film together with DPPC, may compete for surface space. The surface film may therefore contain less DPPC and low surface tensions would be prevented on compression.

The interactions of surfactant and the plama proteins fibrinogen, albumin and the globulins were observed to be different. The differences in these interactions may be due to differences in the hydrophobicity or the degree of denaturation of the protein at the surface. Surface films of pure proteins are suggested to consist of protein unfolded with loss of tertiary structure (Trurnit, 1960; Absolom, van Oss, Zingg & Neumann, 1981). The hydrophobicity of the proteins in native or denatured forms might influence the degree of exclusion of the protein from the surface film into the aqueous hypophase on compression.

Bigelow (1967) developed a theoretical index of hydrophobicity for proteins based on thermodynamic measurements of the energies of hydration of the component amino acids residues. High values of the hydrophobicity index reflect a greater degree of hydrophobicity. There was not a good correlation between the observed inhibitory effects of the various proteins and the hydrophobicity index (Table 4-2). It should be kept in mind that this hydrophobicity index is a theoretical number.

Table 4-3: The hydrophobicity index in kcal/residue of various proteins in native conformation

bovine fibrinogen - 1020

bovine serum albumin - 1120

human gamma globulin - 1090

beta lipoprotein - 1110

alpha lipovitellin (hen egg) - 1110

Other factors such as the degree of protein unfolding at the surface, the shape of the proteins, their relative affinity for the lipid in surfactant and perhaps the inherent surface activity of the proteins likely are involved in the interaction.

4.2.3 Models of mechanisms of protein-surfactant interaction on the surface balance

Introduction

Varying methods have been used on the surface tension balance to test the effect of blood and plasma proteins on surfactant. Investigators have tested mixtures of protein and surfactant on the surface balance i) using surfactant as a subphase solution (Gardner et al, 1962; Mandelbaum & Giammona, 1964; Tierney & Johnson, 1965), ii) by placing the mixtures on the surface of subphase solutions with the glass rod or droplet techniques (Taylor & Abrams, 1966; Miyahara, 1969; Tabak & Notter, 1977; this thesis), and iii) using protein as the subphase solution with surfactant as the surface film (Rufer & Stolz, 1969). In this thesis mixtures of protein and surfactant were placed on the surface of a saline hypophase solution using the droplet technique. It was found that all of the plasma protein fractions, fibrinogen, albumin and the globulins, inhibited the surfactant from reaching low surface tensions on compression of the surface.

The situation of surfactant inactivation in vivo may be represented by the model where both surfactant and protein occupy the air-water interface as employed in this investigation. Other possibilities include: i) the protein might enter the hypophase and have to penetrate the surface film of phopholipid, ii) the protein may combine with

surfactant precursors (eg tubular myelin) in the hypophase and prevent the adsorption of the DPPC to the surface, iii) the inflammatory reaction which injures the capillary endothelium and the alveolar epithelium may also destroy the surfactant with proteases and phospholipases, and iv) in the case of large amounts of leak, the edema fluid may displace the surfactant from the surface by washing it out of the alveoli.

In this section the possibility was tested that protein may enter the hypophase and penetrate a surface film of surfactant to prevent the achievement of low surface tension. The protein was injected into a saline hypophase beneath a preformed surfactant surface film on the surface balance. In another model of protein-surfactant interaction, a surface film of protein was allowed to form from a hypophase solution containing protein before surfactant was layered on the surface.

Methods

In the first model (Figure 4-7) 1 ml of a 25 mg/ml solution of bovine serum albumin (BSA) was injected beneath a preformed surface film of surfactant over 1 minute. Because the ability of the protein to penetrate the surface film might depend on the initial spacing between the surfactant molecules, the surface load of surfactant was tested at 3.9 ug PL and 7.8 ug PL. This corresponds to an area per molecule of 170 angstroms^2/molecule and 85

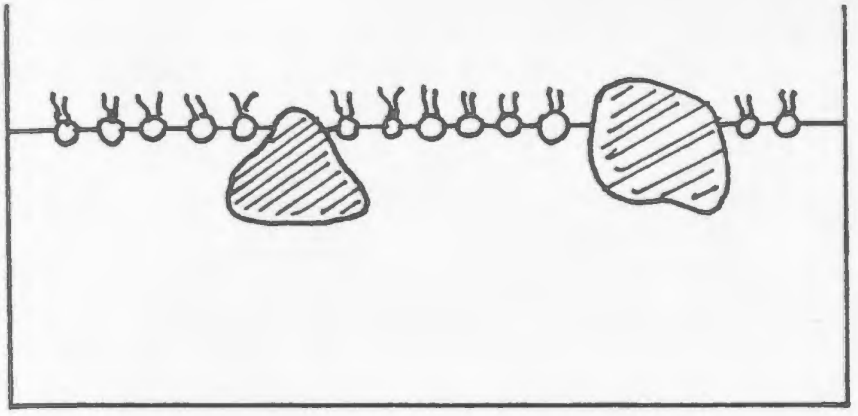
angstroms^2/molecule, respectively. These packing densities do not approach those in the lung where surface tensions are less than 20 mN/m. The area per molecule of DPPC at 20 mN/m is 45 angstroms^2/molecule. Hence these spacings should adequately test penetration of the surface by the protein.

The BSA was injected into the hypophase of solution #1 so that the final concentration was 1 mg/ml. The BSA was first injected into the hypophase solution with no surface film of surfactant to test for mixing and to see if the BSA would reach the surface. The surface tension at maximum surface area was seen to decrease immediately on injection, indicating that the BSA had reached the surface immediately. When testing for the penetration by the BSA of a preformed surfactant surface film, the surface film of surfactant was allowed to equilibrate for 3 minutes before injecting the BSA into the subphase. Five minutes were then allowed before compression was started.

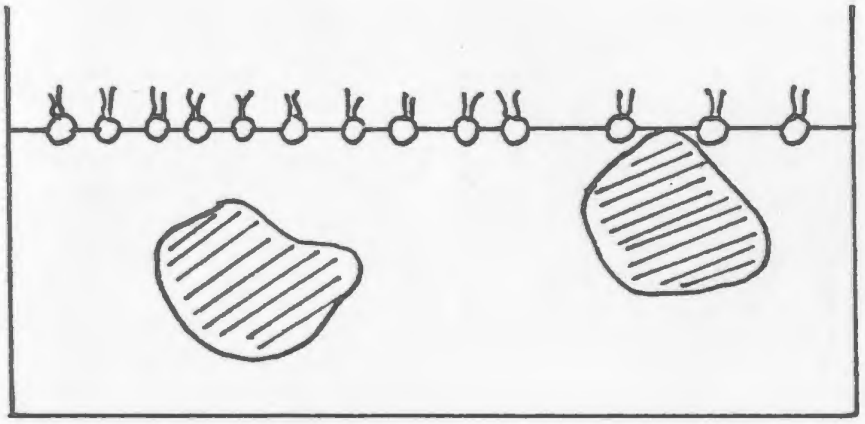
In the second model (Figure 4-7), a solution of BSA, 1 mg/ml in solution #1, was used as the hypophase solution. After 5 minutes, surfactant was placed on the surface with the droplet technique. Compression was started then after 3 minutes.

Results and discussion

The inhibition seen for the model of surfactant layered onto the subphase of protein was greater than that seen for



Protein and surfactant in the surface film



Protein in the subphase beneath the surface film

Figure 4-7 Models of protein-surfactant interaction

Table 4-4: Delta gamma-min for the models on the surface balance

Delta gamma-min, mN/m	surfactant surface load (ug PL)	
	3.9	7.8
surfactant on subphase of protein	7 ±3 (n=2)	11 ±3 (n=2)
protein injected into subphase beneath surfactant	2 ±0 (n=2)	0 ±1 (n=3)

the model of protein injected beneath a preformed surface film of surfactant (Table 4-4). This applied to both surface loads of surfactant. Low minimum surface tension was seen when protein was injected beneath the preformed surfactant surface film even with a low surface load of surfactant. This suggests that two different mechanisms of interaction were involved.

In the model where surfactant was layered onto the surface of a subphase of protein, it was not known that the surfactant would form a complete surface monolayer over the protein hypophase. It was possible also that the surface film would contain both protein and surfactant in the expanded phase of the cycle before compression was started. The surface tension at maximum surface area of a 7.8 ug PL

surfactant load alone on a hypophase of saline was greater than that on a hypophase of BSA, approximately 55-60 mN/m vs 45 mN/m, respectively. The surface tension of the BSA hypophase solution at maximum surface area, after a 5 minute equilibration time, was also about 45 mN/m. This implied that both surfactant and protein coexisted at the surface at maximum surface area before compression was started.

In the surface film of unfolded protein it is likely that the form of protein would be hydrophobic, would not easily be excluded from the surface, and would compete for surface space. This model then, could be considered another test of protein and surfactant coexisting or competing at the surface, rather than a model to test protein penetration of a surfactant surface film.

When protein was injected into the hypophase beneath the preformed surfactant surface films, there was a small decrease in surface tension initially at maximum surface area before compression was started. This increase in surface activity indicated that protein did reach the surface. However the surface tensions at minimum surface area after compression were low, indicating that the surfactant was not inhibited and that the proteins were excluded from the surface. Even though the protein reached the surface it may not have been able to unfold into a more hydrophobic form due to the presence of the lipid at the surface.

The effect of time on the ability of the protein to

penetrate a preformed surfactant surface film was studied. On a hypophase of solution #1 with surface loads of surfactant less than 7.5 ug, and with waiting times greater than 30 minutes, the ability of the surfactant to reach low surface tensions was impaired, ie. gamma-min was greater than 10 mN/m. Perhaps the longer equilibrium times predisposed to oxidation of the phospholipid. In tests of penetration of the surfactant surface layer, BSA was injected into a hypophase of solution #1 to a final concentration of 1 mg/ml beneath a surfactant surface layer with 7.5 ug PL which had been allowed to equilibrate for 3 minutes. 30 minutes were allowed before compression was started. There was no change in minimum surface tension. Gamma-min was less than 4 mN/m, delta gamma-min=0, n=2.

In summary, one model suggested that protein entering the hypophase beneath a surfactant surface film would be easily excluded from the surface. A second model testing protein and surfactant competition for surface space also exhibited surfactant inhibition.

4.2.4 Tests on the pulsating bubble apparatus

The pulsating bubble apparatus, after Enhorning (1977a), may also give clues to the mechanism of inhibition. In this instrument an air bubble is created in a fluid-filled chamber by the application of negative pressure to imitate alveolar opening in the lung. The surface tension at the air-water interface is reflected by the pressure required to

keep the bubble open. The change in surface tension on opening the bubble would indicate the rate at which the surface active molecules were entering the surface film (adsorption to the interface) from the subphase. Changes in this rate in the presence of added protein might indicate that the protein would affect the rate of insertion of the DPPC into the surface.

The bubble can also be pulsated, the effect of which is compression and expansion of the surface area (dynamic alveolar model). The rate at which minimum surface tension was reached after pulsation was started would suggest the rate at which protein or components other than DPPC might be excluded from the surface film on compression.

It was felt that this instrument would provide good models for tests of selective insertion and exclusion of DPPC into and from the air-water interface using a mixture of surfactant and protein.

In preliminary tests however, the pig lung surfactant preparations did not have consistent rates of opening surface tensions or consistent rates of reaching minimum surface tensions on pulsation of the bubble. The time elapsed from the opening of the bubble to an opening pressure of 1 cm H_2O ranged from 73 to 136 seconds (0.65 ug PL/ul) (Figure 4-9). Similar variability was seen in the initial rate of change of opening pressure or in the time taken to reach any given opening pressure. The time elapsed from beginning pulsation to an opening pressure of 0.25 cm

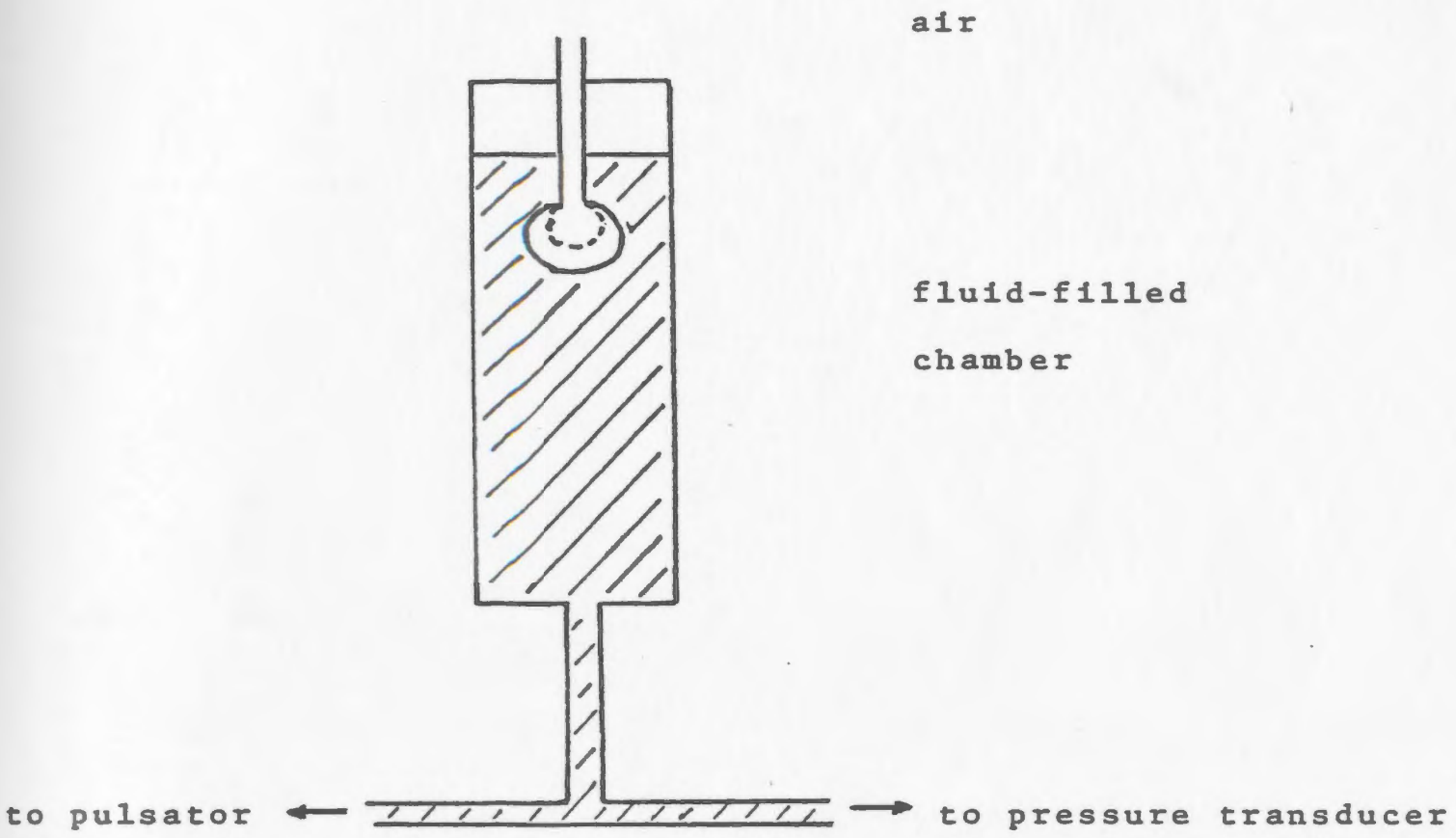


Figure 4-8 The dynamic alveolar model

H_2O ranged from 1.5 sec (first cycle) to 16 minutes (1.3 ug PL/ul). Tests of surfactant-protein interaction were not done at this time.

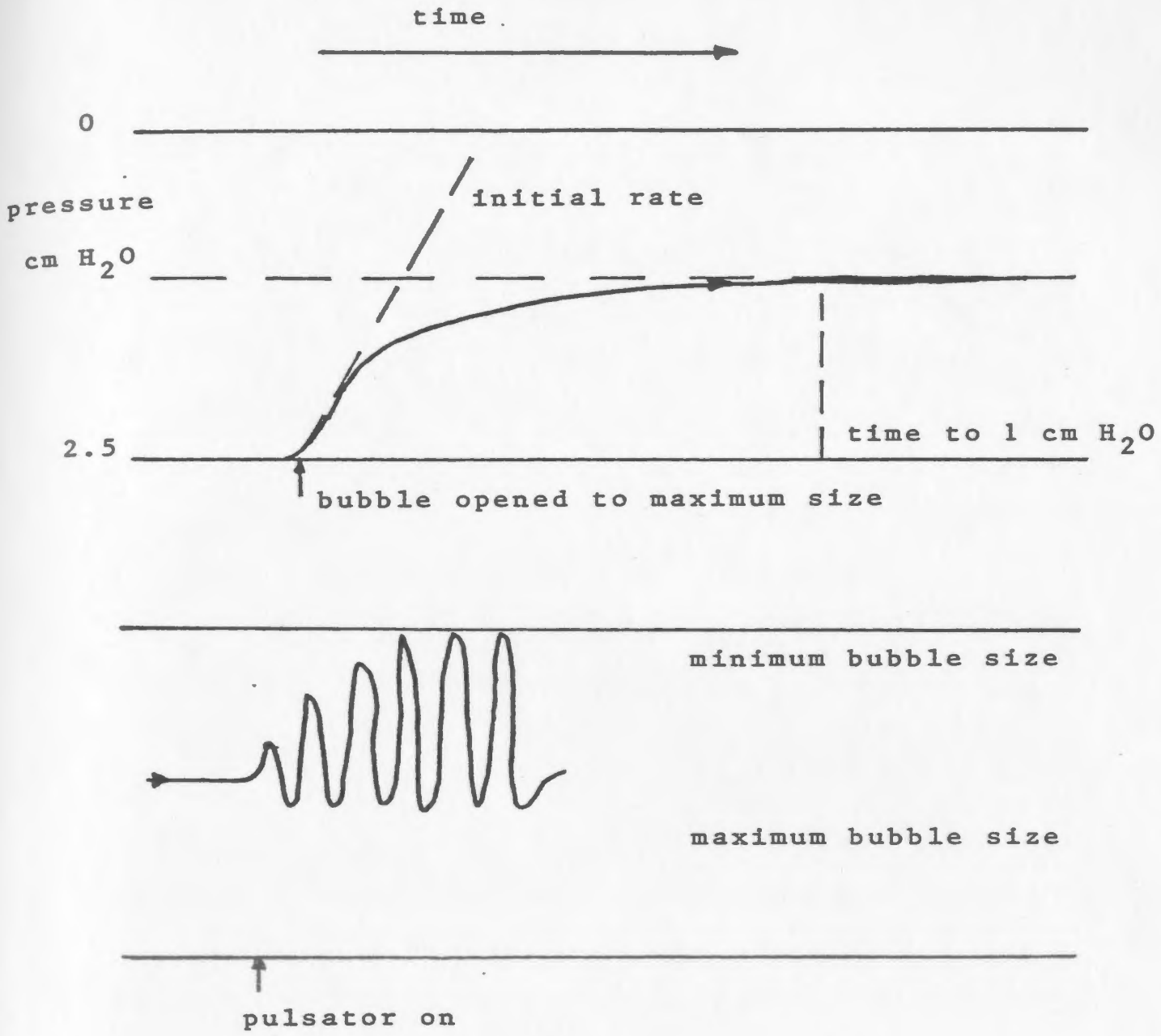


Figure 4-9 Examples of tracings from the pulsating bubble apparatus

Chapter 5

Summary and biologic relevance

The relevance of testing protein-surfactant mixtures on the surface balance has been discussed in relation to surfactant dysfunction in RDS and in particular the RDS following CPB. In RDS an acute lung injury produces leaking blood-air membranes through which plasma constituents enter the alveoli. This thesis addressed the hypothesis that plasma constituents could inhibit surfactant from proper function.

Adult respiratory distress syndrome is a cause of significant morbidity and mortality in today's medicine. Its incidence is increased in the setting of trauma. This condition became well recognized during the Viet Nam War when improved skill in resuscitation prolonged survival from injuries which would have previously been immediately fatal. However with this prolonged survival, ARDS became a major cause of late fatality. Today ARDS is a significant cause of mortality in the victims of motor vehicle accidents. The fatality rate remains at 50% once ARDS develops despite improved critical care medicine.

Much research has been done on this prevalent disease. From a pathophysiologic viewpoint, most studies have addressed the mechanisms of acute injury to the lung and there is a tremendous literature concerning the possible injurious agents, eg. complement, leukocyte products, platelet products, fibrin degradation products, etc. In contrast, studies on surfactant dysfunction have been less extensively investigated.

Surfactant dysfunction is an important result of the acute lung injury. Dysfunctional surfactant would contribute to the clinical problems of atelectasis and pulmonary edema resulting in poor gas exchange. It was important and logical to consider the possibility that plasma constituents which are known to enter the airspaces through leaking blood-air membranes produced by the acute lung injury, might inhibit surfactant from proper function.

Cardiopulmonary bypass is a known etiologic agent of acute lung injury, and although the incidence of RDS following operations involving CPB is small in elective adult cardiac surgery today, RDS remains a significant complication in pediatric cases and in cases involving prolonged bypass. CPB provided a setting where the inflammatory response induced in the blood might produce or increase levels of potential circulating surfactant inhibitors. The induced inflammatory response is a mechanism whereby blood-air membranes could be injured, resulting in leaking membranes and entry of plasma constituents into the alveoli. The

pathophysiologic characteristics of acute lung injury and surfactant dysfunction, and the clinical characteristics of RDS following CPB are similar to those of ARDS. CPB is included in the long list of etiologic factors of ARDS.

The literature presented an inconsistent view of surfactant inhibition by blood and plasma proteins. This inconsistency was also seen in studies of the effect of CPB on surfactant inhbitors in blood. Some of the inconsistency may have resulted from variation in the methods of testing surfactant-potential inhibitor mixtures on the surface balance since different mechanisms of interaction might provide different results. No previous investigation had studied human subjects undergoing CPB.

The task of studying surfactant inhibition in this thesis involved defining one mechanism of surfactant-potential inhibitor interaction, then testing for the presence of potential inhibitors using this assay.

The assay used was a model of protein-surfactant interaction where both protein and surfactant would coexist at the surface. Mixtures of protein and surfactant were placed on the surface of a hypophase solution of saline. The attainment of low surface tension on compression of the surface was used as an indicator of surfactant function. Tests to determine conditions of optimum surface balance function were performed. Test conditions were precisely defined in consideration of the variability in the literature and in contrast to some previous literature cited.

The plasma from normal individuals was found to inhibit surfactant. In a study controlled with prebypass and postbypass samples, CPB did not increase this inhibition in adult patients or in child patients. Surfactant inhibition by plasma taken from child patients was not greater than that taken from adults. Tests of surfactant inhibition by plasma in this assay did not provide reasons for the increased incidence of postpump lung dysfunction seen in children or in prolonged bypass.

Thus the data were consistent with the view that modern CPB itself does not produce surfactant inhibitors in plasma or increase the levels of inhibitors in plasma. Of the patients studied only one child developed RDS as a complication of CPB. The possibility that additional inhibitors may be produced in the group of patients who subsequently develop RDS was not studied. One reason is that the frequency of RDS following CPB in elective adult cardiac surgery is less than 2%.

The data were consistent with the possibility that plasma components entering the alveoli through leaking lung membranes could inhibit surfactant from proper function. In this setting the degree of injury to the blood-air membranes would determine the extent of plasma leakage and the degree of surfactant dysfunction. The increased incidence of postpump lung dysfunction seen in children undergoing CPB and in prolonged bypass would then possibly depend on an increased extent of injury to the lung membranes as a result

of the CPB or an increased susceptibility of the lung to injury. For example, prolonged bypass may induce a greater inflammatory response in the blood, and children may have more fragile lungs.

The raised minimum surface tensions of 20-30 mN/m of the protein-surfactant mixtures seen in the tests of this thesis are similar to the minimum surface tensions seen in tests of lung washings and extracts of lung minces from patients with RDS in adults and infants. In this range of surface tension there is the clinical picture of lung collapse and alveolar edema. In contrast, alveolar surface tension has been measured in vivo in normal cat lungs at 40% total lung capacity as less than 1 mN/m (Schurch, 1982).

In a proposed mechanism of inhibition, when both protein and surfactant exist at the surface, the protein was not excluded from the surface film hence preventing the DPPC component of surfactant from achieving high packing density and low surface tension. No specific surfactant inhibitor was suggested by the data. It seemed unlikely that plasma components other than proteins would contribute to surfactant inhibition. The plasma protein fractions fibrinogen, albumin and globulins were all seen to inhibit surfactant.

Other models of protein-surfactant interaction on the surface balance were studied. Surfactant layered on a hypophase of protein likely represented an alternate model of protein and surfactant at the surface whereas protein

injected beneath a preformed surfactant surface film tested penetration of the surfactant surface film by the protein. While protein and surfactant at the air-water interface in vivo would result in surfactant dysfunction, the actual mechanism whereby plasma protein would reach the surface to interact with the surfactant is unknown.

Mechanisms of the effect of plasma protein on surfactant may possibly be investigated further using the pulsating bubble apparatus. Consideration of the possibility that surfactant inhibitors produced by the acute lung inflammation and injury, and which might be local to the lung (ie. not circulating in the blood) is not without merit.

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Appendix A

Methods and Protocols

A.1 Solutions for BAL protocols

These solutions were given in the paper by King & Clements (1972). The solutions all contained .005M THAM with pH adjusted to 7.35 with concentrated HCl.

	NaCl	NaBr	$MgCl_2$	$CaCl_2$
Solution #1:	.15M	0	.003M	.003M
Solution #2:	.015M	.6M	.004M	.004M
Solution #3:	.15M	1.4M	.031M	.031M
Solution #4:	.15M	1.64M	.035M	.035M

A.2 Modification of the Yu et al (1983) protocol for bronchoalveolar lavage fluid (BAL)

All centrifugations were done at 4 degrees C.

1. The BAL was centrifuged at 800 gav for 10 min (RC-3 Sorvall; Sorvall rotor HG-4L; 1685 rpm).
2. The supernatant from (1) was centrifuged at 8000 gav for 60 min (Beckman J2-21; JA-10 rotor; 6800 rpm).
3. The pellet from (2) was resuspended in solution #1, then dialysed (12,000 MW exclusion membrane) against H_2O for 48 hrs (5 changes in bath, 4 degrees C).
4. The non-diffusable material was lyophilized and stored at -20 degrees C.

A.3 Modification of the King & Clements (1972) protocol for BAL

All centrifugations were done at 4 degrees C.

1. The BAL was centrifuged at 800 gav for 10 min (RC-3 Sorvall; Sorvall rotor HG-4L; 1685 rpm).
2. The supernatant from (1) was centrifuged at 65,900 gav for 90 min (Beckman L3-50; 42.1 rotor; 31,000 rpm).
3. The pellet from (2) was resuspended in solution #4 (78 ml) and centrifuged at 81,500 gav for 15 hrs (Beckman L5-50B; SW 28 rotor; 24,500 rpm).
4. The floating white pellicle from (3) was removed with spatula and pasteur pipette. It was resuspended in solution #1 and centrifuged at 65,900 gav for 2 hrs (Beckman L3-50; 60 Ti rotor; 31,000 rpm).
5. The pellet from (4) was resuspended in solution #3 (78 ml) and left to equilibrate overnight.
6. A continuous density gradient using the suspension from (5) and an equal volume of solution #2 was made, then centrifuged at 81,500 gav for 15 hrs (Beckman L5-50B, SW 28 rotor; 24,500 rpm).
7. The band of white particles about one-third of the way up from the bottom of the centrifuge tubes was aspirated (about 2 ml). The aspirate was diluted with solution #1 (30 ml) and centrifuged at 65,900 gav for 2 hrs (Beckman L5-50B; 60 Ti rotor; 31,000 rpm).

8. The pellet from (7) was resuspended in solution #3 (78 ml) and centrifuged at 65,900 gav for 15 hrs (Beckman L5-50B; SW 28 rotor; 24,500 rpm).

9. The pellicle from (8) was resuspended in H_2O and centrifuged at 65,900 gav for 2 hrs (Beckman L5-50B; 60 Ti rotor; 31,000 rpm).

10. The pellet from (9) was resuspended in solution #1 and dialysed against H_2O for 48 hrs, with 5 changes in water bath, at 4 degrees C.

11. The non-diffusable material was lyophilized and stored at -20 degrees C.

A.4 Modification of the Shelley et al (1977) protocol for BAL

All centrifugations were done at 4 degrees C.

1. The BAL was centrifuged at 800 gav for 10 min (RC-3 Sorvall; Sorvall rotor HG-4L; 1685 rpm).
2. The supernatant from (1) was centrifuged at 8000 gav for 60 min (Beckman J2-21; JA-10 rotor; 6800 rpm).
3. The pellet from (2) was resuspended in solution #4 and centrifuged at 81,500 gav overnight (Beckman L5-50B; SW 28 rotor; 24,500 rpm).
4. The pellicle from (3) floating on top was removed with a spatula and a pipette and resuspended in solution #1. It was then centrifuged at 65,900 gav for 2 hrs (Beckman L3-50; 60 Ti rotor; 31,000 rpm).
5. The pellet from (4) was resuspended in 20 ml of a 16 g/dl NaBr solution. A discontinuous density gradient was formed by layering on 32 ml of a 13 g/dl NaBr solution, then 8 ml of a 0.9 g/dl NaCl solution. The gradient was then centrifuged at 81,500 gav for 3 hrs (Beckman L5-50B; SW 28 rotor; 24,500 rpm).
6. The pellicle from (5) formed at the interface between the 13% NaBr and the saline was removed with spatula and pipette. It was resuspended in solution #1 and centrifuged at 65,900 gav for 90 min (Beckman L5-50B; 60 Ti rotor; 31,000 rpm).

7. The pellet from (6) was resuspended in solution #1 and dialysed against H_2O (12,000 MW exclusion membrane) for 48 hrs, with 5 changes in water bath, at 4 degrees C.

8. The non-diffusable material was lyophilized and stored at -20 degrees C.

A.5 Our protocol for preparation of surfactant from BAL

All centrifugations were done at 4 degrees C.

1. The BAL was centrifuged at 800 gav for 10 min (RC-3 Sorvall; Sorvall rotor HG-4L; 1685 rpm).
2. The supernatant from (1) was centrifuged at 8,000 gav for 60 min (Beckman L3-50; 42.1 rotor; 6800 rpm).
3. The pellet from (2) was resuspended in solution #4 and centrifuged at 81,500 gav overnight (Beckman L5-50B; SW 28 rotor; 24,500 rpm).
4. The pellicle from (3) floating on top was removed with a spatula and a pipette and resuspended in solution #1. It was then centrifuged at 65,900 gav for 2 hrs (Beckman L3-50; 60 Ti rotor; 31,000 rpm).
5. The pellet from (4) was resuspended in solution #1 and dialysed (12,000 MW exclusion membrane) against H_2O for 48 hrs with 5 changes in water bath, at 4 degrees C.
6. The non-diffusable material was lyophilized and stored at -20 degrees C.

A.6 Preparation of surfactant suspensions

The lyophilized pig lung surfactant had been stored at -20 degrees C. Suspensions of 2 mg/ml of surfactant in solution #1 were made for testing on the surface balance. The lyophilized material was suspended by stirring with a glass rod, vortexing, and sonicating (3 bursts of 100 watts of 15 sec duration each; Sonifer Cell Disruptor, Ultrasonics; microprobe with 13 mm horn and 3.2 mm tip). The material was kept cool in an ice bath between sonications. The suspensions of surfactant were divided into 0.5 ml aliquots and stored in glass vials at -20 degrees C.

Tests of surfactant function were made on the surface balance using thawed aliquots of the surfactant suspensions. All comparative tests of surfactant function were performed using the same batch of lyophilized surfactant prepared in one processing protocol, harvest date 1/8.

A.7 Phosphate determination by a modification of the method of Bartlett (1959)

Materials

1. chromic acid-washed glass tubes
2. 70% perchloric acid
3. ANSA
4. molybdate
5. antibumping granules

Methods

1. Add antibumping granules, 50 ul of 2 mg/ml surfactant, 1 ml of perchloric acid
2. Boil for 10 minutes or until clear
3. Add 8.0 ml H_2O, 0.5 ml molybdate, 0.5 ml ANSA
4. Vortex
5. Standard: 2 ml of 2 ug phosphorus/ml standard solution, 1.0 ml of perchloric acid, 6 ml of H_2O, 0.5 ml molybdate, 0.5 ml ANSA
6. Immerse standard and samples in boiling water bath for 12 minutes
7. Read optical density at 815 nm
8. Phospholipid concentration estimated by multiplying the phosphate concentration by 25
9. Linearity of the test was determined using standard phosphate solutions

A.8 Biuret protein determination after Gornall et al (1949)

Materials

Biuret reagent

1.5 g $CuSO_4 .5H_2O$. Dissolve in 500 ml H_2O

Add 6 g NaK Tartarate

Add with constant swirling, 300 ml of 10% NaOH

Dilute to 1 litre and store in dark bottle

5% deoxycholic acid (DOC)

standard bovine serum albumin solution (BSA) - 5 mg/ml;

crystalline BSA from Sigma catalog no. A-7906,

lot 53F-0256, nb. H_2O content 5.6%

Methods

1. Size of test sample = 50 ul
2. Add 0.2 ml of 5% DOC, 0.75 ml H_2O,
   4.0 ml Biuret reagent
3. Vortex, and let stand for 30 min before reading
   optical density
4. Standard curve: 4.0 ml of Biuret reagent, 0.2 ml DOC,
   various amounts of standard protein solution
   0.2, 0.4, 0.6, 0.8 1.0 ml with corresponding amount of
   H_2O to make up 5.0 ml in total
5. Control: 4.0 ml Biuret reagent, 1.0 ml H_2O
6. Read optical density at 550 nm

A.9 Polyacrylamide gel electrophoresis, W.Davidson, personal communication

Materials

Separating gel 60 ml

10 ml Tris-sulfate pH 9.0, 1.5 M Tris, titrated with sulfuric acid

20 ml 0.2% ammonium persulfate

14 ml H_2O

16 ml 30% acrylamide (0.8% Bis)

4 drops Temed

Stacking gel 10 ml

1.25 ml Tris-sulfate pH 7.4, 0.5 M Tris

5.0 ml 0.2% ammonium persulfate

2.5 ml H_2O

1.25 ml 30% acrylamide (0.8% Bis)

2 drops Temed

Methods

A mold for shaping the slab gel was made using two glass plates (20.7 x 26.0 cm) separated with plastic borders (PVC plastic, 1.6 mm thick), sealed with high pressure vacuum grease and clamped with large spring-type paper clips. The separating gel (approximately 20 cm in length) was poured into the mold leaving room for the stacking gel at the top (approximately 5 cm from the top edge of the glass). The separating gel was covered with isopropanol to create a

smooth surface. At least 4-5 hours were allowed for polymerization. The propanol was poured off. A plastic comb (12 tooth PVC comb with tooth measurements 0.65 x 1.9 cm) was inserted above the separating gel as a template for the stacking gel. The stacking gel was poured under the comb and was allowed to polymerize for 1-2 hrs. The comb was removed to expose the wells (0.65 x 1.9 cm) into which the samples were placed. The samples were mixed with 50% glycerol (sample:glycerol, 3:1, v:v) to settle the sample at the bottom of the well. The samples were inserted to a maximum of 30 ul per well. Bromphenol blue was used in the side wells as a migration marker. The gel was run at constant current, 10 mA per gel with the voltage under 200 v. to avoid heat denaturation of the proteins. The running time was about 10-12 hrs. The gel was then removed from between the glass plates and handled with gloves (to avoid protein contamination from the fingers). Coomassie brilliant blue was used to stain the gel for 1 hr.(0.2 g Coomassie G250, 500 ml H_2O, 25 ml 70% perchloric acid). The gel was then destained and fixed with 3-4 rinses of 7% glacial acetic acid over 48 hrs.

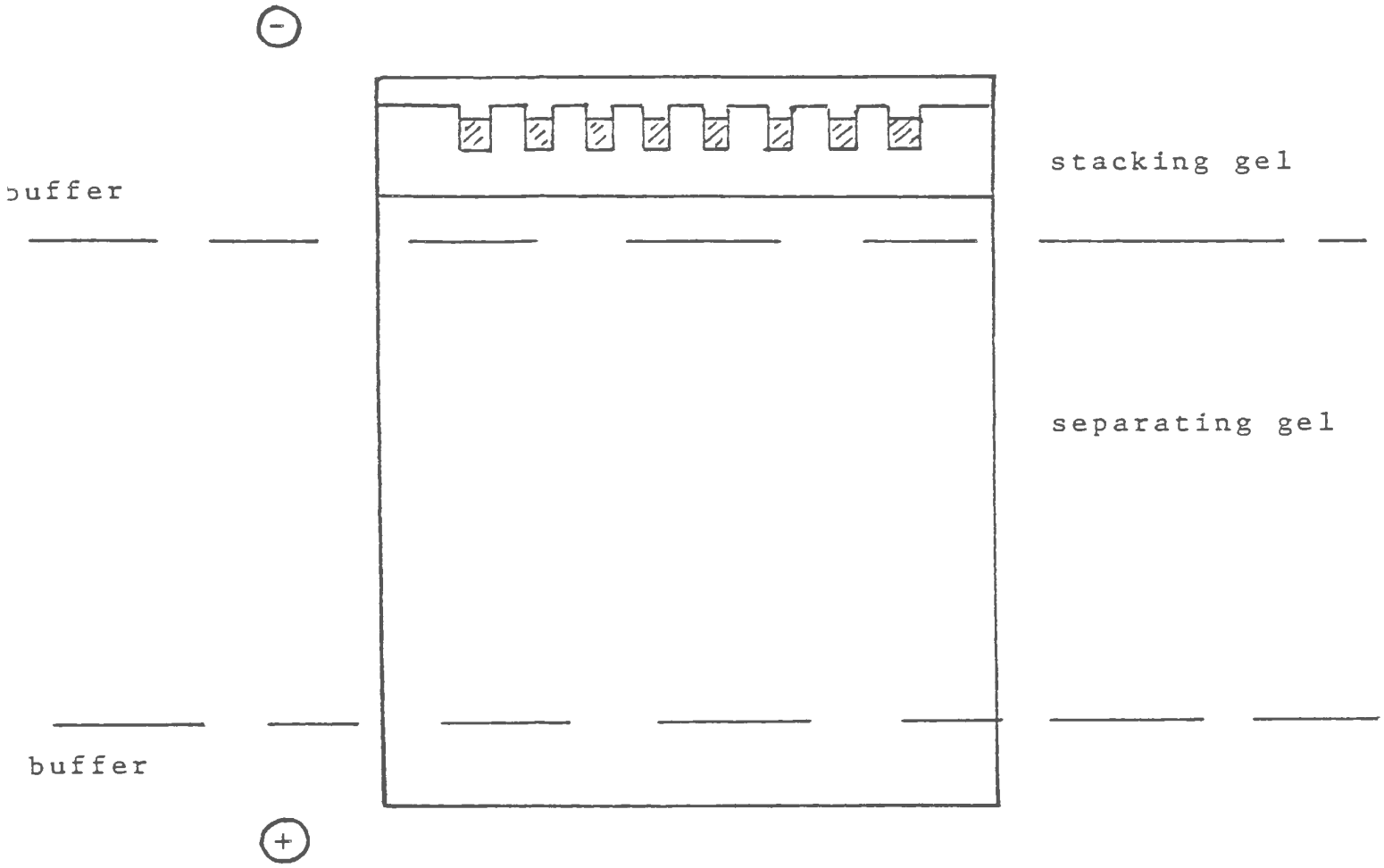

Figure A-1 Apparatus for polyacrylamide gel electrophoresis

A.10 Polyethylene glycol (PEG) fractionation of plasma proteins

Ikegami et al (1983); Polson et al (1964)

Materials

PEG average MW 3350, Sigma P-3640 Lot 43F0292

0.02 M monobasic phosphate and NaOH buffer pH 7.0 titrated with concentrated HCl

Methods

Plasma was prepared with heparin. It was diluted with the phosphate buffer to 0.4 g/dl (5 ml plasma, 95 ml buffer).

PEG was added slowly with stirring at room temperature to 17 g/dl to yield an opaque white liquid. Equilibration was allowed over 20 minutes before centrifugation at 10,000 gav for 15 min at 21 degrees C (Beckman L-50B centrifuge, 60 Ti rotor, 11,800 rpm). This yielded a clear supernatant (PEG 17%) and a white precipitate (expected to contain fibrinogen and the globulins as the major components).

PEG was added slowly to the supernatant from (2) to 25 gm%, (assume volume of solid PEG of 0.66 ml/g). The pH was adjusted to 5.4 with concentrated HCl to yield an opaque yellowish liquid. After equilibration for 20 min, the liquid was centrifuged at 10,000 gav for 15 min at 21 degrees C. This yielded a clear supernatant and a white precipitate (expected to contain albumin).

PEG was added slowly to the supernatant from (3) to 50%, followed by an equilibration time of 20 min. The liquid was centrifuged at 10,000 gav for 15 min. at 21 degrees C. No precipitate was observed by visual inspection, although the fraction was treated as if there was a precipitate. This is the fraction in which the inhibitor of Ikegami and Jobe might be expected.

All of the precipitates from the 3 fractions were resuspended in solution #1. The PEG 25% fraction required adjustment of the pH back to 7.0 with NaOH before it dissolved. With no observable precipitate in the 50% fraction, this fraction was collected by rinsing the inside walls of the centrifuge tubes with solution #1. Minimum amounts of solution #1 were used to redissolve the proteins so that the protein concentration would be comparable to plasma protein concentrations. This was desirable for testing on the surface balance since too low a protein concentration would require high volumes of solution to test equivalent amounts of protein on the surface.

A.11 Refractive index (RI) tables for solutions of bovine serum albumin (BSA) and polyethylene glycol (PEG)

Table A-1: RI vs [BSA]

RI	[BSA]mg/ml
-.3	0.96
-.1	1.90
0	3.85
0.4	7.70
1.2	15.40
2.7	30.80
5.8	61.60

Table A-2: RI vs [PEG], [BSA] = 0 mg/ml

RI	[PEG]g/dl
1.9	2.5
3.3	4.5
4.2	5.0
7.8	10.0
16.2	20.0
21.3	25.0
39.5	50.0

Table A-3: RI vs [PEG], [BSA] = 6.16 mg/ml

RI	[PEG]g/dl
3.2	4.5
7.5	9.0
15.0	18.0
18.9	22.5
34.6	45.0

Table A-4: RI vs [PEG], [BSA] = 10.3 mg/ml

RI	[PEG]g/dl
3.3	4.3
7.3	8.6
14.6	17.1
18.4	21.4
34.8	42.9

Table A-5: RI vs [PEG], [BSA] = 20.5 mg/ml

RI	[PEG]g/dl
4.3	3.0
4.7	3.3
7.1	6.7
12.8	13.3
15.8	16.7
29.6	33.3

Table A-6: RI vs [PEG], [BSA] = 30.8 mg/ml

RI	[PEG]g/dl
4.5	2.3
4.8	2.5
6.8	5.0
11.0	10.0
13.4	12.5
22.7	25.0

Table A-7: RI vs [PEG], [BSA] = 41.1 mg/ml

RI	[PEG]g/dl
5.0	1.7
6.5	3.3
9.4	6.7
11.2	8.3
18.0	16.7

Table A-8: RI vs [BSA], taken from the plots of the data, RI vs [PEG] for various [BSA] at the y-intercepts

RI	[BSA]ug/ml
0.62	6.16
0.37	10.30
1.76	20.50
2.87	30.80
3.66	41.10

Table A-9: Delta gamma-min vs [PEG/PL], san F load = 7.8 ug PL

delta gamma-min (mN/m)	[PEG/PL] (ug/ug)
3.4	1.3
2.3	2.6
3.5	6.4
5.8	12.8
3.4	12.8
5.8	16.0
8.0	32.1
14.9	32.1
15.8	51.3
18.4	64.1

A.12 Ethanol precipitation of the PEG fractions using the Cohn method

Cohn et al (1950); Edsall & Anson (1947)

Materials

1. Acetate buffer pH 4.0 (180 ml 0.2M NaOAc, 820 ml 0.2M HOAc)

Methods

All work was done at 4 degrees C. All solutions, glassware and centrifuge tubes and rotor were prechilled. The temperature of precipitation should be 0 to -5 degrees C.

Each PEG fraction precipitate was resuspended in 10 ml of solution #1 and chilled.

Redistilled 95% ethanol was added drop by drop with a pasteur pipette. The solutions were kept in an ice-water bath.

To the solution containing the PEG 17% precipitate, ethanol was added to 25% v:v (3.5 ml). This was expected to precipitate fibrinogen and the gamma and beta globulins.

To the solution containing the PEG 25% precipitate, ethanol was added to 40% v:v (7.0 ml), then the pH adjusted to 4.8 with the acetate buffer (about 0.63 ml). The pH was checked by taking up 0.2 ml of the ethanol-protein solution in 9 volumes of water (1.8 ml) in order to dilute the ethanol for reading the pH with a glass electrode.

To the solution containing the PEG 50% precipitate,

ethanol was added to 40% v:v (7.0 ml), then the pH adjusted to 4.8 with the acetate buffer.

The fractions were centrifuged at 10,000 gav, at temperature -5 degrees C, for 15 minutes (Beckman L5-50, 60 Ti rotor, 11,800 rpm). About 10 minutes of equilibration time elapsed from the adding of the ethanol and adjustment of the pH to the time of centrifugation.

The precipitates were resuspended in 1 ml of solution #1, then dialysed with an 8,000 MW membrane in solution #1 at 4 degrees C overnight. The microdialysis method of Lau & Fujitaki (1981) was used. This consisted of placing the sample in a 1.5 ml plastic "Eppendorf" centrifuge tube covered by the dialysis membrane which was tied in place with a silk thread (Figure A-2). The tube was taped to the side of a 600 ml glass beaker. The dialysis bath of 500 ml was stirred constantly by magnetic stirrer.

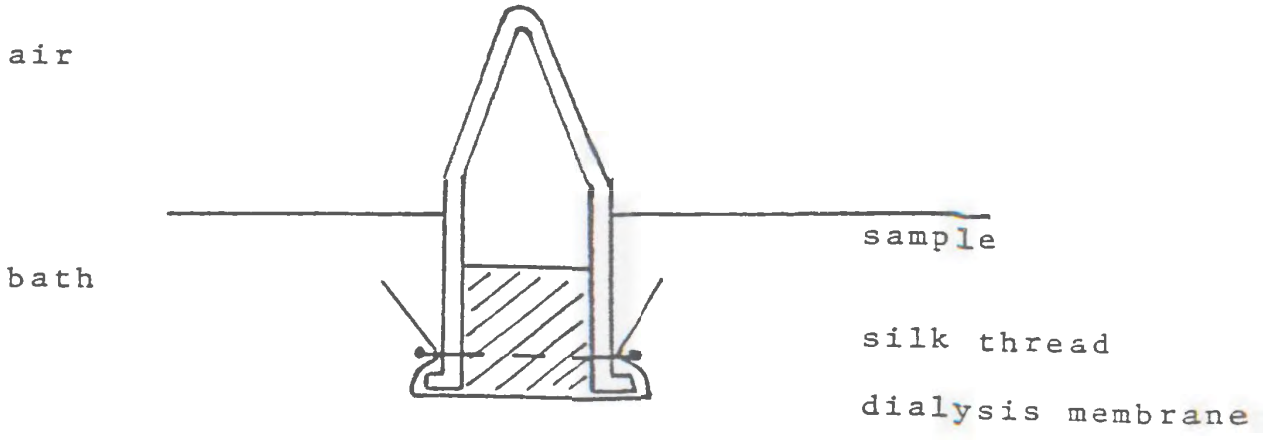

Figure A-2 Method for microdialysis

A.13 Materials and equipment

	Supplier, catalog number, lot number
acetid acid (glacial)	Fisher A-38
acrylamide	BioRad 161-0107
albumin (human)	Red Cross product
ammonium molybdate	Fisher A674
ammonium persulfate	BioRad 161-0700
ANSA (1-amino-2-naphthol-4-sulfonic acid)	Sigma A0505
antibumping granules	BDH 2189
BIS (NN-methylene-bis-acrylamide)	BioRad 161-0200
bromphenol blue	BioRad 161-0404
BSA (bovine serum albumin)	Sigma A7906 lot 53-F-0256
$CaCl_2$ (dihydrate)	Sigma C-3881
chloroform	Fisher C-605
citric acid (trisodium salt dihydrate)	Sigma C7254
Coomassie brilliant blue G250	BioRad 161-0406
$CuSO_4$ (pentahydrate)	Sigma C7631
deoxycholic acid (sodium salt)	Sigma D6750
dialysis membrane	Fisher 8-667B
DPPC	Sigma P6267
DSPC	Sigma P1138
electrophoresis power supply	Hoefer Scientific Instruments PS1200
ethanol (95% redistilled)	Consolidated Alcohols, Toronto
fibrinogen (human)	Sigma F3879 lot 81F-9365
freeze-dryer (lyophilizer)	Labconco
globulins (human)-gamma and beta	Sigma G2388 lot 93F-9365
-alpha	Sigma G2011 lot 11F-9317
HCl (37%)	CanLab C9800-10

heparin (porcine intestinal mucosa)	Organon
hexane (pesticide grade)	Fisher H-300
isopropanol	Fisher A-416B
$LaCl_3$ (7 H_2O)	Sigma L4131
methanol	Fisher A-936
$MgCl_2$	Fisher M-33
NaBr	Fisher S-255
NaCl	Fisher S-271B
NaH_2PO_4	Sigma S0751
NaOH	Fisher S-318B
NaK tartarate	Sigma S-2377
perchloric acid (70%)	Fisher A-229
polyethylene glycol	Sigma P3640
proteinase K	Sigma P-0390 lot 14F-0142
refractometer	Fisher Scientific
sonicator	Sonifer Cell Disruptor Heat Systems-Ultrasonics
spectrophotometer	Unicam (SP500 Series 2)
sucrose	Sigma S9378
sulfuric acid	Fisher A-300
surfactometer	Kimray-Greenfield (surface balance) Surfactometer International (pulsating bubble apparatus)
Temed (tetramethylene diamine)	BioRad 161-0800
THAM (tris hydroxymethyl aminomethane)	Fisher T-395
vortex	Fisher