THE ASSESSMENT OF

FETAL LUNG MATURITY

BY

ANALYSIS OF AMNIOTIC FLUID

A Thesis Presented in Candidature

for the Degree of

Master of Science

by

Anne Isabel Wilson, B.Sc.

Department of Midwifery

and

Department of Biochemistry

University of Glasgow

January 1982

ProQuest Number: 10647035

ProQuest 10647035

Perhaps credit for the earliest observation of mammalian lung surfactant should go not to workers of the present century however, but to Frederic Marten who recorded the following in 1671 under the heading "How they catch the whale":

"When the whales blow up the water, they fling out with it some fattish substance that floats upon the sea like sperm, and this fat the Mallemucks devour greedily, of which several thousands attend him, so that a whale often hath more attendants than a king hath servants."

... cited in Lung Surfactant (Goerke, 1974).

CONTENTS

CHAPTER THREE

CHAPTER FOUR

Discussion

LIST OF FIGURES

LIST OF TABLES

ACKNOWLEDGEMENTS

SUMMARY

This thesis begins with an introduction which describes the
role of pulmonary surfactant and the evidence that a pulmonary
surfactant deficiency in the neonate can lead to Respiratory
Distress Syndrome. Respiratory Distress Syndrome is a serious
problem producing a high incidence of mortality and morbidity
in the premature neonate. Lung maturation and the production
and properties of surfactant are outlined. The synthesis of
two of the more important pulmonary surfactant phospholipids,
lecithin and phosphatidylglycerol, are briefly explained.
Attention is drawn to the fact that fetal lung maturity cannot
be inferred from gestational age, emphasising the necessity
of biochemical tests to assess fetal lung status. The intro-
duction concludes with a brief description of some of the tests
of fetal lung maturity.

The principal aims of the work described in this thesis
were:

(a) to assess the lecithin/sphingomyelin (L/S) ratio, lecithin
concentration, the palmitic acid concentration, percentage
palmitic acid and palmitic acid/stearic acid ratio, using
the acetone precipitable lipids extracted from amniotic
fluid samples, and also to evaluate the more rapid surf-
actant tests, the bubble stability test and the spectro-
photometric analysis of amniotic fluid, and to compare
their reliability for predicting fetal lung maturity.

(b) to assess the value of a test which determines the fetal

lung phospholipid profile, a technique which is used in North American Centres, but as far as is known, not yet used in Britain.

(c) to determine the effect of gestational age on the results obtained from the various tests.

(d) to separate the various phospholipids by h.p.l.c. This is a technique which has as yet only been used to separate lecithin and sphingomyelin and not the other phospholipids. The use of this technique as a clinical tool for determining fetal lung maturity was to be assessed.

(e) to investigate the effect of acetone precipitation on amniotic fluid phospholipids, in terms of selective precipitation of phospholipids containing saturated fatty acids and a comparison of the reliability of the L/S ratio determined on the total lipid extract with the L/S ratio determined on the acetone precipitable extract.

The assessment of the L/S ratio, lecithin concentration, spectrophotometric analysis and bubble stability test confirmed that the problem is not in indicating fetal lung maturity, but rather in identifying fetal lung immaturity which was much more difficult to predict reliably. The fatty acid analysis of amniotic fluid lipids allowed determination of the percentage palmitic acid. palmitic acid concentration, and the palmitic acid/stearic acid ratio, which although numbers were small, gave promising results.

The fetal lung phospholipid profile (the main features of which were the L/S ratio and the presence or absence of phosphatidylglycerol) was determined by a standard 2D t.l.c.

technique and it was found to be most accurate in identifying fetal lung status. Therefore, for laboratories with ready access to t.l.c. techniques its use would be highly recommended for determining fetal lung maturity.

The effect of gestational age on the L/S ratio, lecithin concentration and spectrophotometric analysis showed an exponential relationship with an obvious increase in surfactant around 34 weeks of gestation. No relationship was found between the palmitic acid content of the amniotic fluid lipids and gestational age. However, in all tests there was a considerable individual variation at each gestational age.

With suitable eluting columns and solvents the h.p.l.c. was shown to be effective in separating the amniotic fluid phospholipids and the results compared well with those obtained by t.l.c. and phosphate analysis. However, the h.p.l.c. would be too unreliable for use as a clinical tool, but may have a role as a useful research technique.

Acetone precipitation was found to precipitate the acidic phospholipids, and sphingomyelin. Only 50% of the lecithin was precipitated. Not all the unsaturated fatty acids were removed by acetone precipitation, nor were the saturated fatty acids completely precipitated however, the lipid extract was enriched with saturated fatty acids after this step. It was concluded that the L/S ratio was a more reliable predictor of fetal lung maturity after the amniotic fluid lipid extract had been acetone precipitated.

CHAPTER ONE

INTRODUCTION

CHAPTER ONE

1.1   Introduction

Respiratory Distress Syndrome (RDS), also known as Hyaline Membrane
Disease (HMD), is a serious problem producing a high incidence of
mortality and morbidity in the premature neonate. Although the
paediatric management of the problem has greatly improved, the ability
to predict the risk of the neonate developing RDS prior to delivery has
been one of the most important advances in obstetrics over recent
years (Obstetrics and Gynaecology Survey, 1975).

Surfactant has been shown to be essential in the lung because it
maintains alveolar stability. The development of adequate amounts of
surfactant as the fetus matures is an essential prelude to the
efficient functioning of the neonatal lung, and surfactant deficiency
leads to the development of RDS (Avery and Mead, 1959).

1.2   The Properties and Functions of Lung Surfactant

It was discovered in 1929 that lungs were more difficult to inflate
with air than water, and it was concluded that the natural inflation
of the lung required a low surface tension at the air/lung interface
(von Neergard cited in Goerke, 1974). This work was only developed
much later by Macklin (1954) and Pattle (1955) who described the
properties of fluid surfaces and surface tension forces. Pattle's/

work discussed the nature and extent of surface tension forces in the alveolus in terms of La Place's Law and the specific value of the surface tension of alveolar liquid lining. The pressure (P) required to maintain the alveolus patent is given by equation (i):

$$P = \frac{2\gamma}{r}$$ .... (i)

where r = radius of alveolus and γ = surface tension.

This equation emphasises the fact that during expiration, when the radii of the alveoli decrease, the tension in the alveoli walls increases, tending to cause them to collapse. This tendency could be counteracted by increasing intra-alveolar pressure, by "splinting" the alveolar walls or by reducing the tension in the liquid lining in the walls. The maintenance of an increased intra-alveolar pressure for long periods is impossible although the neonate with RDS does attempt to do this by increasing intrathoracic pressure by exhaling against a partially closed glottis, which produces the characteristic grunting noted in RDS. "Splinting" has been proposed as a possible mechanism in man, this being achieved by solidification of surfactant as the surface film becomes compressed (Morley et al, 1981). However, of crucial importance is the effect of surfactant which reduces the surface tension to below 10 - 12 dynes cm^{-1}. A further important characteristic of lung surfactant is the compressibility of the surface film, surface tension falling as the surface area of the film decreases.

Surfactant is a lipoprotein complex, and careful biochemical analysis /

from mature animals has defined most of its constituents. Although the exact percentages vary from study to study there is general agreement on the basic substances and their approximate proportions in lung surfactant (table 1.1).

Like virtually all naturally occurring phospholipids, pulmonary phospholipids are really families of compounds. Thus phosphatidylcholine, also called lecithin (figure 1.1), consists of a group of compounds, 1,2 diacyl-sn-glycero-3 phosphoryl cholines with different fatty acids attached. The fatty acid ester groups usually comprise either palmitic acid and stearic acid, or unsaturated fatty acids. The importance of the type of fatty acid residue in the function of phospholipids in cell membranes has been extensively reviewed (Quinn, 1981).

1,2-dipalmitoyl-sn-glycero-3-phosphoryl choline is the major surface active compound in lung surfactant. Purified dipalmitoyl lecithin has properties that are very similar to lung surfactant in that it reduces surface tension, and produces a compressible surface film. It appears that only the disaturated lecithins give this dramatic reduction in surface tension and the required dependence of surface tension on the surface area of the film (King et al, 1972). The next most abundant phospholipids present in the mature lung surfactant are phosphatidylglycerol and phosphatidylinositol. The exact function of these phospholipids in lung surfactant is not yet known. However, it is possible that phosphatidylglycerol has an important role in determining the properties of pulmonary surfactant, perhaps by stabilising the surface film (Hallman & Gluck, 1976). Certainly the absence of phosphatidylglycerol is associated with a high risk of RDS (Gluck et al, 1979), a fact confirmed in this thesis. Phosphatidylinositol does not appear to have any important role as a surfactant.

Table 1.1   The constituents of lung surfactant

Careful biochemical analysis from mature animals has defined most
of its constituents.   Although the exact percentages vary from study
to study there is general agreement in the basic substances and their
approximate proportions in lung surfactant.

(from Harwood, J.L. Desai, R. Hext, P. Tetley, T. Richards, R.,

Biochem. J. (1975) 151, 707-714., and from a chapter by Usategui-

Gomez in Amniotic Fluid, John Wiley and Sons, New York).

LIPID 90% w/w

Lipids % w/w total lipid

Phosphatidylcholine	72.6%
Phosphatidylglycerol	4.5%
Phosphatidylinositol	4.5%
Phosphatidylethanolamine	4.1%
Cholesterol	3.2%
Triacylglycerol	4.6%
Sphingomyelin	2.1%
Lysophosphatidylcholine	0.2%
Unidentified Others	1.6%
Unesterified Fatty Acids	2.6%

PROTEIN 10% w/w

Proteins % w/w total protein

Immunoglobulin G	7.3%
Immunoglobulin A	0.6%
Ceruloplasmin	0.4%
Albumin	62.6%
Post Albumins	8.3%
Transferrin	16.9%

The lipid constituents of amniotic fluid are from Harwood _et._ _al_,
(1975), and the protein constituents are from Usategui-Gomez,1974.

<u>Figure 1.1   The stereochemistry of phosphatidylcholine</u>

Phosphatidylcholine also called lecithin, consists
of a group of compounds, 1,2 diacyl-sn-glycero-3 phosphoryl
cholines with different fatty acids.

R and R' represent the different fatty acids.
X represents choline.

$$\begin{array}{c} \overset{\displaystyle O}{\overset{\|}{CH_2 \cdot O \cdot C \cdot R}} \\[2mm] \overset{\displaystyle O}{\overset{\|}{R' \cdot C \cdot O \blacktriangleleft C \blacktriangleright H}} \\[2mm] \overset{\displaystyle O}{\overset{\|}{CH_2 \cdot O \cdot P \cdot OX}} \\[1mm] \overset{\displaystyle |}{OH} \end{array}$$
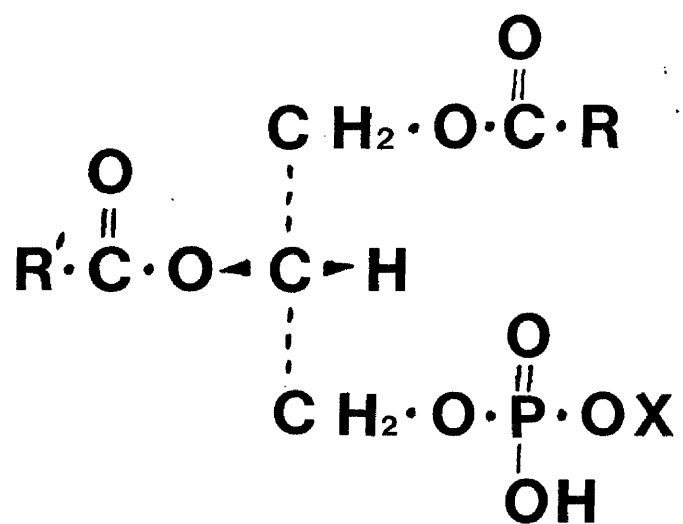
Scarpelli et al (1967) reported that sphingomyelin has a source in the lung, but this phospholipid does not appear to have an important role as a surfactant. However, the sphingomyelin concentration in amniotic fluid is thought to remain relatively constant and, as will be shown later, may be used as an internal standard in the determination of fetal lung maturity (Gluck et al, 1971).

The presence of phosphatidylethanolamine, phosphatidylserine, diacylglycerols, triacylglycerols, unesterified fatty acids and lysophosphatidylcholine in lung material has been established. The concentration of some of these compounds changes as the fetus matures, although their function is generally obscure.

The protein components of amniotic fluid that have been identified are immunoglobulin G, transferrin, post-albumin proteins, group-specific proteins, and albumin, together with other unidentified proteins (Gikas et al, 1977). Certain of these proteins are probably involved in the production, secretion and degradation of surfactant.

1.3   Synthesis of Lecithin and Phosphatidylglycerol in Lung

The type II alveolar epithelial cells are generally believed to be responsible for the production of surfactant (Niden, 1967). The pathways for the synthesis of lecithin in the lung are now fairly well understood (van Golde, 1976; Gurr et al, 1980) and the contribution of these pathways to the synthesis of dipalmitoyl lecithin in surfactant have been investigated and the conclusions drawn from this work are outlined below (van Golde, 1976).

In the de novo synthesis of lecithin, phosphatidic acid (figure 1.2),

may be synthesised either from sn-glycerol-3-phosphate or from

dihydroxyacetone phosphate. Phosphatidate phosphohydrolase (3.1.3.4)

catalyses the conversion of phosphatidic acid into diacylglycerol

(figure 1.3). Diaclyglycerol reacts with CDP-choline, which is synth-

esised from choline by the sequential action of choline kinase (2.7.1.32.)

and choline phosphate cytidylyltransferase (2.7.7.15), to yield

lecithin, a reaction which is catalysed by choline phosph   transferase.

There is abundant evidence that this CDP-choline or activated alcohol

branch of the Kennedy Pathway (1961) is the major pathway for the

de novo synthesis of lecithin in the lung. Although N-methylation of

phosphatidylethanolamine has been demonstrated in the lung, it is now

generally believed that this pathway is of minor significance for the

formation of lecithin in the adult and fetal lung.

The real problem in the synthesis of lecithin, and many phosphoglycer-

ides, is the mechanism(s) which gives   rise to the specific fatty acid

composition and distribution between positions one and two on the

phosphoglycerol unit. The CDP-choline pathway in the lung produces

predominantly phosphatidylcholines containing palmitic acid at the one

position and an unsaturated fatty acid at the two position (van Golde,

1976).

These de novo synthesised unsaturated phosphatidylcholines could be

remodelled into dipalmitoylphosphatidylcholine by means of at least

two possible mechanisms (figure 1.4; figure 1.5):

Figure 1.2 Synthesis of Phosphatidic Acid

In the de novo synthesis of lecithin, phosphatidic acid may be synthesised
either from sn-glycerol-3-phosphate or from dihydroxyacetone phosphate.
Phosphatidate phosphohydrolase catalyses the conversion of phosphatidic
acid into diacylglycerol.

3-sn-glycerol phosphate

or dihydroxyacetone phosphate

+ Acyl-CoA

glycerol - 3 phosphate
acyl - transferases (1 and 2)

Phosphatidic Acid

phosphatidate phosphohydrolase

Pi

1,2-Diacylglycerol

Figure 1.5 Deacylation - Transacylation Process

Unsaturated phosphatidylcholines could be remodelled into dipalmitoyl-
phosphatidylcholine by a deacylation - transacylation cycle. The first
step in this process is the removal of the unsaturated fatty acid by
phospholipase A_2. The lysolecithin acyltransferase catalyses the
transfer of the palmitoyl moiety from one molecule of 1-palmitoyl-sn-
glycero-3-phosphocholine, which results in the formation of dipalmitoyl-
phosphatidylcholine and glycero-3-phosphocholine (van Golde, 1976).

CHOLINE

↓ choline kinase

PHOSPHOCHOLINE

PHOSPHATIDIC ACID

↓ Pi

↓ phosphatecytidylyltransferase

CDP CHOLINE - 1,2 DIACYLGLYCEROL

↓ choline phospho transferase

LECITHIN

9
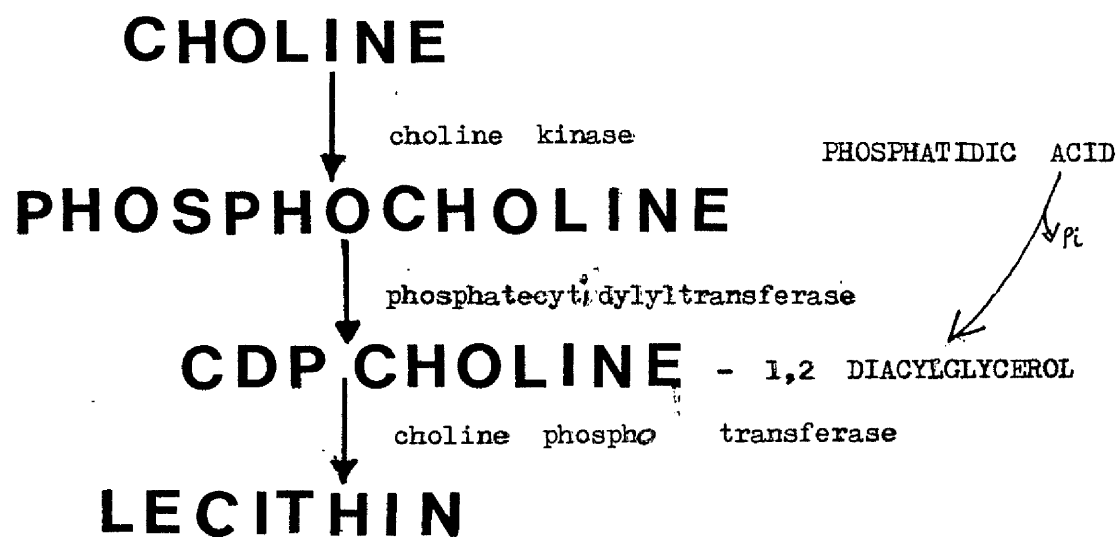
(a)   a deacylation-reacylation cycle

(b)   a deacylation-transacylation process.

The first step in both processes is the removal of the unsaturated fatty acid by phospholipase A_2 (3.1.1.4). In the first process the 1-palmitoyl-sn-glycero-3-phosphocholine produced by the phospholipase A_2 could be reacylated with palmitoyl-CoA to yield dipalmitoylphosphatidylcholine, a reaction which is catalysed by lyso lecithin:acyl CoA acyltransferase (2.3.1.23) (Lands, 1960; Erbland, 1965). In the second possible mechanism lysolecithin acyltransferase catalyses the transfer of the palmitoyl moiety from one molecule of 1-palmitoyl-sn-glycero-3-phosphocholine, which results in the formation of dipalmitoylphosphatidylcholine and glycero-3-phosphocholine. These processes are active in the lung, and are presumed active in the type II alveolar cells, producing lecithin (van Golde, 1976), but detailed studies have indicated that the first process is most important (van Heusden et al, 1980).

Phosphatidylglycerol is synthesised by the pathways shown in figure 1.6. Phosphatidate cytidylyltransferase catalyses the conversion of phosphatidic acid into CDP-diacylglycerol and phosphatidylglycerol is produced by the active CDP-diacylglycerol pathway (van Golde, 1976).

1.4   Lung Maturation and Surfactant Production

Lung development can be divided into anatomical and biochemical aspects. Anatomical development begins in early embryonic life and, in the human, alveolar buds appear at around 24 - 26 weeks of gestation and true alveoli/

Figure 1.3 Synthesis of Lecithin

Phosphatidate phosphohydrolase catalyses the conversion of phosphatidic
acid into diacylglycerol. Diacylglycerol reacts with CDP-choline,
which is synthesised from choline by the sequential action of choline
kinase and choline phosphate citydilyltransferase, to yield lecithin, a
reaction which is catalysed by choline phosphⓐ transferase (van Golde,
1976).
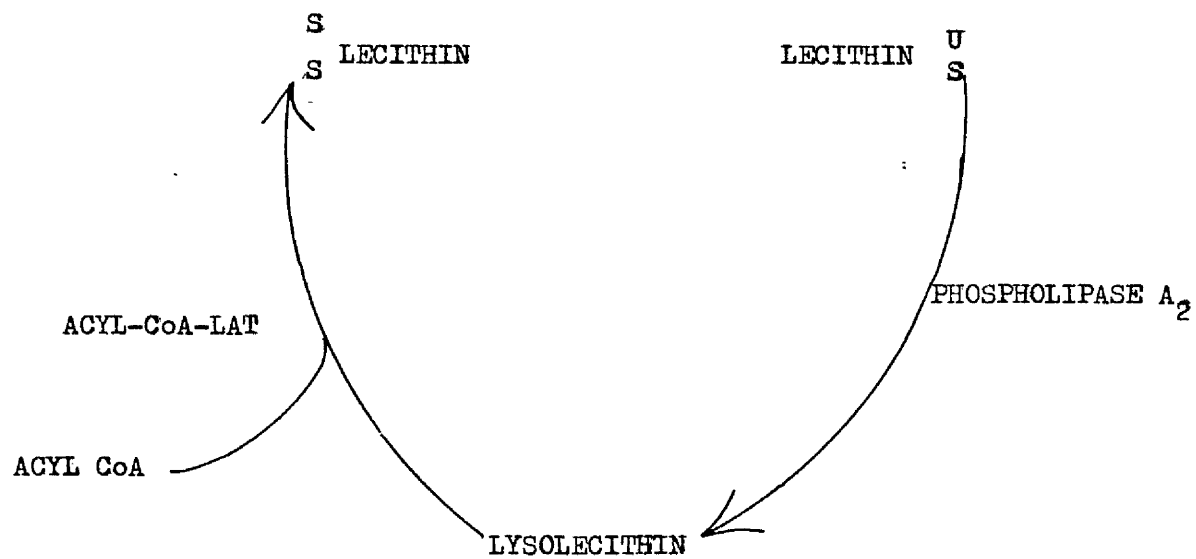
ACYL-CoA-LAT =lysophosphatidylcholine acyltransferase

S = saturated fatty acid   U = unsaturated fatty acid

Figure 1.4 Deacylation - Reacylation Cycle

Unsaturated phosphatidylcholines could be remodelled into dipalmitoyl-
phosphatidylcholine by a deacylation - reacylation cycle. The first
step in this process is the removal of the unsaturated fatty acid by
phospholipase A_2. The 1-palmitoyl-sn-glycero-3-phosphocholine produced
by the phospholipase A_2 could be reacylated with palmitoyl-CoA to yield
dipalmitoylphosphatidylcholine, a reaction which is catalysed by lyso
lecithin:acyl CoA acyltransferase (Lands, 1960; Erbland, 1965).

S

S   LECITHIN   LECITHIN   U

S

LAT   PHOSPHOLIPASE A$_2$

LYSOLECITHIN
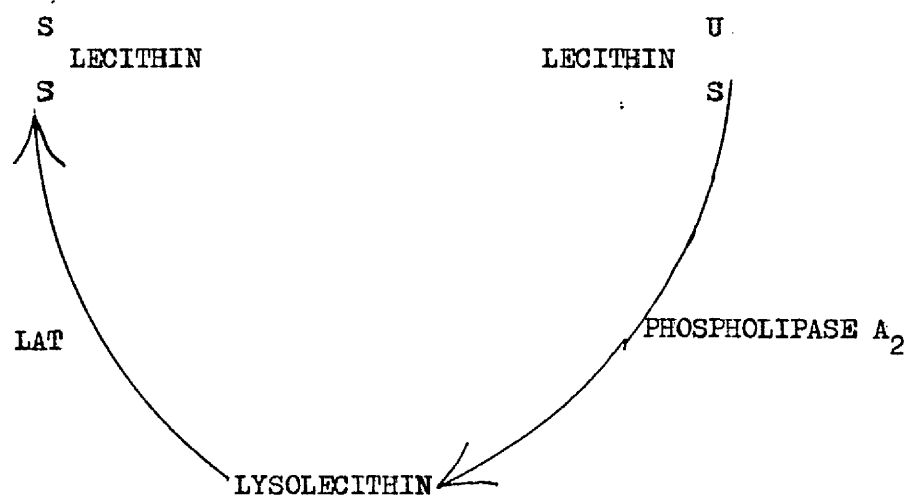
LAT = lysophosphatidylcholine-lysophosphatidylcholine acyltransferase

S = saturated fatty acid   U = unsaturated fatty acid

<u>Figure 1.6</u> <u>Synthesis   of   Phosphatidylglycerol</u>

Phosphatidate cytidylyltransferase (CTP) catalyses the conversion
of phosphatidic acid into CDP-diacylglycerol and phosphatidyl-
glycerol is produced by the active CDP-diacylglycerol pathway
(van Golde, 1976).

PHOSPHATIDIC ACID

CTP

ppi

CDP— DIGLYCERIDE

sn-Glycerol-3-phosphate

CMP

PHOSPHATIDYLGLYCEROL PHOSPHATE

pi

PHOSPHATIDYLGLYCEROL
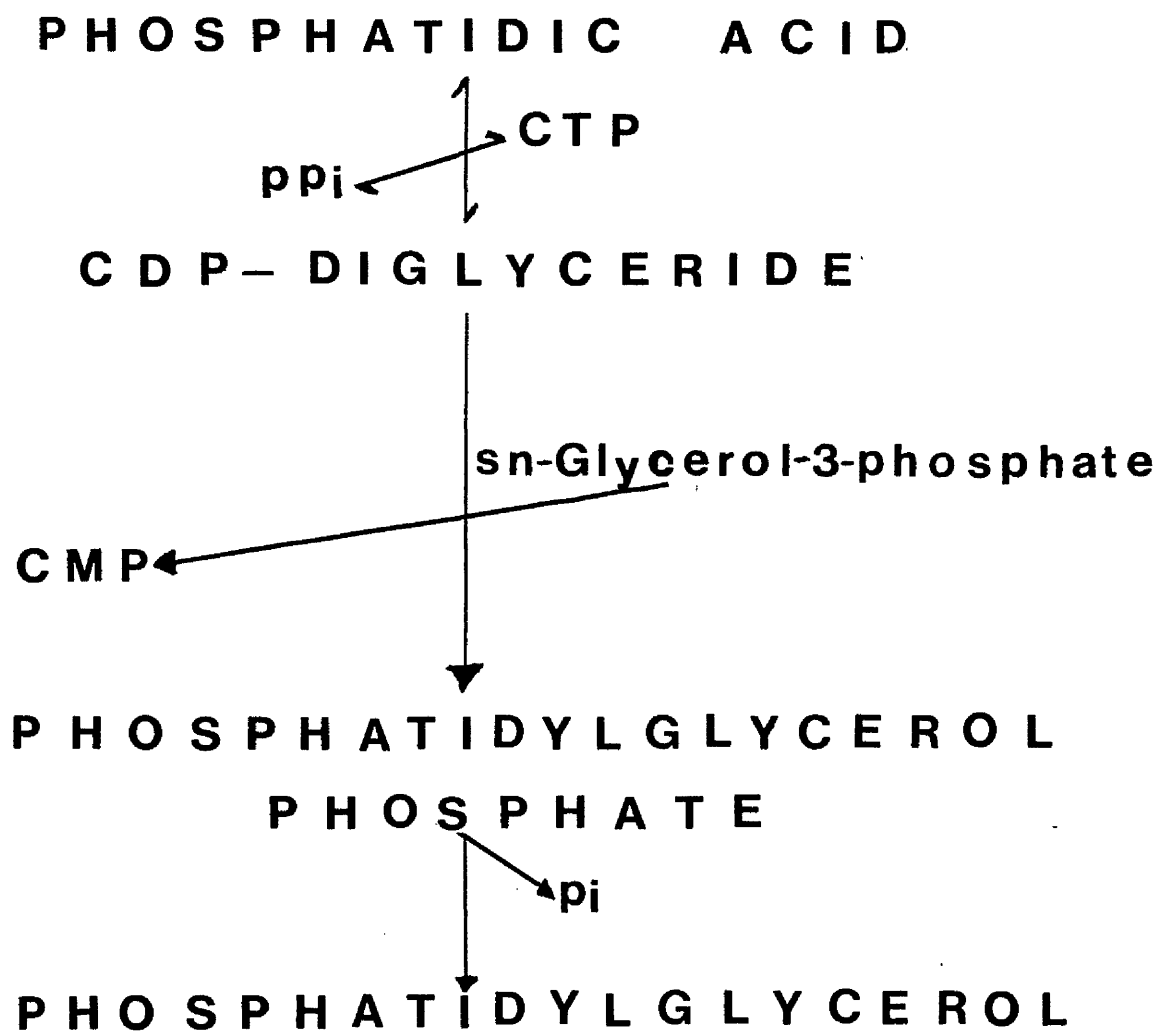
are found from about 34 - 35 weeks. Biochemical development proceeds with the appearance of lecithin at around 24 - 26 weeks. A small but steady increase in lecithin concentration continues until around 34 weeks when there is a sudden rise in the rate of secretion indicating maturation of the lung. Factors such as maternal diabetes mellitus, hypertension, antepartum haemorrhage and intra-uterine growth retardation have been recognised to influence these maturational processes. (Gluck et al, 1973; Obladen et al, 1979). It is this individual variation in the timing of lung maturation which underlines the weakness in using gestational age as an estimate of fetal lung maturity, and strengthens the need for a specific indicator of lung maturity. During the first half of pregnancy the amniotic fluid is formed by diffusion across the fetal skin and fetal membranes. However, after 20 weeks the fetal skin undergoes keratinisation becoming impermeable and fetal urine is the major contributor to the amniotic fluid (figure 1.7). The lung produces a considerable volume of fluid each day and although the bulk of it is swallowed, the remainder, containing lung secretions, passes into the amniotic fluid.

Analysis of amniotic fluid can provide information concerning the state of lung maturation because, as already described, lung secretions enter the amniotic cavity. Amniotic fluid can be obtained either directly by amniocentesis or, when the amniotic membranes are ruptured by drainage from the vagina. Determination of fetal lung maturity may by achieved by an assessment of the surface tension of the amniotic fluid or the identification of substances which are components of the lung surfactant. There are different techniques available to determine/

Figure 1.7 Amniotic Fluid Formation and Removal in Pregnancy

This figure illustrates the known and postulated sites of amniotic fluid formation and removal in pregnancy.
(From Seeds, A. E. in A. C. Barnes, Ed., Intrauterine Development, Lea and Febiger, Philadelphia, 1968, p. 138.)

fetal
swallowing

placenta

fetal
urine
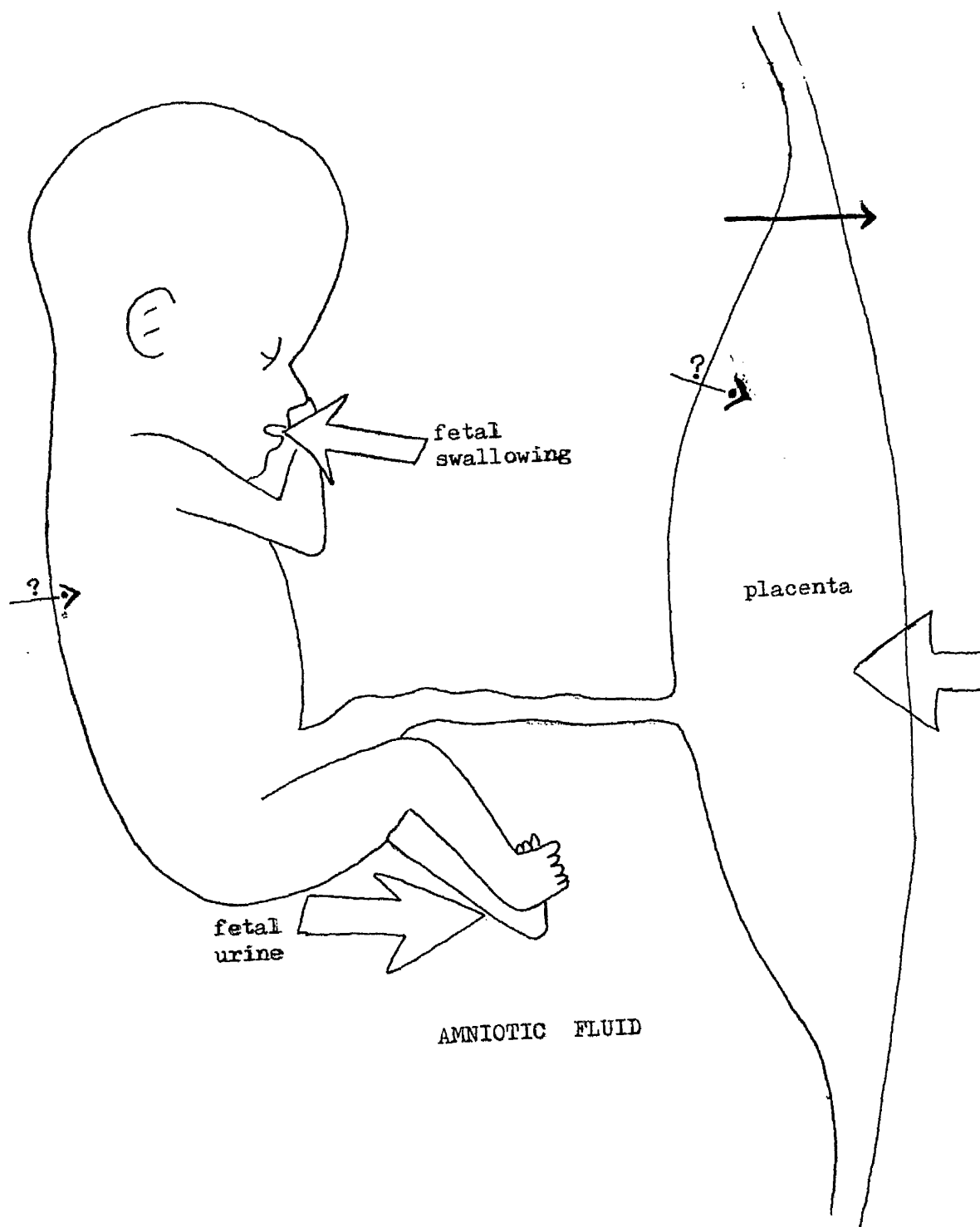
AMNIOTIC   FLUID

fetal lung maturity by analysis of amniotic fluid, some of which will be described in detail later. No single test predicts fetal lung maturity with absolute certainty and it is the overall aim of this thesis to present evidence as to the reliability and diagnostic value of these various techniques.

The tests which were evaluated are described below.

1.5   The Bubble Stability Test

The Bubble Stability Test, which may also be referred to as the Foam Test, Shake Test, or Rapid Surfactant Test, was first introduced in 1972 (Clements et al) and was an attempt to directly determine the surfactant qualities of the amniotic fluid. This inexpensive test is used widely throughout the world, demanding little in the way of expertise. A positive test nearly always indicates the presence of mature lungs, although a negative test is much less reliable.

1.6   Spectrophotometric Analysis of Amniotic Fluid

This procedure, first introduced by Sbarra and co-workers (1976), evaluates the turbidity of the amniotic fluid, which increases with gestational age. Results indicating mature fetal lungs are more reliable than those indicating immature fetal lungs.

Unfortunately, reported studies have only related the spectrophotometric analysis of amniotic fluid to lecithin/sphingomyelin ratios (L/S ratios) (Sbarra et al, 1978); it may be more realistic to correlate the optical density with gestational age and it may be fallacious to/

16

imply that spectrophotometric analysis will necessarily indicate fetal lung maturity. This point has been investigated and discussed in the thesis.

1.7   The Lecithin Concentration

Chemical methods to determine the amniotic fluid lecithin concentration have been developed (Bhagwanani et al, 1972; Biezenski, 1972) but do not appear to be in common use. Recently a promising enzymatic method has been introduced (Beutler et al, 1979) which estimates the total lecithin concentration in amniotic fluid. This enzymatic determination is more rapid and the technique is much less laborious than chemical methods used. The estimation of lecithin concentration is unreliable in the presence of blood or meconium since red blood cells contain lecithin. Further, the concentration of a single substance in the amniotic fluid is affected by changes in amniotic fluid volume.

1.8   The Lecithin/Sphingomyelin Ratio (L/S Ratio)

The estimation of the lecithin/sphingomyelin (L/S) ratio by one-dimensional thin layer chromatography (1 D-t.l.c.) has become a standard test of fetal lung maturity and it has been evaluated by a number of investigators (Gluck et al, 1973; Whitfield et al, 1973; Donald et al, 1973). The difference between this technique and that of evaluating the lecithin concentration is that sphingomyelin acts as an "internal standard" and reduces the effects of changes in amniotic fluid volume on the lecithin concentration. The reliability of the L/S ratio has resulted in the test becoming an important aid in obstetric decision making. The presence of blood or meconium in the amniotic

fluid interferes with the determination of the L/S ratio as it does
with the estimation of lecithin concentration, and this fact reduces
the diagnostic value of the test. The effect of contamination on the
amniotic fluid L/S ratio and the uncertainty of the predictive value
when the L/S ratio indicates immature fetal lungs, has led to the
development of other tests of fetal lung maturity.

1.9   The Lung Phospholipid Profile

As previously mentioned a number of different phospholipids, apart
from lecithin and sphingomyelin, are to be found in the amniotic fluid
and are thought to originate in the fetal lung (Pfleger et al, 1971).
Together they comprise the lung phospholipid profile and have been
identified as phosphatidylinositol, phosphatidylglycerol, phosphatidyl-
ethanolamine and phosphatidylserine. Phosphatidylinositol and phospha-
tidylglycerol are considered to be of significance in lung maturation
(Hallman et al, 1976), the latter being a particularly important
factor. Using the phospholipid profile, it is possible to estimate
the L/S ratio and to quantitate the other phospholipids, especially
phosphatidylinositol and phosphatidylglycerol.

Studies of phosphatidylglycerol are also useful in evaluating phospho-
lipids from amniotic fluid that is contaminated with blood or meconium
because phosphatidylglycerol is found in neither contaminant.

1.10   Fatty Acid Composition of Phospholipids in Amniotic Fluid

Techniques have been developed to estimate the concentration of
palmitic acid in amniotic fluid as an index of fetal lung maturity.

Warren and colleagues (1974) demonstrated a rapid increase in palmitic acid concentrations at a gestational age of about 35 weeks, and there was a reasonable correlation between palmitic acid levels and the L/S ratio.

The estimation of palmitic acid levels in amniotic fluid is invalidated by the presence of blood or meconium, and also by alterations in amniotic fluid volume. To overcome this latter problem stearic acid has been used as reference substance to palmitic acid (O'Neil et al, 1978; Schirar et al, 1975).

CHAPTER TWO

MATERIALS AND METHODS

CHAPTER TWO

2.1   Materials

The routine chemicals used were usually Analar grade (BDH Chemicals Ltd., Poole, Dorset, BH12 4NN) when available or otherwise the best commercial grade available. Only double-distilled water was used.

The following chemicals were obtained from the suppliers listed.

Silica gel 60H (Merck., BDH Chemicals Ltd., Poole, Dorset, BH12 4NN)

Phospholipid and Fatty Acid Standards (Sigma Chemical Company Ltd., Poole, Dorset, BH17 7NH)

Gas Chrom Q (100-120 mesh)(Chromatography Services Ltd., Hoylake, Wirral, Merseyside)

Silar 10C (Applied Science Laboratories, Pierce and Warriner Ltd., Chester)

Porasil column 30cm x 4mm I.D. containing μ Porasil (Waters Associates (Inst.) Ltd., Hartford, Northwich, Cheshire, CW2 2AH).

2.2   Methods

2.2.1   Collection of Amniotic Fluid

Amniotic fluid samples were collected by amniocentesis, by vaginal drainage or at caesarean sections. Samples contaminated by blood or/

20

meconium were not discarded.

2.2.2   Centrifugation

The bulk of the amniotic fluid sample was centrifuged immediately for
five minutes at 1100g (3000 rpm on an MSE minor centrifuge) (Measuring
and Scientific Equipment Ltd., Manor Royal, Crawley, Sussex, England)
and the supernatant removed for analysis.

2.2.3   Storage

In most cases analysis of the amniotic fluid specimen was performed on
the day of collection. When this was not possible the supernatant was
stored at -20°C.

2.2.4   Phospholipid Extraction

The phospholipids were/extracted from 3-4ml amniotic fluid by adding
three volumes of chloroform:methanol (2:1 v/v) (Christie, 1973). The
mixture was shaken vigorously and centrifuged for five minutes at
1100g. This gave a clear separation of the upper aqueous layer,
interfacial protein layer, and the lower chloroform layer containing
the extracted phospholipids. This chloroform layer was transferred
into a centrifuge tube and evaporated to dryness at 60°C using a
stream of nitrogen.

2.2.5   Acetone Precipitation

The acetone precipitation of lipids was carried out as described by
Borer et al (1971).

The test tube containing the extracted lipids was cooled in crushed ice. 0.25 ml of ice-cold acetone was added and the tube agitated slightly. A white material could be seen on the walls and in the tip of the tube. An additional 0.75 ml of ice-cold acetone was then added. The tube was cooled in ice for five minutes and centrifuged at 1100g for one minute and the soluble fraction was either discarded, or transferred to another tube and evaporated to dryness and saved for further analysis. The tube containing the acetone insoluble precipitate was evaporated to dryness in a stream of nitrogen to remove any remaining acetone.

2.3 One-Dimensional Thin Layer Chromatography (1D t.l.c.)

The acetone precipitate (Methods, 2.2.5) was re-suspended in 30 μl of chloroform, 15 μl of which was then spotted onto a thin layer of silica gel H using a Hamilton syringe with a square ended needle. The thin layer was made from a slurry of silica gel H with 5% w/v ammonium sulphate solution and a layer 0.3mm thick spread on to a 20 cm x 20 cm borosilicate glass plate (Anchor Glass Ltd., London). The plates were activated by heating at 110°C for twenty minutes before use. Standard chromatography techniques were used. The solvent was chloroform: methanol:water:glacial acetic acid (65:25:4:8 by vol) and the chromatography plate was developed in this solvent in the ascending direction to 10 cm above the origin in a Shandon t.l.c. tank.

The plate is removed from the tank, dried at 70°C for five minutes and then charred on a hot plate at 250°C to visualise the lipids. The lipids appeared as brown-black spots on a white background after this treatment. The separation of lecithin and sphingomyelin is shown in/

figure 2.1. The phospholipid spots usually appeared with well defined edges and the area of the spots was determined planimetrically by measuring both the maximum length and breadth of the spot (figure 2.1). The area of the lecithin spot divided by the area of the sphingomyelin spot allowed calculation of the L/S ratio. An L/S ratio of 2.0 was used as the critical value, since this was the critical value adopted by Gluck et al (1971).

2.4   Two-Dimensional Thin Layer Chromatography

The technique of two-dimensional thin layer chromatography (2D t.l.c.) has been described by Kulovich et al (1979). The acetone precipitable phospholipids were dissolved in 30 μl of chloroform, 15 μl of which was then spotted onto a silica gel H t.l.c. plate using a Hamilton syringe with a square ended needle. The thin layer is made from a slurry of silica gel H containing 5% w/v ammonium sulphate solution and a layer 0.3 mm thick spread onto 20 cm x 20 cm borosilicate glass plates, and the plates activated by heating at 110°C for twenty minutes before use.

The sample was spotted 3 cm from the right hand side and 3 cm from the bottom of the edge of a freshly activated plate (figure 2.2).

5 μl of 2.5 mg/ml^{-1} phosphatidylglycerol standard was spotted onto the bottom of the left hand side of the plate 3 cm from the side and 3 cm from the bottom edge of the plate, and a further 5 μl of phosphatidyl-glycerol standard was plated at the right hand side 3 cm from the top and side edges as shown in figure 2.2.

Figure 2.1   The L/S Ratio

The amniotic fluid was collected as described in Methods, 2.2.1 and
centrifuged (Methods, 2.2.2). The lipids were extracted (Methods, 2.2.4)
and acetone precipitated (Methods, 2.2.5). The separation of lecithin
and sphingomyelin by one dimensional t.l.c. (Methods, 2.3) is shown.
The area of the spots was determined planimetrically by measuring
both the maximum length and breadth of the spot. The area of the
lecithin spot divided by the area of the sphingomyelin spot allows the
calculation of the L/S ratio.

l represents lecithin

S represents sphingomyelin

a—a represents maximum length of lecithin

b—b represents maximum breadth of lecithin

other
phospholipids
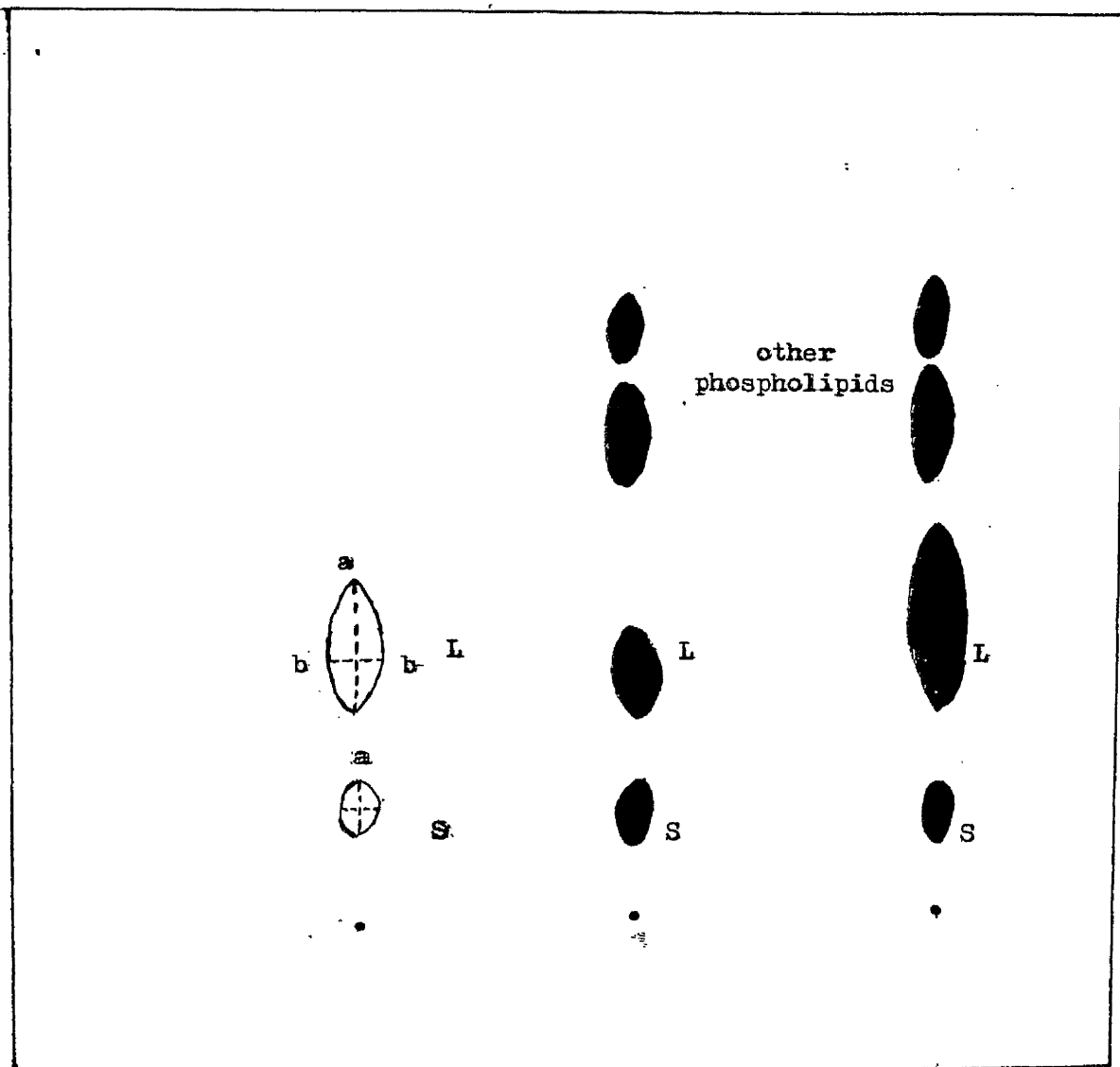
immature
L/S ratio

mature
L/S ratio

Figure 2.2   The Lung Phospholipid Profile

The amniotic fluid was collected as described in Methods, 2.2.1 and

centrifuged (Methods, 2.2.2).   The lipids were extracted (Methods, 2.2.4)

and acetone precipitated (Methods, 2.2.5).   A standard 2D t.l.c.

technique was used to separate the phospholipids (Methods, 2.4).

O represents the origin where the lipid extract was spotted.

PG_1 represents the phosphatidylglycerol standard which has
travelled with the first solvent system.

PG_2 represents the phosphatidylglycerol standard which has
travelled with the second solvent system.

S represents sphingomyelin

L represents lecithin

PS represents phosphatidylserine

PI represents phosphatidylinositol

PE represents phosphatidylethanolamine

PG represents phosphatidylglycerol
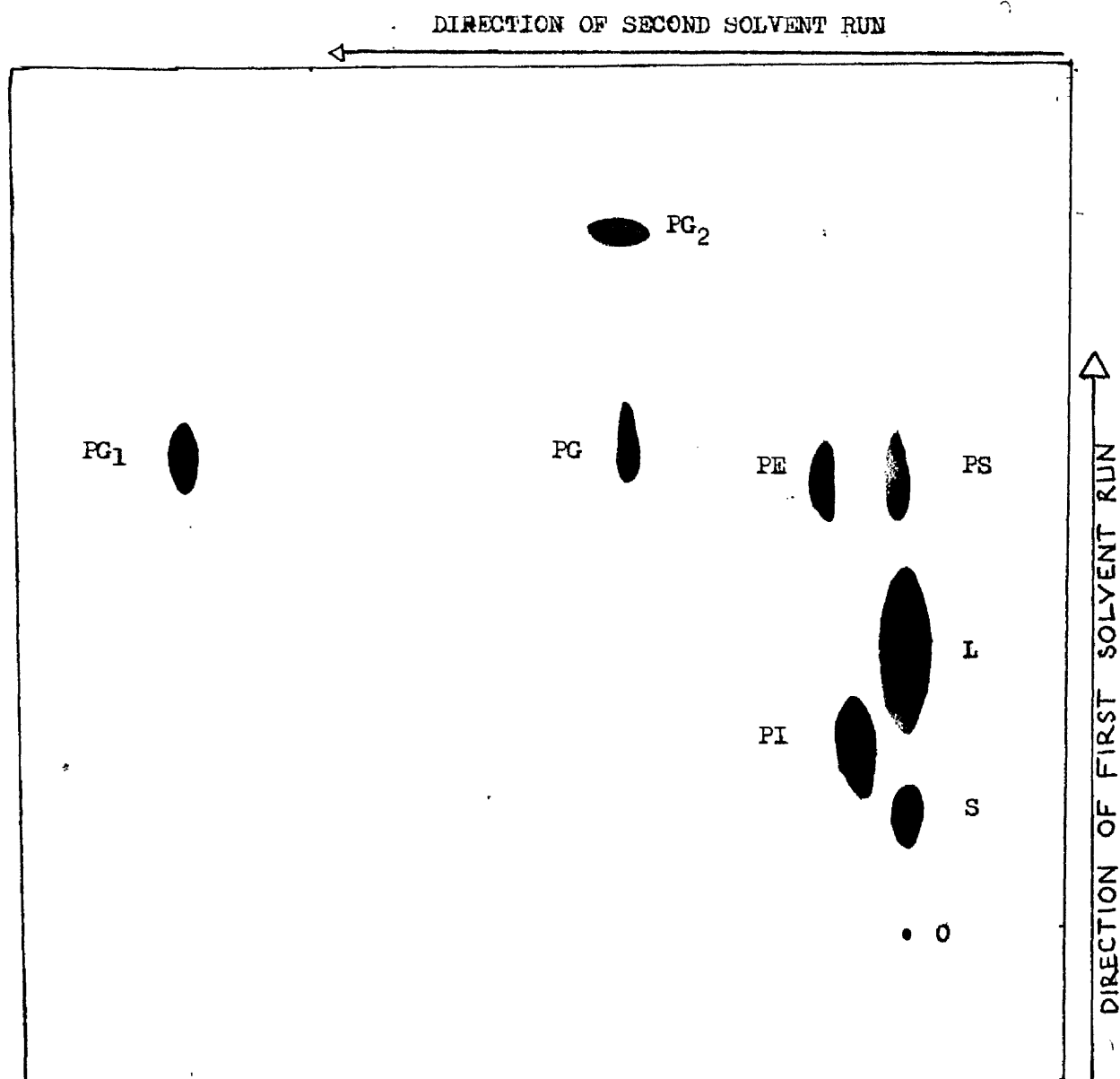
A standard 2D t.l.c. technique was used. The first solvent was chloroform:methanol:water:glacial acetic acid (65:25:4:8 by vol) and the solvent front was allowed to develop in an ascending direction to 10 cm. above the origin. The plate was removed from the chromatography tank, dried at 70°C for five minutes, turned at right angles and placed in the second solvent system of tetrahydrafuran:dimethoxy-methane:methanol:1M ammonium hydroxide (40:28.5:7.8:4.2 by vol). This new solvent front was allowed to develop in an ascending direction for a further 10 cm. The plate was removed from the chromatography tank, dried at 100°C, and then charred at 250°C. The separation of the phospholipids is shown in figure 2.2. They appear with well-defined edges in most cases. The area of the spots was estimated planimetrically and the L/S ratio calculated. If required the phospho-lipid phosphate content was then determined by scraping the spots from the plate into clean tubes and estimating the phosphate in each spot (Methods, 2.5.1).

2.5   Phosphate Assay

The phosphate assay was based on the technique of Kankara et al (1971) and is described below.

2.5.1   Recovery of Phospholipids from t.l.c.

The phospholipids were scraped from the plate into clean tubes ready for digestion, and the gel blank was made from an area of silica gel scraped off from below the origin, where both solvents had been in contact with the gel but not with the lipid extract (figure 2.3). The apparent concentration of phosphate in the gel blank is directly proportional to the area of silica removed, regardless of the presence of phospho-lipid, and therefore a constant area of silica gel is removed for each phospholipid spot and for the blank. This area is equivalent to the area of the largest phospholipid spot present which is usually lecithin.

<u>Figure 2.3</u> <u>Removal of the Gel Blank</u>

The gel blank was made from an area of silica gel scraped off from
below the origin, where both solvents had been in contact with gel but
not with the lipid extract.

The hatched area represents the silica gel in contact with both solvents
and not with the lipid extract.

dimension 2

SILICA GEL ONLY

SOLVENT
TWO
ONLY

SOLVENT ONE
SOLVENT TWO
LIPID

SOLVENT ONE
SOLVENT TWO

O

SOLVENT
ONE
ONLY

SOLVENT ONE
SOLVENT TWO

dimension 1
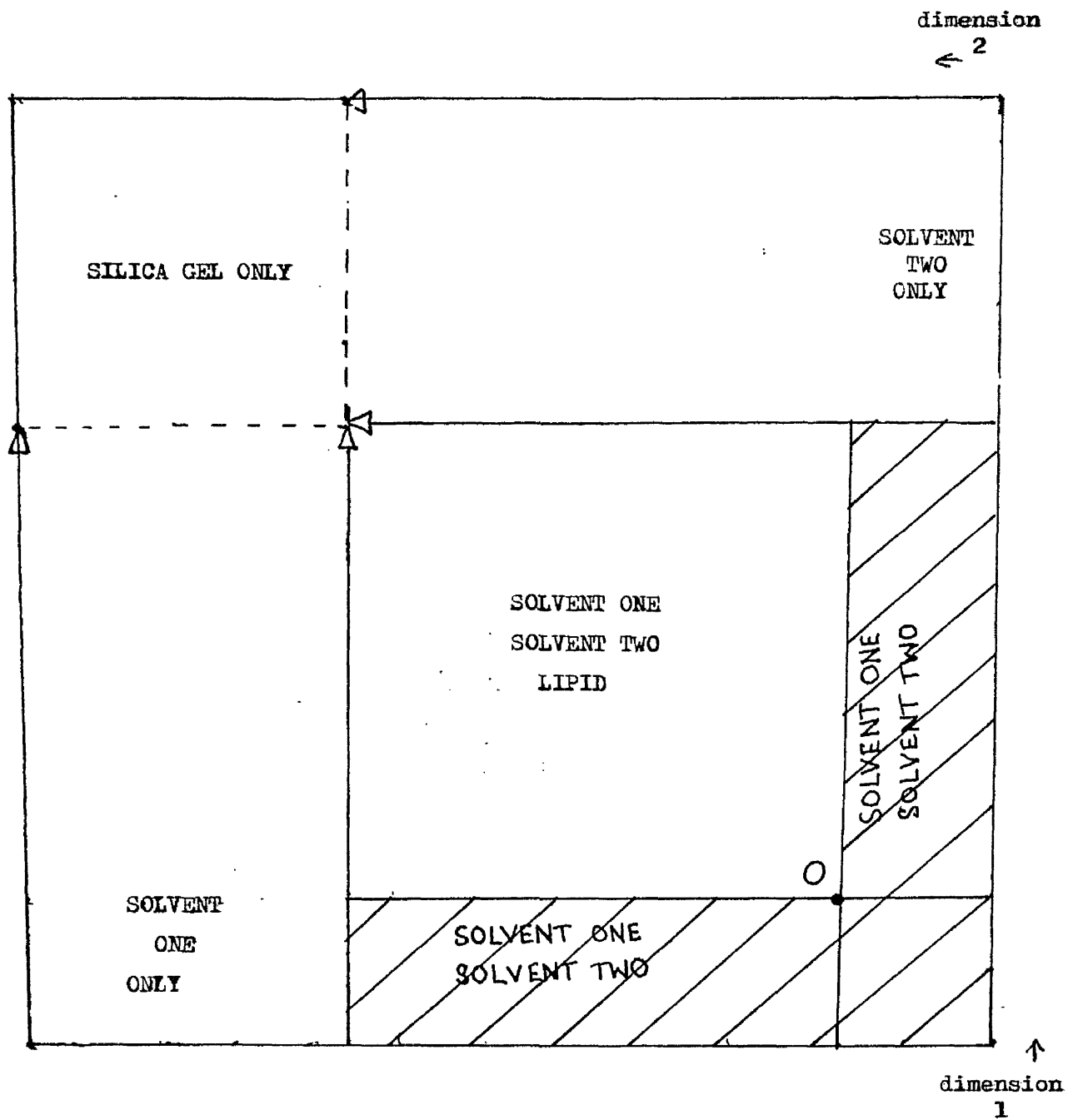
2.5.2   Digestion

2 ml 5M H_2SO_4 was added to each tube. The digestion was carried out at $180^{\circ}C$ for two hours. The tubes were cooled. 0.1 ml of 30% H_2O_2 was added to each tube, and digestion continued at $180^{\circ}C$ for a further two hours. The volume in each tube by the end of the digestion was only 0.5 ml.

2.5.3   Colour Development

9.5 ml double-distilled water was added to each tube and a set of standards, containing from 0 to 0.3μ moles of inorganic phosphate in 10 ml of 1M H_2SO_4, prepared.

2 ml of freshly prepared mixture of equal volumes of 5% w/v ammonium molybdate and Fiske-Subba Row Reagent (as prepared by Bartlett, 1959) were added to each tube.

The contents of each tube were mixed using a vortex mixer. The tubes were placed in a boiling water bath for seven minutes. The tubes were then centrifuged for two minutes at 370g to precipitate the silica gel. A spectrophotometer (Pye Unicam, SP600) was used and the optical density determined at 830 nm in standard 3 ml cuvettes. Reagent blanks, the gel blank, and the eluted lipids were assayed simultaneously.

A typical standard curve is shown in figure 2.4. The phosphate content was determined for each lipid from such a curve after allowance had/

28

Figure 2.4 A Typical Standard Curve

The phosphate content was determined for each lipid from
such a standard curve.
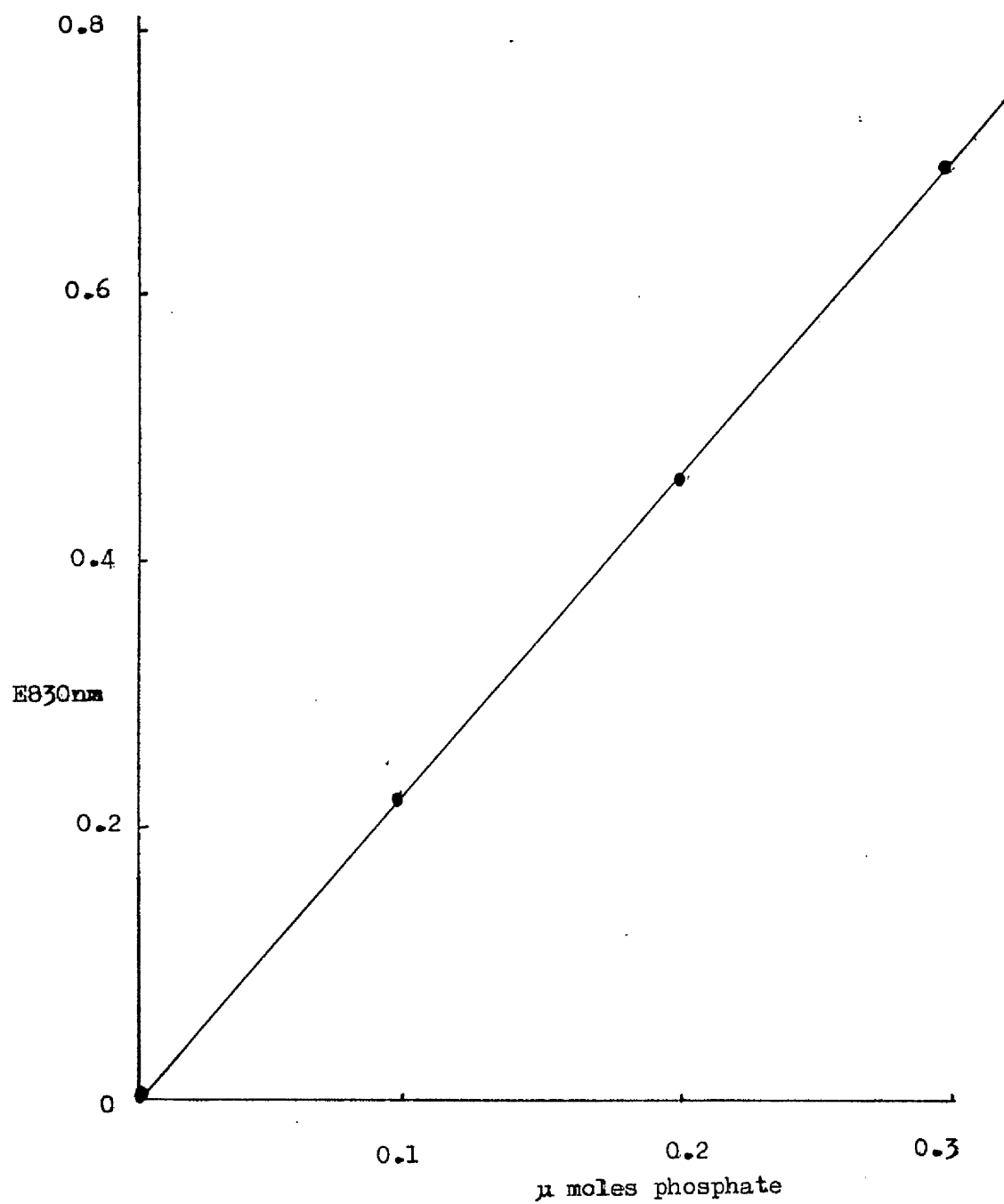
been made for the amount of silica gel containing the lipid. A critical value of 25 μmoll^{-1} amniotic fluid was used, above which no cases of RDS occurred and below which all babies which developed RDS were detected.

2.6   High Performance Liquid Chromatography (h.p.l.c.)

The h.p.l.c. equipment was supplied by Waters Associates Instruments ltd., Hartford, Northwich, Cheshire, CW2 2AH; and the apparatus consisted of the following:-

a Model 6000 A solvent delivery system,

a Model U6K septumless injector,

a μ Porasil column 30 cm x 4mm i.d. containing u Porasil connected to

a guard column packed with Porasil, and

a Model R401 refractometer.

The h.p.l.c. system was set up as follows:

The reference side of the R401 refractometer was flushed well with the solvent system and effluent pumped to the top of a one-litre measuring cylinder placed above the machine, thus providing back pressure when the flow is stopped and the trapped reference established. The solvent which consists of chloroform:methanol:water (714:256:20 by vol) is then allowed to flow through the Porasil column at a rate of 2 ml/min^{-1} and a pressure of 5.5 to 13.8 Pa. The eluted lipids were detected using the R401 refractometer with a positive polarity, and attenuation x 2 (less than this gave an unsatisfactory baseline), chart recorder speed 2.5 min cm^{-1}. When the steady regular baseline with minimal noise was established the sample was injected.

The amniotic fluid phospholipids were dissolved in 35 µl of

chloroform:methanol (95:5 v/v) and a 25 µl sample was then injected

into the U6K injector using a 25 ul Hamilton syringe (Waters Associates

(Inst.) Ltd.) and the chart marker pressed simultaneously. The

separation took approximately forty-five minutes to complete.

The working standard for the h.p.l.c. comprised phosphatidylethanol-
amine (1.0 mg ml^{-1}), phosphatidylglycerol (2.5 mg ml^{-1}), phosphatidyl-
inositol (2.5 mg ml^{-1}), dipalmitoyl lecithin (10.0 mg ml^{-1}), and
sphingomyelin (10.0 mg ml^{-1}) dissolved in chloroform:methanol (95:5 v/v).
The standards were stored at -10°C when not in use.

Phosphatidylserine and lysolecithin were not included in the standard

since neither were detectable, at levels found in amniotic fluid, by

the h.p.l.c. technique. Initially lysolecithin was incorporated in

the standard solution to act as an internal standard. However,

experience showed that the peak heights for each phospholipid were

sufficiently reproducible using 25 µl injections, therefore an internal

standard proved unnecessary, and routinely the amounts of individual

phospholipids were determined by direct comparison of the peak height

with the peak height of the appropriate phospholipid in the chromatog-

rams of the composite working standard. Figures 2.5., 2.6 , 2.7 show

separations from a standard, a mature lung and an immature lung,

respectively.

2.7   Gas Liquid Chromatography Used to Determine the Fatty Acid

Composition of amniotic Fluid Lipids

The fatty acid composition of the whole amniotic fluid fraction, the/

31

Figure 2.5 High Performance Liquid Chromatography

The amniotic fluid was collected as described in Methods, 2.2.1 and centrifuged (Methods, 2.2.2). The lipids were extracted (Methods, 2.2.4) and acetone precipitated (Methods, 2.2.5). The h.p.l.c. was used to separate the phospholipids (Methods, 2.6). Figure 2.5 shows a typical chromatogram obtained using a composite standard.

INJ represents injection marker

PE represents phosphatidylethanolamine

PG represents phosphatidylglycerol

PI represents phosphatidylinositol

LEC represents lecithin

SPH represents sphingomyelin

STANDARDS

LEC

PE

PG

PI

SPH

refractive
index
increment

Inj.

30 minutes

32
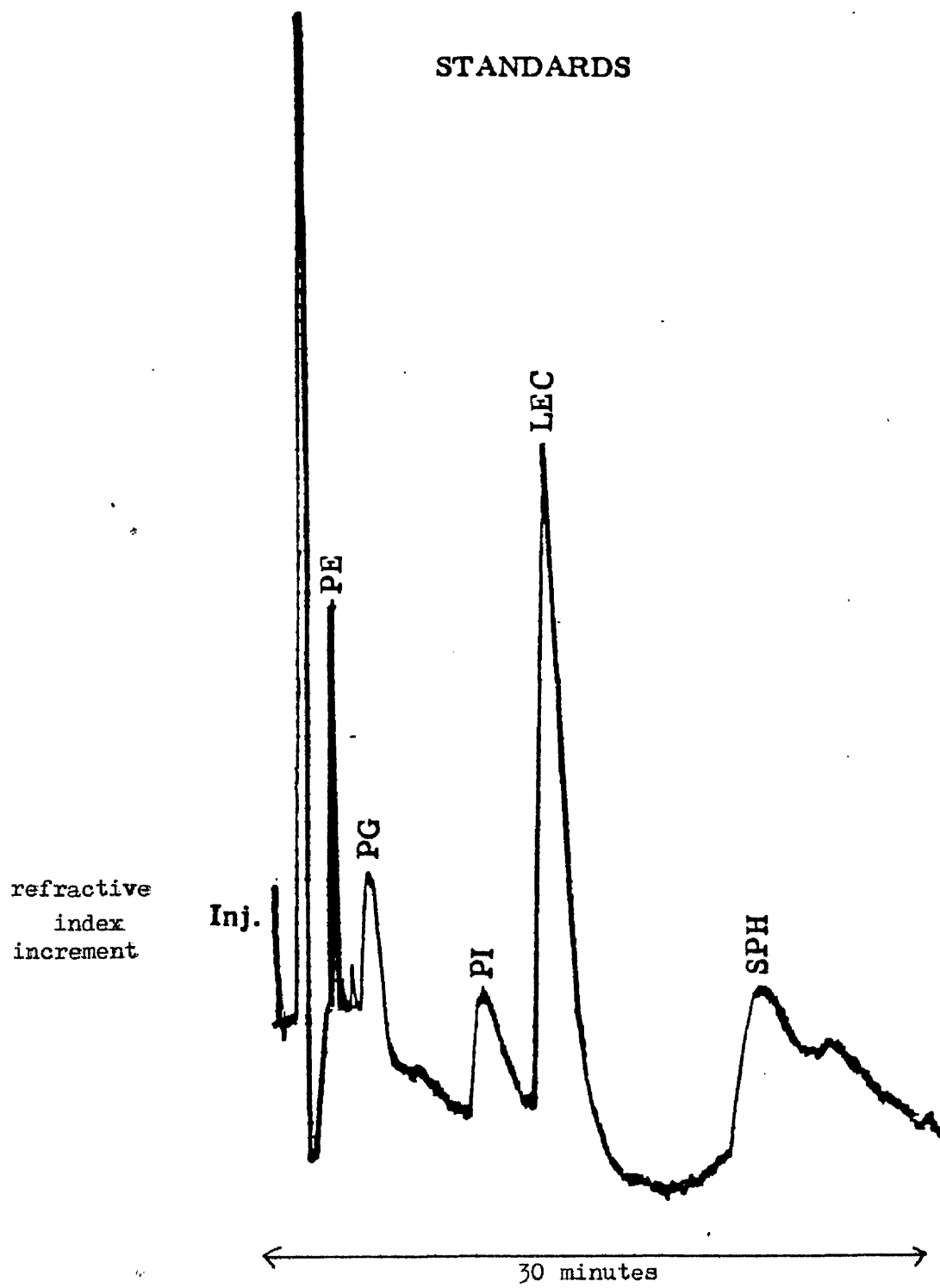
<u>Figure 2.6</u> <u>A Typical Chromatogram Obtained Using a Lipid Extract</u>

 <u>from a Mature Lung</u>

The amniotic fluid was collected as described in Methods, 2.2.1 and

centrifuged (Methods, 2.2.2). The lipids were extracted (Methods, 2.2.4)

and acetone precipitated (Methods, 2.2.5). The h.p.l.c. was used to

separate the phospholipids (Methods, 2.6). This figure shows a

chromatogram obtained using a lipid extract from a mature lung.

INJ represents injection marker

PE represents phosphatidylethanolamine

PG represents phosphatidylglycerol

PI represents phosphatidylinositol

LEC represents lecithin

SPH represents sphingomyelin

MATURE LUNG
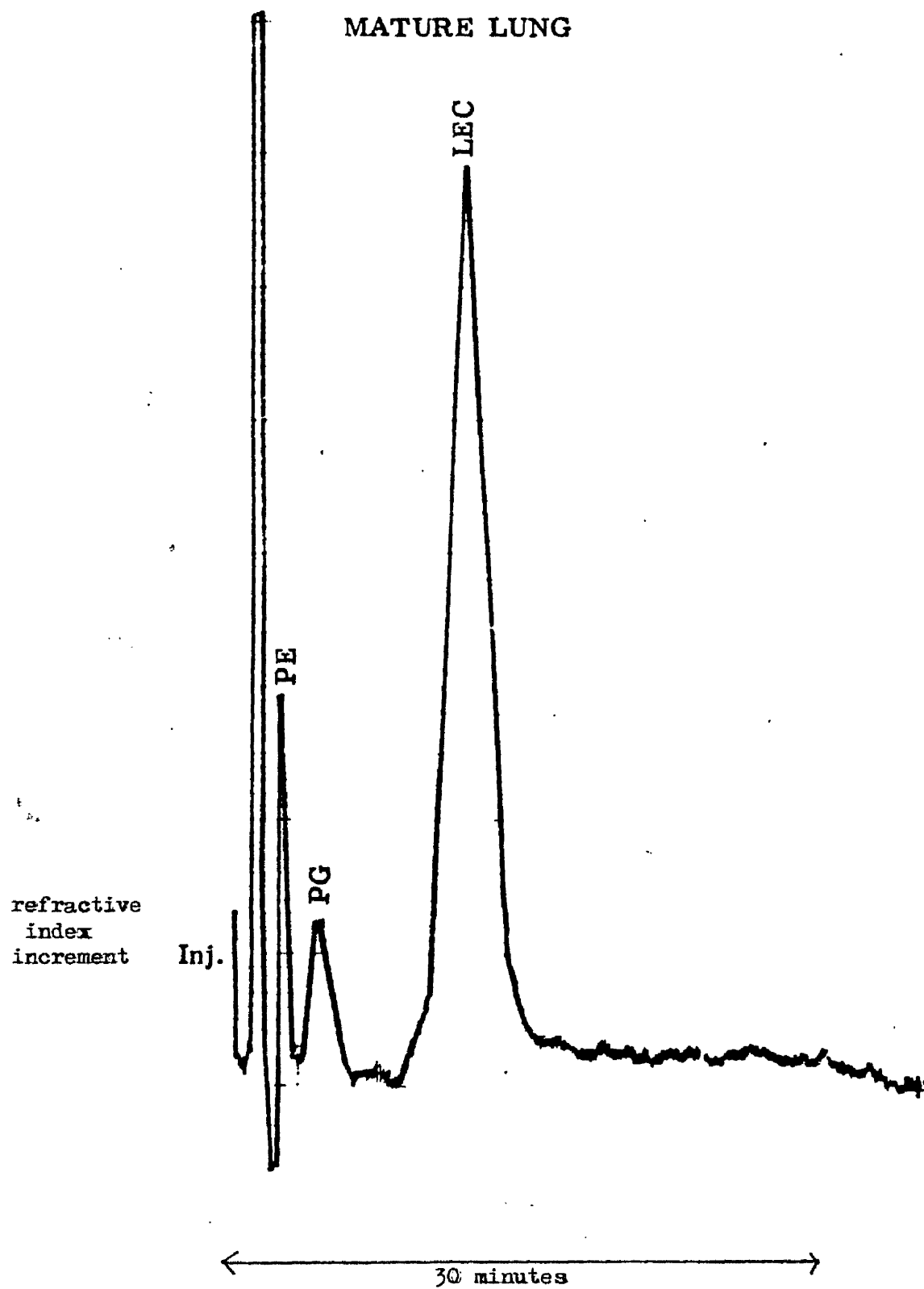
<u>Figure 2.7   A Typical Chromatogram Obtained Using a Lipid Extract</u>

<u>from an Immature Lung</u>

The amniotic fluid was collected as described in Methods, 2.2.1 and

centrifuged (Methods, 2.2.2). The lipids were extracted (Methods, 2.2.4)

and acetone precipitated (Methods, 2.2.5). The h.p.l.c. was used to

separate the phospholipids (Methods, 2.6). This figure shows a

chromatogram using a lipid extract from an immature lung.

INJ represents injection marker

PE represents phosphatidylethanolamine

PG represents phosphatidylglycerol

PI represents phosphatidylinositol

LEC represents lecithin

SPH represents sphingomyelin

IMMATURE LUNG

refractive
index
increment

Inj.

PE

LEC

SPH

← 30 minutes →
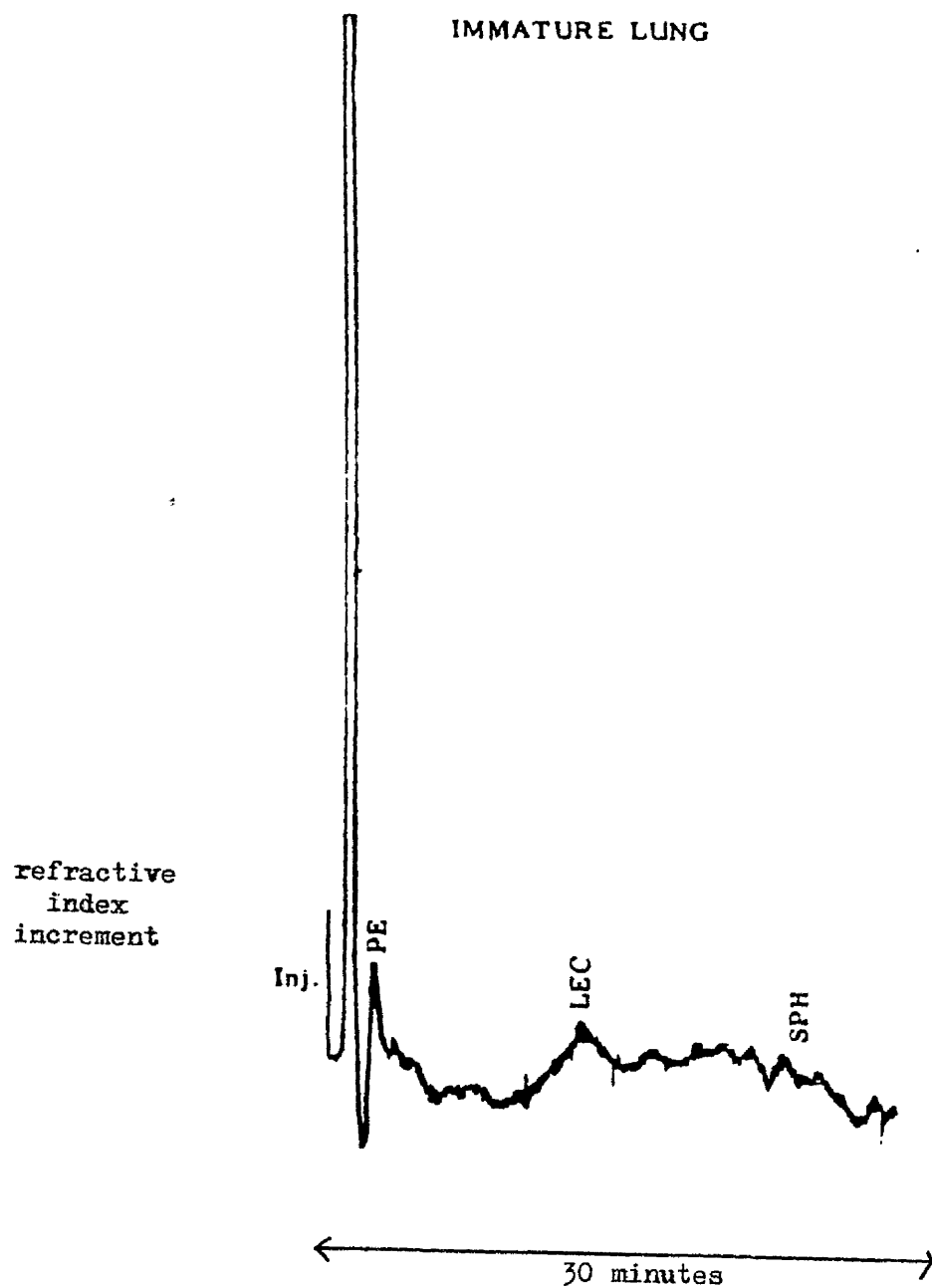
acetone precipitable and the acetone soluble fraction were analysed by gas liquid chromatography (g.l.c.) using a Perkin Elmer F11 gas chromatograph.

0.05 ml of a heptadecanoic acid (17:0) solution containing 14.22 μg heptadecanoic acid was added as an internal standard to the dried lipid extract.

2.7.1   Transesterification of Amniotic Fluid Lipids

The transesterification used to produce the fatty acid methyl esters for g.l.c. was based on that described by Christie (1973). The lipids were extracted from 5 ml of amniotic fluid (Methods, 2.2), were transferred to a stoppered test tube and the solvent removed. 5 ml of 2% v/v sulphuric acid in dry methanol was added and the tube was heated at 60 - 65°C in a water bath for two hours. After cooling the tube, 5 ml of diethyl ether and 5 ml of water were added and the tube vigorously shaken. The upper layer which contained the fatty acid methyl esters was removed and passed through a short column of anhydrous sodium sulphate to dry it. The lower aqueous phase from the transesterification was re-extracted with another 5 ml of diethyl ether. It was important when removing this ether extract not to contaminate it with water from the lower layer. The combined diethyl ether extracts were evaporated to dryness under a stream of nitrogen.

2.7.2   Gas Liquid Chromatography

The fatty acid methyl esters were dissolved in 10 μl of toluene and 1 μl of this solution injected into the port of the gas chromatograph.

Initially methanol was used to dissolve the fatty acids but better results were obtained by using toluene which dissolved more material.

The separation was carried out on a 2 m long x 3 mm i.d. stainless steel column containing Gas Chrom Q (100 - 120 mesh) coated with 10% (w/w) Silar 10C. The column temperature was 165°C with a nitrogen pressure of 69 Pa. Appropriate standards were used to demonstrate the separation of the various fatty acid methyl esters. Retention times were measured simply as the distance of the fatty acid methyl ester peak from the solvent front peak on the g.l.c. chart. The log retention time was plotted against the number of carbon atoms in the standard fatty acid methyl esters to allow identification of most of the fatty acids present in the lipid extract. Using an internal standard it was possible to calculate the absolute concentrations of the fatty acids present in the extract. Heptadecanoic (17:0) acid was used as an internal standard as this fatty acid is not present in amniotic fluid lipids. Figures 2.8a and 2.8b illustrate traces from g.l.c. analysis of amniotic fluid lipids' fatty acid methyl esters to which no internal standard has been added. It can be seen that there is no evidence of heptadecanoic acid methyl ester in the samples. Figure 2.8c shows a tracing where heptadecanoic acid has been added.

The percentage composition by weight, and the concentration of the fatty acids present were then determined from measurement of the area of each peak. The area of each peak was measured by cutting out and weighing the tracings of each peak.

<u>Figure 2.8</u>   <u>Gas Liquid Chromatography of Fatty Acid Methyl Esters</u>

<u>of Amniotic Fluid Lipids</u>

The amniotic fluid was collected as described in Methods 2.2.1 and

centrifuged (Methods, 2.2.2). The lipids were extracted (Methods, 2.2.4)

and acetone precipitated (Methods, 2.2.5). Transesterification was

carried out as described in Methods, 2.7.1 and the fatty acid

composition of lipids analysed using a Perkin Elmer F11 Gas

Chromatograph (Methods, 2.7.2). Figure 2.8a and 2.8b illustrate

traces from g.l.c. analysis of amniotic fluid lipid fatty acid methyl

esters to which no internal standard had been added, whilst figure

2.8c shows a tracing where haptadecanoic acid (17:0) has been added

(Methods, 2.7.2).

2.8. (a)

RECORDER RESPONSE

RETENTION TIME (cm)

14:0   14:1   16:0   18:0
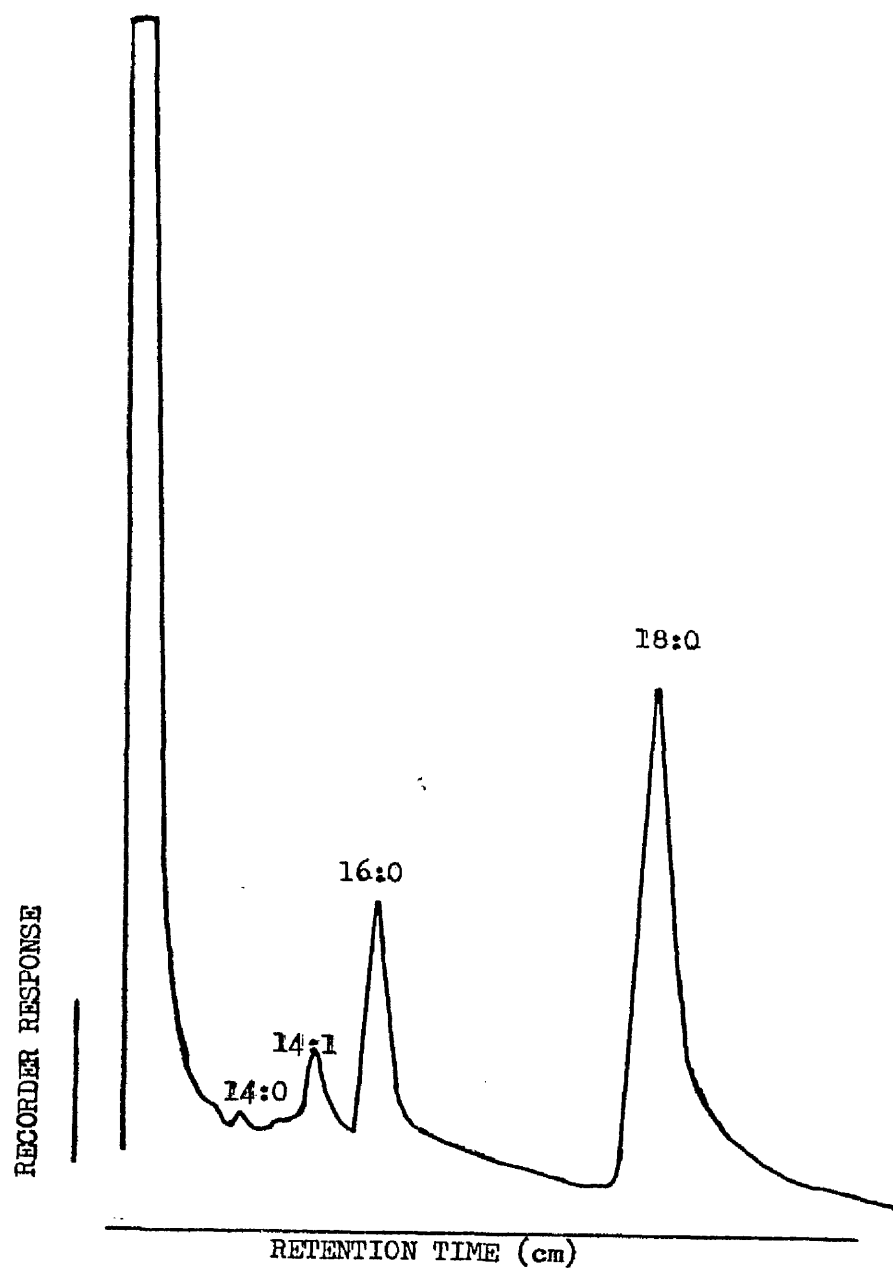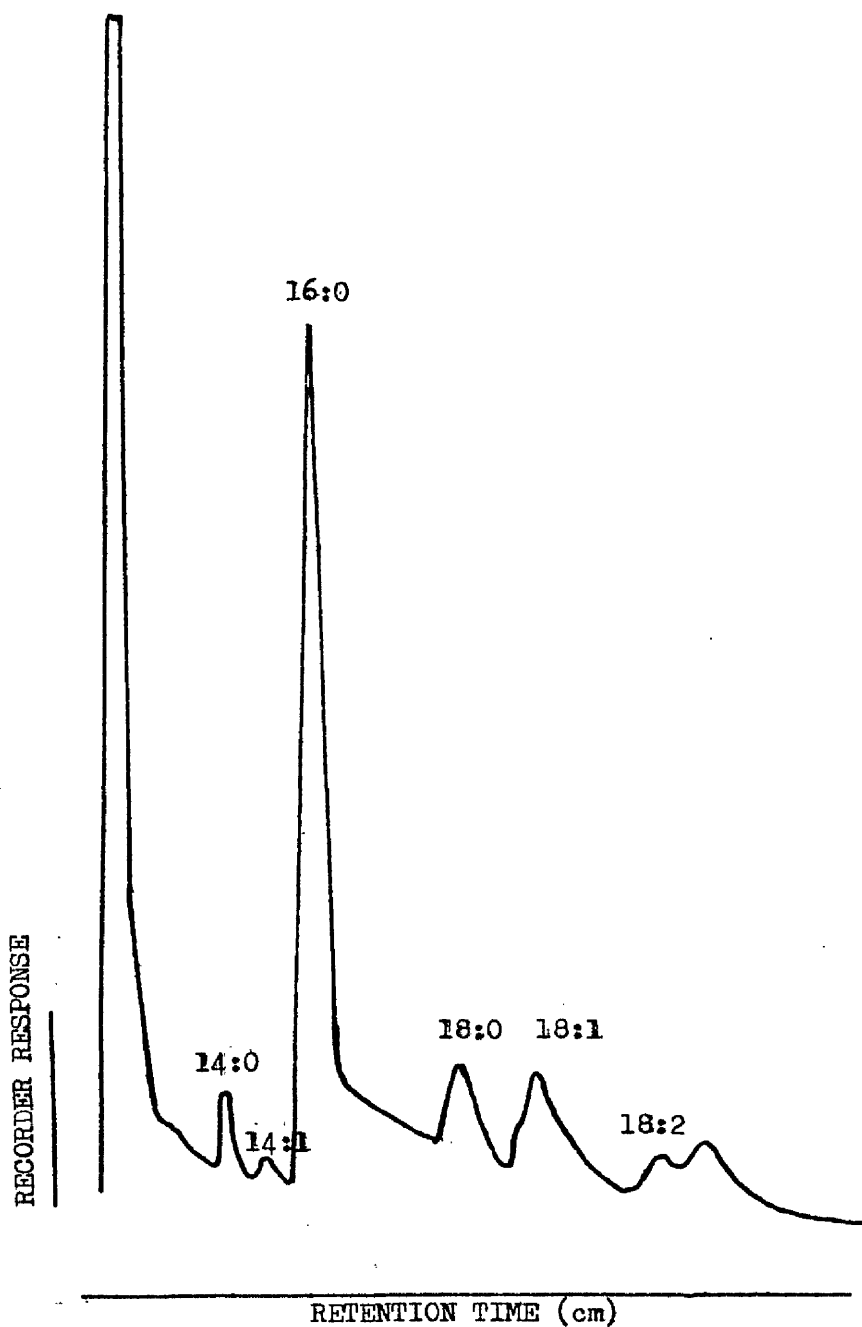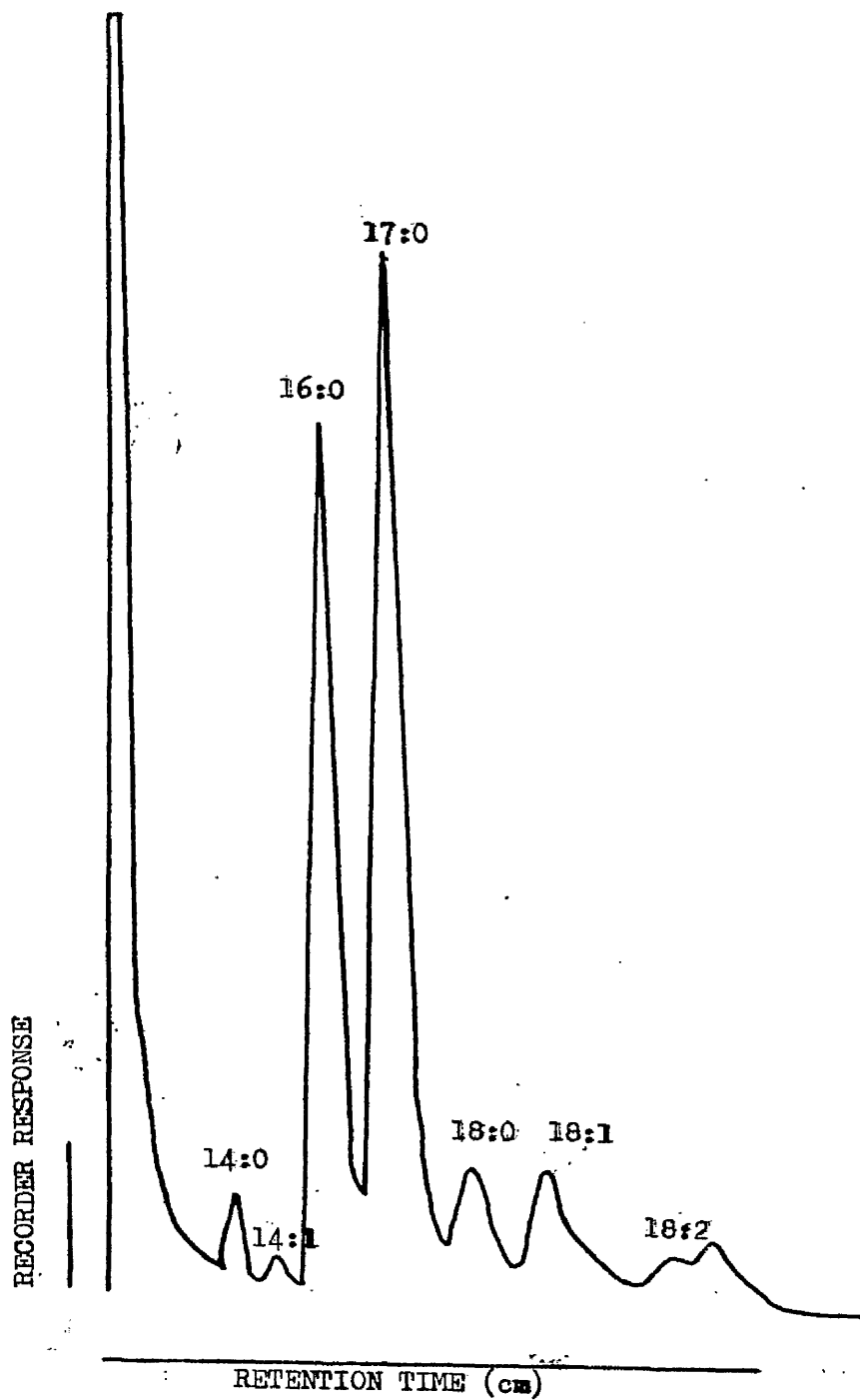
2.8   Amniotic Fluid Optical Density

The technique used was based on the method of Sbarra et al, (1977).
Amniotic fluid samples were analysed within one hour of sample
collection. After centrifugation for five minutes at 1100g the
optical density of the supernatant was determined in a Pye Unicam SP600
spectrophotometer, with appropriate red filter, at 650 nm, using 1 cm
light path cuvette with distilled water as a blank. An optical
density of 0.15 was used as the critical value since this was the
critical value adopted by other laboratories (Sbarra et al, 1977;
Copeland et al, 1978; Spellacy et al, 1979; Hill et al, 1979).

2.9   The Bubble Stability Test

The Bubble Stability Test was performed as described by Clements et al
(1972). All amniotic fluids were tested within one hour of sample
collection. Immediately before analysis the tube containing the fluid
was gently inverted five times to obtain a uniform suspension of
particles without causing the formation of foam.

1.0, 0.75, 0.50 and 0.25 ml of amniotic *fluid* are added to tubes numbered
1, 2, 3 and 4 respectively. 0.25, 0.50 and 0.75 ml of saline are
added to tubes 2, 3 and 4 respectively. One ml of 95% (v/v) ethanol is added
to each tube. The tubes are capped with clean rubber stoppers,
shaken vigorously for exactly 15 seconds and then stood vertically.
15 minutes later the air/liquid interface is examined in each tube for
the presence of small, stable bubbles. To do this the tubes were
viewed against a flat black background with bright overhead lighting.
A tube is recorded positive if it shows a complete ring of bubbles at/

the meniscus. Either or both tubes 3 and 4 being positive was
interpreted as an indication of mature fetal lungs.

Although this test is relatively simple, rigid adherence to the method
is required to obtain reproducible results. It has been shown that
the glassware used must be free of detergents or other surface active
materials; the alcohol solution, the concentration of which is
critical, should be freshly prepared. The test is invalid for amniotic
fluid samples containing blood or meconium as these substances give
rise to false positive results.

2.10   Assessment of Gestational Age

Gestational age was determined by the   obstetricians from the
menstrual history and by clinical and early ultrasonic assessment of
uterine size at the first antenatal visit.

2.11   Assessment of RDS

Evaluation of newborn infants was by the paediatric staff, the
diagnosis of RDS being based on clinical features (tachypnoea, grunting,
sternal recession, and rib retraction) present for at least 24 hours
and the radiological appearance of the lung fields. On the basis of
the ventilatory support required to maintain satisfactory oxygenation,
RDS was assessed as mild when only enriched ambient oxygen was required,
moderate when constant positive airways pressure was needed, and severe
when intermittent positive-pressure ventilation had to be used.

41

2.12 The Sensitivity, Specificity, Predictive Value and Predictive Accuracy of Each Test

Reliability of an immature result was expressed as <u>sensitivity</u> defined as the percentage of babies with RDS and with an immature result.

$$\text{Sensitivity} = \frac{\text{immature result, RDS present}}{\text{immature result, RDS present} + \text{mature result, RDS present.}}$$

Reliability of a mature result was expressed as <u>specificity</u> defined as the percentage of babies with RDS absent.

$$\text{Specificity} = \frac{\text{mature result, RDS absent}}{\text{mature result, RDS absent} + \text{immature result, RDS absent.}}$$

<u>Predictive Value</u> was defined as the percentage of immature results of all immature results, or the percentage of mature results of all mature results.

$$\text{Predictive Value} = \frac{\text{immature result, RDS present}}{\text{all immature results}}$$

OR

$$\frac{\text{mature result, RDS absent}}{\text{all mature results.}}$$

$$\text{Predictive Accuracy} = \frac{\text{correctly predicted results}}{\text{all results}}$$

CHAPTER THREE

RESULTS

CHAPTER THREE

RESULTS

3.1 The Effect of Gestational Age and the Predictive Reliability of Tests for Fetal Lung Maturity Based on Lipid Measurements

3.1.1 The Effect of Gestational Age on the Amniotic Fluid Lecithin/Sphingomyelin Ratio Determined by Two-Dimensional t.l.c.

The Lecithin/Sphingomyelin (L/S) ratio was determined by 2D t.l.c. using the acetone precipitable lipids from 766 amniotic fluid samples (Methods, 2.4). The L/S ratio shows a general increase with gestational age although there is a large individual variation. There was a noticeable increase in the L/S ratio from 35 weeks of gestation as shown in figure 3.1. Of 478 samples which were collected at 35 weeks or greater, 400 (83.7%) had an L/S ratio of 2.0 or more (table 3.1). The remaining 288 samples were collected from neonates with gestational ages of less than 35 weeks, and only 89 of these samples (30.9%) had L/S ratio greater than 2.0.

3.1.2 The Effect of Gestational Age on the Presence of Phosphatidylglycerol in Amniotic Fluid

The presence of phosphatidylglycerol was determined by 2D t.l.c. in 766 amniotic fluid lipid samples (Methods, 2.4). At 35 weeks or later/

44

Figure 3.1 The Effect of Gestational Age on the Amniotic Fluid L/S

Ratio Determined by 2D t.l.c.

The amniotic fluid was collected as described in Methods, 2.2.1 and

centrifuged (Methods, 2.2.2). The lipids were extracted (Methods, 2.2.4)

and acetone precipitated (Methods, 2.2.5). The L/S ratio was determined

by 2D t.l.c. Assessment of gestational age was as described in Methods,

2.10.
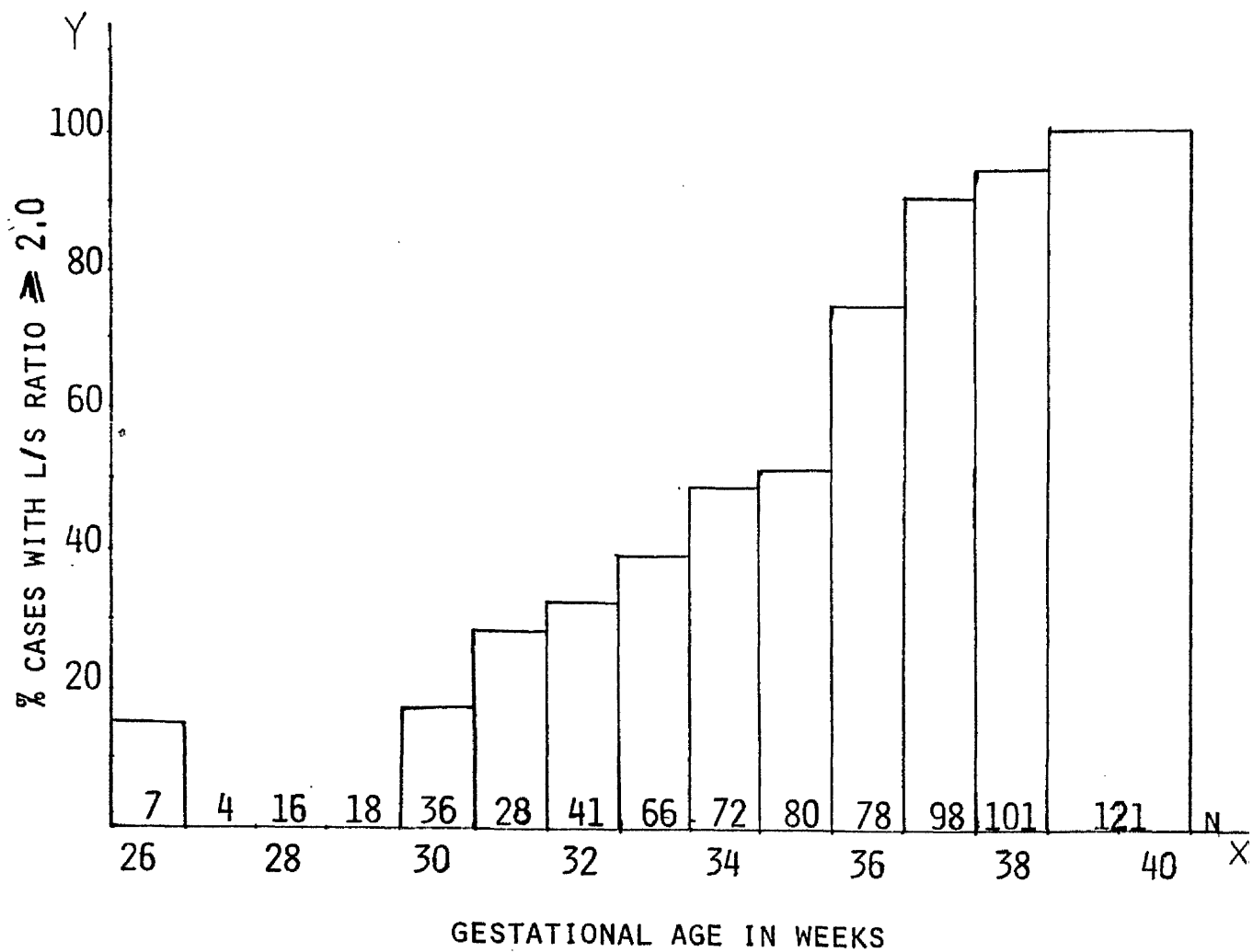
WHERE N EQUALS THE NUMBER OF SAMPLES

Table 3.1 <u>The effect of gestational age on the amniotic fluid</u>

<u>L/S ratio by 2D t.l.c.</u>

Amniotic fluid samples were collected (Methods,2.2.1) and centrifuged

(Methods, 2.2.2). The lipids were extracted (Methods, 2.2.4) and acetone

precipitated (Methods, 2.2.5). The L/S ratio was determined as described

in Methods, 2.4 . Assessment of gestational age was as described in

Methods, 2.10. The number in brackets represents the number of patients.

Gestational Age	No. of Samples	% With L/S $\geqslant$ 2.0	% With L/S $<$ 2.0
$\geqslant$ 35 weeks	478	83.7 (400)	16.3 (78)
$<$ 35 weeks	288	30.9 (89)	69.1 (199)
Total	766	63.8 (489)	36.2 (277)

phosphatidylglycerol was nearly always detected (figure 3.2). From 478 samples of fluid collected at 35 weeks of gestation or later, 438 (91.6%) showed phosphatidylglycerol (table 3.2). In the remaining 288 samples collected before 35 weeks, phosphatidylglycerol was present in 143 (49.7%) of the samples. Using the phosphate assay (Methods, 2.5), the lowest limit of detectability for phosphatidylglycerol by 2D t.l.c. (Methods, 2.4) was 0.4 µmol 1^{-1} amniotic fluid.

3.1.3 The Presence of Phosphatidylglycerol as an Index of Fetal Lung Maturity

A summary of results appears in table 3.3. Five hundred and eighteen patients were studied when the amniotic fluid was collected within 72 hours of delivery. In 492 of these cases phosphatidylglycerol was present and only three of the babies developed RDS (0.6%), each of these three having been delivered by emergency caesarean section because of an acute obstetric complication. In the remaining 26 patients phosphatidylglycerol was absent in the amniotic fluid. The overall incidence of RDS in these patients was 84.6%, nine of them dying as a result. In two of the four instances in which RDS did not occur, the mothers had been treated with betamethasone phosphate (4 mg eight hourly for 48 hours) in an attempt to accelerate fetal lung maturation (Howie et al, 1977). In one of the remaining two patients, premature rupture of the membranes had occurred spontaneously 65 hours before delivery. In the other case the mother was a diabetic, and the L/S ratio of this amniotic fluid was found to be above 2.0.

<u>Figure 3.2</u> <u>The Effect of Gestational Age on the Presence of</u>

<u>Phosphatidylglycerol in Amniotic Fluid</u>

The amniotic fluid was collected as described in Methods, 2.2.1 and
centrifuged (Methods, 2.2.2). The lipids were extracted (Methods, 2.2.4)
and acetone precipitated (Methods, 2.2.5). The presence of
phosphatidylglycerol was determined by 2D t.l.c. (Methods, 2.4).
Assessment of gestational age was as described in Methods, 2.10.
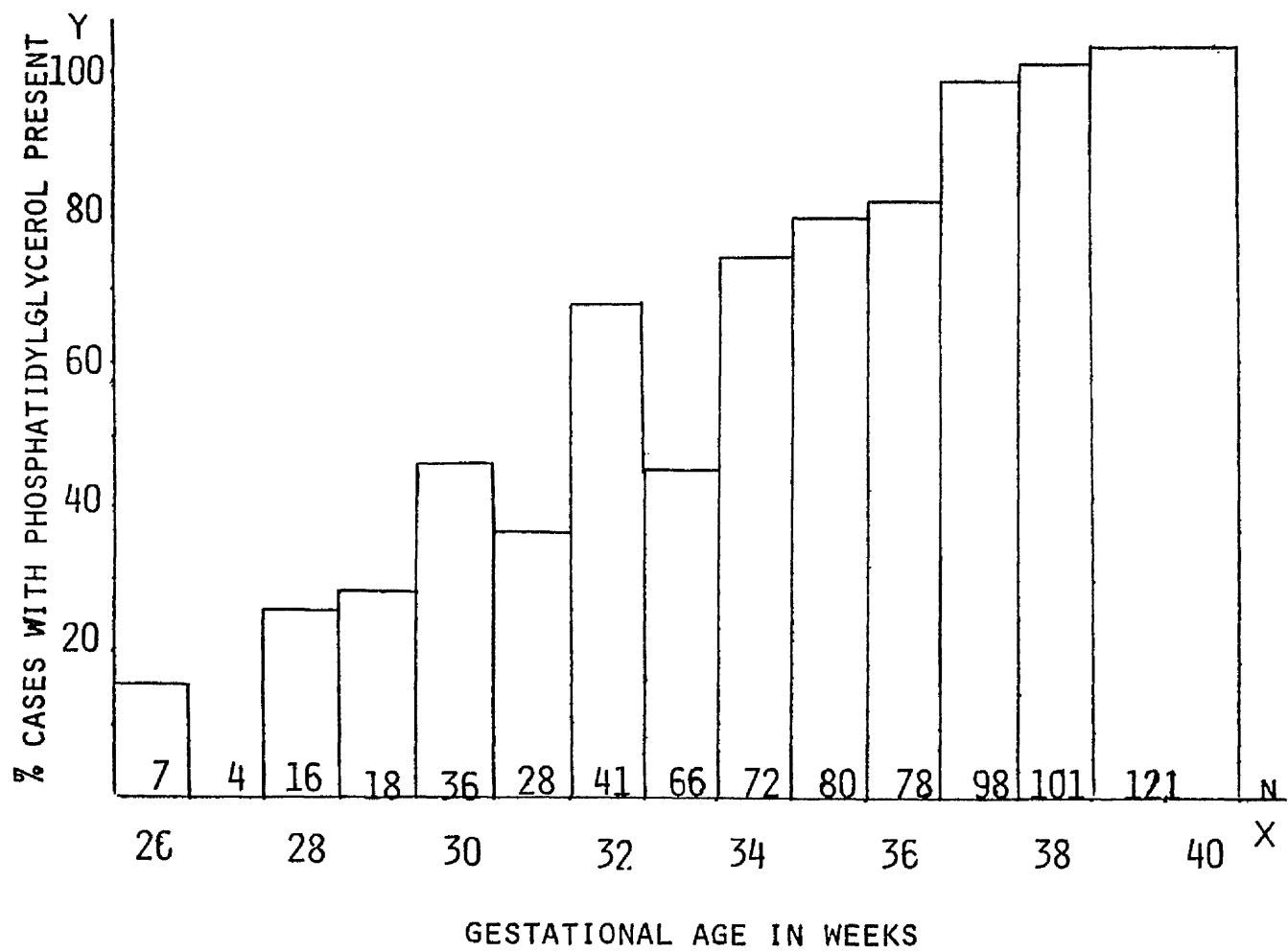
WHERE N EQUALS THE NUMBER OF SAMPLES

Table 3.2 The effect of gestational age on the presence of phosphatidyl-

           glycerol in amniotic fluid

Amniotic fluid samples were collected (Methods, 2.2.1) and centrifuged

(Methods, 2.2.2). The lipids were extracted (Methods, 2.2.4) and acetone

precipitated (Methods, 2.2.5). The lipids were separated by 2D t.l.c.

(Methods, 2.4) and the presence or absence of phosphatidylglycerol

noted. Gestational age was assessed (Methods, 2.10). The number in

brackets represents the number of patients.

Gestational Age	No. of Samples	% With PG Present	% With PG Absent
≥ 35 weeks	478	91.6 (438)	8.4 (40)
≤ 35 weeks	288	49.7 (143)	50.3 (145)
Total	766	75.8 (581)	24.2 (185)

Table 3.3 The presence of phosphatidylglycerol as an index of fetal

lung maturity

Amniotic fluid samples were collected (Methods, 2.2.1) and centrifuged

as described in Methods, 2.2.2. The lipids were extracted (Methods, 2.2.4)

and acetone precipitated (Methods, 2.2.5). The lipids were separated by

2D t.l.c. (Methods, 2.4) and the presence or absence of phosphatidyl-

glycerol noted. All samples were collected within 72 hours of delivery.

Assessment of RDS was made by the paediatric staff as described in Methods,

2.11. The number in brackets represents the number of patients.

	No. of Patients	% RDS Present	% RDS Absent
PG Present	492	0.61 (3)	99.4 (489)
PG Absent	26	84.6 (22)	15.4 (4)
Total	518	4.8 (25)	95.2 (493)

The predictive accuracy was maintained in the 260 amniotic fluids collected by vaginal drainage (table 3.4). Of 246 samples which had phosphatidylglycerol present only one of the babies developed RDS, a false positive prediction rate of 0.4%. In the remaining 14 samples phosphatidylglycerol was absent and 13 babies developed RDS (92.9%), seven being fatally affected. In the instance in which RDS did not occur in this series, the mother had been treated with betamethasone phosphate.

3.1.4   The L/S Ratio and the Detection of Phosphatidylglycerol as an Index of Fetal Lung Maturity

A summary of the results appears in table 3.5. Five hundred and eighteen patients were studied when the amniotic fluid was collected within 72 hours of delivery. In 412 patients the L/S ratio of the amniotic fluid was 2.0 or above suggesting mature fetal lungs and phosphatidylglycerol was absent in only one of these samples collected from a patient who was an insulin dependent diabetic. None of these babies developed RDS.

In the remaining 106 patients an immature amniotic fluid L/S ratio (less than 2.0) was found and the overall incidence of RDS was 23.6%. Eighty-one of these fluids contained phosphatidylglycerol leaving 25 in which it was absent. In relation to the amniotic fluids with immature L/S ratios but detectable phosphatidylglycerol three of 81 babies (3.7%) developed classical RDS, each of these three having been delivered by emergency caesarean section because of an acute obstetric complication. In the remaining 25 patients with immature L/S ratios/

Table 3.4 <u>The presence of phosphatidylglycerol as an index of fetal</u>

<u>lung maturity in samples collected by vaginal drainage</u>

Amniotic fluid samples were collected by vaginal drainage and centrifuged
as described in Methods, 2.2.2. The lipids were extracted (Methods,2.2.4)
and acetone precipitated (Methods, 2.2.5). The lipids were separated by
2D t.l.c. (Methods, 2.4) and the presence or absence of phosphatidyl-
glycerol noted. All samples were collected within 72 hours of delivery.
Assessment of RDS was made by the paediatric staff as described in
Methods, 2.11. The number in brackets represents the number of patients.

	No. of Patients	% RDS Present	% RDS Absent
PG Present	246	0.4 (1)	99.6 (245)
PG Absent	14	92.9 (13)	7.1 (1)
Total	260	5.4 (14)	94.6 (246)

Table 3.5   The L/S ratio and the presence or absence
             of phosphatidylglycerol as an index of fetal
             lung maturity

Amniotic fluid samples were collected (Methods, 2.2.1) and

centrifuged (Methods, 2.2.2). The lipids were extracted (Methods,

2.2.4) and acetone precipitated (Methods, 2.2.5). The lipids were

separated by 2D t.l.c. (Methods, 2.4) and the L/S ratio determined

and the presence or absence of phosphatidylglycerol noted. All

samples were collected within 72 hours of delivery. Assessment

of RDS was made by the paediatric staff (Methods,2.11). The number

in brackets represents the number of patients.

	All Cases		PG Present		PG Absent	
L/S Ratio	Total	No. with RDS	Total	No. with RDS	Total	No. with RDS
≧ 2.0	412	0 (0)	411	0 (0)	1	0 (0)
< 2.0	106	25 (23.6)	81	3 (3.7)	25	22 (88.0)
Total	518	25 (4.8)	492	3 (0.6)	26	22 (84.6)

but no phosphatidylglycerol, 21 of these babies (84.0%) developed RDS, 9 (42.9%) dying as a result. As already described (Results, 3.1.3) in two of the four instances in which RDS did not occur, the mothers had been treated with betamethasone and in another spontaneous premature rupture of the membranes had occurred.

The predictive accuracy was maintained in the 260 amniotic fluids collected by vaginal drainage (table 3.6). Two hundred and sixteen samples showed a mature L/S ratio and the presence of phosphatidylglycerol and none of the babies developed RDS. In the remaining 44 samples the L/S ratio was less than 2.0 and only 14 of these babies (31.8%) developed RDS; phosphatidylglycerol was present in 30 of these samples with L/S less than 2.0 and only one baby developed RDS in this group, a false positive prediction rate by phosphatidylglycerol of 3.3 %. Phosphatidylglycerol was absent in the 14 remaining cases and 13 babies developed RDS (92.9%), 7 being fatally affected. In the instance in which RDS did not occur in this series, the mother had been treated with betamethasone.

3.1.5   The Effect of Gestational Age on the Amniotic Fluid L/S Ratio Determined by One-Dimensional t.l.c.

The L/S ratio was determined by 1D t.l.c. (Methods, 2.3) from the acetone precipitable lipids in amniotic fluid from 93 patients and their results plotted in relation to their gestational age (figure 3.3). The L/S ratio increased with gestational age but there was a wide scatter throughout the gestation. The best fitting curve was/

Table 3.6 The L/S ratio and the presence or absence of

phosphatidylglycerol in samples collected by

vaginal drainage as an index of fetal lung maturity

Amniotic fluid samples were collected by vaginal drainage and cent-

rifuged (Methods, 2.2.2). The lipids were extracted (Methods, 2.2.4)

and acetone precipitated (Methods, 2.2.5). The lipids were separated by

2D t.l.c. (Methods, 2.4) and the L/S ratio determined and the presence

or absence of phosphatidylglycerol noted. All samples were collected

within 72 hours of delivery. Assessment of RDS was made by the

paediatric staff (Methods, 2.11). The number in brackets represents

the number of patients.

	All Cases			PG Present			PG Absent		
L/S Ratio	Total	No. with RDS		Total	No. with RDS		Total	No. with RDS	
$\geqslant$ 2.0	216	0	(0)	216	0	(0)	0	0	(0)
$<$ 2.0	44	14	(31.8)	30	1	(3.3)	14	13	(92.9)
Total	260	14	(5.4)	246	1	(0.4)	14	13	(92.8)

<u>Figure 3.3</u> <u>The Effect of Gestational Age on the Amniotic Fluid</u>

   <u>L/S Ratio</u>

The amniotic fluid was collected as described in Methods, 2.2.1 and

centrifuged (Methods, 2.2.2). The lipids were extracted (Methods, 2.2.4)

and acetone precipitated (Methods, 2.2.5). The L/S ratio was

determined by 1D t.l.c. as described in Methods, 2.3. Assessment of

gestational age was as described in Methods, 2.10. Equation (ii)

fits the data (r = 0.49, P < 0.001, n = 93).

$$y = 0.09e^{0.09x} \quad \ldots\ldots\ldots\ldots\ldots\ldots \text{(ii)}$$

   where x is the gestational age in weeks
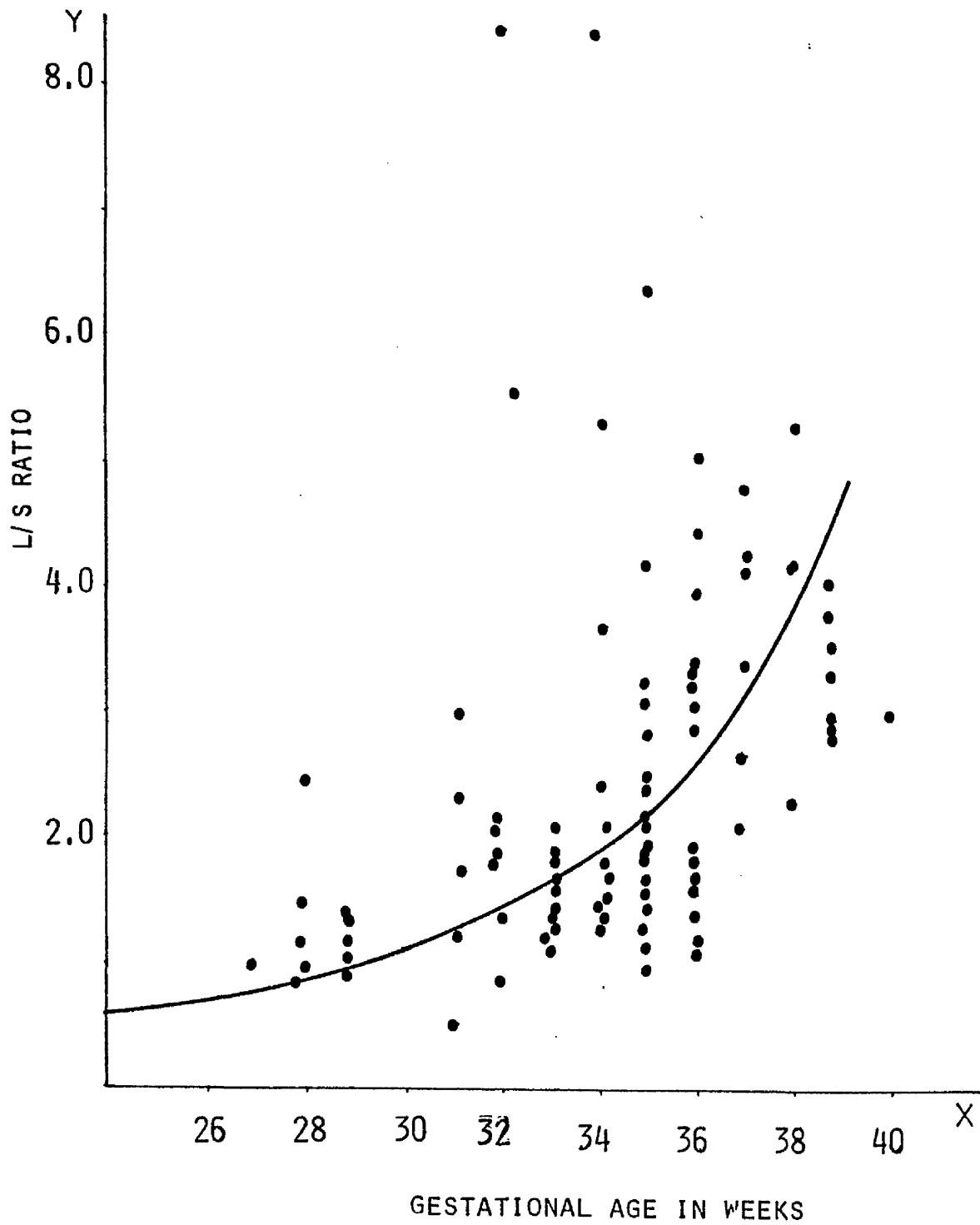
   and y is the L/S ratio.

exponential (r = 0.49, P < 0.001).

$$y = 0.09e^{0.09x} \quad \dots\dots\dots\dots\dots\dots\dots \quad (ii)$$

where x is the gestational age in weeks and

y is the L/S ratio.

The L/S ratio was less than 2.0 in 16 of the 50 samples (32.0%)
collected at 35 weeks of gestation or later (table 3.7). The mean L/S
ratio of these 50 samples was 3.0. Samples were obtained from 43
patients whose babies had gestational ages of less than 35 weeks and
the mean L/S ratios of these samples was 2.0. Thirty-one of these 43
samples (72.1%) had L/S ratios below 2.0. There was a noticeable incr-
ease in the L/S ratio at 35 weeks of gestation when the mean L/S ratio
rose above 2.0.

3.1.6   The L/S Ratio Determined by One-Dimensional t.l.c. as an
         Index of Fetal Lung Maturity

A summary of results appears in table 3.8. Amniotic fluid samples
were collected from 69 patients within 72 hours of delivery. In 45
patients the L/S ratio of amniotic fluid was 2.0 or greater, suggesting
mature fetal lungs, and none of the babies developed RDS. In the
remaining 24 patients an immature L/S ratio was found but only six
babies (25.0%) developed RDS. Thus the criterion of immaturity
(L/S < 2.0) gives a false negative prediction rate of 75.0%.

<u>Table 3.7</u> <u>The effect of gestational age on the amniotic fluid</u>

   <u>L/S ratio determined by 1D t.l.c.</u>

The amniotic fluids were collected as described in Methods, 2.2.1

and centrifuged (Methods, 2.2.2). The lipids were extracted

(Methods, 2.2.4) and acetone precipitated (Methods, 2.2.5). The

L/S ratio was determined by 1D t.l.c. as described in Methods, 2.3.

A critical value of 2.0 was used (Gluck <u>et al</u>, 1971). Assessment

of gestational age was as described in Methods, 2.10.

Gestational Age	No. of Samples	% with L/S $\geq$ 2.0	% with L/S $<$ 2.0
$\geq$ 35 weeks	50	68.0 (34)	32.0 (16)
$<$ 35 weeks	43	72.1 (31)	38.7 (12)
Total	93	69.9 (65)	30.1 (28)

Table 3.8   The L/S ratio determined by 1D t.l.c. as an index of

    fetal lung maturity

The amniotic fluids were collected as described in Methods, 2.2.1

and centrifuged (Methods, 2.2.2). The lipids were extracted :

(Methods, 2.2.4) and acetone precipitated (Methods,2.2.5). The

L/S ratio was determined by 1D t.l.c. as described in Methods, 2.3.

A critical value of 2.0 was used (Gluck et al, 1971). All samples

were collected within 72 hours of delivery. Assessment of RDS was

made by the paediatric staff as described in Methods, 2.11. The

number in brackets represents the number of patients.

L/S Ratio	No. of Patients	% RDS Present	% RDS Absent
$\geqslant$ L/S 2.0	45	0 (0)	100 (45)
$<$ L/S 2.0	24	25.0 (6)	75.0 (18)
Total	69	8.7 (6)	91.3 (63)

3.1.7   The Effect of Gestational Age on the Amniotic Fluid Lecithin

Concentration

The amniotic fluid lecithin concentration was determined by the
phosphate assay (Methods, 2.5). The results of 50 determinations of
lecithin concentration in the acetone precipitable lipids are plotted
against gestational age in figure 3.4. The lecithin concentration
increased during pregnancy but variations were considerable at all
gestational ages. The best fitting curve was exponential.
($r = 0.66$, $P < 0.001$)

$$y = 103.18e^{3.88x} \qquad \ldots\ldots\ldots\ldots\ldots\ldots \qquad (iii)$$

where x is the gestational age in weeks and

y is the lecithin concentration.

There is an obvious increase from 35 weeks of gestation. Twenty-eight
amniotic fluid specimens were collected from patients with a
gestational age of less than 35 weeks and only 2 of the 28 samples had
lecithin concentrations greater than or equal to 25 μmol 1^{-1} amniotic
fluid (table 3.9). When the gestational age was 35 weeks or greater
only 4 of the 22 samples (18.2%) had lecithin concentrations less than
25 μmol 1^{-1} amniotic fluid.

3.1.8   Lecithin Concentration as an Index of Fetal Lung Maturity

The lecithin concentration was determined in 28 amniotic fluids
collected within 72 hours of delivery. A critical value of 25 μmol 1^{-1}
amniotic fluid was used, above which no cases of RDS occurred and/

60

<u>Figure 3.4</u> <u>The Effect of Gestational Age on the Amniotic Fluid</u>

<u>Lecithin Concentration</u>

The amniotic fluid was collected as described in Methods, 2.2.1 and

centrifuged (Methods, 2.2.2). The lipids were extracted (Methods, 2.2.4)

and acetone precipitated (Methods, 2.2.5). The lecithin concentration

was then measured by the phosphate analysis technique as described in

Methods, 2.10. Equation (iii) fits the data ($r = 0.66$, $P < 0.001$,

$n = 50$).

$$y = 103.18e^{3.88x} \quad \cdot \quad \ldots\ldots\ldots\ldots \quad (iii)$$

where x is the gestational age in weeks

and y is the lecithin concentration.
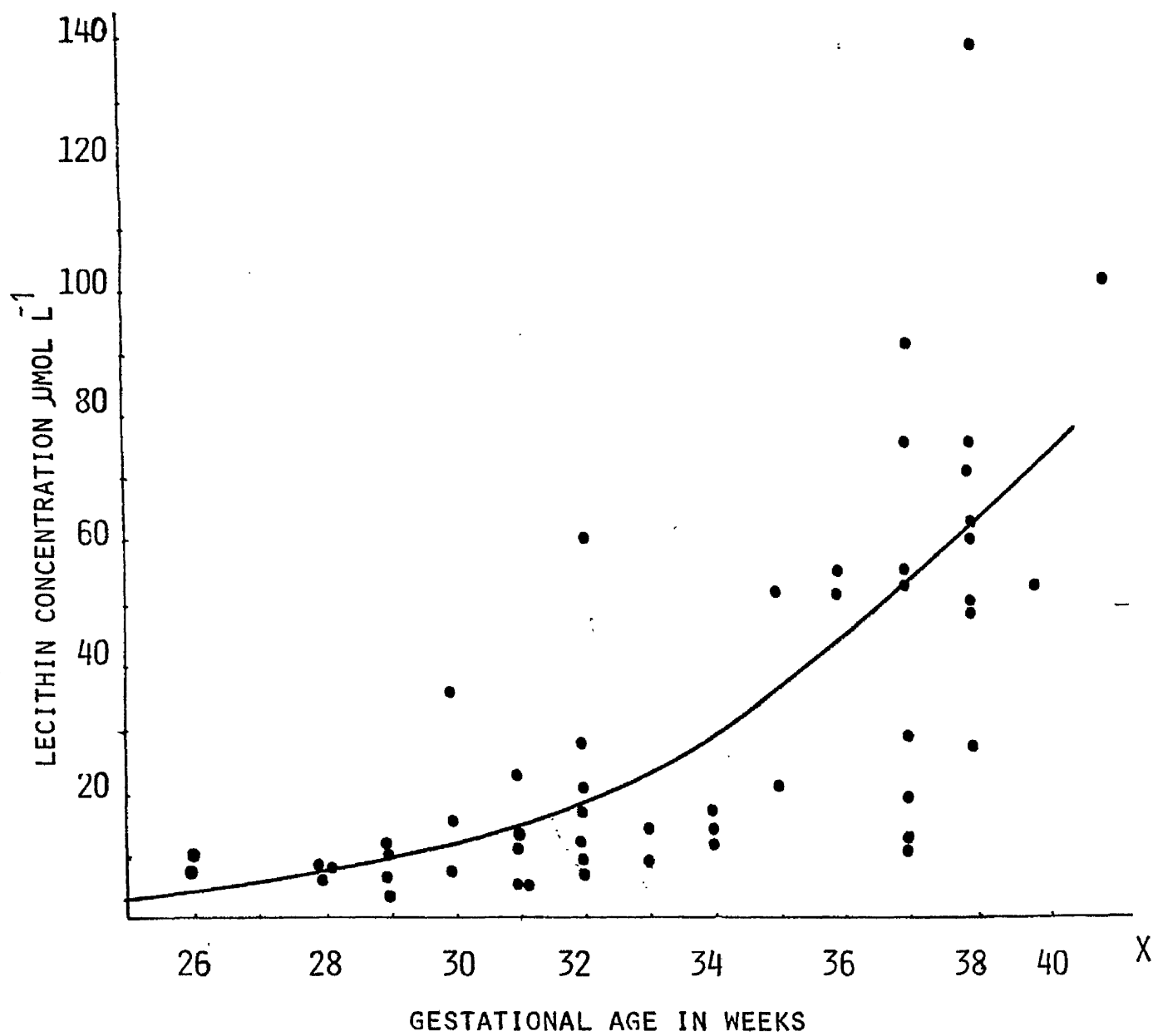
Table 3.9   The effect of gestational age on the amniotic fluid

lecithin concentration

The amniotic fluids were collected as described in Methods, 2.2.1

and centrifuged (Methods, 2.2.2). The lipids were extracted (Methods,

2.2.4) and acetone precipitated (Methods, 2.2.5). The lecithin

concentration was measured by the phosphate analysis technique

(Methods, 2.5) following 2D t.l.c. A critical value of 25 μmol^{-1}

was used (Results, 3.1.8). Assessment of gestational age was

as described in Methods, 2.10. The number in brackets represents

the number of patients.

Gestational Age	No. of Samples	% With Lecithin $\geqslant 25$ umol 1^{-1}	% With Lecithin < 25 umol 1^{-1}
$\geqslant 35$ weeks	22	81.8 (18)	18.2 (4)
< 35 weeks	28	7.1 (2)	92.9 (26)
Total	50	40.0 (20)	60.0 (30)

below which all babies which developed RDS were detected.

However the lack of predictive accuracy when the lecithin concentration is less than the critical value is obvious (table 3.10). Of 16 samples with a lecithin concentration of less than 25 μmol l^{-1} amniotic fluid, only 6 of the 16 babies developed RDS. The predictive accuracy was only 37.5%.

3.1.9   The Effect of Gestational Age on the Phosphatidylglycerol Concentration in Amniotic Fluid

Forty-one determinations of phosphatidylglycerol concentration were made using t.l.c. (Methods, 2.4) followed by phospholipid phosphate determination (Methods, 2.5). Twenty-five of the 41 samples had phosphatidylglycerol present. The 25 determinations of phosphatidylglycerol concentration are plotted against gestational age in figure 3.5 and as illustrated the results are widely scattered. The concentration of phosphatidylglycerol was determined in 28 amniotic fluid samples collected before 35 weeks of gestation and phosphatidylglycerol was measurable in 12 of these samples with an average concentration of 4.58 μ moles l^{-1} amniotic fluid. At 35 weeks of gestation or later phosphatidylglycerol was present in each of the 13 specimens with an average concentration of 8.03μ mol l^{-1} amniotic fluid. The concentration of phosphatidylglycerol present before and after 35 weeks of gestation was not found to be significantly different. The timing of the appearance of phosphatidylglycerol is very variable, especially before 35 weeks of gestation as can be seen in figure 3.2.

Table 3.10   The lecithin concentration as an index of fetal lung maturity

The amniotic fluid samples were collected (Methods, 2.2.1) and centrifuged
(Methods, 2.2.2). The lipids were extracted (Methods, 2.2.4) and
acetone precipitated (Methods, 2.2.5). The lecithin concentration was
determined by phosphate analysis technique (Methods, 2.5) following
2D t.l.c. (Methods, 2.4). All samples were collected within 72 hours
of delivery. Assessment of RDS was made by the paediatric staff as
described in Methods, 2.11. The number in brackets represents the number
of patients.

Lecithin Concentration	No. of Patients	% RDS Present	% RDS Absent
Lecithin $\geqslant$ 25 μmol 1^{-1}	12	0 (0)	100 (12)
Lecithin $<$ 25 μmol 1^{-1}	16	37.5 (6)	62.5 (10)
Total	28	21.4 (6)	78.6 (22)

The Effect of Gestational Age on the Concentration of

Phosphatidylglycerol

The amniotic fluid was collected as described in Methods, 2.2.1 and

centrifuged (Methods, 2.2.2). The lipids were extracted (Methods, 2.2.4)

and acetone precipitated (Methods, 2.2.5). The concentration of

phosphatidylglycerol was then determined by the phosphate analysis

technique as described in Methods, 2.5 following 2D t.l.c. (Methods, 2.4).

Assessment of gestational age was made as described in Methods, 2.10.

65
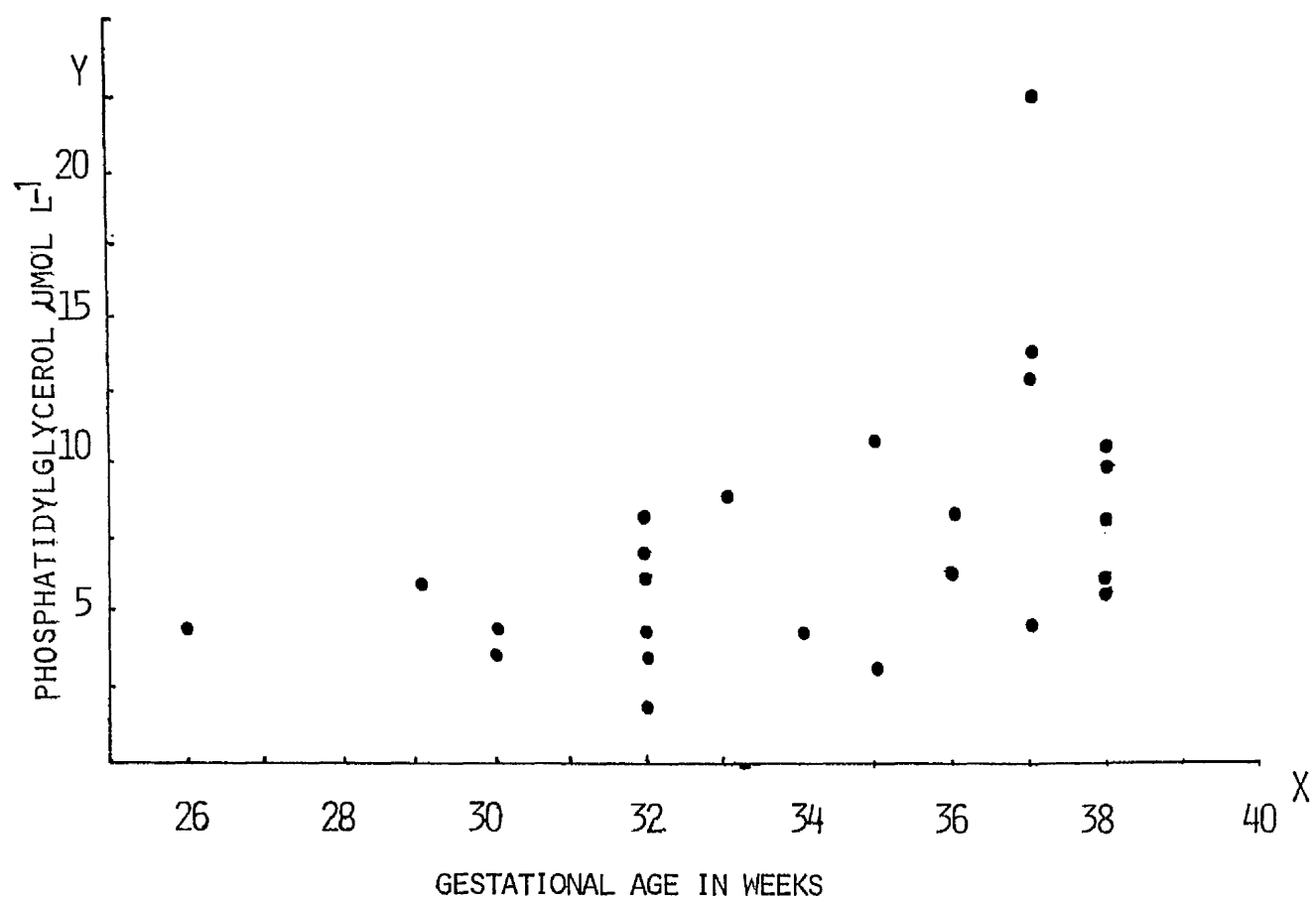
3.1.10 : Phosphatidylglycerol Concentration and Fetal Lung Maturity

Phosphatidylglycerol was present in 23 of the 28 amniotic fluid specimens which were collected within 72 hours of delivery. There was no RDS in these 23 babies whose phosphatidylglycerol concentrations varied considerably and ranged from 1.9 μ mol l^{-1} amniotic fluid to 21.8 μ mol l^{-1} amniotic fluid. However, in the 5 remaining samples with no detectable phosphatidylglycerol all 5 babies developed RDS.

3.1.11 The Effect of Gestational Age on the Percentage Palmitic
Acid in Fatty Acids of Acetone Precipitable Amniotic Fluid
Lipids

Figure 3.6 shows the percentage palmitic acid in the acetone precipitated lipids in amniotic fluids from 37 patients against gestational age. The lipids were isolated from fluid samples collected within 72 hours of delivery. There is a great deal of individual variation in the percentage palmitic acid found in the amniotic fluid taken from 28th week of gestation to term. The mean percentage palmitic acid content of the eighteen samples collected at less than 35 weeks was 53.0% but at 35 weeks or greater, the mean percentage palmitic acid content of the twenty samples analysed was only 54.0%, not a statistically significant difference.

3.1.12 The Percentage Palmitic Acid Content of Amniotic Fluid
as an Index of Fetal Lung Maturity

Thirty-seven babies were delivered within 72 hours of collecting the amniotic fluid and 4 of them developed RDS. A summary of results is/

Figure 3.6 The Effect of Gestational Age on the Percentage

　　　　　　Palmitic Acid Content of the Fatty Acid Residues

　　　　　　in Acetone Precipitable Amniotic Fluid Lipids

The amniotic fluid was collected as described in Methods, 2.2.1 and

centrifuged (Methods, 2.2.2). The lipids were extracted (Methods, 2.2.4)

and acetone precipitated (Methods, 2.2.5). The percentage of palmitic

acid measured as described in Methods, 2.7 was compared with

gestational age. Gestational age was assessed as described in

Methods, 2.10. There was no correlation found (P > 0.1).

GESTATIONAL AGE IN WEEKS
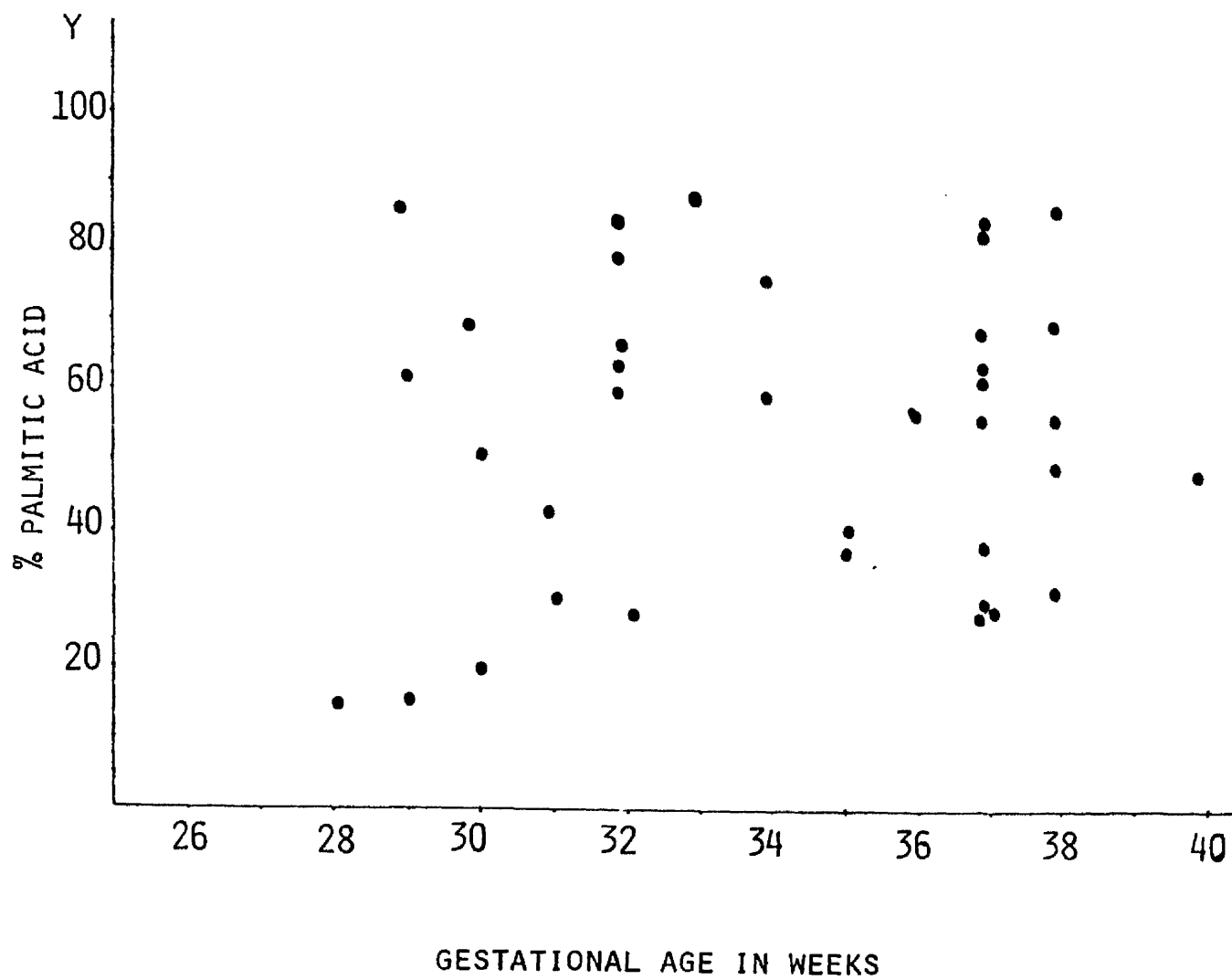
shown in table 3.11. When 25% or more of the fatty acid in the acetone precipitable lipids was palmitic acid only one of the 33 babies developed RDS. However, with a value of less than 25 % of palmitic acid present, 3 out of 4 babies developed RDS. The mother of the one baby which developed RDS but with an amniotic fluid palmitic acid level above 25% received betamethasone phosphate and the actual <u>concentration</u> of palmitic acid was very low.

3.1.13   The Effect of Gestational Age on the Concentration of
Palmitic Acid in Acetone Precipitable Amniotic Fluid Lipids

The results of 37 determinations of the absolute concentrations of palmitic acid are plotted against gestational age in figure 3.7. Eighteen samples, collected from patients with a gestational age of less than 35 weeks had a mean palmitic acid concentration of 1.9 μmol l^{-1} amniotic fluid. There was a noticeable increase from 35 weeks of gestation when the mean value rose to 6.9 mmol l^{-1}.

3.1.14   Palmitic Acid Concentration as an Index of Fetal Lung
Maturity

The palmitic acid concentration found in the acetone precipitable lipids was determined in 37 amniotic fluids obtained 72 hours before delivery. Four of the 37 babies developed RDS. The palmitic acid concentration was found to be less than 3 mmol l^{-1} in each sample. Of the remaining 33 amniotic fluid samples, with no associated cases of RDS, 12 had a palmitic acid concentration less than 3 mmol l^{-1} and the remaining 21 had values above this (table 3.12).

Table 3.11 The percentage palmitic acid residues in amniotic

fluid lipids as an index of fetal lung maturity

The amniotic fluid samples were collected (Methods, 2.2.1) and

centrifuged (Methods, 2.2.2). The lipids were extracted (Methods,

2.2.4) and acetone precipitated (Methods, 2.2.5). The lipids

were transesterified (Methods, 2.7.1) and the fatty acid com-

position analysed by gas liquid chromatography (Methods, 2.7.2)

and the percentage palmitic acid calculated. All samples were

collected within 72 hours of delivery. Assessment of RDS was

made by the paediatric staff (Methods, 2.11). The number in brac-

kets represents the number of patients.

Percentage of Palmitic Acid Residue	No. of Patients	% RDS Present	% RDS Absent
≥ 25.0	33	3.0 (1)	97.0 (32)
< 25.0	4	75.0 (3)	25.0 (1)
Total	37	10.8 (4)	89.2 (33)

Figure 3.7   The Effect of Gestational Age on the Concentration of

Palmitic Acid in Acetone Precipitable Amniotic Fluid

Lipids

The amniotic fluid was collected as described in Methods, 2.2.1 and

centrifuged (Methods, 2.2.2). The lipids were extracted (Methods, 2.2.4)

and acetone precipitated (Methods, 2.2.5). The concentration of

palmitic acid was determined as described in Methods, 2.7, and the

results compared with gestational age. Gestational age was assessed

as described in Methods, 2.10.

GESTATIONAL AGE IN WEEKS
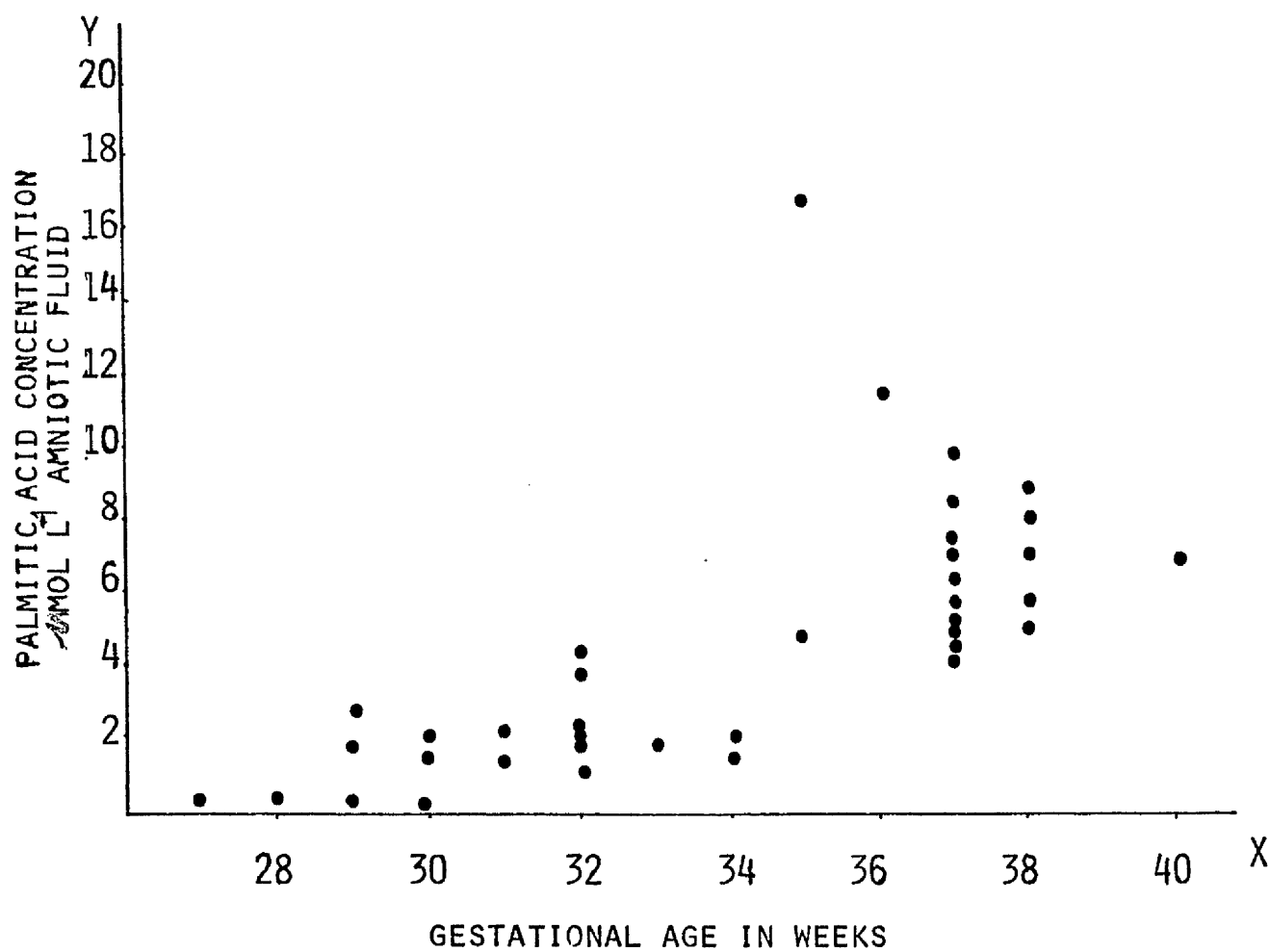
Table 3.12   The palmitic acid residue concentration as an index

of fetal lung maturity

The amniotic fluid samples were collected (Methods, 2.2.1) and

centrifuged (Methods, 2.2.2). The lipids were extracted (Methods,

2.2.4) and acetone precipitated (Methods, 2.2.5). The lipids were

transesterified (Methods, 2.7.1) and the fatty acid composition

analysed by gas liquid chromatography (Methods, 2.7.2). An

internal standard was used (Methods, 2.7) allowing estimation

of the palmitic acid concentration. All samples were collected

within 72 hours of delivery. Assessment of RDS was made by the

paediatric staff (Methods, 2.11). The number in brackets represents

the number of patients.

Palmitic Acid Concentration	No. of Patients	% RDS Present	% RDS Absent
$\geqslant 3.0 \, \mu\text{mol l}^{-1}$	21	0 (0)	100 (21)
$< 3.0 \, \mu\text{mol l}^{-1}$	16	25.0 (4)	75.0 (12)
Total	37	10.8 (4)	89.2 (33)

3.1.15   The Effect of Gestational Age on the Palmitic Acid/Stearic

Acid Ratio in the Acetone Precipitable Amniotic Fluid Lipids

The results of 37 determinations of palmitic acid/stearic acid ratio

in the acetone precipitable lipids of amniotic fluid are plotted

against gestational age in figure 3.8. Eighteen samples were collected

from patients with a gestational age of less than 35 weeks, and the

mean palmitic acid/stearic acid ratio was 7.3. The mean palmitic acid/

stearic acid ratio found in the 19 samples collected at 35 weeks of

gestation or greater was 12.4. There is a wide scatter of palmitic acid/

stearic acid ratios from 28 weeks to term as is shown in figure 3.8.

3.1.16   Palmitic Acid/Stearic Acid Ratio as an Index of Fetal Lung

Maturity

The palmitic acid/stearic acid ratio of the acetone precipitable

lipids was determined in 37 amniotic fluids taken less than 72 hours

prior to delivery (table 3.13). Four of the 37 babies developed RDS

and the palmitic acid/stearic acid ratio was less than 2.0 in three of

these four babies. In the remaining case the palmitic acid/stearic

acid ratio was 29 (the percentage palmitic acid was 86.0 and the

palmitic acid concentration was $< 3\mu$mol l^{-1}). Betamethasone phosphate

had been administered to this mother in an attempt to accelerate fetal

lung maturity. Of the 33 babies with no RDS the palmitic acid/stearic

acid ratio was less than 2.0 in only five samples. The remaining 29

samples all had palmitic acid/stearic acid ratios of two or above (87.9%).

Figure 3.8   The Effect of Gestational Age on the Palmitic Acid/

                Stearic Acid Ratio in the Acetone Precipitable Amniotic

                Fluid Lipids

The amniotic fluid was collected as described in Methods, 2.2.1 and

centrifuged (Methods, 2.2.2).   The lipids were extracted (Methods, 2.2.4)

and acetone precipitated (Methods, 2.2.5).   The palmitic acid/stearic

acid ratio was determined by gas liquid chromatography as described

in Methods, 2.7, and the results compared with gestational age.

Gestational age was assessed as described in Methods, 2.10.   There was

no correlation found $(P > 0.1)$.
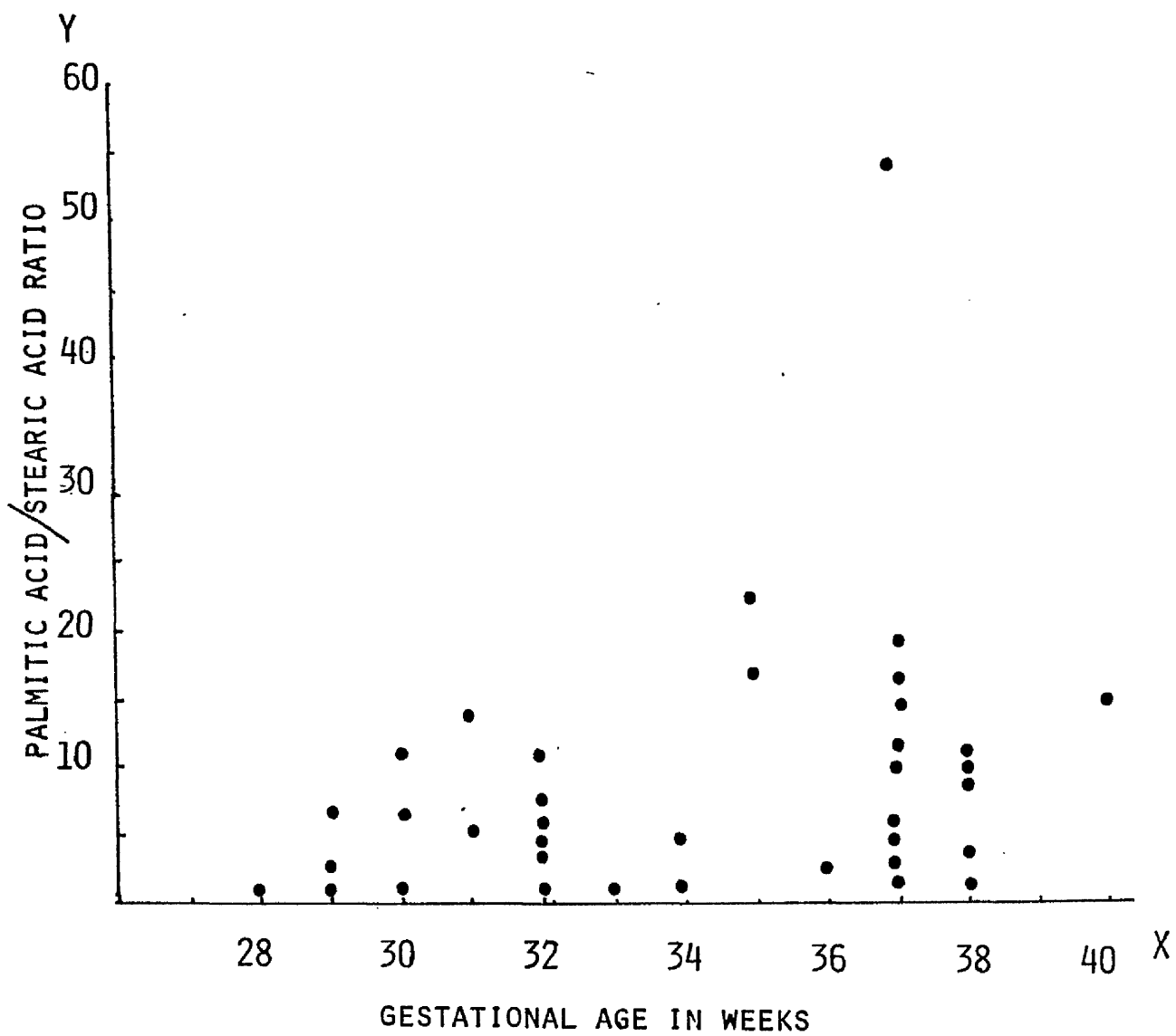
<u>Table 3.13</u> <u>The palmitic acid / stearic acid ratio as an index of</u>

<u>fetal lung maturity</u>

The amniotic fluid samples were collected (Methods,2.2.1) and

centrifuged (Methods, 2.2.2). The lipids were extracted (Methods,

2.2.4) and acetone precipitated (Methods, 2.2.5). The lipids were

transesterified (Methods, 2.7.1) and the fatty acid composition

analysed by gas liquid chromatography (Methods, 2.7.2). The palmitic

acid / stearic acid ratio was then calculated. All samples were

collected within 72 hours of delivery. Assessment of RDS was made

by the paediatric staff (Methods, 2,11). The number in brackets

represents the number of patients.

Palmitic Acid/ Stearic Acid Ratio	No. of Patients	% RDS Present	% RDS Absent
$\geqslant 2.0$	29	3.5 (1)	96.5 (28)
< 2.0	8	37.5 (3)	62.5 (5)
Total	37	10.8 (4)	89.2 (33)

3.2   Rapid Surfactant Tests

3.2.1   The Effect of Gestational Age on the Amniotic Fluid Optical Density

The optical density of the amniotic fluid was measured (Methods, 2.8) in 120 samples. The optical density increases exponentially rather than linearly with gestational age (figure 3.9).

$(r = 0.66, \quad P < 0.001)$. The equation that fits the results is

$$y = 1.186 \times 10^{-3} e^{\, 0.15x} \quad \ldots\ldots\ldots\ldots\ldots\ldots \quad (iv)$$

where x is the gestational age in weeks and

y is the optical density.

There is an obvious abrupt increase at 35 weeks of gestation or greater. Fifty-eight specimens were collected from patients with a gestational age of less than 35 weeks and 38 of these samples (65.5%) had an optical density less than 0.15 (at 650 nm). When the gestational age was 35 weeks or greater only 4 of the 62 samples (6.5%) analysed had optical densities below 0.15 (table 3.14).

3.2.2   Optical Density Measurements as an Index of Fetal Lung Maturity

The optical density of amniotic fluid was measured from 85 patients within 72 hours of delivery. An optical density of 0.15 was used as the critical value, since this was the critical value adopted by other laboratories (Sbarra et al, 1977; Copeland et al, 1978; Spellacy et al, 1979; Hill et al, 1979). A summary of results is shown in/

Figure 3.9 The Effect of Gestational Age on the Amniotic Fluid
          Optical Density

The amniotic fluid was collected by amniocentesis, and centrifuged
(Methods, 2.2.2). The optical density of the amniotic fluid was
measured as described in Methods, 2.8. Assessment of gestational age
was made as described in Methods, 2. . Equation (iv) fits the data
$(r = 0.66, \ P < 0.001, \ n = 120)$.

$$y = 1.186 \times 10^{-3} e^{0.15x} \ \ldots\ldots\ldots\ldots \quad \text{(iv)}$$

where x is the gestational age in weeks

and y is the optical density of the
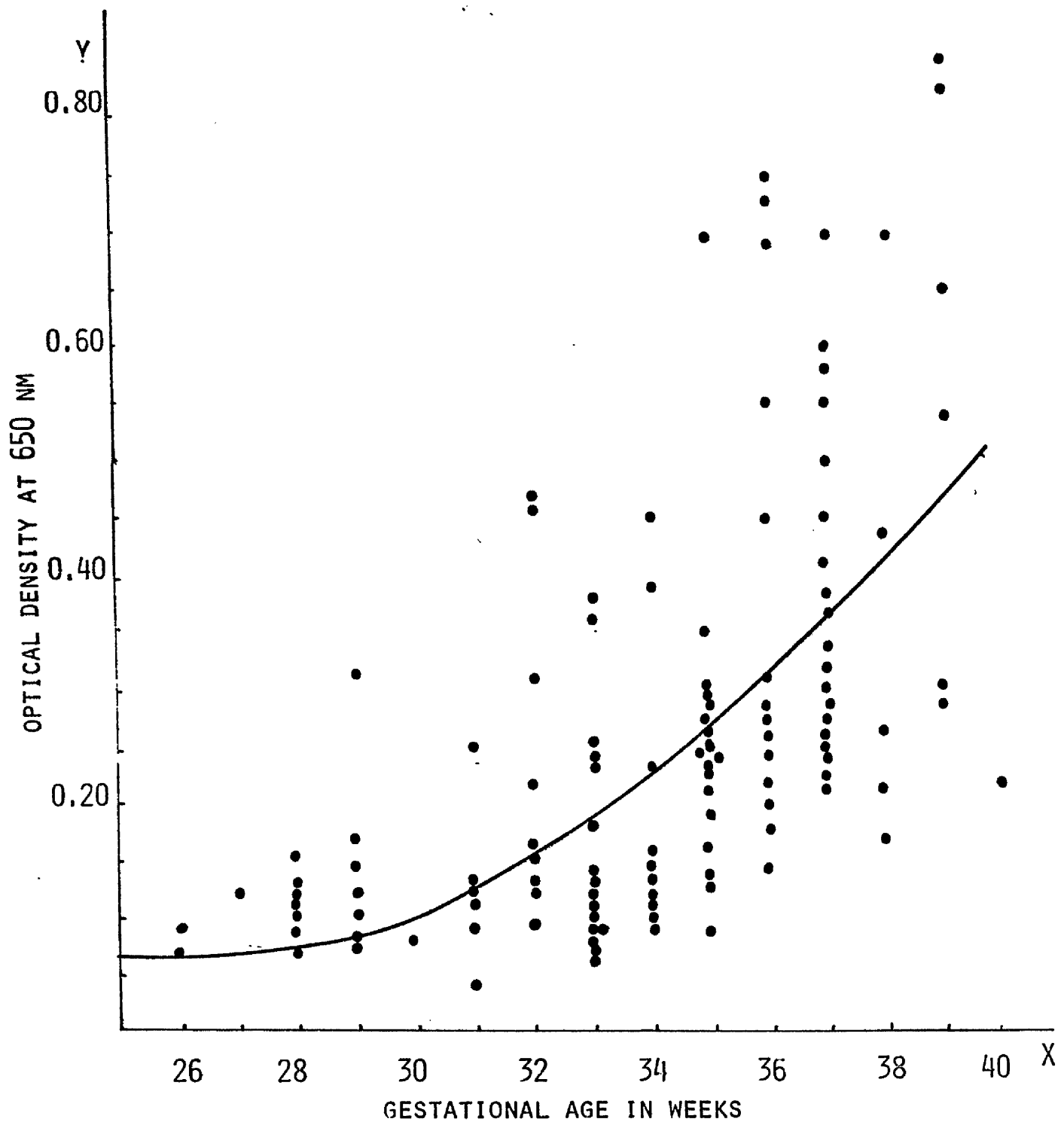
amniotic fluid.

<u>Table 3.14 The effect of gestational age on the amniotic</u>
<u>fluid optical density</u>

The optical density of amniotic fluid samples was
determined as described in Methods 2.8. Only samples
collected by amniocentesis were analysed. The gestational
age was assessed as described in Methods 2.10. The number
in brackets represents the number of patients.

Gestational Age	No. of Samples	% with Optical Density $\geqslant 0.15$	% with Optical Density < 0.15
$\geqslant$ 35 weeks	62	93.5 (58)	6.5 (4)
$<$ 35 weeks	58	34.5 (20)	65.5 (38)
Total	120	65.0 (78)	35.0 (42)

table 3.15. In 70 patients the optical density of the amniotic fluid was 0.15 or greater and none of these babies developed RDS. In the remaining 15 patients an immature optical density measurement (less than 0.15) was found in the amniotic fluid. Four of these babies developed RDS (27.0%). Three of these babies were delivered at 28 weeks of gestation because of acute obstetric complications, and the fourth was delivered at 35 weeks of gestation.

3.2.3 The Effect of Gestational Age on the Amniotic Fluid Bubble Stability Test

The Bubble Stability Test was performed on 190 amniotic fluid samples. Eighty-five samples were collected from patients with a gestational age of less than 35 weeks, and 65 of these had negative results (76.5%). When the gestational age was 35 weeks or greater, only 8 of the 105 samples analysed had negative results (7.6%), (table 3.16).

3.2.4 The Bubble Stability Test as an Index of Fetal Lung Maturity

The Bubble Stability Test was performed on the amniotic fluid taken from 155 patients within 72 hours prior to delivery. A summary of results is shown in table 3.17. One hundred and seventeen amniotic fluid samples gave a positive result suggesting mature fetal lungs and only one baby, delivered at 32 weeks of gestation because of an antepartum haemorrhage, developed RDS, an incidence of 0.7%. In the remaining 38 patients the amniotic fluid bubble stability test was negative and 19 of these babies developed RDS (50.0%).

78

Table 3.15   Optical Density Measurements as an Index of Fetal Lung
              Maturity

The optical density of amniotic fluid was measured as described in

Methods 2.8. A critical value of 0.15 was used (Methods, 2.8). All

samples were collected within 72 hours of delivery. Only samples

collected by amniocentesis were analysed. Assessment of RDS was made

by the paediatric staff as described in Methods 2.11. The number in

brackets represents the number of patients.

Optical Density	No. of Patients	% RDS Present	% RDS Absent
$\geqslant$ 0.15	70	0 (0)	100 (70)
< 0.15	15	26.7 (4)	75.3 (11)
Total	85	4.7 (4)	95.3 (81)

<u>Table 3.16   The effect of gestational age on the amniotic</u>
<u>fluid bubble stability test</u>

The bubble stability test was performed as described
in Methods 2.9.   Samples collected by amniocentesis,
vaginal drainage or at caesarian section were analysed.
Assessment of gestational age was as described in
Methods 2.10.   The number in brackets represents the
number of patients.

Gestational Age	No. of Samples	% With Bubble Stability Test +ve	% With Bubble Stability Test -ve
⩾ 35 weeks	105	92.4 (97)	7.6 (8)
< 35 weeks	85	23.5 (20)	76.5 (65)
Total	190	61.6 (117)	38.4 (73)

<u>Table 3.17   The Bubble Stability Test as an Index of Fetal Lung</u>

<u>Maturity</u>

The Bubble Stability Test was performed as described in Methods 2.10.

All samples were collected within 72 hours of delivery. Amniotic

fluid samples were collected by amniocentesis, vaginal drainage or

at time of caesarean section. Assessment of RDS was made by the

paediatric staff as described in Methods 2.11. The number in brackets

represents the number of patients.

Bubble Stability Test	No. of Patients	% RDS Present	% RDS Absent
+ve	117	0.8 (1)	99.2 (116)
-ve	38	50.0 (19)	50.0 (19)
Total	155	12.90 (20)	87.1 (135)

3.3 Amniotic Fluid Phospholipid Concentrations Obtained by High Performance Liquid Chromatography Compared with the Results Derived from Phosphate Analysis Following 2D t.l.c.

The concentrations of acetone precipitable lecithin, phosphatidylglycerol, phosphatidylinositol, phosphatidylethanolamine and sphingomyelin in amniotic fluid were determined by both h.p.l.c. (Methods, 2.6) and by inorganic phosphate estimation (Methods, 2.5) following 2D t.l.c. (Methods, 2.4). The results show that the values obtained from the two methods are in good agreement.

3.3.1 Lecithin Concentration

A comparison of the results obtained from 40 amniotic fluid samples is shown in figure 3.10 The equation (v) fits the data ($r=0.99$, $P<0.001$).

$$y = 0.98x + 3.0 \qquad \dots\dots\dots\dots\dots\dots\dots\dots\dots \quad (v)$$

where x is the lecithin concentration

determined by phosphate analysis following 2D t.l.c. and

y is the lecithin concentration determined by h.p.l.c.

3.3.2 Phosphatidylglycerol Concentration

A comparison of the results obtained from 11 amniotic fluid samples is shown in figure 3.11. Phosphatidylglycerol was not detected in 4 samples by both techniques. Half the limit of sensitivity was used for the samples where phosphatidylglycerol was not detected. Equation (vi) fits the data ($r = 0.93$, $P<0.001$).

Figure 3.10 The Concentration of Lecithin Obtained by h.p.l.c.

Compared with the Results Derived from Phosphate Analysis

Following 2D t.l.c.

The amniotic fluid was collected as described in Methods, 2.2.1 and

centrifuged (Methods, 2.2.2). The lipids were extracted (Methods, 2.2.4)

and acetone precipitated (Methods, 2.2.5). A comparison of the

concentration of lecithin determined by h.p.l.c. as described in

Methods, 2.6 with the results derived from the phosphate estimation

(Methods, 2.5) following 2D t.l.c. (Methods, 2.4) is shown. Equation

(v) fits the data ($r = 0.99$, $P < 0.001$, $n = 40$).

$$y = 0.98x + 3.0 \quad \dots\dots\dots\dots\dots \quad (v)$$

where x is the lecithin concentration obtained

by phosphate analysis following 2D t.l.c.

and y is the concentration of lecithin determined

by h.p.l.c.

Lecithin concentration determined by phosphate analysis
μmol/l amniotic fluid (x)

Lecithin concentration determined by HPLC
μmol/l amniotic fluid (y)
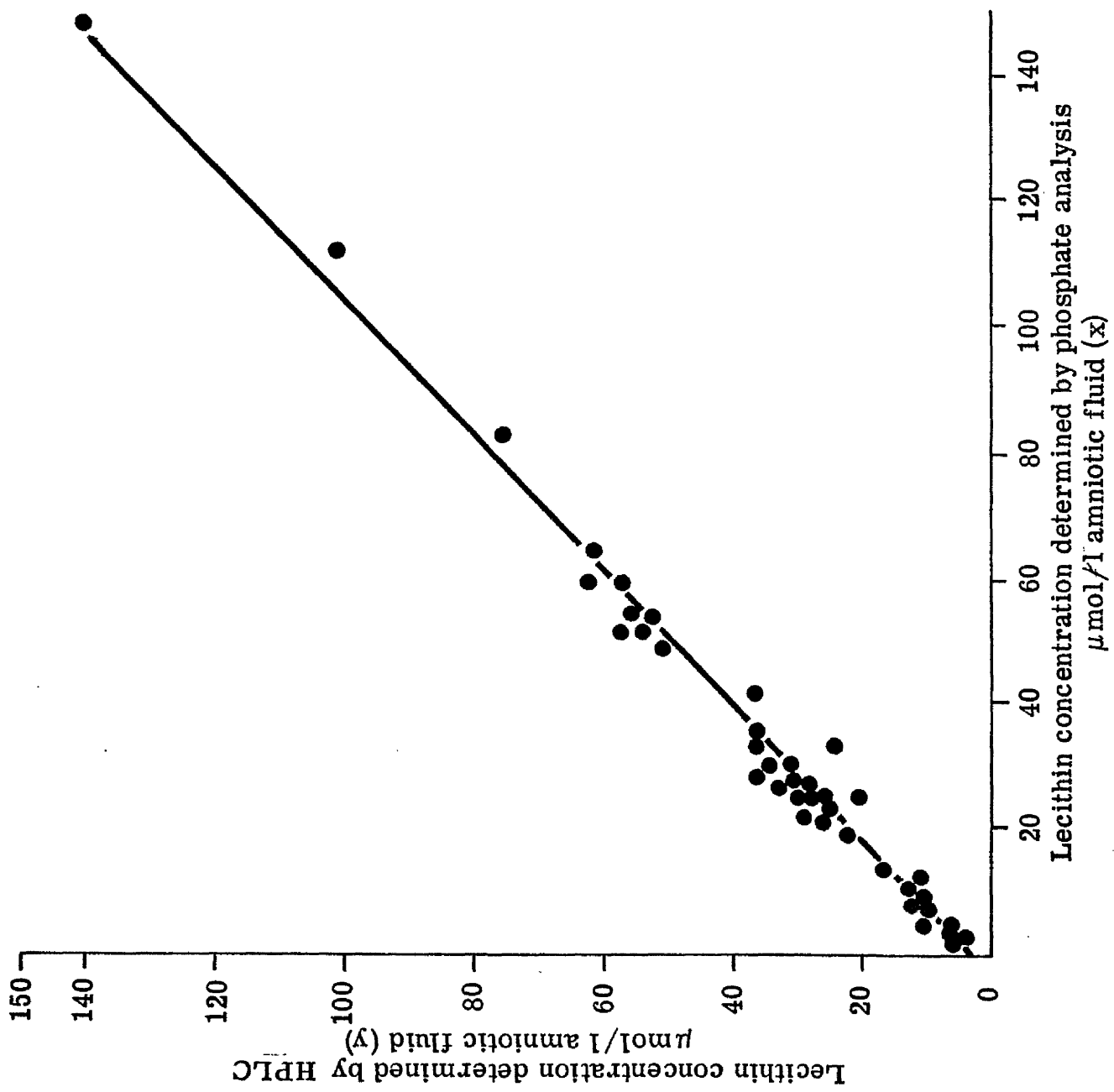
Figure 3.11   The Concentration of Phosphatidylglycerol Obtained

by h.p.l.c. Compared with the Results Derived from

Phosphate Analysis Following 2D t.l.c.

The amniotic fluid was collected as described in Methods, 2.2.1 and

centrifuged (Methods, 2.2.2). The lipids were extracted (Methods, 2.2.4)

and acetone precipitated (Methods, 2.2.5). A comparison of the

concentration of phosphatidylglycerol determined by h.p.l.c. as

described in Methods, 2.6 with the results derived from the phosphate

estimation (Methods, 2.5) following 2D t.l.c. (Methods, 2.4) is shown.

The equation (vi) fits the data ($r = 0.93$, $P < 0.001$, $n = 11$).

$$y = 0.44x + 0.75 \qquad \ldots\ldots\ldots\ldots \qquad (vi)$$

where x is the phosphatidylglycerol concentration

obtained by phosphate analysis following 2D t.l.c.

and y is the concentration of phosphatidylglycerol

determined by h.p.l.c.

Phosphatidyl glycerol determined by phosphate analysis μmol/l amniotic fluid (x)

Phosphatidyl glycerol concentration determined by HPLC μmol/l amniotic fluid (y)

84
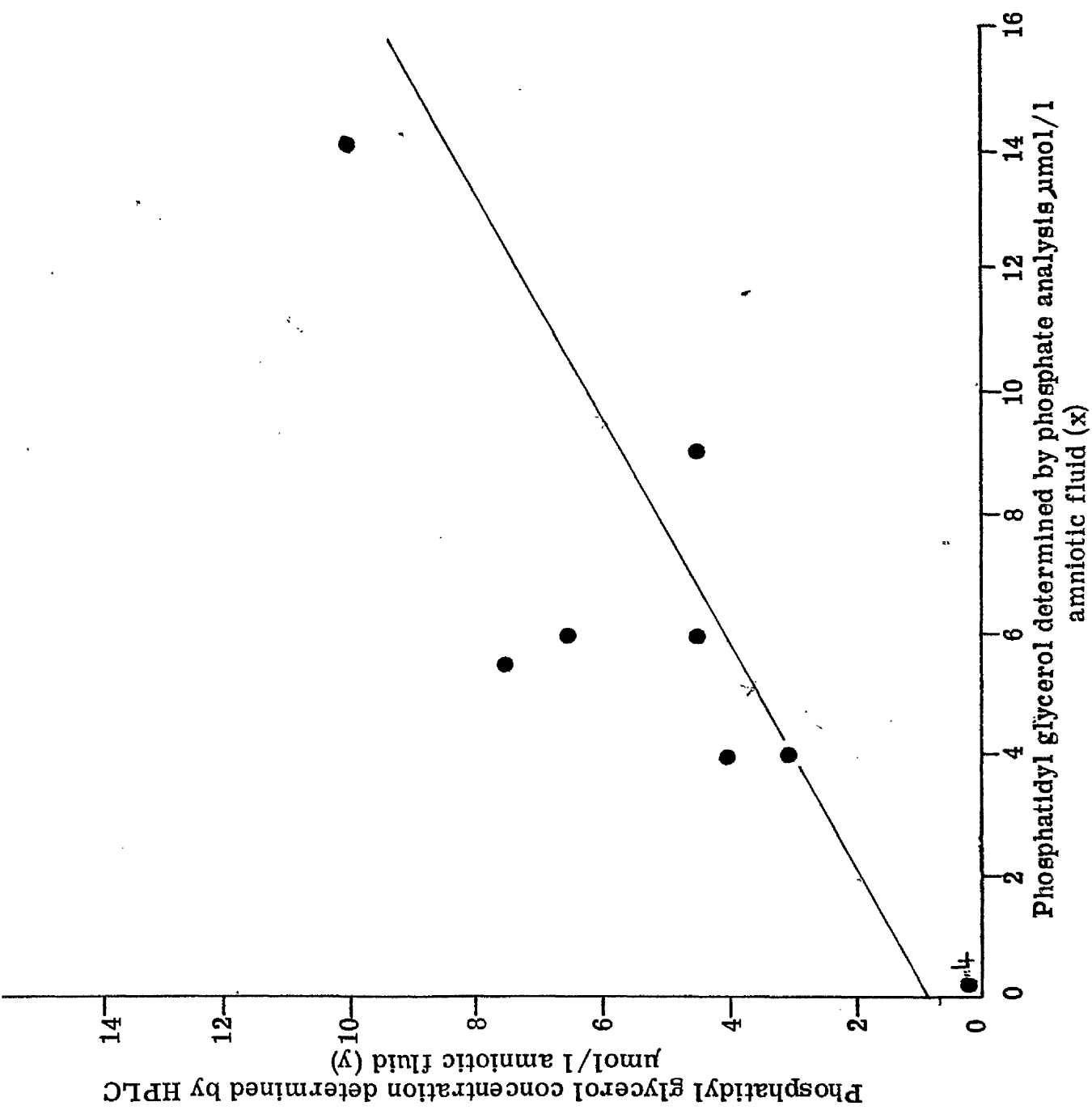
$$y = 0.44x + 0.75 \quad \ldots\ldots\ldots\ldots\ldots\ldots \quad (vi)$$

where x is the concentration of phosphatidylglycerol

determined by phosphate analysis following 2D t.l.c. and

y is the phosphatidylglycerol concentration determined by h.p.l.c.

3.3.3   Phosphatidylinositol Concentration

The concentration of phosphatidylinositol was determined by both

techniques in 11 amniotic fluid samples (figure 3.12). The equation

(vii) fits the data (r = 0.94, P < 0.001).

$$y = 1.18x - 3.08 \quad \ldots\ldots\ldots\ldots\ldots\ldots \quad (vii)$$

where x is the concentration of phosphatidylinositol

determined by 2D t.l.c. and

y is the concentration of phosphatidylinositol

determined by h.p.l.c.

3.3.4   Phosphatidylethanolamine Concentration

The concentration of phosphatidylethanolamine was determined by both

techniques in 24 amniotic fluid samples (figure 3.13). The equation

(viii) fits the data (r = 0.97, P < 0.001).

$$y = 0.97x - 0.49 \quad \ldots\ldots\ldots\ldots\ldots\ldots \quad (viii)$$

where x is the concentration of phosphatidylethanolamine

determined by phosphate analysis following 2D t.l.c. and

y is the concentration of phosphatidylethanolamine

determined by h.p.l.c.

Figure 3.12   The Concentration of Phosphatidylinositol Obtained by

　　　　　　h.p.l.c. Compared with the Results Derived from Phosphate

　　　　　　Analysis Following 2D t.l.c.

The amniotic fluid was collected as described in Methods, 2.2.1 and

centrifuged (Methods, 2.2.2). The lipids were extracted (Methods, 2.2.4)

and acetone precipitated (Methods, 2.2.5). A comparison of the

concentration of phosphatidylinositol determined by h.p.l.c. as

described in Methods, 2.6 with the results derived from the phosphate

estimation (Methods, 2.5) following 2D t.l.c. (Methods, 2.4) is shown.

Equation (vii) fits the data ($r = 0.94$, $P < 0.001$, $n = 11$).

$$y = 1.18x - 3.08 \quad \cdots\cdots\cdots\cdots \quad \text{(vii)}$$

where x is the phosphatidylinositol concentration

obtained by phosphate analysis following 2D t.l.c.

and y is the concentration of phosphatidylinositol
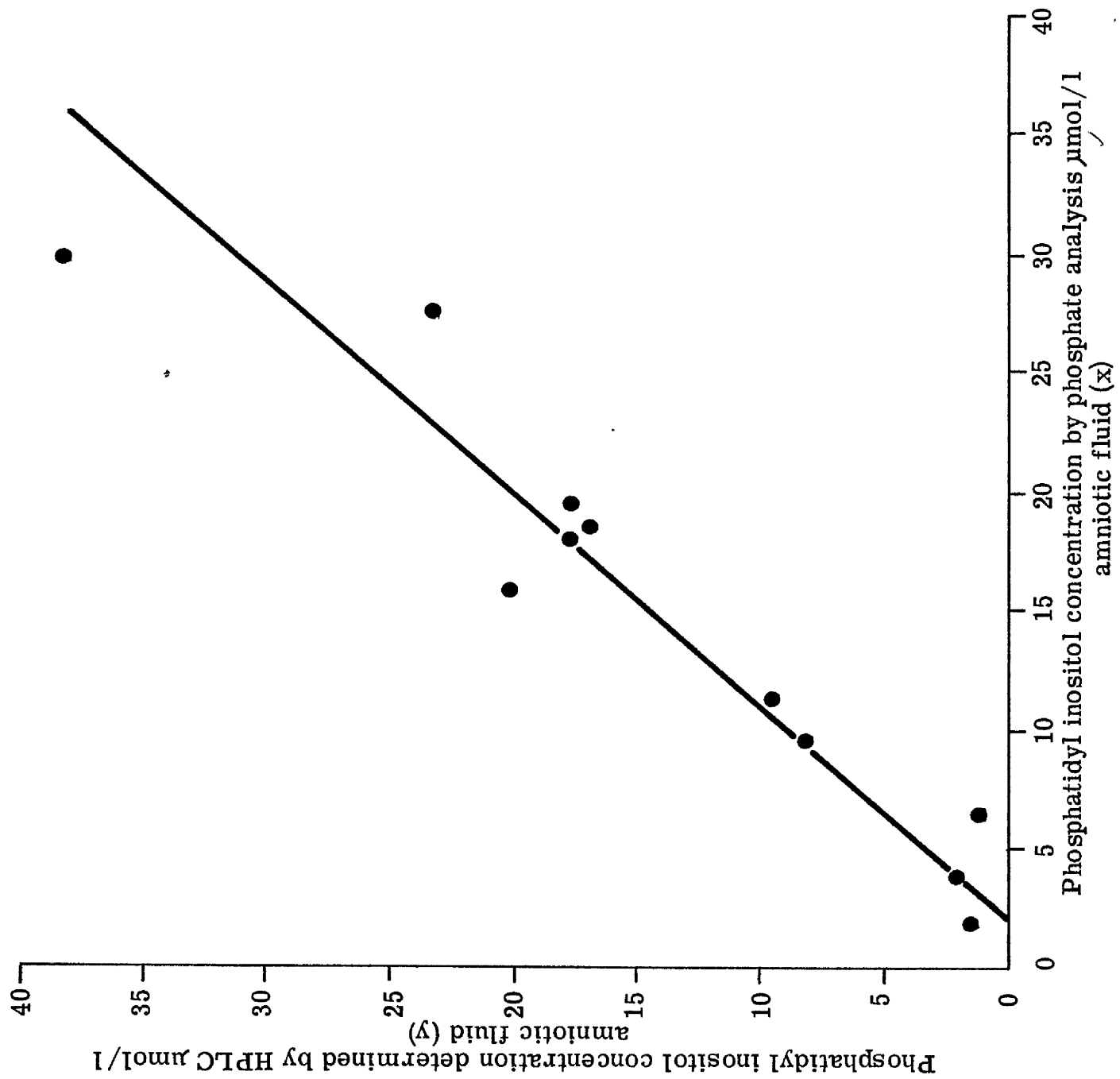
determined by h.p.l.c.

Figure 3.13   The Concentration of Phosphatidylethanolamine Obtained

by h.p.l.c. Compared with the Results Derived from

Phosphate Analysis Following 2D t.l.c.

The amniotic fluid was collected as described in Methods, 2.2.1 and

centrifuged (Methods, 2.2.2). The lipids were extracted (Methods, 2.2.4)

and acetone precipitated (Methods, 2.2.5). A comparison of the

concentration of phosphatidylethanolamine determined by h.p.l.c. as

described in Methods, 2.6 with the results obtained from the phosphate

estimation (Methods, 2.5) following 2D t.l.c. (Methods, 2.4) is shown.

Equation (viii) fits the data ($r = 0.97$, $P < 0.001$, $n = 24$).

$$y = 0.97x - 0.49 \quad \ldots\ldots\ldots\ldots \quad \text{(viii)}$$

where x is the concentration of phosphatidylethanolamine

obtained by the phosphate analysis following 2D t.l.c.

and y is the concentration of phosphatidylethanolamine

determined by h.p.l.c.

Phosphatidyl ethanolamine concentration determined by phosphate analysis μmol/l amniotic fluid (x)

Phosphatidyl ethanolamine concentration determined by HPLC μmol/l amniotic fluid (y)
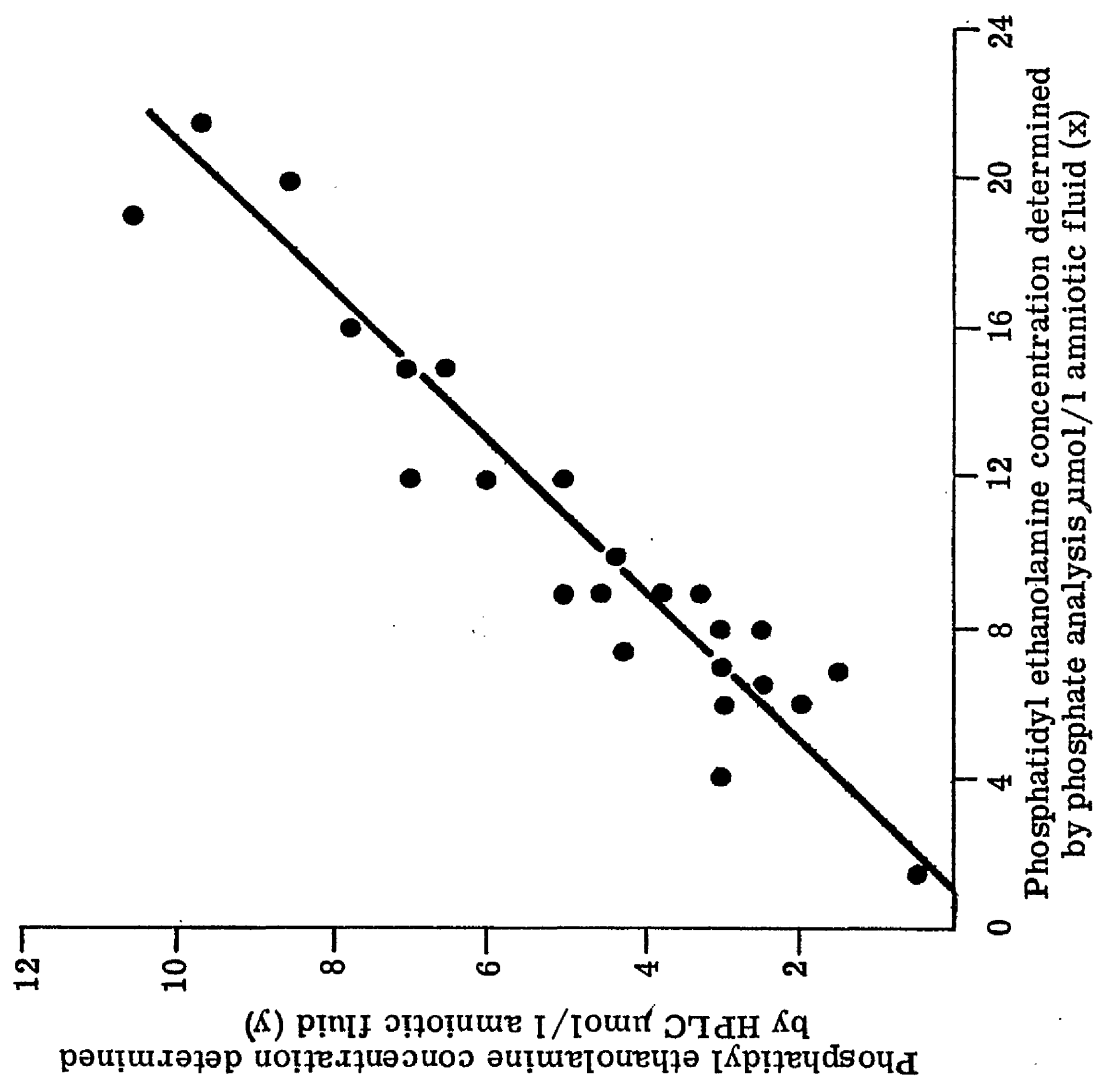
3.3.5   Sphingomyelin Concentration

The concentration of sphingomyelin was determined by both techniques
in 11 samples (figure 3.14). The equation (ix) fits the results
($r = 0.89$, $P < 0.001$).

$$y = 0.68x + 1.51 \qquad \ldots\ldots\ldots\ldots\ldots\ldots \qquad (ix)$$

where x is the concentration of sphingomyelin

determined by phosphate analysis following 2D t.l.c.

and y is the concentration of sphingomyelin

determined by h.p.l.c.

3.4   Comparison of Analytical Methods

3.4.1   A Comparison of the Lecithin Concentration and the L/S Ratio
in Amniotic Fluid

The lecithin concentration of amniotic fluid measured either by
h.p.l.c. (Methods, 2.6) or phosphate analysis (Methods, 2.5) following
2D t.l.c. (Methods, 2.4) was compared with the L/S ratio. A comparison
of the L/S ratios against the lecithin concentration in 88 amniotic
fluids is illustrated in figure 3.15. The equation (x) fits the
results ($r = 0.58$, $P < 0.001$).

$$y = 0.02x + 1.74 \qquad \ldots\ldots\ldots\ldots\ldots\ldots \qquad (x)$$

where x is the lecithin concentration

and y is the L/S ratio.

<u>Figure 3.14</u> <u>A Comparison of the Concentration of Sphingomyelin</u>

<u>by h.p.l.c. and by the Phosphate Assay after 2D t.l.c.</u>

The amniotic fluid was collected as described in Methods, 2.2.1 and

centrifuged (Methods, 2.2.2). The lipids were extracted (Methods, 2.2.4)

and acetone precipitated (Methods, 2.2.5). A comparison of the

sphingomyelin concentration determined by h.p.l.c. (Methods, 2.6) and

by the phosphate assay after 2D t.l.c. (Methods, 2.5) is illustrated.

Equation (ix) fits the data ($r = 0.89$, $P < 0.001$, $n = 11$).

$$y = 0.68x + 1.51 \quad \text{............} \quad (ix)$$

where x is the concentration of sphingomyelin

determined by the phosphorus technique after 2D t.l.c.

and y is the concentration of sphingomyelin

determined by h.p.l.c.

Sphingomyelin determined by phosphate analysis μmol/1 amniotic fluid (x)

Sphingomyelin determined by HPLC, μmol/1 amniotic fluid (y)

89
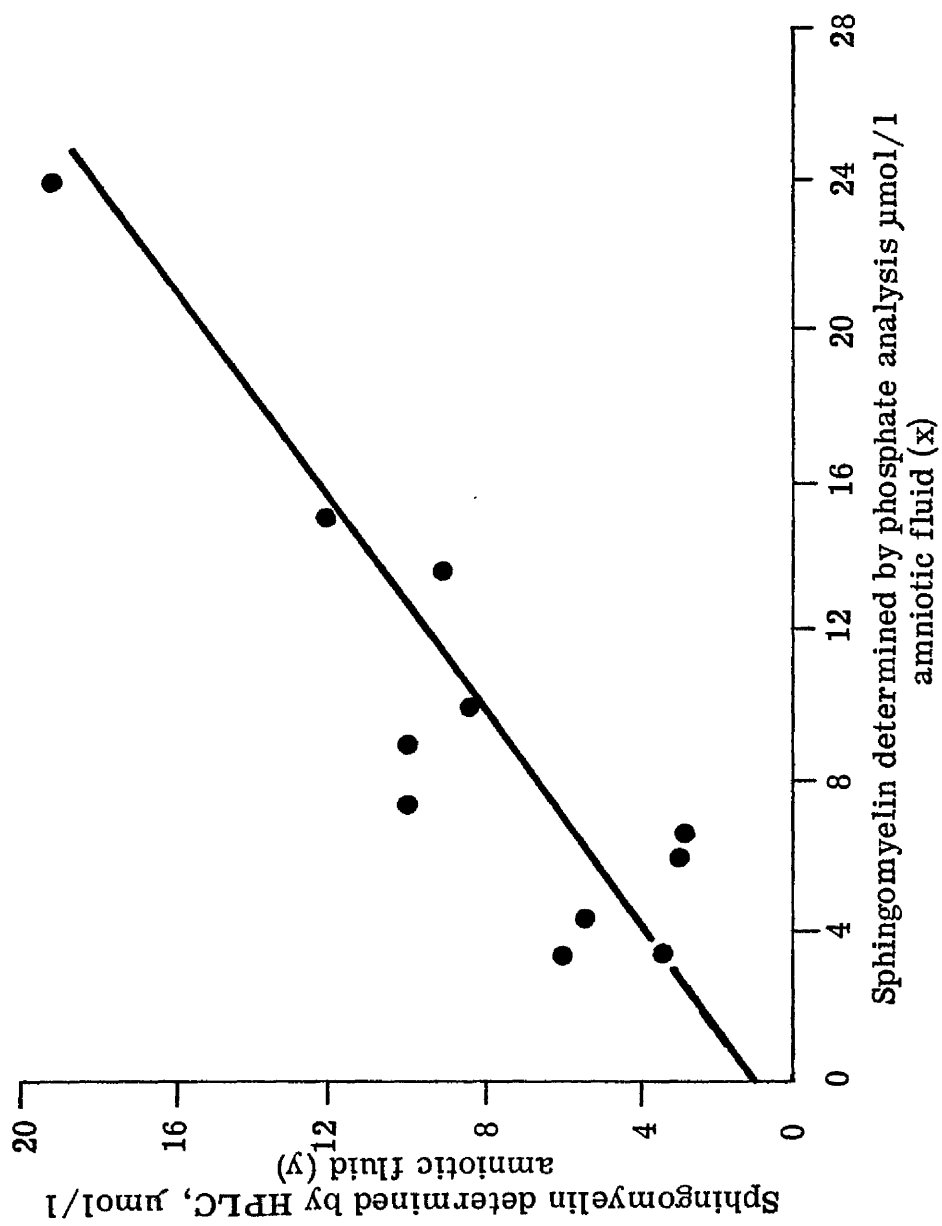
Figure 3.15 A Comparison of the L/S Ratio and the Lecithin

Concentration in Amniotic Fluid

The amniotic fluid was collected as described in Methods, 2.2.1 and

centrifuged (Methods, 2.2.2). The lipids were extracted (Methods, 2.2.4)

and acetone precipitated (Methods, 2.2.5). The L/S ratio was

determined by 2D t.l.c. as described in Methods, 2.4 and the results

compared with the lecithin concentration obtained by either h.p.l.c.

(Methods, 2.6) or phosphate assay (Methods, 2.5) following 2D t.l.c.

Equation (x) fits the data ($r = 0.58$, $P < 0.001$, $n = 88$).

$$y = 0.02x + 1.74 \quad \ldots\ldots\ldots\ldots\ldots \quad (x)$$

where x is the concentration of lecithin

and y is the L/S ratio.

LECITHIN CONCENTRATION μMOL L⁻¹ AMNIOTIC FLUID
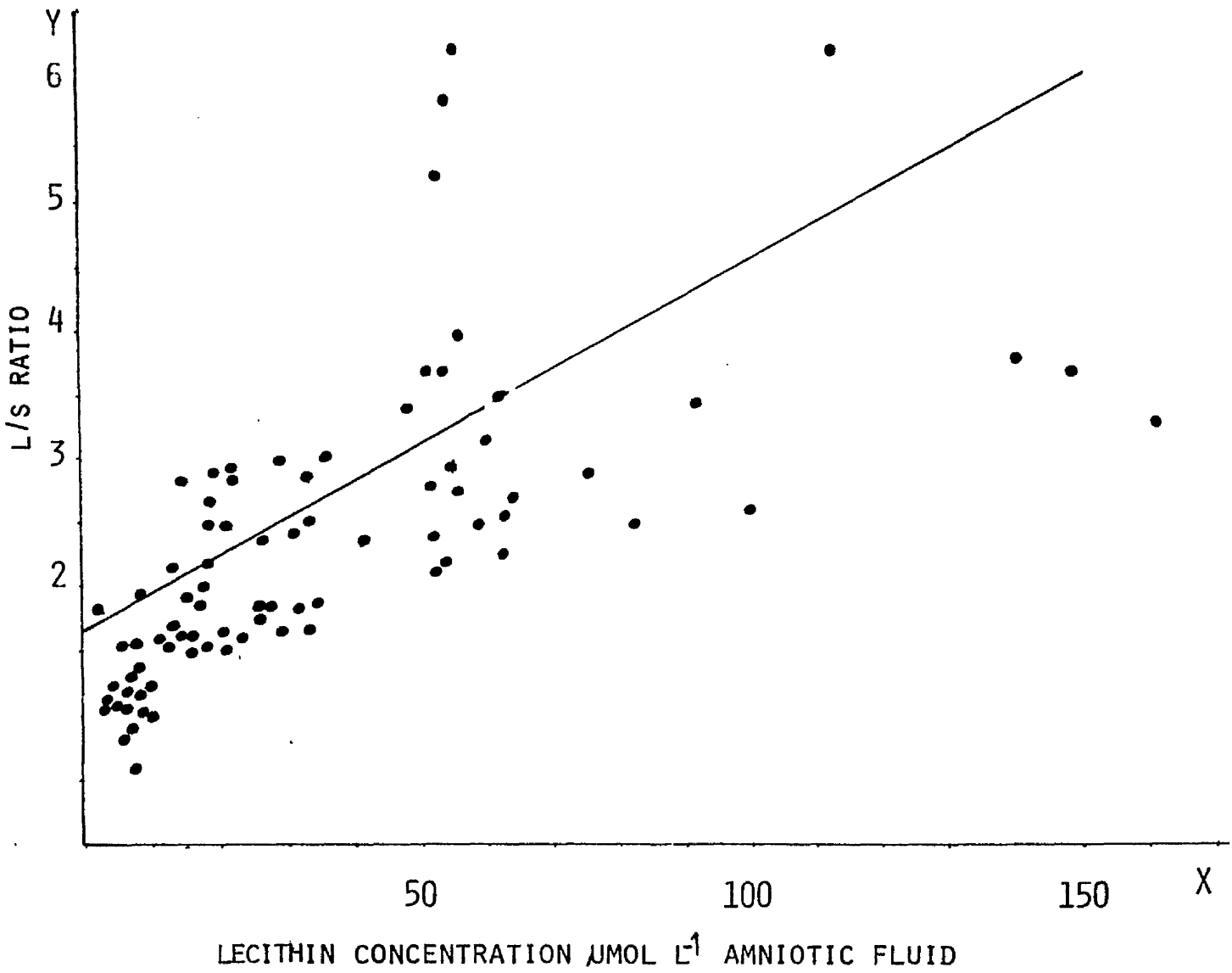
Using critical values of 25 μ mol l^{-1} for lecithin concentration

(results 3.1.8) and an L/S ratio of 2.0 (results 3.1.1)

72 of the 88 results (86.8%) were in agreement leading to the same

interpretation of fetal lung maturity (table 3.18). Thirty-four

amniotic fluids had both lecithin concentration and the L/S ratio

above their critical values and 38 had both measurements below their

critical values. Of the remaining 16 amniotic fluids the two

techniques gave differing results, 9 samples having L/S ratios of 2.0

or above and a lecithin concentration below its critical value. In

the remaining 7 amniotic fluid samples the lecithin concentration was

above the critical value and the L/S ratio was less than 2.0.

The occurrence of RDS in 28 babies when delivery was within 72 hours

of sample collection is shown in table 3.19. When both lecithin

concentration and L/S ratio measurements were below their critical

values 60% of the babies developed RDS. However, no baby developed

RDS if either parameters were above their critical values.

3.4.2   A Comparison of the Percentage Palmitic Acid of the Fatty

         Acids in the Acetone Precipitable Lipids of Amniotic Fluid

         and the Concentration of Acetone Precipitable Lecithin in

         Amniotic Fluid

No relationship was found between the percentage palmitic acid and the

lecithin concentration in the acetone precipitable lipids from 26

amniotic fluids (r = 0.14,   P > 0.1).

Using critical values of 25.0% for the percentage palmitic acid/

Table 3.18 A comparison of the lecithin concentration and

the L/S ratio in amniotic fluid

The amniotic fluids were collected as described in Methods, 2.2.1
and centrifuged (Methods, 2.2.2). The lipids were extracted as des-
cribed in Methods, 2.2.4 and acetone precipitated (Methods, 2.2.5).
The lecithin concentration was determined by h.p.l.c. (Methods, 2.6)
or by phosphate analysis technique (Methods, 2.5) following 2D t.l.c.
A critical value of 25 μmol l^{-1} was used (Results, 3.18). The L/S
ratio was determined by 2D t.l.c. An L/S ratio of 2.0 was used as
the critical value. The interpretation of the results was then
compared.

	Lecithin $\geqslant$ 25 μmol 1^{-1}	Lecithin $<$ 25 μmol 1^{-1}
L/S $\geqslant$ 2.0	34	9
L/S $<$ 2.0	7	38

Table 3.19 Lecithin concentration and L/S ratio as an index of fetal

lung maturity

The amniotic fluid was collected (Methods, 2.2.1) and centrifuged

(Methods, 2.2.2). The lipids were extracted (Methods, 2.2.4) and

acetone precipitated (Methods, 2.2.5). The lecithin concentration

was determined by phosphate analysis technique (Methods, 2.5) following

2D t.l.c. (Methods, 2.4) or by h.p.l.c. (Methods, 2.6). The L/S ratio

was determined by 2D t.l.c. (Methods, 2.4). All samples were collected

within 72 hours of delivery. Assessment of RDS was made by the paediatric

staff (Methods, 2.11). The number in brackets represents the number of

patients.

	No. of Patients	% with RDS
Lecithin $\geq$ 25 μmol 1^{-1} and L/S $\geq$ 2.0	15	0 (0)
Lecithin $\geq$ 25 μmol 1^{-1} and L/S $<$ 2.0	4	0 (0)
Lecithin $<$ 25 μmol 1^{-1} and L/S $\geq$ 2.0	4	0 (0)
Lecithin $<$ 25 μmol 1^{-1} and L/S $<$ 2.0	5	60 (3)
Total	28	10.7 (3)

(results 3.1.12) and 25 μ mol 1^{-1} for the concentration of lecithin

(results 3.1.8)   15 of the 26 results (57.7%) were in

agreement leading to the same interpretation of fetal lung maturity

(table 3.20). Twelve samples indicated mature lungs and none of the

babies developed RDS. In the remaining 3 both tests indicated

immature fetal lungs and all 3 babies developed RDS. Of the 11 samples

in which the two techniques gave differing results all had the

proportion of palmitic acid above 25.0% while the concentration of

lecithin in these samples was below its critical value. One baby in

this group was delivered at 28 weeks of gestation because of an acute

obstetric complication, and this baby developed severe RDS.

3.4.3   A Comparison of Palmitic Acid as a Percentage of the Fatty
Acids and the L/S Ratio of Amniotic Fluid

The percentage palmitic acid was compared with the L/S ratio in the

acetone precipitated fraction from 37 amniotic fluids and no

correlation was found ($r = 0.12$, $P > 0.1$).

Using critical values of 2.0 and 25% for the L/S ratio (results 3.1.1)

and the percentage palmitic acid (results 3.1.12) respectively, 24 of

the 37 results (64.9%) were in agreement leading to the same

interpretation of fetal lung maturity (table 3.21). Twenty-one

amniotic fluids had results above their critical values and there were

no cases of RDS. In the remaining 3 amniotic fluid samples the

results were below their critical values and all three babies developed

RDS.

Table 3.20 A comparison of the percentage palmitic acid residues
in amniotic fluid lipids and the concentration of
lecithin in amniotic fluid

The amniotic fluids were collected (Methods, 2.2.1) and centrifuged
(Methods, 2.2.2). The lipids were extracted (Methods, 2.2.4) and
acetone precipitated (Methods, 2.2.5). The percentage palmitic
acid was determined by transesterifying the lipids (Methods, 2.7.1)
and analysing the fatty acid composition after gas liquid chromato-
graphy (Methods, 2.7.2). The lecithin concentration was determined
by phosphate analysis technique (Methods, 2.5) following 2D t.l.c.
(Methods, 2.4). The results of each test were compared. All
samples were collected within 72 hours of delivery. Assessment of
RDS was made by the paediatric staff (Methods, 2.11). The number
in brackets represents the number of patients.

	Palmitic Acid ≥ 25%	RDS Present	Palmitic Acid < 25%	RDS Present
Lecithin ≥ 25 μmol l^{-1}	12	0	0	0
Lecithin < 25 μmol l^{-1}	11	0	3	3

Table 3.21 A comparison of the percentage palmitic acid residues

in amniotic fluid lipids and the L/S ratio of amniotic

fluid

The amniotic fluid samples were collected (Methods, 2.2.1) and

centrifuged (Methods, 2.2.2). The lipids were extracted (Methods,

2.2.4) and acetone precipitated (Methods, 2.2.5). The lipids were

transesterified (Methods, 2.7.1) and the fatty acid composition

analysed by gas liquid chromatography (Methods, 2.7.2) and the

percentage palmitic acid calculated. The L/S ratio was determined

by 2D t.l.c. (Methods, 2.4). The results of each test were compared.

Each sample was collected within 72 hours of delivery. Assessment

of RDS was made by the paediatric staff (Methods, 2.11). The

number in brackets represents the number of patients.

	No. of Patients	% with RDS
L/S $\geqslant$ 2.0 and palmitic acid $\geqslant$ 25%	21	0 (0)
L/S $<$ 2.0 and palmitic acid $\geqslant$ 25%	13	7.5 (1)
L/S $\geqslant$ 2.0 and palmitic acid $<$ 25%	0	0 (0)
L/S $<$ 2.0 and palmitic acid $<$ 25%	3	100 (3)
Total	37	10.8 (4)

In the remaining 13 cases the two techniques gave differing results, all having the proportion of palmitic acid above 25% while the L/S ratios were below 2.0. One baby in this group did develop RDS.

3.4.4   A Comparison of the Percentage Palmitic Acid in Acetone
Precipitable Lecithin and the Concentration of Acetone
Precipitable Lecithin in Amniotic Fluid

A comparison of the percentage of palmitic acid in the acetone precipitable lecithin and the concentration of acetone precipitable lecithin in six specimens of amniotic fluid is illustrated in figure 3.16   As can be seen the percentage palmitic acid in lecithin increases with the lecithin concentration. The equation (xi) fits the results (r = 0.97, 0.01 > P > 0.001).

$$y = 1.08x - 0.54 \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (xi)$$

where x is the concentration of lecithin and y is the percentage of palmitic acid in lecithin.

Using critical values of 25% and 25 μ mol 1^{-1} for the percentage of palmitic acid and the concentration of lecithin respectively all 6 samples predicted fetal lung maturity. The 6 babies were delivered within 72 hours of sample collection and none of the babies developed RDS.

3.4.5   A Comparison of the Percentage of Palmitic Acid in the Acetone
Precipitable Lecithin and the Acetone Precipitable L/S Ratio
of Amniotic Fluid

The percentage of palmitic acid in the lecithin fatty acids was compared with the L/S ratio in 6 amniotic fluids. As is illustrated/

Figure 3.16   A Comparison of the Percentage Palmitic Acid in Lecithin

and the Concentration of Lecithin in Amniotic Fluid

The amniotic fluid was collected as described in Methods 2.2.1, and

centrifuged (Methods, 2.2.2). The lipids were extracted (Methods, 2.2.4)

and acetone precipitated (Methods, 2.2.5). The percentage of palmitic

acid in lecithin fatty acid residues (Methods, 2.7) was compared

with the lecithin concentration (Methods, 2.5 or 2.6). Equation (xi)

fits the data ($r = 0.97$, $0.01 > P > 0.001$).

$$y = 1.08x - 0.54 \quad \ldots\ldots\ldots\ldots \quad (xi)$$

where x is the concentration of lecithin

and y is the percentage of palmitic acid in lecithin.
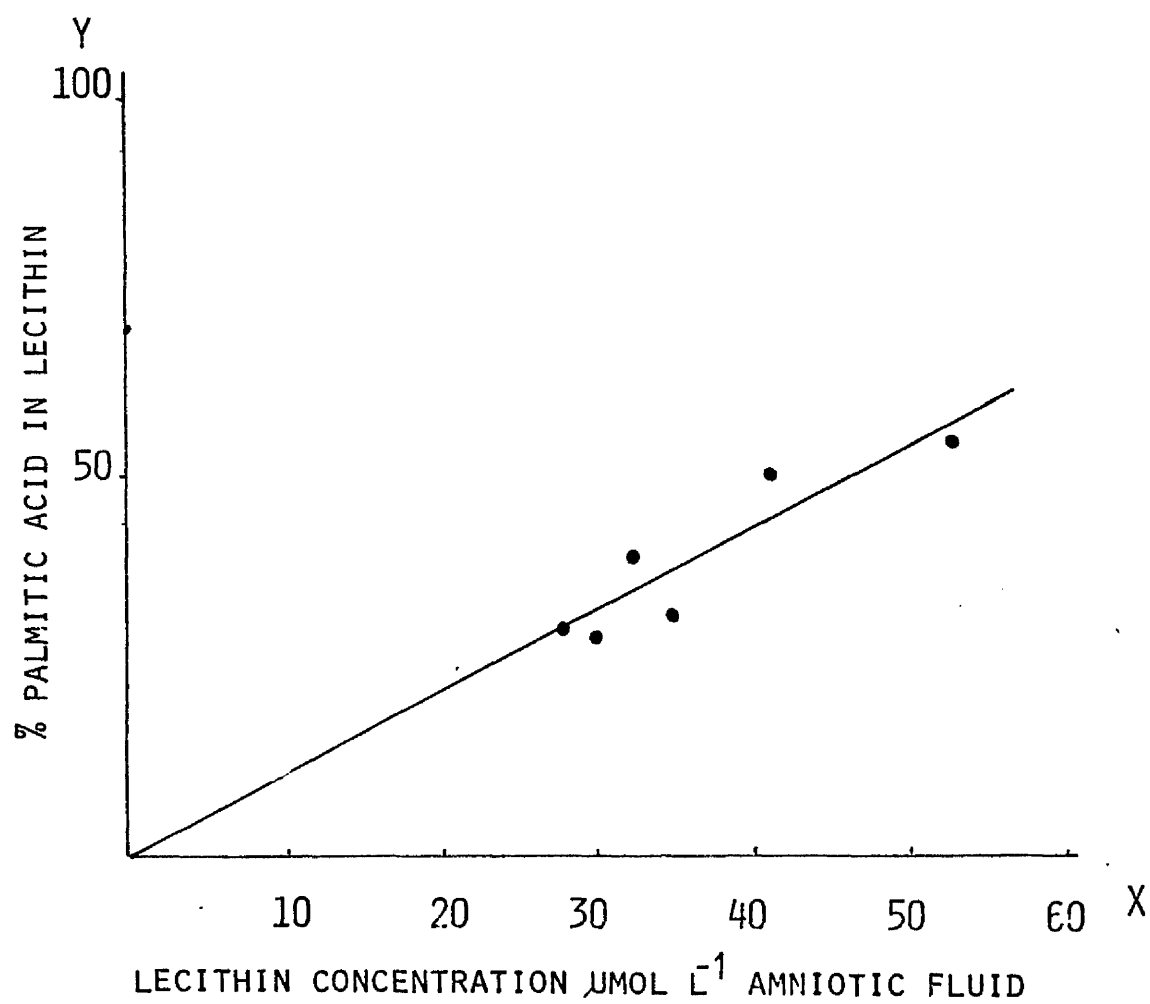
in figure 3.17 the proportion of palmitic acid tends to increase with L/S ratio. The equation (xii) fits the results ($r = 0.75$, $0.1 > P > 0.05$).

$$y = 1.55x + 12.77 \quad \text{.....................} \quad (xii)$$

where x is the L/S ratio and y is the percentage of palmitic acid in lecithin.

Using critical values of 25.0% and 2.0 for the percentage palmitic acid and the L/S ratio respectively all 6 samples led to the correct interpretation of fetal lung maturity.

3.4.6 A Comparison of the Lecithin Concentration and the Optical Density of Amniotic Fluid

The acetone precipitable lecithin concentration of amniotic fluid was compared with the optical density of amniotic fluid.

The relationship of the optical density measurements to the lecithin concentration in 30 amniotic fluids is illustrated in figure 3.18. The optical density increases with lecithin concentration ($r = 0.66$, $P < 0.001$).

Using critical values of 25 μ moles 1^{-1} amniotic fluid and 0.15 for the concentration of lecithin (results 3.1.8) and the optical density respectively, 20 of the 30 results (66.7%) were in agreement, indicating fetal lung maturity (table 3.22). Seven amniotic fluids had both the lecithin concentration and the optical density above critical values whilst 13 amniotic fluids had lecithin concentrations and the optical density measurement below critical values. Of the remaining 10 amniotic fluids the results would have led to different/

Figure 3.17 A Comparison of the Percentage Palmitic Acid in Lecithin

and the L/S Ratio of Amniotic Fluid

The amniotic fluid was collected as described in Methods, 2.2.1 and

centrifuged (Methods, 2.2.2). The lipids were extracted (Methods, 2.2.4)

and acetone precipitated (Methods, 2.2.5). The percentage of palmitic

acid in lecithin fatty acid residues (Methods, 2.7) was compared with

the L/S ratio (Methods, 2.4). Equation (xii) fits the data (r = 0.75,

0.1 > P > 0.05).

$$y = 1.55x + 12.77 \quad \ldots\ldots\ldots\ldots \quad (xii)$$

where x is the L/S ratio

and y is the percentage of palmitic acid in lecithin.

Figure 3.18 A Comparison of the Lecithin Concentration and the Optical Density of Amniotic Fluid

The amniotic fluid was collected by amniocentesis and centrifuged (Methods, 2.2.2). The optical density was determined as described in Methods, 2.8. The phospholipids were then extracted (Methods, 2.2.4) and acetone precipitated (Methods, 2.2.5). The lecithin concentration was determined as described in Methods, 2.5 or 2.6. Equation (xiii) fits the data ($r = 0.66$, $P < 0.001$).

$$y = 2.88 \times 10^{-3} e^{0.14x} \quad \dots\dots\dots\dots\dots(xiii)$$

where x is the lecithin concentration (μmol l^{-1})

and y is the optical density of the amniotic fluid.

<u>Table 3.22 A comparison of the lecithin concentration and the</u>

<u>optical density of amniotic fluid</u>

The amniotic fluids were collected by amniocentesis and centrifuged
(Methods, 2.2.2). The optical density was measured at 650nm as des-
cribed in Methods 2.8. A critical value of 0.15 was used (Results,
3.2.2). The lipids were then extracted (Methods, 2.2.4) and acetone
precipitated (Methods, 2.2.5), and the lecithin concentration was det-
ermined by h.p.l.c. (Methods, 2.6) or by phosphate analysis technique
(Methods, 2.5) following 2D t.l.c. (Methods, 2.4). A critical value of
25 μmol l^{-1} (Results, 3.18) was used.

	Optical density $\geqslant 0.15$	Optical density $\leqslant 0.15$
Lecithin $\geqslant$ 25 μmol 1^{-1}	7	2
Lecithin $<$ 25 μmol 1^{-1}	8	13

interpretations of fetal lung maturity, eight samples having an optical density above the critical value, but lecithin concentrations below 25 μ moles l^{-1} amniotic fluid. In the other 2 amniotic fluid samples the lecithin concentration was above the critical value but the optical density was less than 0.15.

The occurrence of RDS in 16 babies when delivery was within 72 hours of sample collection is shown in table 3.23. When both lecithin concentration and optical density measurements were below their critical values only 25% of the babies developed RDS. However, when either lecithin concentration or optical density (singly or in combination) was above the critical value, no baby developed RDS.

3.4.7   A Comparison of the L/S Ratio and the Optical Density of Amniotic Fluid

The L/S ratio determined by 2D t.l.c. in the acetone precipitable amniotic fluid lipids was compared with the optical density of amniotic fluid measured at 650 nm.

Figure 3.19   illustrates the relationship between the optical density measurements the the L/S ratio in 120 samples. The equation (xiii) fits the data ($r = 0.40$, $P < 0.001$).

$$y = 0.09x + 0.08 \quad \ldots\ldots\ldots\ldots\ldots\ldots \quad (xiii)$$

where x is the L/S ratio and y is the optical density.

The optical density increases with L/S ratio; however, there is a wide scatter.

Table 3.23 The Lecithin Concentration and the Optical Density as an

Index of Fetal Lung Maturity

The amniotic fluids were collected by amniocentesis and centrifuged (Methods,2.2.2.). The optical density was measured at 650nm as described in Methods,2.8. A critical value of 0.15 was used (Results, 3.2.2). The lipids were then extracted (Methods,2.2.4) and acetone precipitated (Methods,2.2.5), and the lecithin concentration was determined by h.p.l.c. (Methods,2.6), or by phosphate analysis technique (Methods,2.5) following 2Dt.l.c. (Methods,2.4). A critical value of 25 μmol l^{-1} (Results, 3.18) was used. Assessment of RDS was made by the paediatric staff as described in Methods, 2.11.

	No. of Patients	% with RDS
Lecithin $\geqslant$ 25 μmol l^{-1} and O.D. $\geqslant$ 0.15	7	0 (0)
Lecithin $\leqslant$ 25 μmol l^{-1} and O.D. $\geqslant$ 0.15	4	0 (0)
Lecithin $\geqslant$ 25 μmol l^{-1} and O.D. $\leqslant$ 0.15	1	0 (0)
Lecithin $<$ 25 μmol l^{-1} and O.D. $<$ 0.15	4	25 (1)
Total	16	6.3 (1)

Figure 3.19 A Comparison of the L/S Ratio and the Optical Density

of Amniotic Fluid

The amniotic fluid was collected by amniocentesis and centrifuged
(Methods, 2.2.2). The optical density was determined as described in
Methods, 2.8. The phospholipids were then extracted (Methods, 2.2.4)
and acetone precipitated (Methods, 2.2.5). The L/S ratio was
determined by 2D t.l.c. as described in Methods, 2.4, and the results
compared with the optical density of amniotic fluid. Only samples
collected by amniocentesis were used. Equation (xiv) fits the data
$(r = 0.40, P < 0.001, n = 120)$.

$$y = 0.09x + 0.08 \qquad \ldots\ldots\ldots\ldots \qquad (xiv)$$

where x is the L/S ratio
and y is the optical density of the amniotic fluid.
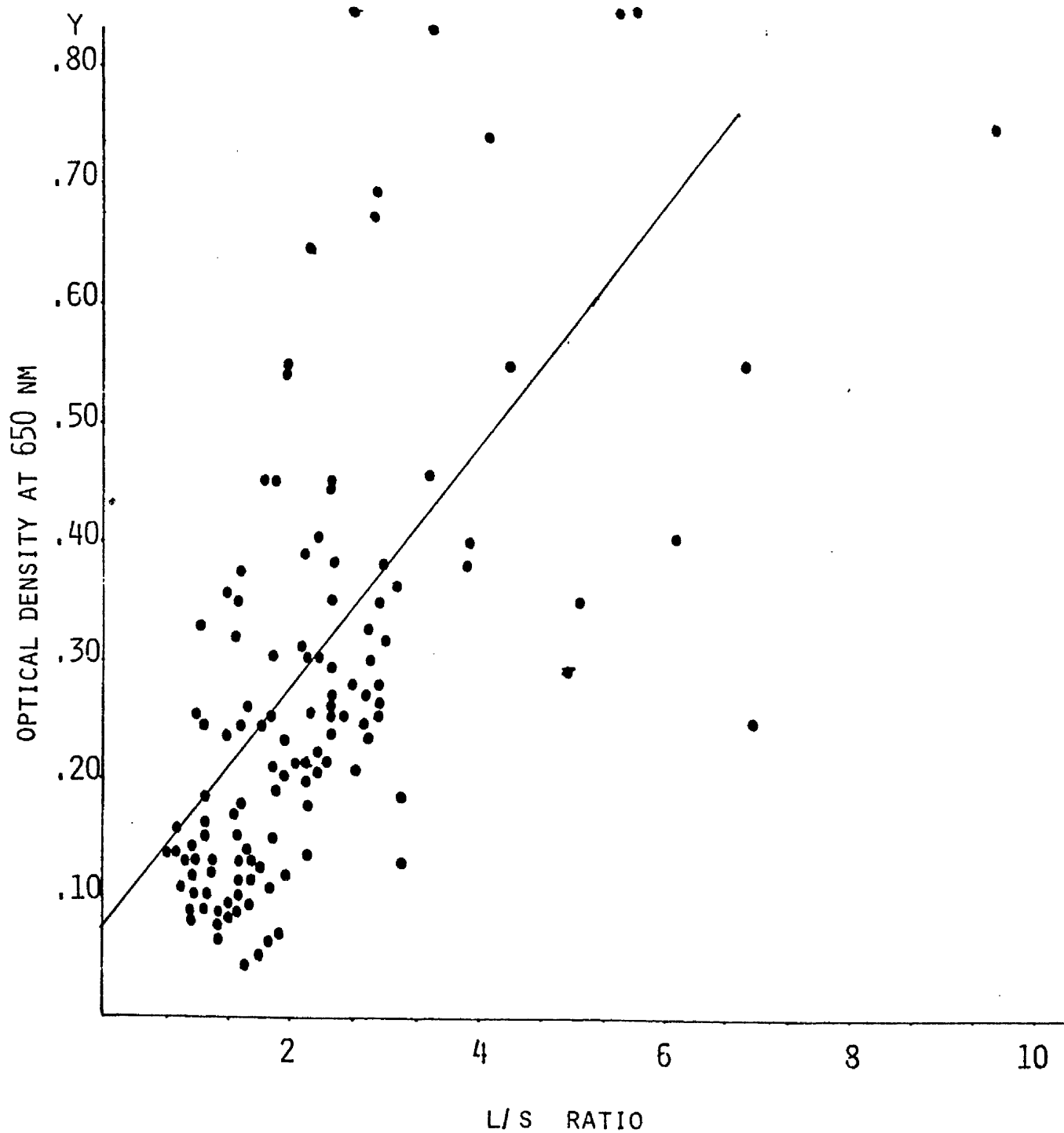
Using critical values of 2.0 and 0.15 for the L/S ratio (results 3.2.2) and the optical density respectively, 93 of the 120 results (77.3%) would have resulted in the same interpretation of fetal lung maturity (table 3.24). 33 amniotic fluids analysed had both an L/S ratio and an OD less than their critical values, and 60 amniotic fluids had an L/S ratio and the optical density above their critical values. Of the remaining 27 the results would have led to different interpretations of fetal lung maturity, 25 samples were found to have an optical density above the critical value and an L/S ratio of less than 2.0. In the other 2 amniotic fluid samples the L/S ratio was above the critical value and the optical density was less than o.15.

The occurrence of RDS in 85 babies when delivery was within 72 hours of sample collection is shown in table 3.25. When both tests indicated fetal lung immaturity 30.8% of the babies developed RDS. However, when either the L/S ratio or the optical density (singly or in combination) was above the critical value no baby developed RDS.

3.5   The Effect of Acetone Precipitation on Measurement of Lung Amniotic Fluid Lipids and Lipid Components

3.5.1   The Effect of Acetone Precipitation on Amniotic Fluid Lecithin

The acetone precipitable lecithin concentration in amniotic fluid is plotted against the total lecithin concentration in amniotic fluid in figure 3.20. In each of the 15 samples analysed about 50 per cent of the lecithin is acetone precipitable as can be seen from the slope of the graph.
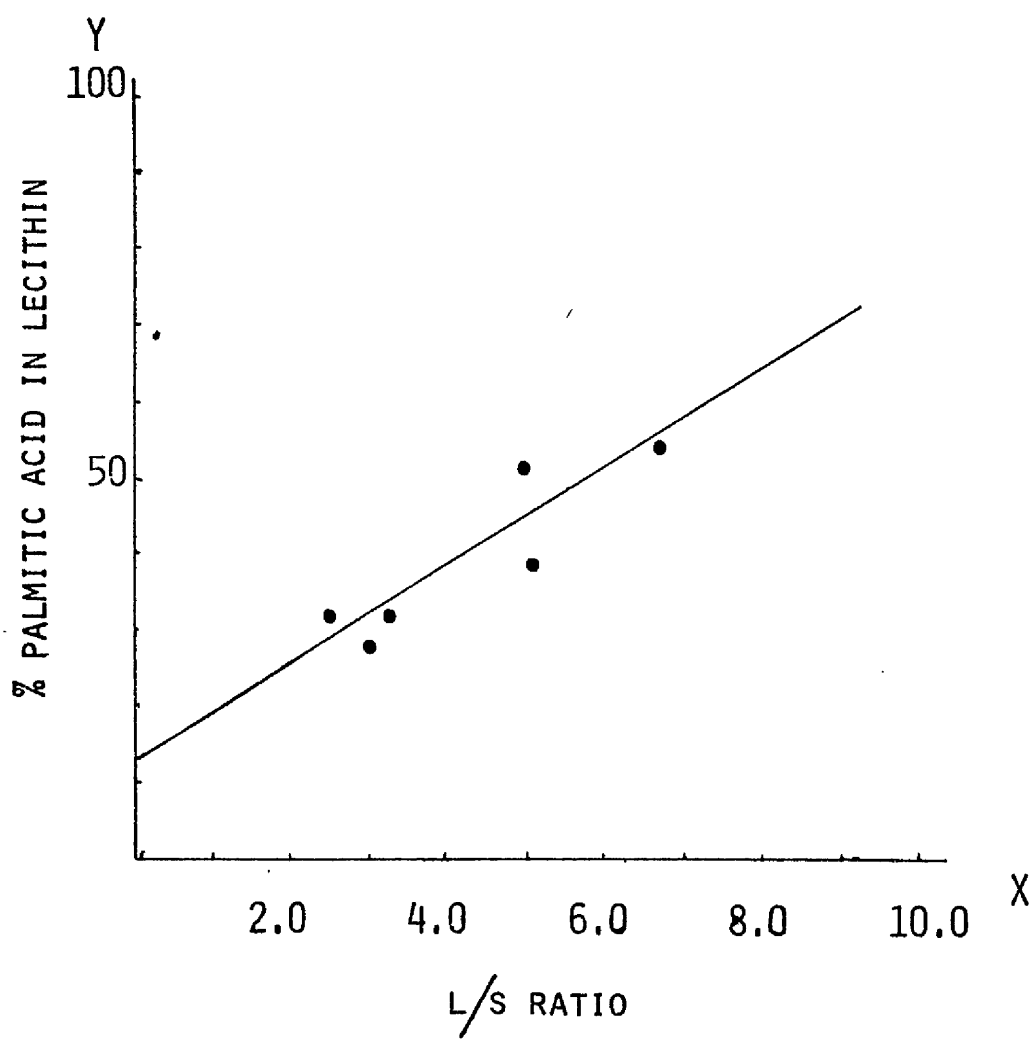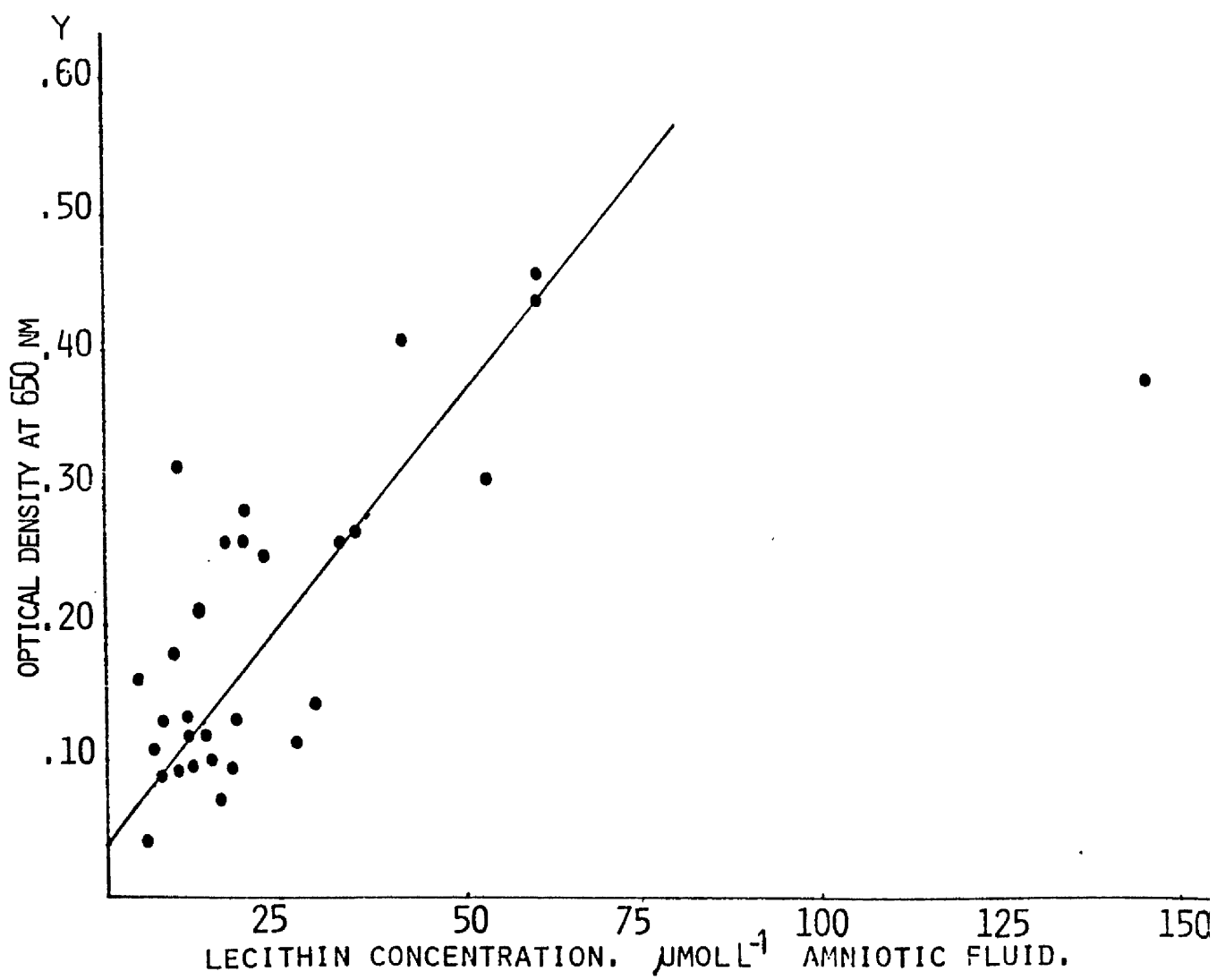
Table 3.24 A comparison of the L/S ratio and the optical density of
amniotic fluid

The amniotic fluids were collected by amniocentesis and centrifuged
(Methods, 2.2.2). The optical density was measured at 650nm as des-
cribed in Methods 2.8. A critical value of 0.15 was used (Results,
3.2.2). The lipids were then extracted (Methods, 2.2.4) and acetone
precipitated (Methods, 2.2.5), and the L/S ratio determined by 2D t.l.c.
(Methods, 2.4). An L/S ratio of 2.0 was used as the critical value.
The interpretation of the results was then compared.

	Optical Density $\geqslant 0.15$		Optical Density $\leqslant 0.15$
L/S $\geqslant$ 2.0	60	;	2
L/S $<$ 2.0	25		33

Table 3.25 The L/S ratio and optical density measurements as an index

of fetal lung maturity

The amniotic fluid samples were collected by amniocentesis and centrifuged

(Methods, 2.2.2). The optical density was measured at 650nm (Methods, 2.8).

The lipids were then extracted (Methods, 2.2.4) and acetone precipitated

(Methods, 2.2.5). The L/S ratio was determined by 2D t.l.c. (Methods, 2.4).

All samples were collected within 72 hours of delivery. Assessment of RDS

was made by the paediatric staff (Methods, 2.11).

	No. of Patients	% with RDS
L/S $\geqslant$ 2.0 and O.D. $\geqslant$ 0.15	55	0 (0)
L/S $<$ 2.0 and O.D. $\geqslant$ 0.15	15	0 (0)
L/S $\geqslant$ 2.0 and O.D. $<$ 0.15	2	0 (0)
L/S $<$ 2.0 and O.D. $<$ 0.15	13	30.7 (4)
Total	85	4.2 (4)

<u>Figure 3.20</u>   <u>The Effect of Acetone Precipitation on the Amniotic Fluid</u>

<u>Lecithin Concentration</u>

The amniotic fluid was collected as described in Methods, 2.2.1 and

centrifuged (Methods, 2.2.2). The lipids were extracted (Methods, 2.2.4).

Two aliquots of each amniotic fluid sample were used. The extracted

lipids from one aliquot were acetone precipitated (Methods, 2.2.5). In

the other aliquot this step was omitted. The concentration of lecithin

was measured by the phosphate technique (Methods, 2.5) following

2D t.l.c. (Methods, 2.4). Equation (xiv) fits the data ($r = 0.95$,

$P < 0.001$, $n = 15$).

$$y = 0.53x + 3.29 \quad \ldots\ldots\ldots\ldots \quad (xiv)$$

where x is the lecithin concentration

of the total lipid fraction (umol l^{-1})

and y is the lecithin concentration

of the acetone precipitable fraction (umol l^{-1}).
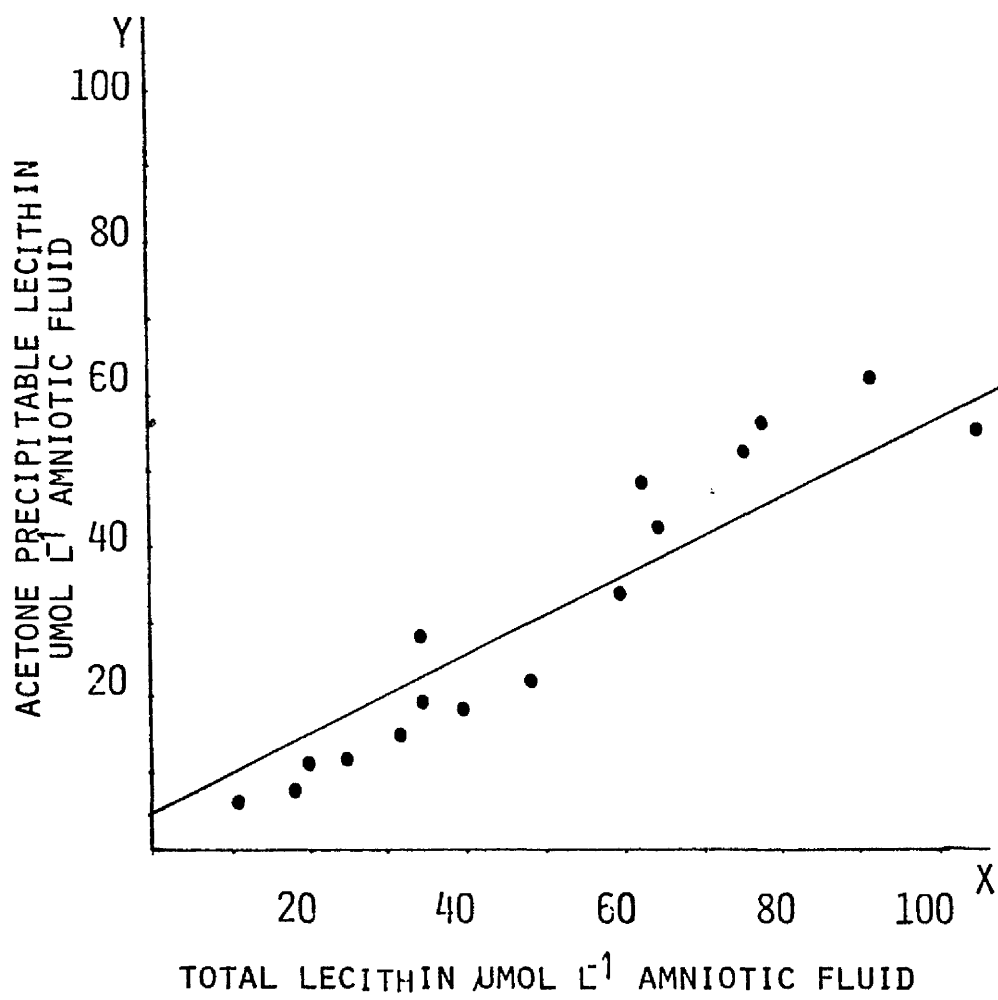
Equation (xiv) fits the data (r = 0.95, P < 0.001).

$$y = 0.53x + 3.29 \quad \dots\dots\dots\dots\dots\dots \quad \text{(xiv)}$$

where x is the lecithin concentration of the total fraction and

y is the lecithin concentration of the acetone precipitable fraction.

3.5.2   The Effect of Acetone Precipitation on the Amniotic Fluid

         L/S Ratio

Twenty-eight determinations of the L/S ratio of the acetone

precipitable lipids in amniotic fluid are plotted against the L/S

ratio in the total amniotic fluid (figure 3.21). In each sample the

L/S ratio was reduced by acetone precipitation. The L/S ratio in

acetone precipitable material is approximately half the value in the

whole amniotic fluid lipids as can be seen from equation (xv).

Equation (xv) fits the data (r = 0.91, P < 0.001).

$$y = 0.53x + 0.37 \quad \dots\dots\dots\dots\dots\dots \quad \text{(xv)}$$

where x is the L/S ratio of the whole fraction and

      y is the L/S ratio of the acetone precipitable fraction.

The slope is very similar to that obtained from a plot of acetone

precipitable lecithin against the total lecithin (see figure 3.20).

3.5.3   The Effect of Acetone Precipitation on Amniotic Fluid

         Sphingomyelin

The acetone precipitable sphingomyelin concentration in 15 amniotic/

110

<u>Figure 3.21</u>   <u>The Effect of Acetone Precipitation on the L/S Ratio</u>

The amniotic fluid was collected as described in Methods, 2.2.1 and

centrifuged (Methods, 2.2.2). The lipids were extracted (Methods, 2.2.4).

Two aliquots of each amniotic fluid sample were used. The extracted

lipids from one aliquot were acetone precipitated (Methods, 2.2.5).

In the other aliquot this step was omitted. The L/S ratio was measured

as described by 2D t.l.c. (Methods, 2.4). Equation (xv) fits the data

(r = 0.95, P < 0.001, n = 28).

$$y = 0.53x + 0.37 \quad \ldots\ldots\ldots\ldots \quad (xv)$$

where x is the L/S ratio of the total lipid fraction

and y is the L/S ratio of the acetone precipitable fraction.
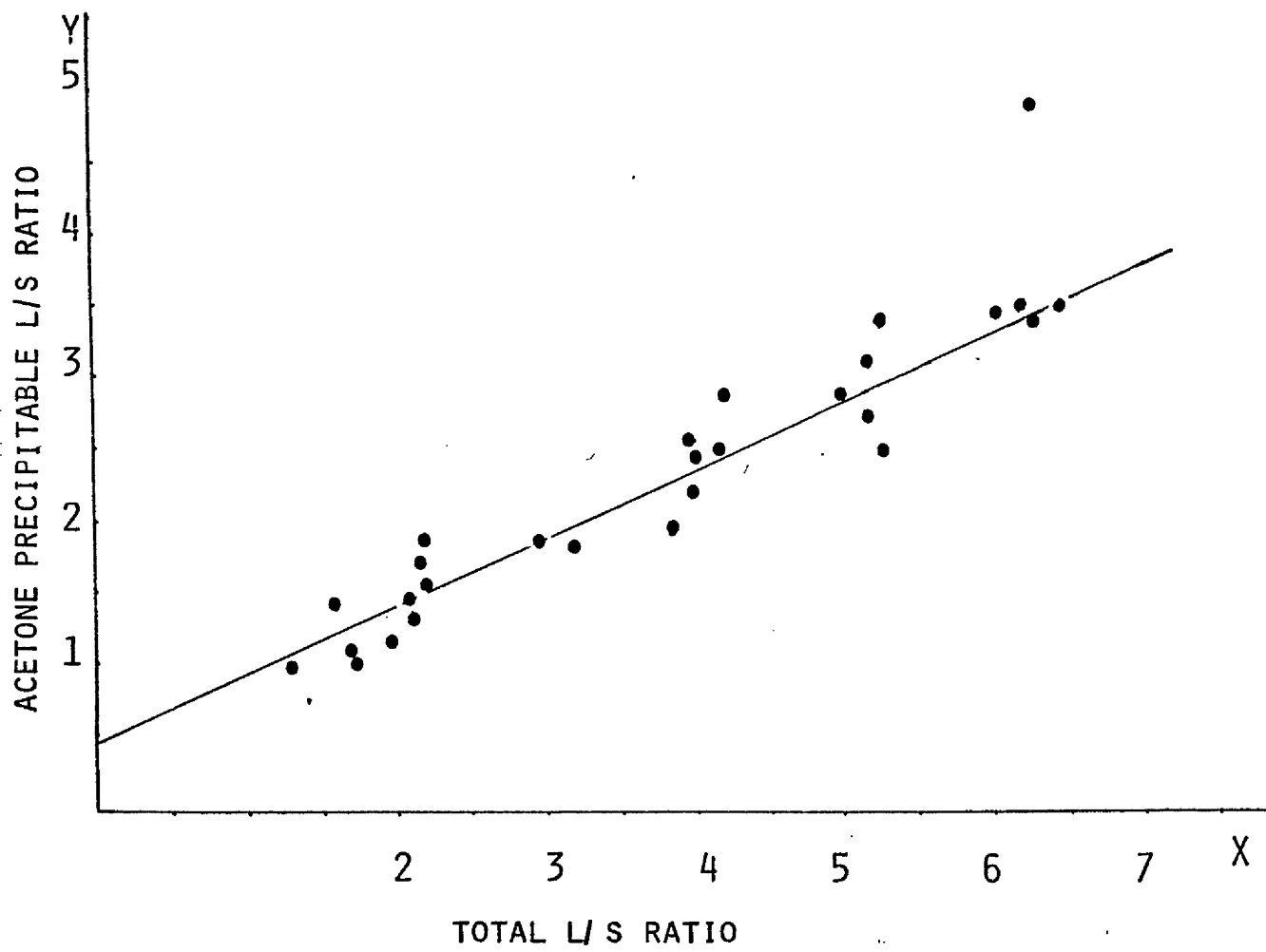
fluid samples is plotted against the total sphingomyelin concentration
in these amniotic fluids (figure 3.22). Equation (xvi) fits the
results (r = 0.87, P < 0.001).

$$y = 1.00x - 0.28 \qquad \dots\dots\dots\dots\dots\dots\dots \qquad \text{(xvi)}$$

where x is the concentration of sphingomyelin of the

total lipid fraction

and y is the sphingomyelin concentration of the acetone

precipitable fraction.

The results indicate that virtually all the sphingomyelin is
precipitated.

3.5.4   The Effect of Acetone Precipitation on the Phosphatidylglyc-
erol in Amniotic Fluid

The content of phosphatidylglycerol in the acetone precipitable
fraction was determined in 10 amniotic fluids and plotted against the
total phosphatidylglycerol concentration (figure 3.23). The equation
(xvii) fits the data (r = 0.98, P < 0.001).

$$y = 0.94x - 1.04 \qquad \dots\dots\dots\dots\dots\dots\dots \qquad \text{(xvii)}$$

where x is the phosphatidylglycerol concentration

of the total lipid fraction

and y is the phosphatidylglycerol concentration

of the acetone precipitable fraction.

Figure 3.22 The Effect of Acetone Precipitation on the Sphingomyelin

Concentration

The amniotic fluid was collected as described in Methods, 2.2.1 and

centrifuged (Methods, 2.2.2). The lipids were extracted (Methods, 2.2.4).

Two aliquots of each amniotic fluid sample were used. The extracted

lipids from one aliquot were acetone precipitated (Methods, 2.2.5).

In the other aliquot this step was omitted. The concentration of

sphingomyelin was measured by the phosphate technique (Methods, 2.5)

following 2D t.l.c. (Methods, 2.4). Equation (xvii) fits the data

$(r = 0.98, P < 0.001, n = 15)$.

$$y = 0.94x - 1.04 \qquad \ldots\ldots\ldots\ldots \qquad (xvii)$$

where x is the sphingomyelin concentration

of the total lipid fraction (umol l^{-1})

and y is the sphingomyelin concentration

of the acetone precipitable fraction (umol l^{-1}).

113
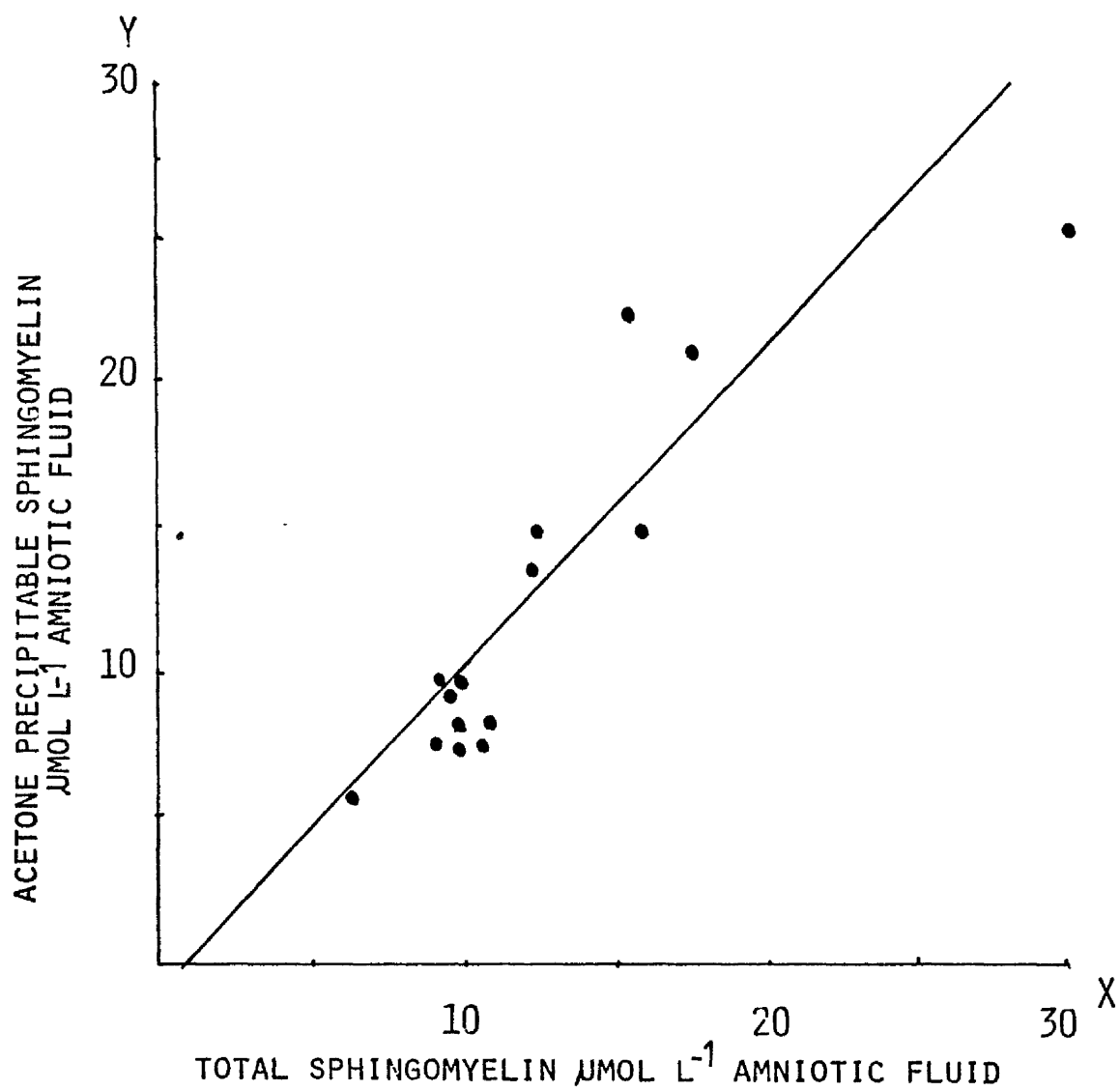
Figure 3.23   The Effect of Acetone Precipitation on the Phosphatidyl-

                glycerol in Amniotic Fluid

The amniotic fluid was collected as described in Methods, 2.2.1 and

centrifuged (Methods, 2.2.2). The lipids were extracted (Methods, 2.2.4).

Two aliquots of each amniotic fluid sample were used. The extracted

lipids from one aliquot were acetone precipitated as described in

Methods, 2.2.5. In the other aliquot this step was omitted. Phospha-

tidylglycerol was separated by 2D t.l.c. as described in Methods, 2.4

and the phosphate concentration determined as described in Methods, 2.5.

Equation (xvii) fits the data ($r = 0.98$, $P < 0.001$, $n = 10$).

$$y = 0.94x - 1.04 \quad \ldots\ldots\ldots\ldots \quad \text{(xvii)}$$

where x is the phosphatidylglycerol concentration

of the total lipid fraction (umol l^{-1})

and y is the phosphatidylglycerol concentration

of the acetone precipitable fraction (umol l^{-1}).
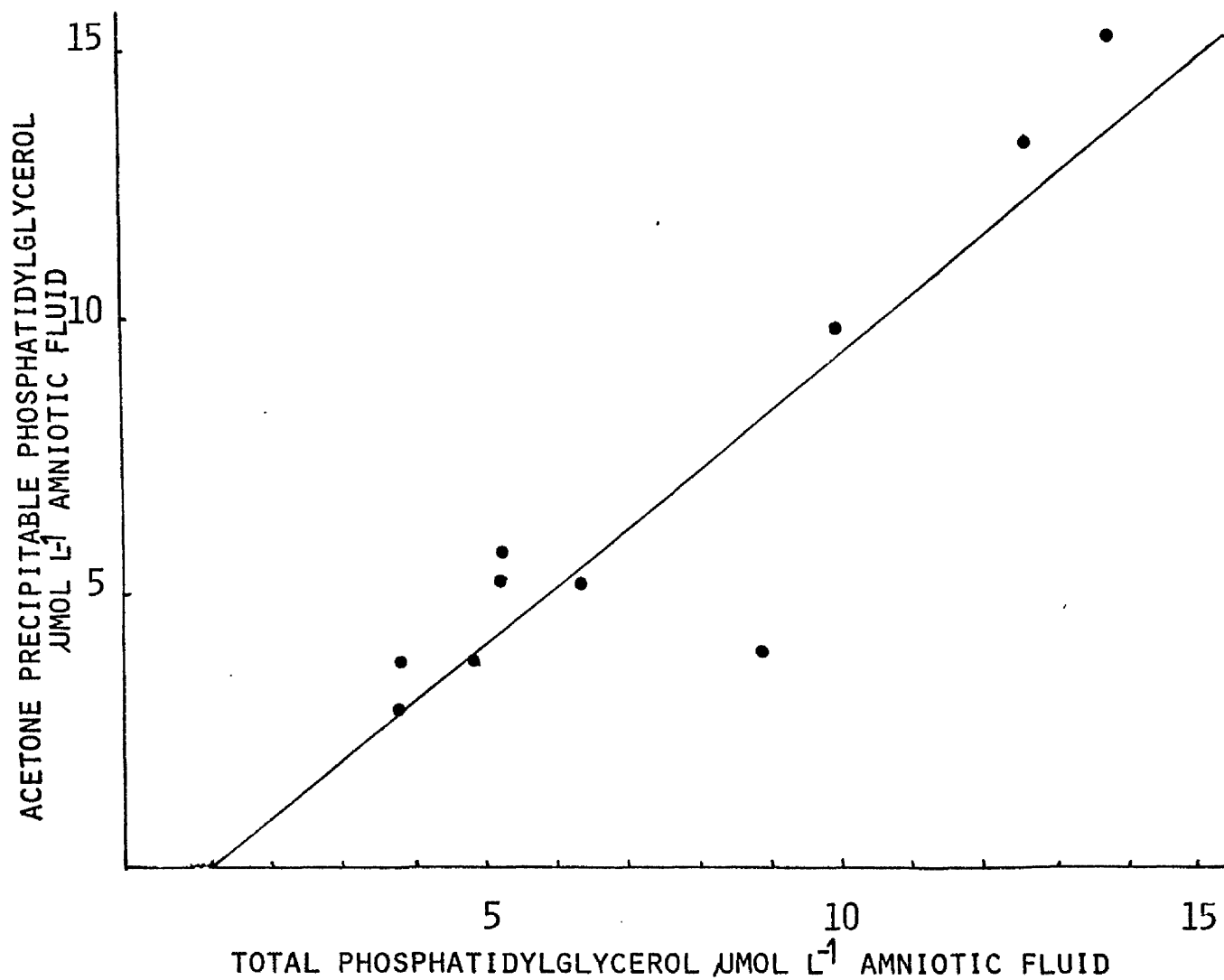
It can be seen from the results that about 90 per cent of
phosphatidylglycerol is precipitated.

3.5.5   The Effect of Acetone Precipitation on the Phosphatidylinosit-
         ol Concentration

The content of phosphatidylinositol in the acetone precipitable fraction
was determined in 6 amniotic fluids and plotted against the total
phosphatidylinositol concentration (figure 3.24). The equation (xviii)
fits the results (r = 0.98, P < 0.001).

$$y = 0.91x + 1.38 \quad \ldots\ldots\ldots\ldots\ldots\ldots \quad (xviii)$$

    where x is the phosphatidylinositol concentration
            of the total lipid fraction
    and y is the phosphatidylinositol concentration
            of the acetone precipitable fraction.

It can be seen from the equation that about 90 per cent of the
phosphatidylinositol is precipitated.

3.5.6   The Concentration of the Fatty Acid Residues Determined in
         the Total Amniotic Fluid Lipids, the Acetone Precipitable
         Lipids and the Acetone Soluble Lipids

The concentrations of the fatty acid residues (from myristic (14:0) to
linoleic (18.2)) were determined for the total amniotic fluid lipids,
the acetone precipitable fraction and the acetone soluble fraction
from 37 amniotic fluid samples. Figure 3.25 shows that myristic acid/

<u>Figure 3.24</u> <u>The Effect of Acetone Precipitation on the Phosphatidyl-</u>

          <u>inositol Concentration</u>

The amniotic fluid was collected as described in Methods, 2.2.1 and

centrifuged (Methods, 2.2.2). The lipids were extracted (Methods, 2.2.4).

Two aliquots of each amniotic fluid sample were used. The extracted

lipids from one aliquot were acetone precipitated as described in Methods,

2.2.5. In the other aliquot this step was omitted. Phosphatidylinositol

was separated by 2D t.l.c. as described in Methods, 2.4 and the

phosphate concentration determined as described in Methods, 2.5.

Equation (xviii) fits the data ($r = 0.98$, $P < 0.001$).

$$y = 0.91x + 1.38 \quad \ldots\ldots\ldots\ldots \quad \text{(xviii)}$$

      where x is the phosphatidylinositol concentration

         of the total lipid fraction (umol 1^{-1})

        and y is the phosphatidylinositol concentration

      of the acetone precipitable fraction (umol 1^{-1}).
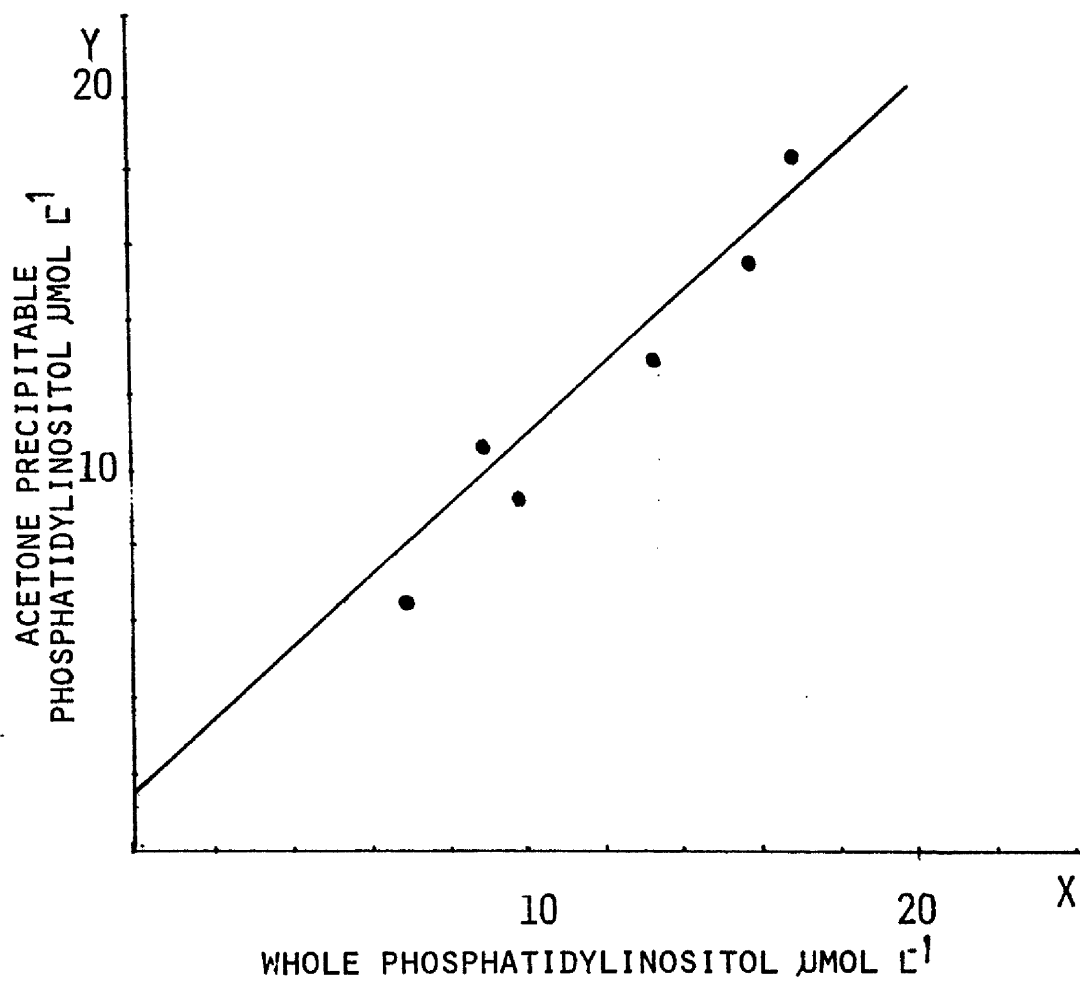
Figure 3. 25 The concentrations of the fatty acid
residues determined in the whole amniotic
fluid lipids, the acetone precipitable
lipids and the acetone soluble lipids

Two aliquots of each amniotic fluid sample were
used. The extracted lipids from one aliquot were
acetone precipitated as described in Methods 2.2.5.
The acetone soluble lipids were retained for analysis.
In the other aliquot this step was omitted. The con-
centrations of the fatty acid residues were determined
for the whole amniotic fluid lipids, the acetone
precipitable fraction and the acetone soluble fraction
as described in Methods 2.7 and the results are
shown.

■   represents the total lipid fraction

▨   represents the acetone precipitable fraction

□   represents the acetone soluble fraction
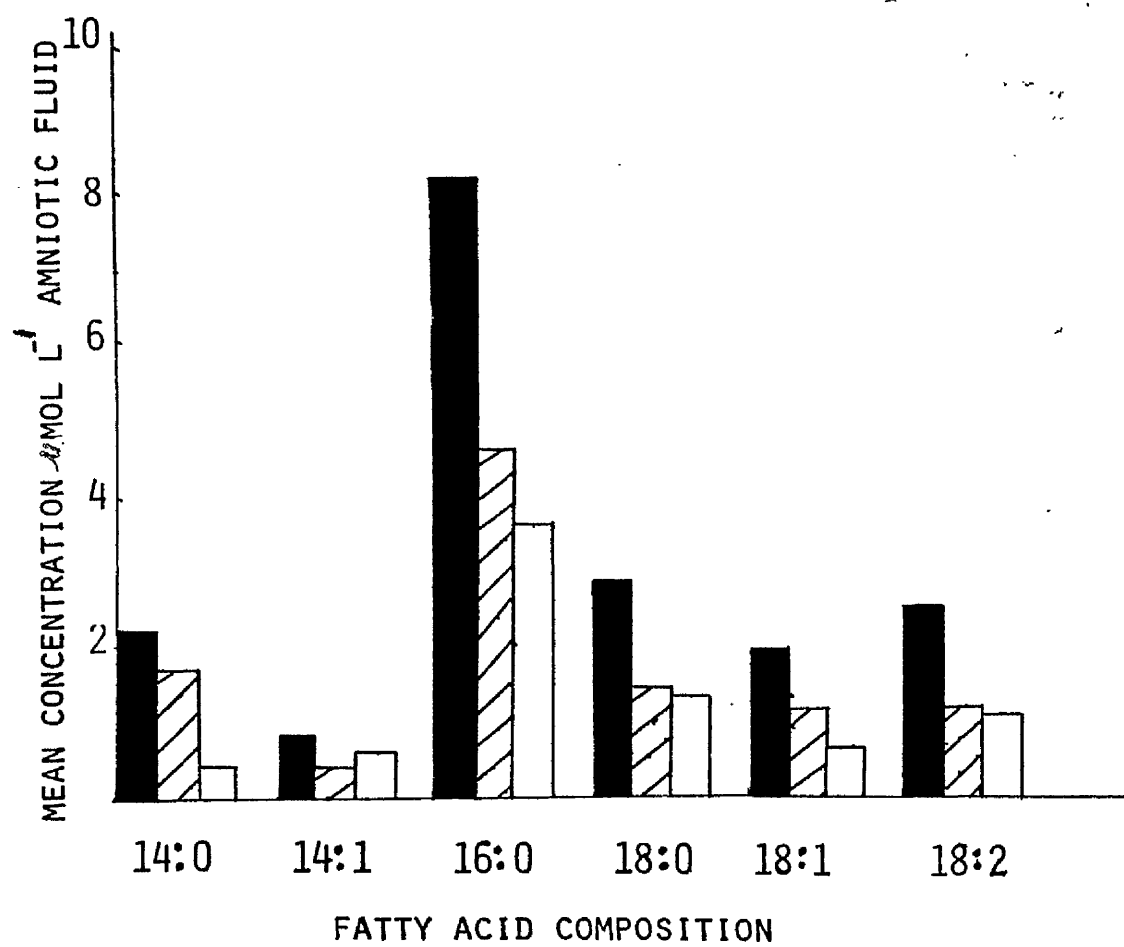
residues are the only fatty acid residues which are almost completely precipitated by acetone. Figure 3.25 also demonstrates that the concentration of any particular fatty acid residue in the amniotic fluid lipid is equal to the sum of the concentration of that fatty acid residue in the acetone precipitable and acetone soluble lipids, indicating the reliability of the analytical technique.

3.5.7   The Fatty Acid Residue Composition of Total Lipids, Acetone Precipitable Lipids and Acetone Soluble Lipids of Amniotic Fluid

The fatty acid residue composition was determined using 37 amniotic fluid lipids for the total lipid fraction, the acetone precipitable lipid fraction and the acetone soluble fraction. The fatty acid composition of fatty acid residues (from myristic acid (14:0) to linoleic acid (18:2)) of each of the lipid fractions is shown in figure 3.26. It can be seen that the acetone insoluble fraction of the lipids contains a greater proportion of myristic acid. The proportion of stearic acid is also increased in the acetone precipitable fraction. The proportion of palmitic acid is similar in each fraction, as is the proportion of oleic. The proportions of linoleic acid, and myristoleic acid are reduced in the acetone precipitable fraction. It can be seen that the soluble fraction contains both unsaturated and saturated fatty acids and that the major fatty acid in each of the fractions is palmitic acid.

<u>Figure 3 26</u> <u>The Proportions of the Fatty Acid Residues Determined in</u>

<u>the Whole Amniotic Fluid Lipids</u>

Two aliquots of each amniotic fluid sample were used. The extracted

lipids from one aliquot were acetone precipitated as described in

Methods, 2.2.5. The acetone soluble lipids were retained for analysis.

In the other aliquot this step was omitted. The percentage composition

of the fatty acid residues were determined for the whole amniotic fluid

lipids, the acetone precipitable fraction and the acetone soluble

fraction as described in Methods, 2.7.

■   represents the total lipid fraction.

▨   represents the acetone precipitable fraction.

☐   represents the acetone soluble fraction.

FATTY ACID COMPOSITION
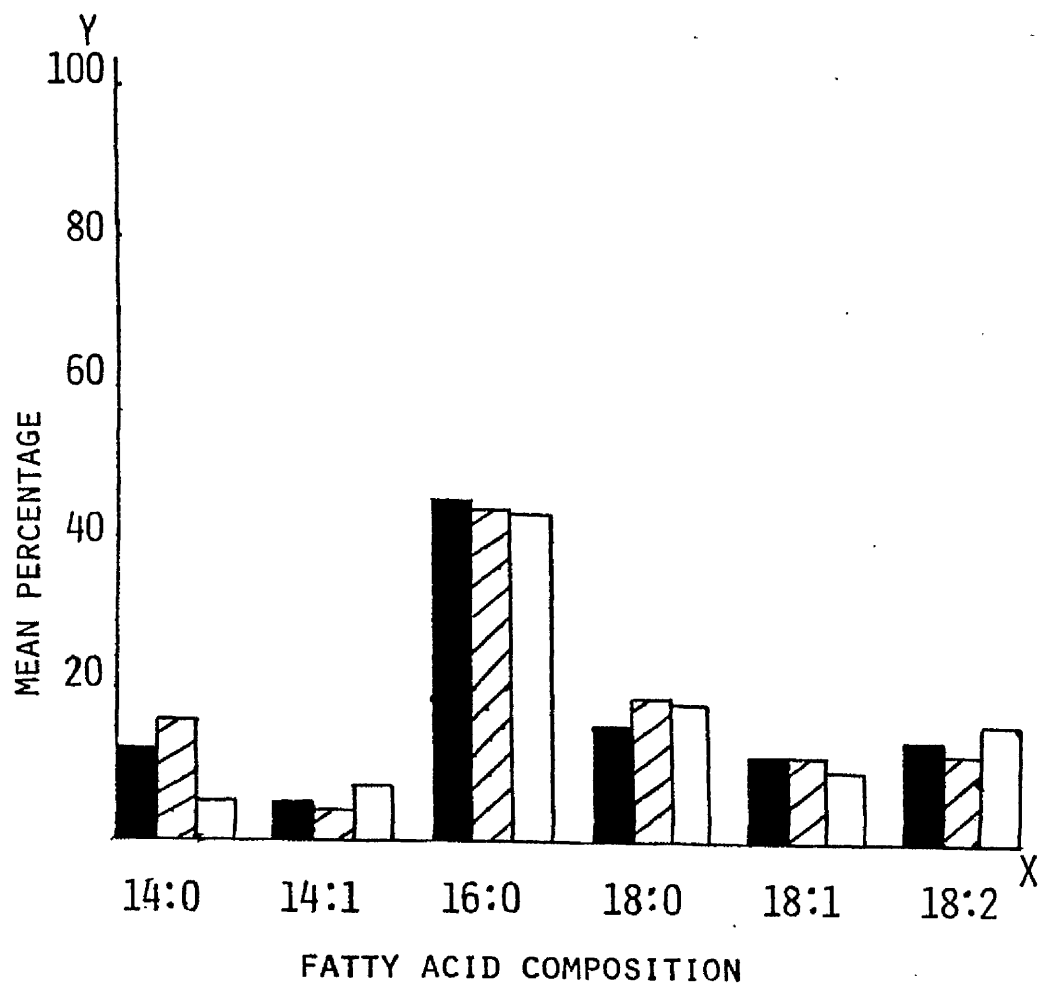
3.5.8 The Saturated Fatty Acid/Unsaturated Fatty Acid Ratio in the Total Lipid Fraction, the Acetone Precipitable and Soluble Fractions in Amniotic Fluid Lipids

The ratio of saturated fatty acids to unsaturated fatty acids in amniotic fluid lipids was calculated for the whole lipid fraction, the acetone precipitable fraction and the acetone soluble fraction. The average ratios obtained for each fraction from 37 amniotic fluid samples are shown in figure 3.27. It can be seen that the acetone precipitated fraction is enriched with saturated fatty acid residues (P < 0.001), while the acetone soluble fraction contains less saturated fatty acid residues (P < 0.001).

3.5.9 The Proportion of Fatty Acid Residues as Palmitic Acid in Total Amniotic Fluid and Acetone Precipitated Lipids

Thirty-seven determinations of the percentage palmitic acid in the acetone precipitable lipids are plotted against the percentage palmitic acid of the total lipids (figure 3.28). Equation (xix) fits the results (r = 0.72, P < 0.001).

$$y = 0.83x + 10.27 \quad \dots\dots\dots\dots\dots\dots \quad \text{(xix)}$$

where x is the percentage of palmitic acid of the
whole lipid fraction
and y is the percentage of palmitic acid of the
acetone precipitable lipid fraction.

Figure 3.27   The Mean Saturated/Unsaturated Fatty Acid Ratio in the

Whole Amniotic Fluid Lipids, the Acetone Precipitable

Lipids and the Acetone Soluble Lipids

Two aliquots of each amniotic fluid sample were used. The extracted

lipids from one aliquot were acetone precipitated as described in

Methods, 2.2.5. The acetone soluble lipids were retained for analysis.

In the other aliquot the acetone step was omitted. The palmitic acid/

stearic acid ratios were determined for each sample, (Methods, 2.7) and

the mean ratio calculated for each fraction.

■   represents the total lipid fraction.

▨   represents the acetone precipitable fraction.

▢   represents the acetone soluble fraction.
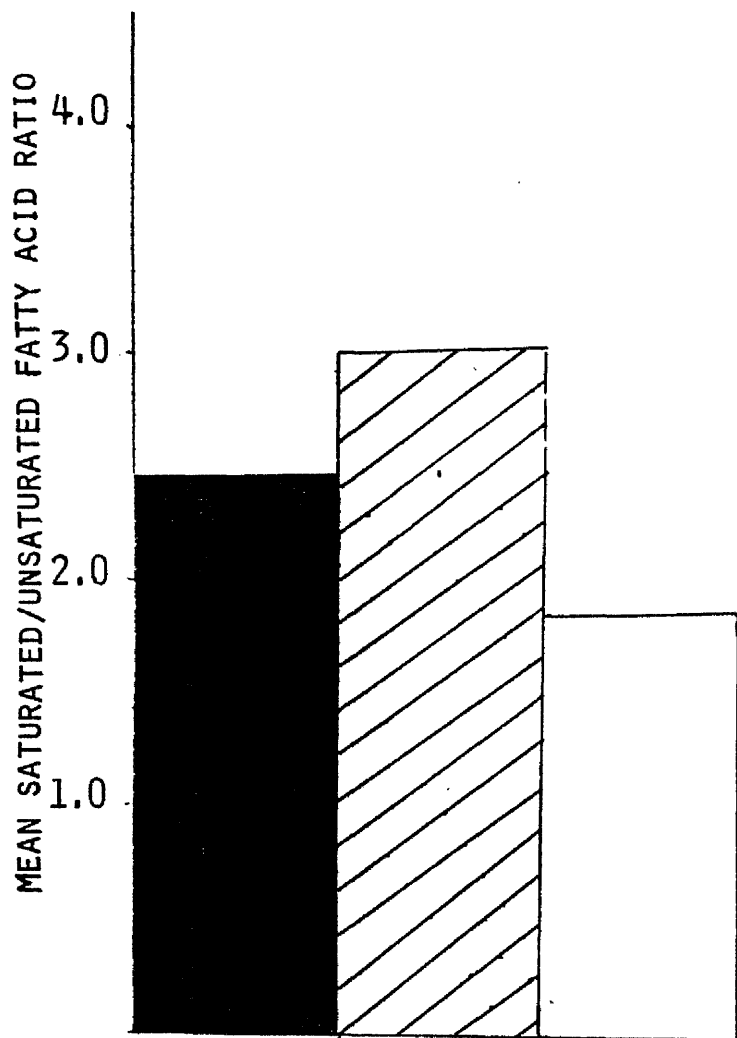
Figure 3.28 The Proportion of Fatty Acid Residues as Palmitic Acid

in Whole Amniotic Fluid and Acetone Precipitated Lipids

The amniotic fluid was collected as described in Methods, 2.2.1 and
centrifuged (Methods, 2.2.2). The lipids were extracted (Methods, 2.2.4).
Two aliquots of each sample were used. The extracted lipids from one
aliquot were acetone precipitated (Methods, 2.2.5). In the other
aliquot this step was omitted. The percentage palmitic acid in the
acetone precipitable lipids and the percentage palmitic acid of the
total lipids' fatty acid residues in the amniotic fluid were determined
using the technique described in Methods, 2.7, and the results
compared. Equation (xix) fits the data ($r = 0.72$, $P < 0.001$, $n = 37$).

$$y = 0.83x + 10.27 \quad \ldots\ldots\ldots\ldots \quad (xix)$$

where x is the percentage of palmitic acid

of the whole lipid fraction

and y is the percentage of palmitic acid

in the acetone precipitable fraction.
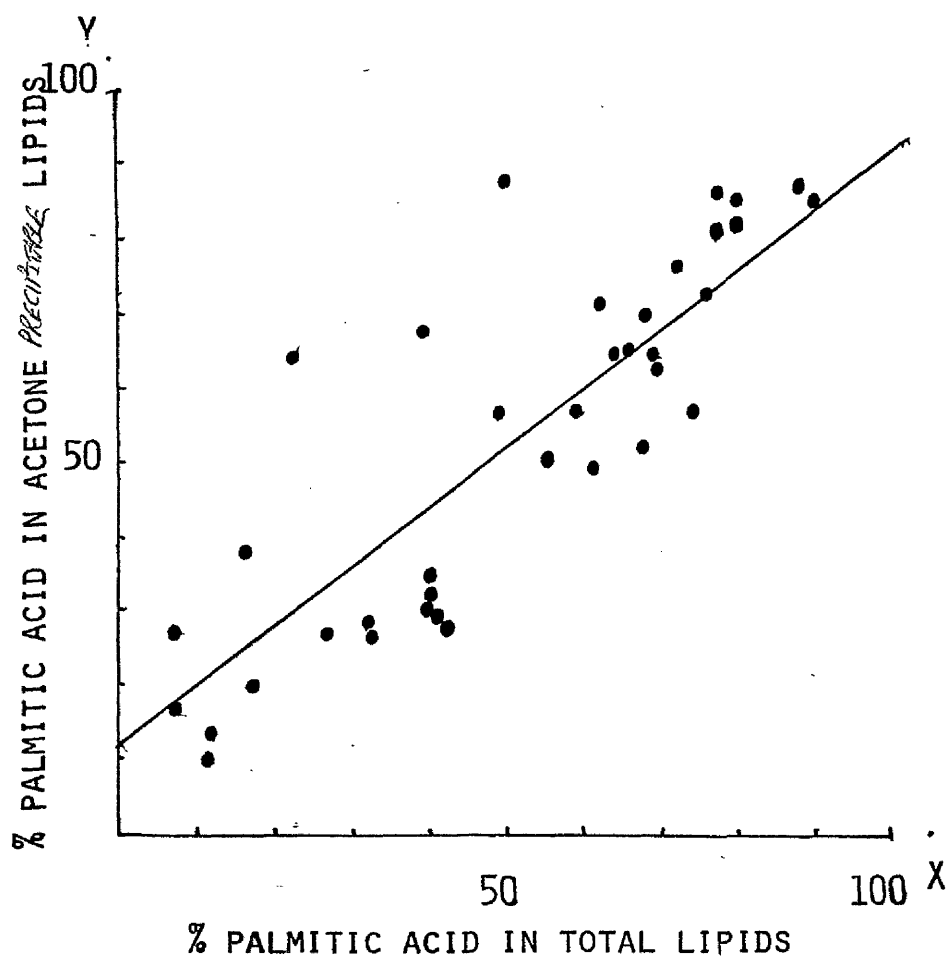
It can be seen from the results that the proportion of fatty acid residues as palmitic acid does not change greatly after acetone precipitation of the lipids in the amniotic fluid sample.

3.5.10   The Concentration of Palmitic Acid Residues in the Acetone Precipitable Fraction in Relation to the Palmitic Acid Residue Concentration of the Total Lipids

The results of 37 determinations of the palmitic acid concentration of the acetone precipitable lipids' fatty acid residues in amniotic fluid are plotted against the palmitic acid concentration of the total lipids (figure 3.29). The palmitic acid concentration in acetone precipitable material is approximately half the value of the whole amniotic fluid lipids, as can be seen from equation (xx). Equation (xx) fits the results ($r = 0.79$, $P < 0.001$).

$$y = 0.47x + 1.26 \quad \ldots\ldots\ldots\ldots\ldots\ldots \quad (xx)$$

where x is the palmitic acid concentration of the
whole lipid fraction
and y is the palmitic acid concentration of the
acetone precipitable lipid fraction.

The slope obtained is very similar to those obtained from plots of acetone precipitable lecithin and L/S ratio against total lecithin and L/S ratio respectively (see figures 3.1 and 3.2 respectively).

123

Figure 3.29 The Palmitic Acid Concentration of the Acetone Precipitable

　　　　　　　Lipids' Fatty Acid Residues in Relation to the Palmitic

　　　　　　　Acid Concentration of the Total Lipids

The amniotic fluid was collected as described in Methods, 2.2.1 and

centrifuged (Methods, 2.2.2). The lipids were extracted (Methods, 2.2.4).

Two aliquots of each amniotic fluid sample were used. The extracted

lipids from one aliquot were acetone precipitated (Methods, 2.2.5).

In the other aliquot this step was omitted. The palmitic acid

concentration of the acetone precipitable lipids' fatty acid residues

in amniotic fluid and the palmitic acid concentration of the total

lipids were determined using the technique described in Methods, 2.7,

and the results compared. Equation (xx) fits the data (r = 0.79,

P < 0.001, n = 37).

$$y = 0.47x + 1.26 \quad \ldots\ldots\ldots\ldots \quad (xx)$$

where x is the palmitic acid concentration

of the whole lipid fraction (mmol l^{-1})

and y is the palmitic acid concentration

of the acetone precipitable lipid fraction (mmol l^{-1}).

CONCENTRATION PALMITIC ACID IN ACETONE PRECIPITABLE LIPIDS (M $MOLL^{-1}$)

PALMITIC ACID CONCENTRATION IN TOTAL LIPIDS (μ $MOLL^{-1}$)
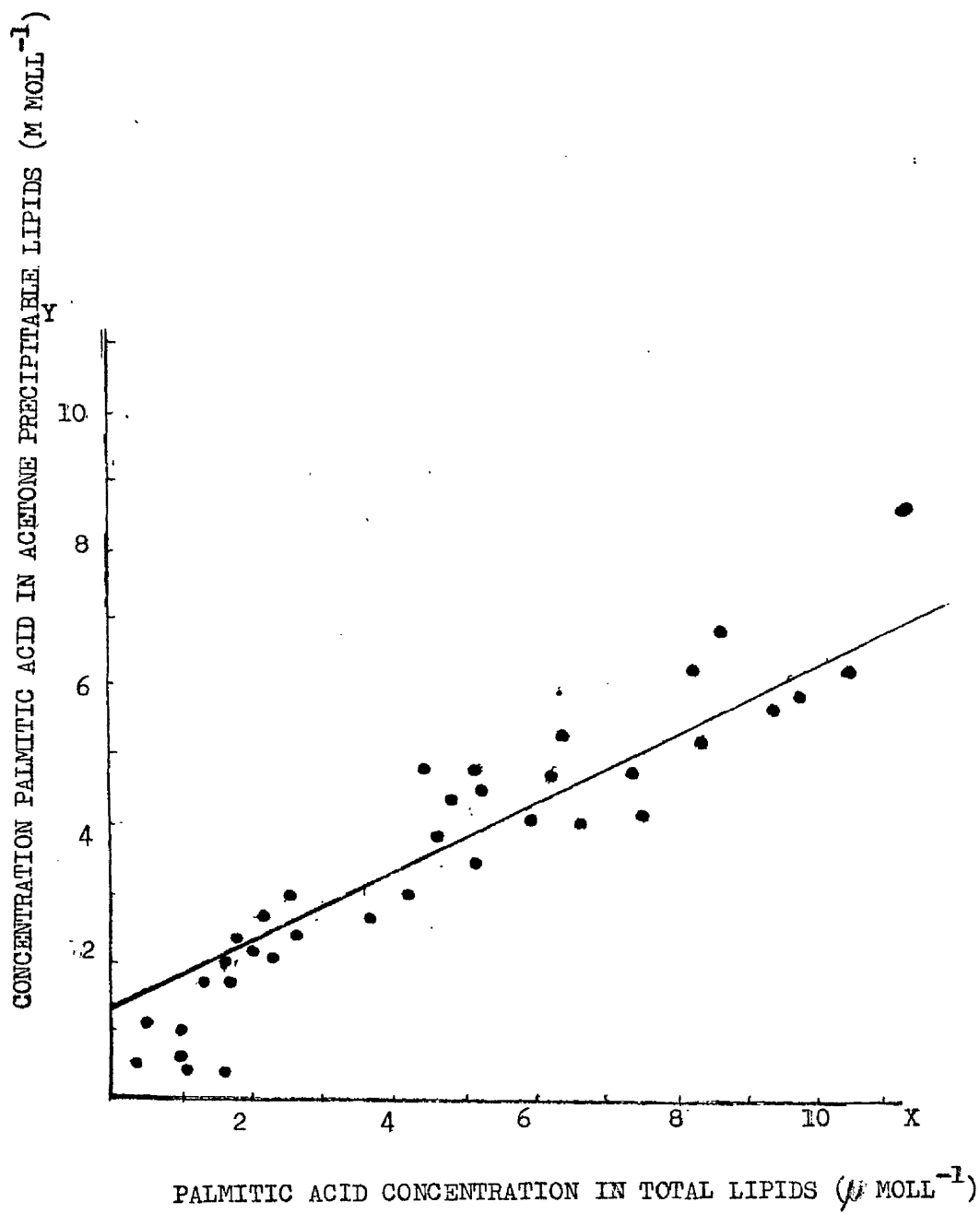
3.5.11　The Palmitic Acid/Stearic Acid Ratio in the Acetone

　　　　Precipitable Lipids in Relation to the Palmitic Acid/

　　　　Stearic Acid Ratio in Total Lipids

The results of 37 determinations of the palmitic acid/stearic acid
ratio of the acetone precipitable lipids in amniotic fluid are plotted
against the palmitic acid/stearic acid ratio of the total lipids
(figure 3.3⊘). Equation (xxi) fits the results (r = 0.62,　P < 0.001).

$$y = 0.90x + 2.20 \quad \dots\dots\dots\dots\dots\dots \quad (xxi)$$

　　where x is the palmitic acid/stearic acid ratio of

　　　　　　the whole lipid fraction

　　and y is the palmitic acid/stearic acid ratio of

　　　　　　the acetone precipitable fraction.

It can be seen from the results that the palmitic acid/stearic acid
ratio in acetone precipitable lipids is approximately 85 per cent of
the value in total amniotic fluid lipids.

3.5.12　The Percentage of Oleic Acid of the Acetone Precipitable

　　　　Lipid Fatty Acid Residues in relation to the Per Cent Oleic

　　　　Acid Total Lipids

The results of 17 determinations of percentage oleic acid of the
acetone precipitable lipids' fatty acids in amniotic fluid are plotted
against the percentage oleic acid of the total lipids' fatty acids in
figure 3.3⅃. Equation (xxii) fits the results (r = 0.84,　P < 0.001).

Figure 3.30 The Palmitic Acid/Stearic Acid Ratio in the Acetone
              Precipitable Lipids in Relation to the Palmitic Acid/
              Stearic Acid Ratio in Total Lipids

The amniotic fluid was collected as described in Methods, 2.2.1 and
centrifuged (Methods, 2.2.2). The lipids were extracted (Methods, 2.2.4).
Two aliquots of each sample were used. The extracted lipids from one
aliquot were acetone precipitated (Methods, 2.2.5). In the other
aliquot this step was omitted. The palmitic acid/stearic acid ratio
of the acetone precipitable lipids in amniotic fluid and the palmitic
acid/stearic acid ratio of the total lipids were determined using the
technique described in Methods, 2.7, and the results compared. Equation
(xxi) fits the data ($r = 0.62$, $P < 0.001$, $n = 37$).

$$y = 0.90x + 2.20 \quad \ldots\ldots\ldots\ldots \quad (xxi)$$

where x is the palmitic acid/stearic acid ratio
of the whole lipid fraction
and y is the palmitic acid/stearic acid ratio
of the acetone precipitable fraction.

PALMITIC ACID / STEARIC ACID RATIO IN TOTAL LIPIDS
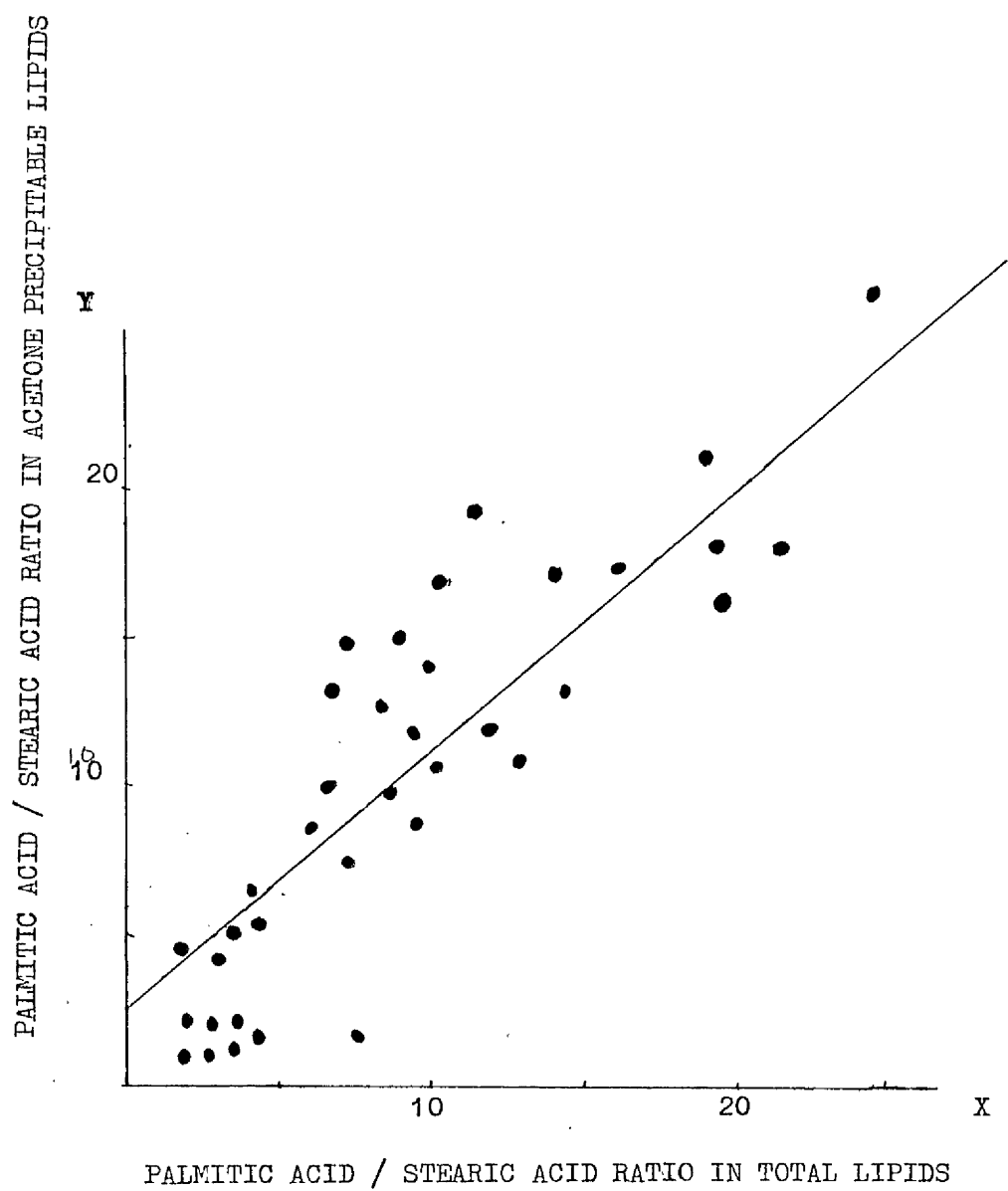
<u>Figure 3.31</u> <u>The Percentage of Oleic Acid of the Acetone Precipitable</u>

<u>Lipid Fatty Acid Residues in Relation to the Percentage</u>

<u>Oleic Acid in Total Lipids</u>

The amniotic fluid was collected as described in Methods, 2.2.1 and

centrifuged (Methods, 2.2.2). The lipids were extracted (Methods, 2.2.4).

Two aliquots of each sample were used. The extracted lipids from one

aliquot were acetone precipitated (Methods, 2.2.5). In the other

aliquot this step was omitted. The percentage of oleic acid of the

acetone precipitable lipids' fatty acid residues in amniotic fluid and

the percentage of oleic acid of the total lipids was determined using

the technique described in Methods, 2.7, and the results compared.

Equation (xxii) fits the data. $(r = 0.84, \quad P < 0.001, \quad n=17)$.

$$y = 0.52x + 3.32 \quad \ldots\ldots\ldots \quad (xxii)$$

where x is the percentage of oleic acid

of the whole lipid fraction

and y is the percentage of oleic acid

of the acetone precipitable fraction.

% OLEIC ACID IN TOTAL LIPID
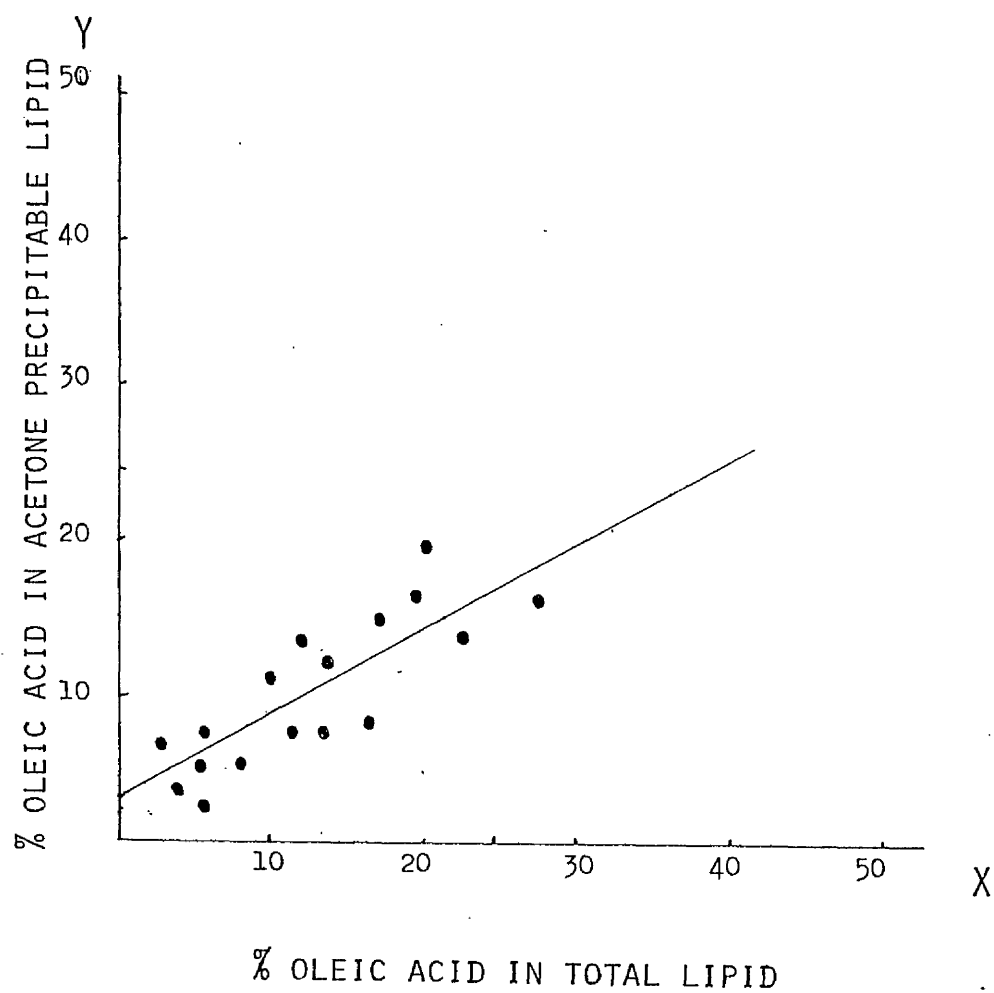
$$y = 0.52x + 3.32 \quad \ldots\ldots\ldots\ldots\ldots\ldots \quad \text{(xxii)}$$

where x is the percentage of oleic acid of the

whole lipid fraction

and y is the percentage of oleic acid of the

acetone precipitable fraction.

The results indicate that the acetone precipitable lipids contain about half the percentage of oleic acid compared with the whole amniotic fluid lipids.

3.5.13 The Absolute Concentration of Oleic Acid in the Acetone Precipitable Lipids in Relation to the Absolute Concentration of Oleic Acid in the Total Lipids

The results of 17 determinations of oleic acid (18:1) concentration of the acetone precipitable lipids' fatty acids in amniotic fluid are plotted against the oleic acid concentration of the total lipids' fatty acids (figure 3.32). In each sample the oleic acid concentration was reduced by acetone precipitation. Equation (xxiii) fits the results (r = 0.84, P < 0.001).

$$y = 0.88x - 0.63 \quad \ldots\ldots\ldots\ldots\ldots\ldots \quad \text{(xxiii)}$$

where x is the oleic acid concentration of the

total lipid fraction

and y is the oleic acid concentration of the

acetone precipitable fraction.

Figure 3.32   The Absolute Concentration of Oleic Acid in the Acetone

Precipitable Lipids' Fatty Acids in Relation to the

Absolute Concentration of Oleic Acid in the Total Lipids'

Fatty Acids

The amniotic fluid was collected as described in Methods, 2.2.1 and

centrifuged (Methods, 2.2.2). The lipids were extracted (Methods, 2.2.4).

Two aliquots of each sample were used. The extracted lipids from one

aliquot were acetone precipitated (Methods, 2.2.5). In the other

aliquot this step was omitted. The oleic acid concentration of the

acetone precipitable lipids' fatty acids in amniotic fluid and the oleic

acid concentration of the total lipids' fatty acids were determined

using the technique described in Methods, 2.7, and the results compared.

Equation (xxiii) fits the data ($r = 0.84$, $P < 0.001$, $n = 17$).

$$y = 0.88x - 0.63 \quad \ldots \qquad \text{(xxiii)}$$

where x is the oleic acid concentration

of the total lipid fraction ($mmol\ l^{-1}$)

and y is the oleic acid concentration

of the acetone precipitable fraction ($mmol\ l^{-1}$).
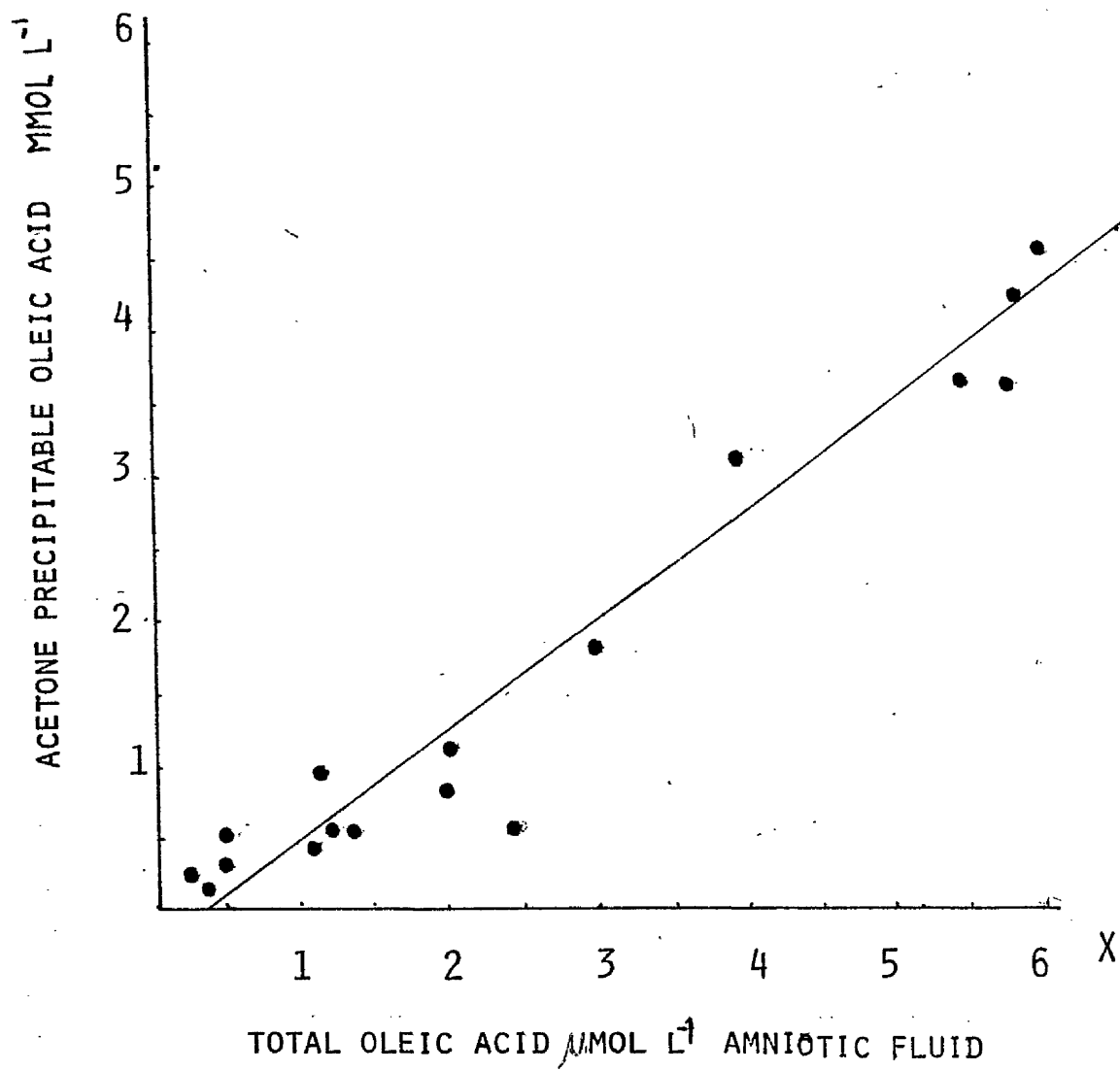
The results indicate that approximately 80 per cent of the oleic acid is precipitated.

3.5.14   The Effect of Omitting Acetone Precipitation in Determining Fetal Lung Maturity Using the L/S Ratio Technique

In 23 amniotic fluid samples the L/S ratio had been determined on both the acetone precipitable lipid fraction and the whole lipid fraction.

All the babies were delivered within 72 hours after sample collection, the presence or absence of RDS was noted. A summary of the results appears in table 3.26. When acetone precipitation was used the L/S ratio was above 2.0 in 16 cases and none of the babies developed RDS. In the remaining 7 patients the L/S ratio was found to be below 2.0 and 4 of the babies developed RDS (57.1%). However, when the acetone precipitation was omitted from the procedure on the same amniotic fluid samples, 20 of the 23 samples had an L/S of 2.0 or above, and 3 of these babies were found to have RDS (15.0%). In the remaining 3 patients an immature L/S ratio was found and one baby developed RDS (33.3%).

Table 3.26 The effect of omitting acetone precipitation in
          determining fetal lung maturity by the L/S ratio test

The amniotic fluids were collected as described in Methods,2.2.1,
and centrifuged (Methods,2.2.2). Two aliquots of each sample were
used. The lipids were extracted as described in Methods,2.2.4
and one aliquot was acetone precipitated (Methods, 2.2.5). The
L/S ratio was determined (Methods, 2.4) on both the acetone pre-
cipitable fraction and the total lipid fraction. All samples
were collected within 72 hours of delivery. Assessment of RDS
was made by the paediatric staff as described in Methods,2.11.
The number in brackets represents the number of patients.

Acetone Precipitable L/S Ratio	No. of Patients	% RDS Present	% RDS Absent
L/S $\geqslant$ 2.0	16	0 (0)	100 (16)
L/S $<$ 2.0	7	57.1 (4)	42.9 (3)
Total	23	17.4 (4)	82.6 (19)

Total Amniotic Fluid L/S Ratio	No. of Patients	% RDS Present	% RDS Absent
L/S $\geqslant$ 2.0	20	15.0 (3)	85.0 (17)
L/S $<$ 2.0	3	33.3 (1)	66.7 (2)
Total	23	17.4 (4)	82.6 (19)

CHAPTER FOUR

DISCUSSION

CHAPTER FOUR

DISCUSSION

4.1   The Lecithin Concentration

A number of chemical methods have been developed to determine the amniotic fluid lecithin concentration but they do not appear to be in common use (Bhagwanani et al, 1972; Biezenski, 1967). The technique is laborious, involving the extraction of the lipids from the amniotic fluid, followed by their separation by t.l.c. Elution of the lecithin from the chromatogram and estimation of the phosphorus concentration in the eluted fluid finally allows the determination of the amount of lecithin present. Recently Beutler et al (1979) developed a specific enzymatic assay for the quantitative determination of lecithin in amniotic fluid. This assay gives absolute concentrations of lecithin, and does not require extraction of lipids from the amniotic fluid and separation of lipids by t.l.c. The enzymatic determination requires about 45 minutes and the technique is much less laborious than the phosphorus assay following 2D t.l.c. However, it is the total lecithin concentration and not the acetone precipitable lecithin concentration that is measured, and the omission of this step causes inaccuracies when predicting fetal lung maturity (Results, 3.5.14).

Bhagwanani et al (1972) demonstrated that amniotic fluid lecithin concentrations increase gradually with gestational age and they found a critical value for lecithin of 3.5 mg% (47.6 μ mol l^{-1}) above which no cases of RDS occurred. However, although Biezenski (1972) also observed a gradual increase in lecithin concentration with gestational age he observed a different range of values, emphasising the importance of establishing normal values for each laboratory.

In this thesis the finding of a higher concentration of amniotic fluid lecithin near term than earlier in gestation is in agreement with the reports of Gluck and colleagues (1971), Bhagwanani et al (1972) and Biezenski (1972). A critical value for lecithin of 25 μ mol l^{-1} amniotic fluid was established above which no cases of RDS occurred, although when the lecithin concentration was below this critical value only 38% of the babies developed RDS.

Various factors may interfere with the reliability of the lecithin concentration estimation. Blood or meconium in the amniotic fluid invalidates the results, whilst the concentration of a single substance in the amniotic fluid is seriously affected by changes in volume. Falconer et al (1973) found that the estimation of lecithin was more useful when related to the volume of amniotic fluid present measured by dye dilution techniques, although this involved additional time in an already time-consuming technique.

4.2   The L/S Ratio

The estimation of the L/S ratio by one-dimensional t.l.c. has become
a standard test of fetal lung maturity, and it has been evaluated by a
number of investigators (Gluck et al, 1973; Whitfield et al, 1974;
Donald et al, 1973). Sphingomyelin, which has a source in the lung
(Scarpelli, 1967), acts as an internal standard reducing the effects
of amniotic fluid volume on the lecithin concentration.

Standardisation of the test procedure is important and failure to do
this leads to unsatisfactory results, and the influence upon the results
of the preparation and storage of amniotic fluids prior to processing
has been examined extensively by Wagstaff et al (1974); of particular
importance are the duration and force of centrifugation used to remove
the particulate matter from the fluid. Gluck and his colleagues (1974)
have reviewed various aspects of the technique and as already reported
(Results, 3.5.14) recognised the need for acetone precipitation. They
established an L/S ratio of 2.0 as the critical value above which RDS
rarely occurred. A review of the world literature (Harvey, 1975)
found 25 papers in which delivery had been effected within 72 hours of
amniocentesis, and the L/S ratio had been performed as originally
described by Gluck (1974). In a total of 2524 patients, ratios greater
than 2.0 were associated with an incidence of RDS of 2.2%. Ratios of
below 2.0 were associated with an incidence of 56.5%.

The results reported here, using the technique described by Gluck and
a critical L/S ratio of 2.0 substantiates the remarkable predictive
accuracy of the L/S ratio test in confirming fetal lung maturation/

(Results, 3.1.6). The much less satisfactory predictive accuracy

(25%) of ratios below the critical value for the method is also

confirmed. The incidence of neonatal RDS was lower than the 56.5%

incidence reported in the review (Harvey, 1975). The reason for the

lower incidence of neonatal RDS when the L/S ratio was less than 2.0

could be that the samples which were analysed in this series were

collected not only by amniocentesis but also by vaginal drainage, a

route known to be associated with unreliability in the determination

of the L/S ratio (Dombroski et al, 1981).

Many of these drainage samples were collected when the mother had

spontaneous premature ruptured membranes. The data reported here show

that even at less than 35 weeks of gestation, an L/S *ratio* greater than or

equal to 2.0 indicates that RDS will be unlikely, making it a most

useful test in patients in whom premature delivery may be indicated by

signs of maternal or fetal jeopardy. Conversely many neonates did not

develop RDS even with an amniotic fluid L/S *ratio* less than 2.0 so that the

test is a poor predictor of lung immaturity. However, the overall

effectiveness of the L/S ratio has resulted in the test becoming an

important aid in obstetric decision-making.

Although good prediction of neonatal respiratory problems seems

feasible using the L/S ratio, blood admixed with amniotic fluid is

most often associated with a false elevation in L/S ratio (Wagstaff,

1974; Gluck, 1974), although the opposite effect has been observed

(Spellacy, 1972). The L/S ratio of red blood cells is 1.5 (Ansell and

Hawthorne, 1973) and plasma has an L/S ratio of 6.0 (Ansell and

Hawthorne, 1973). It is the high L/S ratio in plasma which would result

in the elevation of the L/S ratio in amniotic fluid contaminated by/

blood resulting merely in an unreliable L/S ratio. Meconium has also been found to elevate the L/S ratio (Wagstaff, 1974) although again the converse opinion has been stated (Spellacy, 1972).

The effect of contamination on the amniotic fluid L/S ratio and the uncertainty of the predictive value when the L/S ratio is below 2.0 has led to the development of other tests of fetal lung maturity.

4.3   The Lung Phospholipid Profile

The value of 2D t.l.c. in analysing amniotic fluid phospholipids has been reported by Kulovich et al (1979) to be useful in determining fetal lung maturity. A number of different phospholipids, apart from lecithin and sphingomyelin, are to be found in the amniotic fluid and are thought to originate in the fetal lung (Pfleger et al, 1971). Together with lecithin and sphingomyelin they comprise the lung phospholipid profile and have been identified as phosphatidylinositol, phosphatidylglycerol, phosphatidylethanolamine and phosphatidylserine. Hallman et al (1976) consider phosphatidylinositol and phosphatidylglycerol to be of significance in terms of fetal lung maturation, phosphatidylglycerol in particular being important in improving surfactant efficiency. Gluck et al (1979) demonstrated that phosphatidylglycerol first appeared at 35 to 36 weeks gestation, its concentration rising steadily towards term.

In agreement with Gluck and his colleagues (1979) the results in this thesis indicate that phosphatidylglycerol is a particularly important indication of fetal lung maturity and is a critical component of the/

136

lung surfactant. About 50% of the amniotic fluid specimens collected
at gestational ages of less than 35 weeks have phosphatidylglycerol
present (Results, 3.1.2), the earliest gestational age at which
phosphatidylglycerol appeared being 26 weeks. The patient had a
premature rupture of membranes and delivered a boy weighing 0.8 kg
within 72 hours of sample collection, the baby not developing RDS.
In contrast, an amniotic fluid sample collected by vaginal drainage
within 72 hours of delivery from a patient at 35 weeks of gestation
contained no phosphatidylglycerol and the patient delivered a baby
boy weighing 2.51 kg less than 72 hours after the analysis of amniotic
fluid. This baby, weighing 2.51 kg, developed severe RDS and died.
It is this individual variation in the timing of fetal lung
maturation which emphasises the usefulness of an objective, specific
indicator of fetal lung maturity.

The L/S ratio combined with the detection of phosphatidylglycerol
proved to be of great predictive value in assessing fetal lung status.
Quantitation of phosphatidylglycerol appeared to be an unnecessary
refinement since the mere presence of this phospholipid in the amniotic
fluid regardless of the L/S ratio accurately predicts lung maturity
(Results, 3.1.3). By relying on the presence of phosphatidylglycerol
in the amniotic fluid whenever the L/S ratio was less than the critical
value the only instances of unpredicted RDS would have been in three
small babies whose immediate delivery was deemed essential because of
obstetric complications, a false positive prediction rate by
phosphatidylglycerol of 0.61%. In contrast when phosphatidylglycerol
was absent and the L/S ratio was less than 2.0, 80.8% of the babies
developed RDS. By excluding from consideration the two mothers/

treated by glucocorticoid therapy and the patient with prolonged
spontaneous membrane rupture, on the grounds that these factors may
accelerate fetal lung maturation (Howie et al, 1977), the incidence of
RDS when phosphatidylglycerol was absent is increased to 24 out of 26
babies (92.3%), virtually the same as the 93% incidence by Kulovich
et al (1979). In practical terms an amniotic fluid L/S ratio below
2.0 with no phosphatidylglycerol therefore indicates the near
certainty of RDS if delivery cannot be postponed. On the other hand,
knowledge that phosphatidylglycerol is present despite an immature L/S
ratio should encourage immediate delivery of even a very small preterm
fetus judged to be at very serious risk in utero.

The lung phospholipid profile is also of use when the amniotic fluid is
contaminated with blood or meconium or when the fluid has been
collected by vaginal drainage. The reason is that phosphatidylglycerol
originates specifically from the lung (Kulovich et al, 1979) and
phosphatidylglycerol was absent in plasma and in only negligible
amounts in the red blood cells (Ansell and Hawthorne, 1973). The
presence of phosphatidylglycerol in amniotic fluid therefore indicates,
more reliably than the L/S ratio, fetal lung maturity regardless of
the state of the amniotic fluid. In contrast, lecithin concentration
and the L/S ratio would be changed in the case of blood stained
amniotic fluid.

4.4   Palmitic Acid Content of Acetone Precipitable Amniotic Fluid
Lipids

Other workers have reported the practicability of using palmitic acid/

concentration to assess fetal lung maturity (Ekelund et al, 1973; Warren et al, 1973; Arvidson et al, 1972). In babies with RDS Ekelund et al (1973) found a significantly lower level of palmitic acid in amniotic fluid fatty acid residues. The amount of palmitic acid was found to increase with gestational age (Frantz et al, 1975; Ekelund et al, 1973; Arvidson et al, 1972).

The results in this thesis (Results, 3.1.11 to 3.1.16) are consistent with the findings of these workers, and suggest that analysis of the composition of the fatty acid residue could be of diagnostic value. However, such an analysis takes about four hours, no more than six specimens being processed daily. As with the determination of the lecithin concentration, the concentration of palmitic acid in amniotic fluid is invalidated by alterations in fluid volume (O'Neil, 1978; Shirar, 1975). To overcome this problem, these authors used stearic acid as a reference substance to palmitic acid. The overall predictive accuracy of the palmitic acid/stearic acid ratio (83.78%) (Results, 3.1.16) is better than the palmitic acid concentration (67.57%) (Results, 3.1.14) alone. However, the percentage of palmitic acid in the total lipid fatty acid residues gives the best predictive accuracy (97.3%) (Results, 3.1.12). Possibly this is because the percentage of palmitic acid in total fatty acid residues of the amniotic fluid takes all the unsaturated fatty acid residues into consideration, allowing for changes in the relative fatty acid residue composition of amniotic fluid phospholipids as well as changes in fluid volume. These changes may occur because the other fatty acids may act as substrates in the manufacture of the surfactant phospholipids (van Golde, 1976). It is possible that the palmitic acid/stearic acid ratio is affected/

by such changes to the fatty acid residue composition. The results demonstrate that, although numbers are small, the palmitic acid concentration, the proportion of palmitic acid and the palmitic acid/ stearic acid ratio were all good predictors of lung maturity.

4.5   Optical Density

Measurement of optical density at 650 nm has been compared with the L/S ratio and 92% of the results were in agreement on amniotic fluids collected by amniocentesis (Sbarra et al, 1978). At 650 nm an optical density reading of 0.15 or above is said to be equivalent to an L/S ratio of 2.0 or above and therefore indicates fetal lung maturity. The method has also been recommended by Copeland et al (1978) as being highly accurate and extremely useful for laboratories ill-equipped to perform the L/S ratio. Hill et al (1979) suggested that it might prove a useful screening technique and that by adopting a critical value greater than 0.15, in an attempt to exclude any false positives, a laboratory might significantly reduce its work load.

The results reported here agree with those of others, that the test acts as a simple, reasonably reliable screening technique. An amniotic fluid optical density above the critical value was rarely associated with RDS, whilst below the critical value 37.0% of the babies developed RDS. Therefore, as with most other tests of fetal lung maturity the much less satisfactory predictive accuracy when the result is below the critical value is again confirmed.

Arias et al (1978), however, did not find the test reliable, and encountered cases of RDS when the amniotic fluid specimens had optical/

density values greater than the critical value.

Reported studies have only related the optical density measurements
to L/S ratios, whilst it might have been more realistic to correlate
it with gestational age. The results (3. 4.7) demonstrated that the
optical density measurements correlate~poorly~linearly with the L/S ratios
(r = 0.40, P < 0.001), and as with the L/S ratio the optical density
also has an exponential correlation with gestational age (r = 0.66,
P < 0.001) (Results, 3.. 2.1). Therefore, the optical density clearly
does correspond to the L/S ratio.

4.6   The Bubble Stability Test

The basis of this test, originally described in 1972 by Clements et al
is that the surfactant material from the lung alveoli reduces the
surface tension of the amniotic fluid. It has been suggested that the
shake test is best used as a screening test because when it is positive
RDS is rare. The results of this simple test obtained from other
laboratories were reviewed by Harvey et al (1978) and when delivery
was within 72 hours of amniocentesis, a positive test indicated a risk
to the neonate of developing RDS of 0.65%. When the test was negative
Harvey's review showed that about 50% of the babies develop RDS. The
results reported (Results, 3.2.4) indicate a risk to the neonate of
0.85% when the test is positive. When the results were negative 50.0%
of the babies developed RDS. Therefore these results confirm that the
test is most useful when it is positive.

Unfortunately, blood and meconium will interfere with the validity of
the test and its value in estimating fetal lung maturity from amniotic/

fluid collected by vaginal drainage is uncertain.

Attempts to improve the predictive accuracy of the test have been made. When the quantity of bubbles remaining following agitation was used, so that an incomplete ring did not necessarily represent a negative test, a totally negative test (i.e. no bubbles in any tube) was associated with a 79% incidence of RDS (Schleuter et al, 1979), a considerable improvement in prediction.

4.7   The Separation of Phospholipids by h.p.l.c.

Despite the success of t.l.c. for the separation and measurement of the amniotic fluid phospholipids of interest in fetal lung maturity, the application of h.p.l.c. to lung maturity studies has as yet resulted in very limited progress.

Jungalwala et al (1976) described a technique using u.v. absorption at 203 nm and h.p.l.c. for the separation and quantitation of lecithin and sphingomyelin which has been applied to amniotic fluid to provide estimation of the lecithin/sphingomyelin ratio, but as yet no method has been described which gives, in one chromatogram, separation and quantitation of phosphatidylethanolamine, phosphatidylglycerol, phosphatidylinositol, phosphatidylserine, lecithin, sphingomyelin and lysolecithin.

In order to use the kind of solvents used widely in the t.l.c. of phospholipids, a means of detection different from u.v. absorption was necessary and in this thesis the results from the use of differential/

refractometry as a means of detection and quantitation of amniotic fluid phospholipids separated by h.p.l.c. are shown (Results, 3.3).

The h.p.l.c. method provides a comparable technique for determining clinically important amniotic fluid phospholipids. It takes only two hours compared with up to eight hours when quantitation by phospholipid phosphorus method following 2D t.l.c. is performed. However, against this advantage must be placed the fact that many samples were wasted during analysis by h.p.l.c. because of baseline drift, noise, or peak fusion due to column failure. Further, a great deal of technical time was expended in solving these various problems. As a clinical technique, the h.p.l.c. apparatus was too unreliable to be acceptable. Further, it would seem that quantitation of phospholipids such as phosphatidylglycerol is not clinically necessary. However, the technique may have a potential research role since it provides a more rapid method of quantitation than that possible by the phosphate analysis technique.

U.v. absorption, as a method of detection, would be desirable, being devoid of the problems of variable sensitivity, noisy baseline at highest sensitivities, susceptibility to temperature fluctuations and draughts, which arise during refractometric detection. However, no u.v. transparent solvent mixture has been devised which would carry out the separation of the amniotic fluid phospholipids. Until this is achieved, a very sensitive, clinically reliable h.p.l.c. method appears elusive since the detection and quantitation of the separated phospholipids in a sufficiently sensitive and reliable manner for routine clinical use appears not to be possible by refractometry.

4.8   The Effects of Acetone Precipitation on Amniotic Fluid Lipids

The effect of acetone precipitation at $0^{\circ}C$ on the amniotic fluid L/S ratio is still a controversial subject, and opinions differ concerning the effect of this step in the precipitation of surface-active lipids. Further, there is controversy surrounding the role of acetone precipitation in improving the predictive ability of the L/S ratio determination. Gluck et al (1972) reported that ice-cold acetone precipitated surface-active lecithin. These authors also postulated a complete precipitation of sphingomyelin with cold acetone. Olson (1975); Smith (1978) and Torday (1979) produced evidence suggesting that this step could be omitted from the analysis of the lipids because they found that the L/S ratio was unchanged by cold acetone precipitation, while other authors have recommended that acetone precipitation be retained as a step in the L/S ratio procedure as it improves the reliability of the method (Gluck et al, 1974; Parkinson and Harvey, 1973; Wagstaff et al, 1974; Jackson et al, 1975; Renney et al, 1976).

This thesis partly involved a study of the effect of cold acetone precipitation on amniotic fluid lipids and omission of this step resulted in an L/S ratio about twice that obtained when acetone precipitation was included, because most of the sphingomyelin and only 50% of the lecithin is precipitated by cold acetone. Thus these results confirm Gluck's postulate of an almost complete precipitation of sphingomyelin and they disagree with those authors who report an unchanged L/S ratio after acetone treatment. In contrast with the fact that 50% of the lecithin is acetone precipitable, the indications/

144

are that, as with sphingomyelin, phosphatidylglycerol and phosphatidylinositol are almost completely precipitated. It appears that by using acetone and reducing the amount of lecithin, the predictive accuracy of the L/S ratio technique is improved (Results, 3.5.14) and these results are in agreement with the authors who report that inaccuracies arise in the prediction of fetal lung maturity if the acetone step is omitted.

It can be seen from figure 3.21 that the higher the lecithin concentration in the amniotic fluid the greater the decrease in the L/S ratio in the acetone precipitable fraction. Controversial results could therefore be obtained if the majority of samples analysed had L/S ratios in the region of less than 3.0 with only a few with higher L/S ratios when the change in the L/S ratio in the acetone precipitable fraction is more dramatic. Thus, correlation analysis of the results obtained show a trend which would not be obvious when comparing an average change in the L/S ratio on acetone precipitation. However, it is not possible to tell from the results of those authors who report an unchanged L/S ratio after acetone precipitation what range of L/S ratios they were analysing, or what statistical analysis they used.

The question of whether the acetone precipitation selectively precipitates 1,2 dipalmitoyllecithin cannot be answered unequivocally from the results reported in this thesis (Results, 3.5) because this species of lecithin was not determined specifically. However, the total lipid fraction, the acetone precipitable fraction and the acetone soluble fraction were all analysed and the fatty acid composition determined. The results (3.5.8) indicate that the acetone precipitation/

of the lipids in amniotic fluid does enrich the fraction with
saturated fatty acids, although not all are precipitated (Results,
3.5.6). In addition, there are unsaturated fatty acids in the acetone
precipitated fraction. As can be seen in figure 3.26, the
concentration of the fatty acids in the soluble fractions added to that in
the insoluble fraction, results in a value almost equal to the
concentration of the fatty acids in the total lipid fraction,
indicating the reliability of the technique.

The use of acetone precipitation to separate phosphatides from neutral
lipids (Kates, 1972) depends upon the general insolubility in cold
acetone of most phosphatides, particularly acid phosphatides. It is
interesting that an almost complete precipitation of the acidic
phospholipids, phosphatidylglycerol and phosphatidylinositol was found
(Results, 3.5.4 and 3.5.5). The procedure described by Kates (1972)
involves precipitation of acidic phospholipids by acetone in the
presence of magnesium ions, but the extent of magnesium salt formation
by the acidic lipids in the procedures used is not known. On the
other hand, in most papers acetone precipitation is considered to be
selective not for acidic phospholipids but for lipids containing
saturated fatty acids or a preponderance of saturated fatty acids.
The selective precipitation of saturated free fatty acids has been
extensively reviewed by Brown et al, 1955.

One of the main problems in the acetone precipitation of fatty acids
is that the solubility of each component of a mixture in acetone can
be affected by the other components of the solution. Therefore the

presence of unsaturated fatty acids may for example increase the solubility of other fatty acids in a mixture. The precipitation of phospholipids which contain two fatty acid residues which may or may not be both saturated is thus complex.

CONCLUSIONS

(1) Fetal lung status cannot be inferred from gestational age as there is a great individual variation.

(2) The acetone precipitation step selectively precipitates the acidic phospholipids and sphingomyelin. Only 50% of the lecithin is precipitated. Although the lipid extract was enriched with saturated fatty acids after acetone precipitation not all the unsaturated fatty acids were removed, nor were the saturated fatty acids completely precipitated. The acetone precipitation step should be included in the determination of the L/S ratio as it improves the predictive reliability of this procedure.

(3) Separation of the phospholipids by h.p.l.c. proved to be too unreliable as a clinical tool for assessing fetal lung maturity but may have a possible role as a research technique.

(4) The assessment of some of the various tests available for predicting fetal lung maturity has confirmed that with most tests the problem is not in identifying mature fetal lungs but rather in confirming immature fetal lungs (table 4.1). However, use of the lung phospholipid profile, the main features of which are the L/S ratio and the presence or absence of phosphatidylglycerol, was shown to be a reliable technique for predicting fetal lung status (table 4.1). If phosphatidylglycerol is found to be present there is very little chance that the baby will develop RDS. On the other hand, if phosphatidylglycerol is absent from the amniotic fluid it is very likely that the baby will develop RDS (table 4.1). In addition, this technique maintains its

148

Table 4.1 The sensitivity, specificity, predictive value and the predictive accuracy of the various tests assessed

The sensitivity, specificity, predictive value and the predictive accuracy of the various tests assessed were calculated (Methods, 2.12).

	Immature Result		Mature Result		Predictive Accuracy (%)
Test	Sensitivity (%)	Predictive Value (%)	Specificity (%)	Predictive Value (%)	
L/S and PG	100	88.0	99.3	100	98.8
L/S (2D t.l.c.)	100	23.6	83.6	100	84.4
L/S (1D t.l.c.)	100	25.0	71.4	100	73.9
L/S (2D t.l.c. total lipid)	25.0	33.3	89.5	85	48.7
PG	88.0	84.6	99.2	99.4	98.7
Lecithin Concentration	100	37.5	54.6	100	64.3
% Palmitic Acid	75.0	75.0	97.0	97	94.6
Palmitic Acid Concentration	100	25.0	63.6	100	67.6
Palmitic Acid/ Stearic Acid Ratio	75.0	37.5	84.9	96.5	83.8
Optical Density	100	26.7	86.4	100	87.1
Bubble Stability Test	95.0	50.0	85.9	99.2	87.1

predictive accuracy even when the samples are collected by vaginal drainage and contaminated with blood or meconium.

For laboratories with ready access to t.l.c. techniques the determination of the fetal lung phospholipid profile would be highly recommended for an accurate prediction of fetal lung status.

BIBLIOGRAPHY

ANSELL, G.B., DAWSON, R.M.C. and HAWTHORNE, J.N. (1973). Form and Function of Phospholipids. Elsevier Publishing Co., Amsterdam.

ARIAS, F., ANDRINOPOULOS, G. and PINEDA, J. (1978). Obstet. Gynecol. 51; 152-155.

ARVIDSON, G., EKELUND, H., ASTEDT, B. (1972). Acta Obstet. Gynecol. Scand. 51:71-75.

AVERY, M.E., and MEAD, J. (1959). Amer. J. Dis. Chil. 97; 517-523.

BARTLETT, G.R. (1959). J. Biol. Chem. 234; 466-467.

BEUTLER, H.O., MICHAL, G., DIEDRICH, K. (1980). The Boehringer Corporation (LONDON) Ltd.

BEAGWANANI, S.G., and FAHMY, D., and TURNBULL, A.C. (1972). Lancet 1; 159-162.

BIEZENSKI, J.J., POMERANCE, W. and GOODMAN, J. (1968). Am. J. Obstet. Gynecol 102; 853-861.

BIEZENSKI, J.J. (1972). Obstet. Gynecol. 41; 825-828.

BORER, R.C. and GLUCK, L. (1971). Laboratory Schedule, Department of Paediatrics, University of California, San Diego, U.S.A.

BROWN, J.B. Progress Chemistry, Fats, Other Lipids. (1955). Vol. 3. 57-94

CLEMENTS, J.A. et al. (1972). N. Engl. J. Med., 286; 1077-1081.

CHRISTIE, W.W. (1973). Lipid Analysis. Pergamon Press, Oxford.

COPELAND, W., STEMPEL., LOTT, J.A. et al. (1978). Am. J. Obstet. Gynecol. 130; 225-226.

DONALD, I.R., FREEMAN, R.K., GOEBELSMANN, U., CHAN, W.C. and NAKAMURA, R.M. (1973). Amer. J. Obstet. Gynecol. 115; 547-552.

DOMBROSKI,R.A., MACKENNA, J. and BRAME, R.G. (1981). Amer. J. Obstet. Gynecol. 140; 461-464.

EKELUND, L., ARVIDSON, G. and ASTEDT, B. (1973). J. Obstet. Gynaec. Brit. Cwlth. 80; 912-917.

....../

ERBLAND, J.F. and MARINETTI, G.V. (1965). Biochim. Biophys. Acta 106; 128-138.

FALCONER, G.F., HODGE, J.S. and GADD, R.L. (1973). B.M.J. 2; 689-691.

FRANTZ, T., LINDBACK, T., SKJAERAASEN, J. and GRAVEN, S. (1975). Acta Obstet. Gynec. Scand. 54; 33-39.

GIKAS, E.G., KING, R.J., MESCHER, E.J., PLATZKER, A.G.G., et al. (1977). Am. Rev. Resp. Dis. 115; 587-593.

GLUCK, L., KULOVICH, M.V., BORER, R.C., BRENNER, P.H., ANDERSON, G.G. and SPELLACY, W.N. (1971). Am. J. Obstet. Gynecol. 109; 440-445.

GLUCK, L., KULOVICH, M.V., EIDELMAN, A.I., CORDERO, L. and KHAZIN, A.F. (1972). Pediatr. Research 6; 81-99.

GLUCK, L., KULOVICH, M.V. (1973). Amer. J. Obstet. Gynecol. 115; 539-546.

GLUCK, L., KULOVICH, M.V., BORER, R.C. and KEIDEL, W.N. (1974). Amer. J. Obstet. Gynecol. 120; 142-155.

GOERKE, J. (1974). Biochim. Biophys. Acta, 344; 241-261.

GURR, M.I. and JAMES, A.T. (1980). Lipid Biochemistry, Chapman and Hall, London.

HALLMAN, M., KULOVICH, M.V., KIRKPATRICK, E., SUGARMAN, R.G. and GLUCK, L. (1976). Amer. J. Obstet. Gynecol. 125; 613-617.

HARVEY, D., PARKINSON, C.E., and CAMPBELL, S. (1975). Lancet 1; 42.

HARVEY, D., PARKINSON, C.E. (1981). Chapter 12 in Laboratory Investigation of Fetal Disease; Barson, John Wright and Sons Ltd.

HILL, C.M., HARKES, A., DONNAI, P., FERGUSON, A. and TINDALL, V.R. (1979). Brit. J. Obstet. Gynaecol. 86; 773-776.

HOWIE, R.N. and LIGGINS, G.C. (1977). In Pre-Term Labour, 5th Study Group of Royal College of Obstetricians & Gynaecologists p. 281-289.

JACKSON R.W. ANDERSON G.D. and HELD B. (1975). Amer. J. Obstet. Gynecol. 121; 1095-1099.

JUNGALWALA, F.B., EVAN, J.E. and McCLUER, R.E. (1976). Biochem. J. 155, 55-60.

KANKARE, P. and SUOVANIEMI, O. (1971). J. Chromatogr. 62, 485-486.

KATES , M. (1975). Techniques of Lipidology; Isolation, Analysis and Identification of Lipids, American Elsevier, New York.

KENNEDY, E.P. (1961). Fed. Proc. 20; 934-940.

KING, R.J. and CLEMENTS, J.A. (1972). Am. J. Physiol. 223; 727-733.

KULOVICH, M.V., HALLMAN, M.B. and GLUCK, L. (1979). Amer. J. Obstet. Gynecol. 135; 57-63.

LANDS, W.E.M. (1960). J. Biol. Chem; 2233-2237, 235.

MACKLIN, C.C. (1954). Lancet 266; 1099-1104, 1954.

MORLEY, C.J., MILLER, N., BANGHAM, A.D., et al. (1981). Lancet, 1; 64-68.

NIDEN, A.H. (1967). Science 158, 1323-1324.

OBLADEN, M., MERRITT, T.A. and GLUCK, L. (1979). Amer. J. Obstet. Gynecol. 135; 1079-1085.

OLSON, E.B., et al. (1975). Pediatr. Res., 9; 65-69.

O'NEIL, G.J., DAVIES, I.J. and SIU, J. (1978). Am. J. of Obs. & Gyne. 132; 519-523.

PARKINSON, C.E. and HARVEY, D.R. (1973). J. Obstet. Gynecol. Br. Cwlth., 80; 406-411.

PATTLE, R.E. (1955). Nature 175; 1125-1126.

PFLEGER, R.C. and THOMAS, H.G. (1971). Arch. Int. Med. 127; 863.

QUINN, P.J. (1981). Progress Biophys. Molec Biol. 38; 91-104.

PENNEY, L.L., HAGERMAN, D.D. and SEI, C.A. (1976). Clin. Chem. 22 / 5; 681-682.

SBARRA, A.J., MICHLEWITZ, H., SELVARAJ, R.J., et al. (1976). Obstet. Gynecol. 48; 613-615.

SBARRA, A.J., MICHLEWITZ, H., SELVARAJ, R.J., et al. (1977). Obstet. Gynecol. 50; 723-724.

SBARRA, A.J., SELVARAJ, R.J., CETRULO, C.L., et al. (1978). Am. J. Obstet. Gynecol. 130; 788-790.

SCARPELLI, E.M. (1967). Ped. 40; 951-961.

SCHLUETER, M.A., PHIBBS, R.H., CREASY, R.K., et al. (1979). Am. J. Obstet. Gynecol. 134; 761-767.

SOURAR, A., VIELH, J.O., ALCINDOR, L.G. and GAUTRAY, J.P. (1975).
Amer. J. Obstet. Gynecol. 121; 653-663.

SMITH, D.E. (1978). Can. J. Med. Tech. 40; 97-108.

SPELLACY, W.N., BUHI, W.C., CRUZ, A.C., et al. (1979). Am. J. Obstet.
Gynecol. 134, 528-531.

TORDAY, J., CARSON, L. and LAWSON, E.E. (1979). N. Engl. J. Med. 301;
1013-1018.

VAN GOLDE, L.M.G. (1976). Am. Rev. of Resp. Disease 114; 977-1000.

VAN GRAFF, J. DE and GUNSTON, K.D. (1978). S. African Med. J. 54;
1145-6.

VAN HEUSDEN, G.P.H., VIANEN, G.M. and VAN DEN BOSCH, H. (1980). J.
Biol. Chem. 255; 9312-9318.

WAGSTAFF, T.I., WHYLEY, G.A. and FREEDMAN, G. (1974). J. Obstet.
Gynecol. Brit. Cwlth. 81; 264-277.

WARREN, C., ALLEN, J.T. and HOLTON, J.B. (1973). Clin. Chim. Acta. 44;
457-459.

WARREN, C., HOLTON, J.B. and ALLEN, J.T. (1974). B.M.J. 1; 94-96.

WHITFIELD, C.R., SPROULE, W.B. (1974). Br. J. Hosp. Med. 12; 678-690.

WHITTLE, M.J., WILSON, A.I., WHITFIELD, C.R., PATON, R.D., LOGAN, R.W.
(1981). British Medical Journal, 282; 428-430.