CTP:CHOLINE PHOSPHATE CYTIDYLYLTRANSFERASE IN HUMAN LUNG

A thesis presented for the degree of

Doctor of Philosophy

by

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ABSTRACT

FACULTY OF MEDICINE

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Human lung cytidylyltransferase was found associated with both 'soluble' (S_{100}) and membrane-rich particulate (P_{100}) fractions of Tris-buffered saline homogenates. S_{100} enzyme activities in 15 - 16 week fetal and adult human lungs represented a constant proportion of overall recovery, (66.8 ± 4.8% vs 66.1 ± 7.5%, means ± standard error). A lack of support for the regulatory translocation of human lung cytidylyltransferase at these extremes of development was unable to rule out a transient change in distribution near term. Rat lung P_{100} enzyme increased from 31% to 40% between d18 gestation and term, d22, but a concomitant increase in total S_{100} cytidylyltransferase, measured in the presence of the lipid activator PG, questioned the physiological significance of the apparent translocation.

Cytidylyltransferase from human and rat lung S_{100} were resolved into a high molecular weight H form (>10^6 daltons) and a lower molecular weight L form (~ 200,000 daltons). Incubation of S_{100} at 37°C for 2 hours yielded insoluble, protein-rich aggregates which were strongly associated with rat H form cytidylyltransferase, while less strongly with the human H form. The principal, 43,000 dalton, protein in these aggregates was identified as a cytoplasmic actin on the basis of its properties, amino acid composition and Western blot analysis. The association of H form cytidylyltransferase with cytoskeletal F-actin containing fractions *in vitro* was disrupted by the detergent CHAPS, which was also able to release a portion of P_{100} enzyme. Separation of human S_{100} H and L form enzyme, by gel filtration or ultracentrifugation, revealed the presence of latent cytidylyltransferase, often as high as 3 fold, which questioned activity determinations in fresh S_{100}. Within the framework of an emerging concept of a highly ordered aqueous cytoplasm, the incorporation of these results suggested that a portion of human lung cytidylyltransferase might be cytoskeletally bound *in vivo*, as has been described for many enzymes or enzyme systems.

The use of conventional purification techniques, including affinity chromatography, with a view to testing these ideas in defined systems, met with little success. Low yields or highly unstable enzyme characterised many individual steps, especially where cytidylyltransferase was separated from F-actin enriched fractions. A number of triazine dyes screened as pseudoaffinity ligands revealed a rapid inhibition with Procion Green H-4G and a partial protection with MgCTP. Sepharose CL4B-immobilised Green H-4G bound cytidylyltransferase, but MgCTP was unable to effect elution. Increasing ionic strength eluted some activity but also inhibited enzyme irreversibly, while CHAPS at 1% released a maximum of only 18% bound enzyme and SDS PAGE revealed a relatively non-specific binding. The use of the dye-affinity matrix offered the potential of a useful purification step with partially purified enzyme if suitable elution conditions could be devised.

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For JESUS, without whose help none
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Postle, A.D., Hunt, A.N. and Normand, I.C.S. (1985) Biochem.Biophys.Acta. 837 , 305 - 313.

Hunt, A.N. and Postle, A.D. (1986) Biochem.Soc.Trans. 14 , 1279 - 1280.

CONTENTS

ABBREVIATIONS

ACC	Acetyl CoA carboxylase
CHAPS	3-(3-Cholamidopropyl) dimethylamino-1-propane sulphate
CK	Choline kinase
DPPC	1,2 Dipalmitoyl-sn-glycerophosphocholine
DTT	Dithiothreitol
HK	Hexokinase
MTL	Microtrabecular lattice
P_{100}	10,000 x g x 10 minute - 100,000 x g x 60 minute pellet
PAGE	Polyacrylamide gel electrophoresis
PAP	Phosphatidate phosphohydrolase
PC	Phosphatidylcholine
PEG	Polyethyleneglycol
PFK	Phosphofructokinase
PG	Phosphatidylglycerol
PMSF	Phenylmethylsulphonyl fluoride
S_{100}	100,000 x g x 60 minute supernatant
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean

CHAPTER 1

INTRODUCTION

1.1 Structure and functions of phospholipids

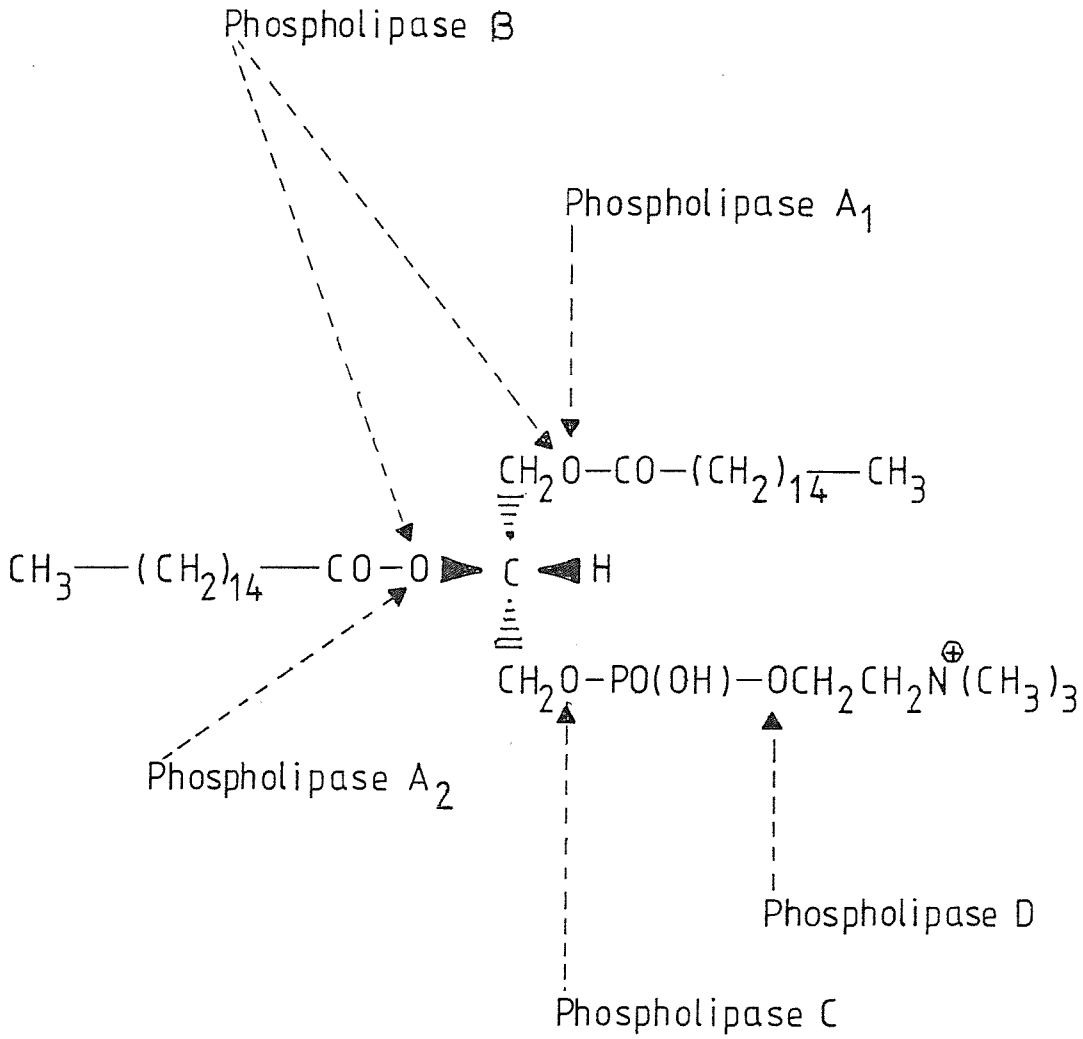
Phospholipids are a ubiquitous, heterogenous collection of lipids chemically characterised as mixed esters of fatty acid(s) and phosphoric acid with alcohols. Glycerophospholipids have glycerol as their alcohol and are the most abundant of the phospholipids present in every cell. Subdivision into classes is made according to the organic base, amino acid or alcohol at the sn-3 position. Choline, ethanolamine, serine, glycerol or myo-inositol produce phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylglycerol (PG) and phosphatidylinositol (PI), respectively. Molecular variation is provided by different combinations of fatty acids which may be saturated (no double bonds) or unsaturated (one or more double bonds).

1.1.1 Phospholipid functions

Phospholipids perform a variety of roles in mammalian tissues. Their quantitatively most important function is structural, and phospholipids are the major component of biological membranes. PC, the most abundant mammalian phospholipid has also been found in high concentration, 3-4mM, in bile (Kawamoto et al 1980). It is the principle phospholipid associated with plasma lipoprotein being particularly enriched in high density lipoproteins (HDL). This plasma PC provides the fatty acyl component of plasma cholesterol ester in a reaction catalysed by Lecithin cholesterol acyltransferase (LCAT).

Messenger functions and some signal transduction functions are also mediated by phospholipids. One molecular species of PC, incorporating an ether linkage in place of an ester, 1-alkyl-2-acyl PC is a potent biological agent responsible for changing platelet shape, causing aggregation and the liberation of 5-hydroxytryptamine. This platelet activating factor is active at concentrations below 10^{-9}M (Blank et al 1979). Arachadonic acid, precursor of prostaglandins,leukotrienes, thromboxanes and related compounds, is usually found esterified to the sn-2 position (section 1.1.2) of cellular phospholipid. Under the action of phospholipase A_2, fig 1.1, arachidonate may be released in response to an appropriate stimulus. Membrane-associated phosphatidylinositol, 4,5-bisphosphate is hydrolysed by a specific phospholipase C, fig 1.1, in a receptor mediated process to yield two intracellular messengers

Fig 1.1 The structure of dipalmitoyl-sn-glycerophosphocholine



(Berridge and Irvine 1984). One of these, diacylglycerol, activates protein kinase C while Inositol (1,4,5) trisphosphate mobilises intracellular Ca^{++}. Another specific phospholipase C has recently been identified which acts on PC (Besteman et al 1986). PC is hydrolysed to diacylglycerol which may activate protein kinase C while simultaneously producing a rapid increase in the choline phosphate pool, which may also act as an intracellular messenger in an as yet unidentified manner (Besterman et al 1986).

A further specialised function of PC is as a major component of pulmonary surfactant, the lipid-protein mixture which lines the alveoli of the mammalian lung and maintains alveolar stability (Pattle 1955).

1.1.2 The Structure and Nomenclature of Phospholipids

Glycerophospholipids are routinely named according to the stereospecific numbering (sn) system proposed by Hirschman (Hirschman 1960) and recommended by the IUPAC-IUB Commission on Biochemical Nomenclature, preventing anomalous classifications inherent in D or L designations. Carbon atoms are numbered according to the system of priorities assigned under the R/S system of Cahn (Cahn et al 1956). In a Fisher projection of the substituted glycerol molecule, fig 1.1, the fatty acid linked to carbon 2 (C-2) is placed on the left and the top carbon designated C-1 and the bottom C-3. Numbering remains constant irrespective of hydroxyl substitution. Under this system the optically active glycerol phosphate from biological sources is designated sn-glycerol-3-phosphate.

The structure of 1,2 dipalmitoyl-sn-glycero-3-phosphorylcholine is shown in fig 1.1. The points of action of phospholipases A_1, A_2, B, C and D are shown. PC is a zwitterion, in that it bears both a negative and positive charge but is electrically neutral. The choline phosphate headgroup, which bears the charged groups, is hydrophilic while the acyl chains are hydrophobic. A consequence of this property is that it is relatively insoluble in aqueous media although most PCs can be dispersed into a micellular form. PC from most tissues possess an unsaturated fatty acid at the sn-2 position and either a saturated or unsaturated sn-1 fatty acid.

Table 1.1

Phospholipid compositions of rat tissues (%).

	Lung(a)	Liver(b)	Heart(c)	Kidney(b)
Phosphatidylcholine	46.6	48.2	40.9	34.3
Phosphatidylethanolamine	23.1	23.6	33.4	27.1
Phosphatidylserine	9.3	3.4	3.6	7.3
Phosphatidylinositol	4.0	8.3	4.2	5.9
Phosphatidic acid	0.3	0.3	0.2	0.3
Cardiolipin	-	4.5	12.6	6.5
Phosphatidylglycerol	2.2	0.3	1.1	0.3
Sphingomyelin	10.4	4.1	3.5	12.1
LysoPC	1.3	0.8	0.6	1.0
Others	2.6	6.4	-	-

Sources

(a) Baxter et al 1969

(b) Rouser et al 1969

(c) Simon and Rouser 1969

1.1.3 Phosphatidylcholine in lung Tissue

A comparison of phospholipid compositions of four rat tissues is shown in table 1.1. Phosphatidylcholine and phosphatidylethanolamine represent the major phospholipids from each tissue. Liver phospholipids are generally similar to lung with the exceptions that sphingomyelin and phosphatidylglycerol are lower and cardiolipin higher. Cardiolipin is also enriched in heart tissue. Little variation is apparent between liver and lung PC percentages, but at the level of individual molecular species a major difference is apparent. PC comprises a family of compounds with different acyl substitutions at the sn-1 and sn-2 positions of the glycerol backbone. Analysis of total fatty acids in lung PC has shown that the saturated palmitate (C16) at 60% (Naimark and Klass 1967) is the major fatty acid present. When the saturated stearate (C18), at 10.3%, is included it is obvious that a significant proportion of lung PC is of a disaturated form. Liver, by comparison, has only 4% of the PC as disaturated species (Shimojo et al 1974). The significance of the high disaturated PC species in lung lies in their surface tension lowering properties (section 1.2).

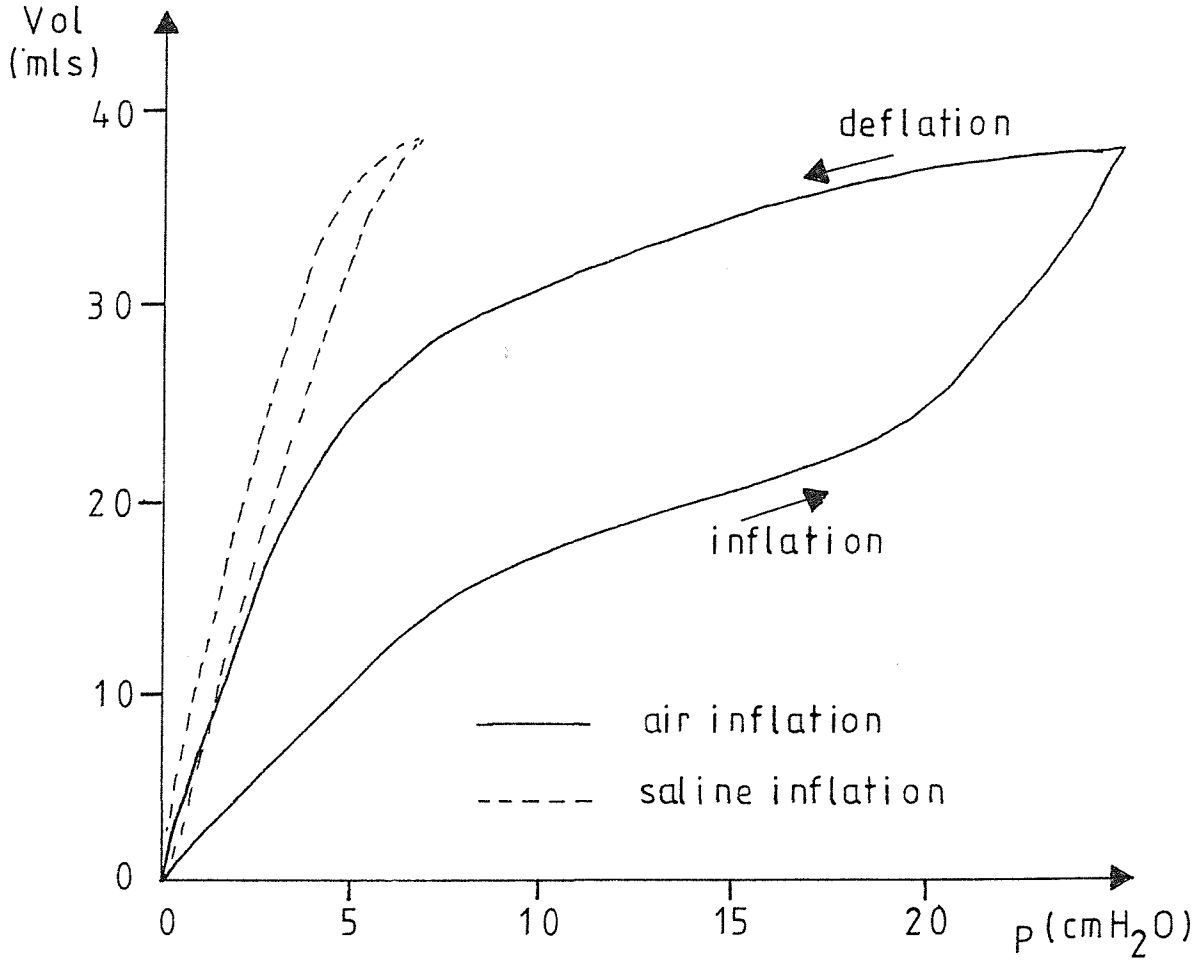
1.2 The Form and Function of Pulmonary Surfactant.

Pulmonary surfactant is a complex material consisting mainly of phospholipids and other lipids, but which also contains specific proteins. Dipalmitoyl phosphatidycholine (DPPC) is the major phospholipid present and largely responsible for its surface proporties (King 1982). The major clinical condition involving a defect in the surfactant system is respiratory distress syndrome (RDS) or hyaline membrane disease, which is due to lung immaturity with consequent insufficient surfactant (Avery and Mead 1959). A surfactant insufficiency is also seen in some adult lung diseases, particulary the Adult Respiratory Distress syndrome which may result from toxic chemical disruption of pulmonary surfactant.

1.2.1 Surface Tension in Lungs

When an excised mammalian lung is inflated with either air or saline, it shows a difference in derived static pressure-volume characteristics, fig 1.2. Saline inflation produces a ready, uniform

Fig 1.2 Static pressure/volume curves of newborn mini pig lungs (after Rufer 1981)



The p/v diagram of an excised mini pig lung at term(112 days) When compared to liquid,the inflation with air needs approximately 3 times higer pressure for the expansion of the lungs to the same total volume.

expansion and deflation, while inflation with air requires a greater initiating pressure, above which the lung expands readily to maximum volume. As air is allowed to leak from the system a hysteresis effect is noted. At any given pressure a greater volume of air is retained than is seen during inflation. The observed retractile forces have been interpreted as due in a large part to the presence of an air/liquid interface which results in a large 'surface tension' effect (von Neergaard 1929). The work done against surfact tension forces appears to constitute a major part of the effort required to inflate the lung. Differences in the pressure/volume relationship of the inflating and deflating lung show that the surface tension of the alveoli must change during the cycle. Air retention at the end expiratory volume suggest that surface tension is low with respect to saline. Surface tension effects at air/water interfaces are the result of the affinity of surface water molecules for each other and the bulk phase water. The extensive hydrogen bonding and van der Waals forces produce a surface pressure effect, which may be thought of as analogous to a 'skin' of water molecules. The operation of surface tension phenomena at the pulmonary air/liquid interfaces, in the absence of surfactant, creates a large contractile force along the surface of the alveoli.

As alveoli become smaller the contractile force increases as a consequence of surface tension effects described by Laplace's Law:

$$P = \frac{2\sigma}{R}$$

where P=pressure across the surface, σ =surface tension and R=radius. While alveoli are not exactly spherical, some variation of the equation applies. P varies inversely with alveolar radius and small diameter alveoli exert great contractile forces. If unchecked, collapse of small alveoli into larger airways would result, decreasing the area available for gas exchange. The presence of a surface active material, a pulmonary surfactant, was postulated to explain the difference in pressure/volume characteristics between the air- and fluid-filled lung (von Neergaard 1929).

1.2.2 The composition of Pulmonary Surfactant

The composition of mammalian and non-mammalian surfactants has been reviewed and found to be broadly similar (Sanders 1982). The fluid obtained from lung lavage varies from 53% to 93% lipid, with the remainder being primarily protein. Between 70% and 90% of the lipid is phospholipid, with the other lipid being neutral lipid, consisting mainly of di- and tri- acyl glycerols and cholesterol. The phospholipid composition of human lung surfactant obtained from lung lavage (Gilfillan et al 1983) is shown in table 1.2. PC (67.5%) comprises the major phospholipid, with 73.1% of this present as disaturated species. The second major phospholipid, PG represents 10% of phospholipid which is considerably higher than found in other tissues, table 1.1. PE, PS and sphingomeyelin, substantial components of lung tissue are present in low concentrations in surfactant preparations. PI is present and an inverse relationship between PI and PG concentrations has been shown to exist (Hallman and Gluck 1980). Disaturated PCs are not exclusive to surfactant being found in smaller concentrations in other non-pulmonary tissues (White 1973, Mason 1973). A recent report has revealed substantial enrichment of DPPC in rat eustachian tube (Wheeler et al 1984) where it may serve to maintain the integrity of the tube in the face of adverse surface tension effects.

Protein may comprise 47% of pulmonary surfactant (Sanders 1982) with particularly high concentrations in alveolar proteinosis (Sahu et al 1976). Two principle surfactant-associated non-serum proteins have been described. In addition a number of others have been identified by non-reducing PAGE which may be resolved into combinations of smaller proteins. The precise function of these proteins is unclear but they have been postulated as either surface active themselves or able to promote lipid adsorption to the air/alveolar interface.

Two dimensional SDS PAGE analysis of a diffuse surfactant-associated protein band of between 30,000 and 38,000 daltons has shown a group of glycosylated derivatives from a 26,000 dalton core protein (Flores et al 1985, Whitsett et al 1985(a)). The glycosylation appears to occur just prior to secretion (Whitsett et al 1985(b)). Sequences are known for the human gene and gene product (White et al 1985, Hawgood et al 1985) and it possesses some very hydrophobic regions, consistant with its proposed lipid interactions. The purified protein does not have surface active properties and has only a small effect on the promotion of DPPC

Table 1.2

Phospholipid composition of human lung lavage surfactant.
(from Gilfillan et al 1983)

Phospholipid	% Total phospholipid
Phosphatidylcholine	67.5
Saturated phosphatidylcholine	49.7
Unsaturated phosphatidylcholine	17.7
Phosphatidylglycerol	10.0
Phosphatidylethanolamine	5.3
Sphingomyelin	4.0
Phosphatidylserine	1.6
Phosphatidylinositol	3.6
Others	8.0
Percentage of total PC as saturated species	73.1

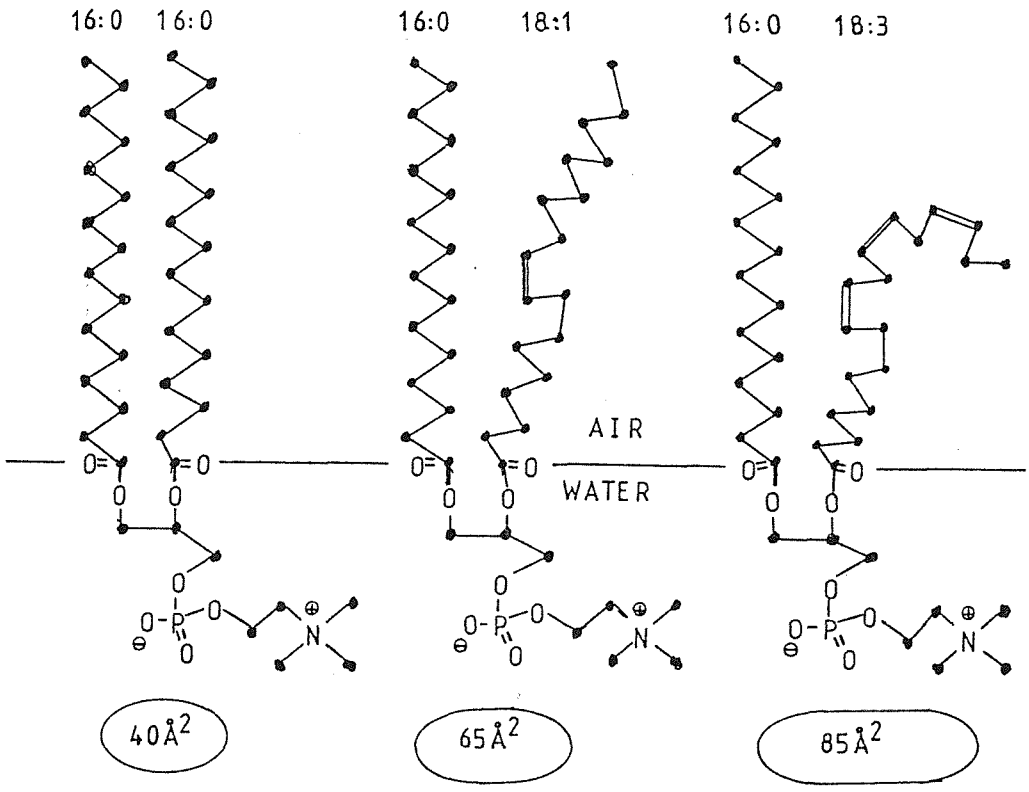


Fig 1.3 Average molecular cross-sectional areas of PC species at the air/water interface (after Possmayer 1981)

The average cross-sectional area occupied by individual molecular species of PC was calculated from the area occupied by a known quantity of each lipid at the air/liquid interface. The flexibility of the unsaturated C16 acyl groups permits a greater degree of compression before monolayer collapse. The introduction of double bonds into the acyl group at the sn2 position confer a rigidity which prevents the same amount of compression being achieved. Increasing unsaturation increases surface area occupation.

adsorption (Suzuki 1982).

A second, highly lipophillic, protein of between 6,000 and 14,000 daltons has been described, which remains tightly lipid bound during extraction (Phizackerly et al 1979). It is enriched in lamellar bodies of pig lung (Phizackerly et al 1979) and humanlung (Suzuki et al 1986) where the use of specific monoclonal antibodies has shown its presence in the alveoli and at the periphery of lamellar bodies. These antibodies do not cross-react with the 30,000-38,000 dalton glycoprotein implying that the smaller protein is not derived from the larger. This lipophillic protein promotes DPPC adsorption to their air/liquid interface in reconstituted surfactant systems (Suzuki 1982, Whitsett et al 1986, Yu and Possmayer 1986).

In our laboratories, human surfactant from bronchio-alveolar lavage has been shown to contain actin, in addition to other proteins, which we believe may be involved in the secretion process (Postle et al 1985).

1.2.3 The Mechanism of Action of Pulmonary Surfactant

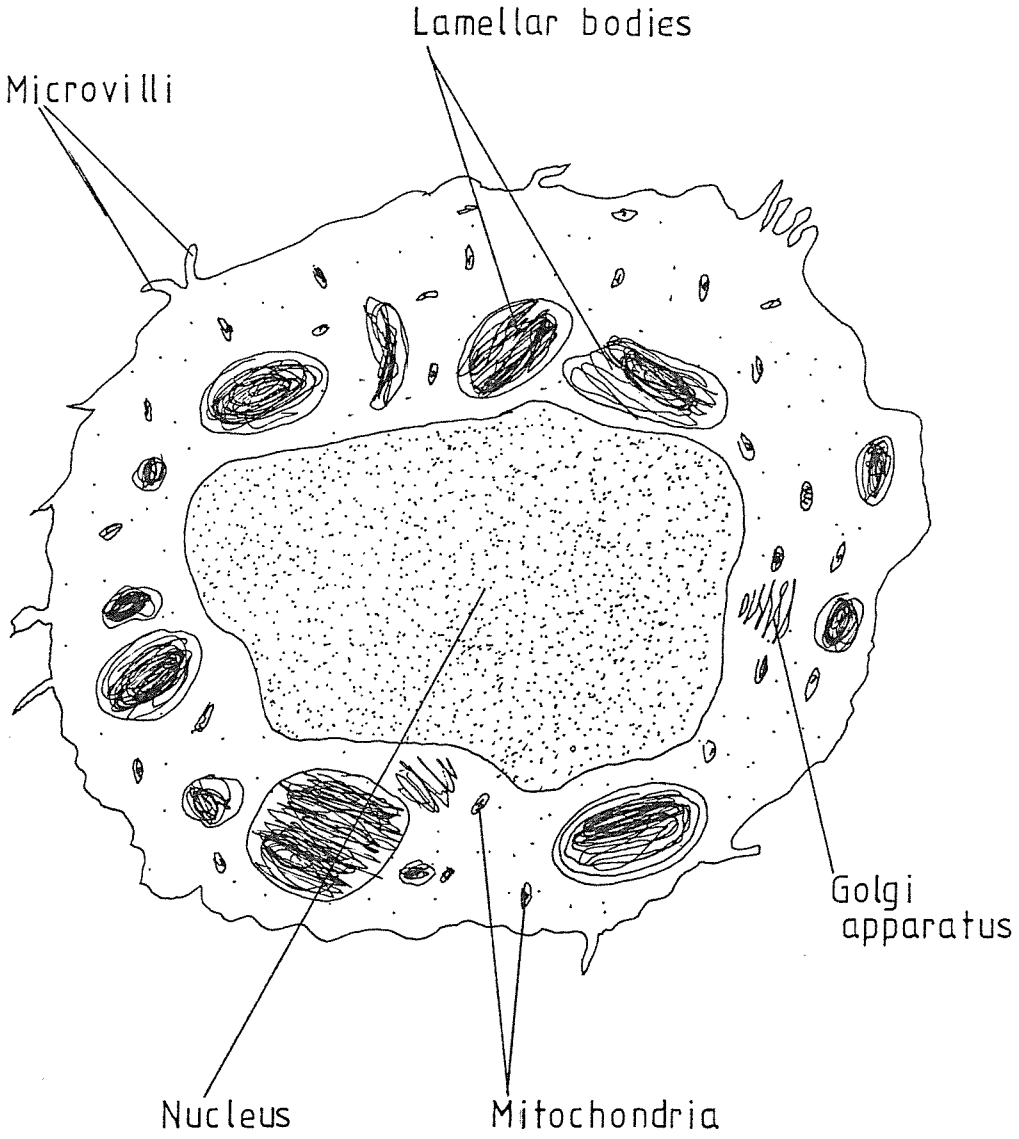
The surface tension lowering proporties of pulmonary surfactants are dependant on the zwitterionic nature of the surfactant phospholipids (section 1.1.2). The polar head-groups bind, and disrupt the interactions between, water molecules at the air/liquid surface, forming a monolayer. The hydrophobic fatty acids extend outwards away from the water surface. When the monolayer is compressed the surface phospholipids draw closer and reduce the surface tension as the charge density from the polar head groups increases. The limit of monolayer compression before collapse depends on the surface area occupied by the phospholipid molecule. DPPC occupies a lower average molecular cross-section, at the air/water interface, than unsaturated PCs, fig1.3 as a consequence of the flexibility of saturated acyl chains. Conventional theory states that pulmonary surfactant produces very low surface tensions when compressed, which serves to counteract the forces leading to alveolar collapse (Pattle 1958). This view represents an over simplification, however, since the molecules of a liquid surfactant would quickly move from areas of high compression to low compression, equilibrating surface tension and allowing alveolar collapse (Morley and Bangham 1981). Repeated compression and expansion of a monolayer of purified surfactant produces a change in chemical composition with a

stable DPPC/PG mixture at the interface and removal of other less stable components to the subphase (Bangham et al 1979, Morley and Bangham 1981). The demonstration that this refined surfactant solidifies under compression (Morley and Bangham 1981) indicates that the surfactant serves to 'splint open' alveoli against adverse surface tension effects. The liquid/crystalline transition temperature of pure DPPC is 41°C, and consequently at 37°C it would be expected to be crystalline. The other surfactant components, in particular PG and the lipophillic protein, appear to allow the rapid, spontaneous, surface spreading of DPPC in surfactant preparations. This is supported by recent observations of repeated compression of the DPPC/PG mixture, which led to a progressive squeezing out of the unsaturated PG from the mixed layer (Bangham 1987). This left a residual DPPC which, at 37°C, then condensed out as a solid phase so rigid that it may prevent alveolar collapse *in vivo*.

1.2.4 The site of pulmonary surfactant synthesis

Over 40 cell types have been shown in the lung (Crapo et al 1982) although the alveolar lining comprises few cell types. The isolated alveolar type II cell, fig. 1.4., has ben identified as the source of pulmonary surfactant (Kikkawa and Yoneda 1974, Mason et al 1977). When compared with lung tissue, isolated type II cells are enriched in both DPPC and PG (Gilfillan et al 1983, Mason and Williams 1980). The material secreted by isolated rat lung type II cells in culture has the same phospholipid composition, morphological appearance, surface activity and surfactant-associated protein as lung lavage surfactant (Dobbs et al 1982). Estimates of the rate of disaturated PC species secreted in adult rat type II cell cultures gave figures of 1-2% of total cellular PC per hour (Dobbs and Mason 1979, Brown and Longmore 1981). Autoradiographic studies, which followed the passage of ^{3}H choline through the mouse lung type II cells (Chevalier and Collet 1972) showed a progression from endoplasmic reticulum, through the golgi apparatus and mitochondria to the lamellar bodies. A developmental increase in lamellar bodies accompanied the appearance of surfactant in fetal mice and sheep (Buckingham and Avery 1962, Orzalesi et al 1965). RDS in lambs was shown to be characterised by a great dimunition or even absence of lamellar bodies (Kikkawa et al 1965).

Fig 1.4 The alveolar type II cell



The stylised isolated rat alveolar type II cell above is one of over 40 cell types present in the mammalian lung. They characteristically possess dense cytoplasmic inclusions called lamellar bodies. These appear to be storage sites for pulmonary surfactant prior to secretion and labelling experiments have shown the passage of radiolabelled choline through the endoplasmic reticulum, golgi apparatus and lamellar bodies.

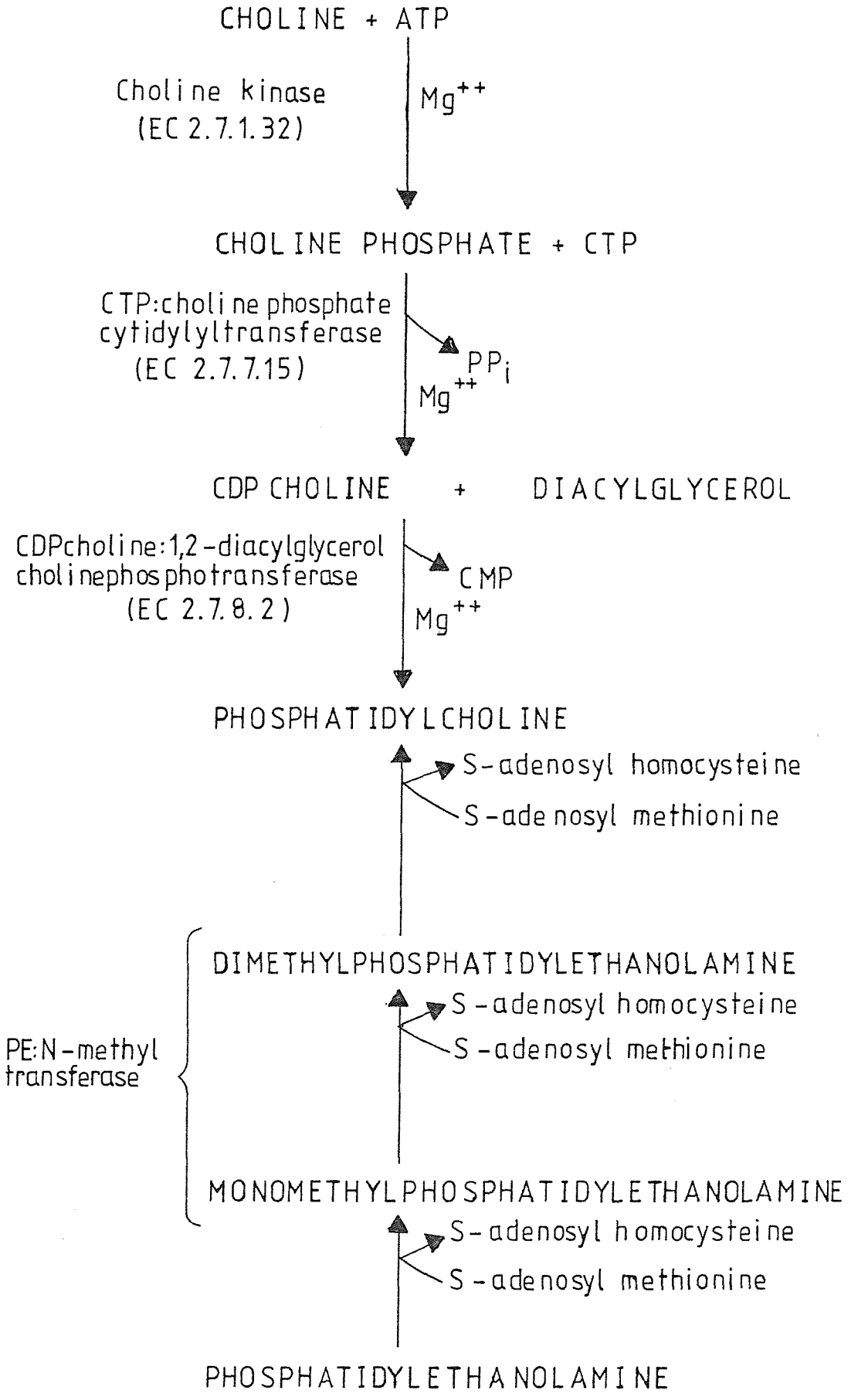
1.3.1 Phosphatidyl ethanolamine N-methylation

The N-methylation pathway of PC synthesis is shown in fig 1.5 S-adenosyl-L-methionine acts as the donar for methyl groups, successively introduced into the terminal amino group of PE. Uncertainty has surrounded whether or not one or two methyl transferases are involved (Mato and Alemany 1983). Resolution of the question for rat liver enzyme was apparently provided by the purification of the enzyme, a heterodimer which catalyses all three additions (Pajares et al 1984, Varela et al 1984). The 25,000 dalton subunit appears to be the catalytic subunit (Pajares et al 1984), while the 50K subunit seems to be a substrate for phosphorylation (Varela et al 1984). The enzyme appears to be closely associated with microtubules in phagocytes. Antitubulins have been shown to decrease N-Methylation while stimulating the CDPcholine pathway (Pike et al 1980). The activity of the N-methyltransferase in liver is between 10 and 1,000 times higher than that in most tissues and the relative contribution of this pathway to total PC synthesis in non-liver cells is negligible (Vance and Kruijff 1980). In liver, phospholipid turnover is rapid with a half life of between 1 and 10 hours (Arvidson 1968) and it has been suggested that high N-methyl transferase activity may serve to maintain a steady state level of PC synthesis under different metabolic conditions (Mato and Alemany 1983). Investigation of N-methylation activity in the primate lung has indicated that less than 3% of total PC synthesis is provided by this pathway (Epstein and Farrell 1975).

1.3.2 The CDPcholine or Kennedy Pathway of Phosphatidyl choline synthesis

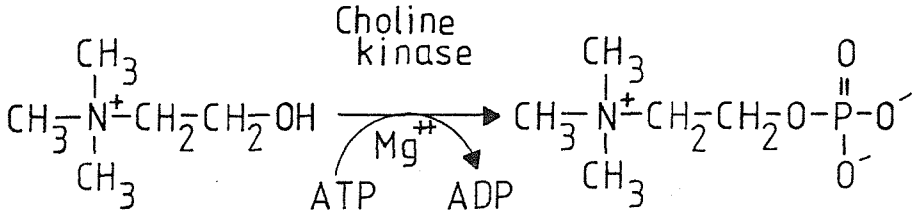
The principle route for PC synthesis in mammalian cells is the CDPcholine pathway, fig 1.5. The enzymic conversion of choline to PC occurs at the expense of activation by cleavage of three phosphate bonds. One is derived from ATP, in the phosphorylation of choline and a further two from CTP during the conversion of choline phosphate to CDPcholine. During the reaction scheme choline is incorporated, from an aqueous environment, into a lipid compartment, with the final enzyme in the pathway, cholinephosphotransferase, being an integral membrane protein (Kennedy and Weiss 1956).

Fig 1.5 Pathways of de novo phosphatidylcholine biosynthesis



1.3.2.1 The Properties of isolated Choline Kinase

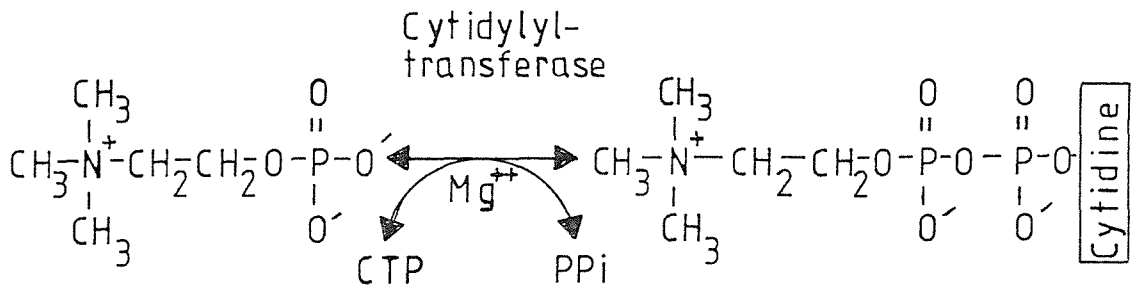
Choline Kinase (CK) (EC 2.7.1.32) catalyses the phosphorylation of choline, yielding choline phosphate, and has a requirement for Mg^{++}.



Its action commits choline to the CDPcholine pathway (Kennedy and Weiss 1956) CK has been found in the cytosolic compartment following fractionation of yeast, liver, brain, intestinal mucosa and kidney (Wittenburg and Kornberg 1953). Rat liver ethanolamine Kinase (EK) activity partially co-purifies with CK (Weinhold and Rethy 1974). By contrast EK from lactating bovine mammary gland could not be separated from CK activity (Infante and Kinsella 1976) leading to the suggestion that both activities reside on one enzyme. CK from monkey lung purified 1,000 fold possessed both activities (Ulane et al 1978) unresolvable by polyacrylamide gel electrophoresis (PAGE) of homogenates. When rat kidney CK was purified, to a dimeric structure of 42,000 dalton subunit, EK activity was present (Ishidate et al1984). Co-purification ofEK/CK with ratios of 0.29-0.42 (Ishidate 1985) provided further evidence of a common enzyme. Kinetic analysis suggested separate active sites on the enzyme. Polyclonal antibody to kidney CK gave a dose-dependant inhibition of CK and EK activities in rat liver, lung and intestine (Ishidate et al 1985). This suggested the bifunctional kinase was common to all these tissues. Kinetic analysis is consistant with a sequential order mechanism of choline binding followed by MgATP (Infante and Kinsella 1976).

1.3.2.2 The Properties of Isolated CTP:choline phosphate cytidylyltransferase

CTP:choline phosphate cytidylyltransferase (EC 2.7.7.15) catalyses the conversion of choline phosphate and MgCTP to CDPcholine and pyrophosphate and has a requirement for Mg^{++}.

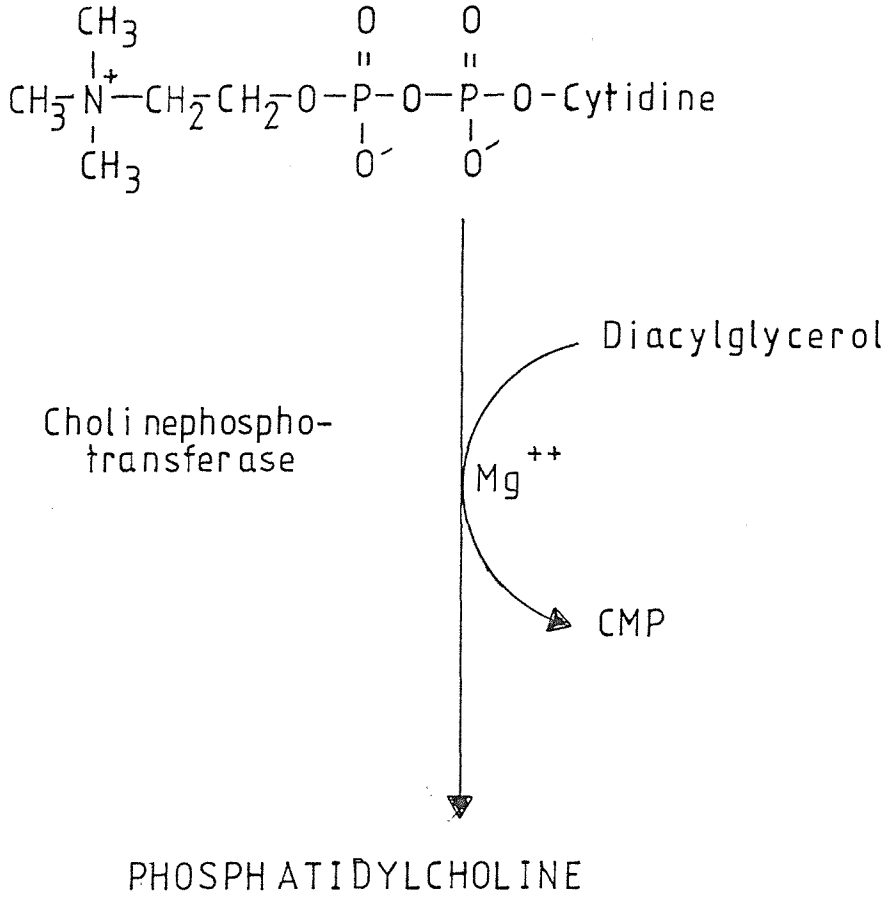


The pyrophosphate is a product of the cleavage of the α and β phosphates of CTP while the γ-phosphate is coupled to choline phosphate to yield CDPcholine. The enzyme is readily reversible in vitro and may be measured in either direction (Borkenhagen and Kennedy 1957). Cytidylyltransferase has been found associated with both cytosolic and microsome compartments of liver, lung, brain and kidney in vitro (Schneider 1963, Stern et al 1976, Feldman et al 1978). The ratio of enzyme in both compartments varied with homogenising conditions (Schneider 1963, Stern et al 1976). Low ionic strength promoted recovery of the membrane-associated enzyme while high ionic strength favoured the cytosolic enzyme. This has been interpreted as indicative of an interaction with membrane which is sufficiently weak to be manipulated by ionic strength variation. Gel filtration of the cytosolic enzyme resolved it into two forms, a low molecular weight (L) form of 190,000 - 200,000 daltons and a higher molecular weight (H) form with a range of molecular weights above 10^6 daltons (Feldman et al 1978, Choy et al 1977). Storage of liver cytosols increased the proportion of high molecular weight enzyme (Choy et al 1977), while high ionic strength or detergent converted H to L form. Low yields of unstable enzyme have characterised purification attempts (Choy and Vance 1976, Choy et al 1977, Vance et al 1981). Recently a 2,180 fold purification of rat liver enzyme has been recorded, with yields of 6.4% (Weinhold et al 1986). SDS PAGE analysis suggested the native enzyme is a tetramer of two 39,000 dalton subunits and two of 48,000 daltons providing a basic unit of 174,000 daltons. Both cytosolic and partially purified L form enzymes required lipid for maximum activity (Stern et al 1976, Choy et al 1979, Weinhold et al 1986). An essential thiol appeared necessary for catalytic function in liver enzyme (Weinhold et al 1986) with sulphydryl reagents N-ethylmaleimide, 5',5'-dithio bis-(-2-nitrobenzoic acid) and p-chloro mercuribenzoate irreversibly inhibiting the enzyme. CTP, and to a lesser extent choline phosphate provided protection from inhibition.

The losses inherent with most purification protocols have generally prevented successful purification of cytidylyltransferase from non-hepatic tissue due to the lower total activities present and consequent necessary scaling up of schemes.

1.3.2.3 Properties of isolated CDPcholine:1,2-diacylglycerol cholinephosphotransferase

CDPcholine; 1,2-diacyglycerol cholinephosphotransferase (EC 2.7.8.2) (choline phosphotransferase) catalyses the esterification of 1,2-diacyl-sn-glycerols with the choline phosphate component of CDPcholine to yield PC and CMP.



The liver enzyme is an integral membrane protein, largely associated with the microsomal fraction(Jelsenna and Morré 1978). It has been suggested that the enrichment of saturated acyl chains in lung PC might be controlled at the cholinephosphotransferase level by selective incorporation of 1,2-dipalmitoyl-sn-glycerol. Whole rat lung microsomes

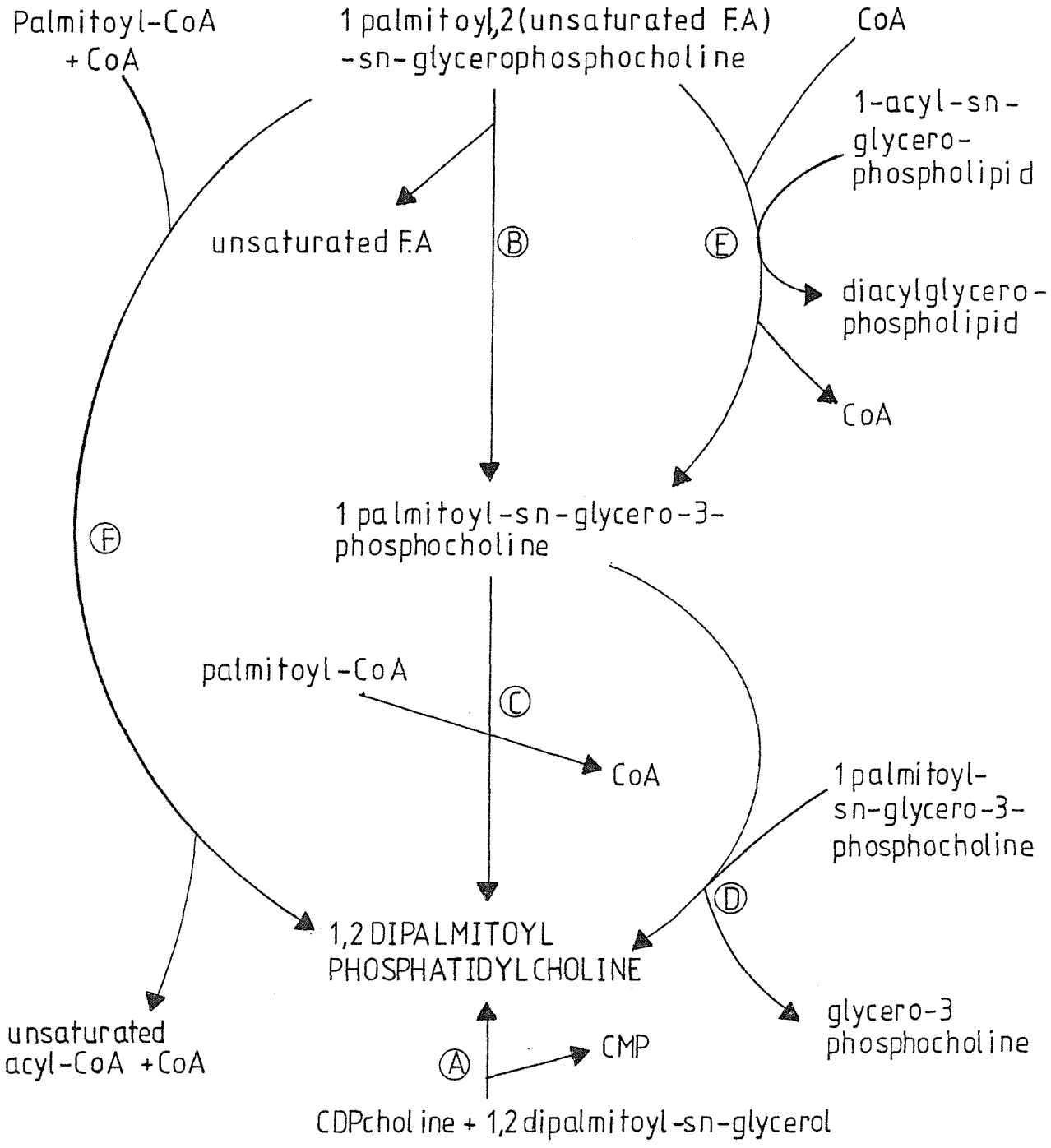


Fig 1.6 Pathways of DPPC synthesis in pulmonary tissue

ENZYMES

(A) Cholinephosphotransferase(EC 2.7.8.2)

(B) Phospholipase A_2(EC 3.1.1.4)

(C) & (F) Acyl-CoA:lysophosphatidylcholine acyltransferase(EC 2.3.1.23)

(D) Lysophosphatidylcholine:lysophosphatidylcholine acyltransferase

(E) ATP independant CoA mediated acyltransferase

were shown to produce only 17% of de novo PC as the dipalmitoyl species (Moriya and Kanoh 1974) showing that post synthesis remodeling must occur. Others have been unable to demonstrate incorporation of exogenous dipalmitoylglycerol (Oldenberg and Van Golde 1976), possibly the result of presentation without PG or excess diacylglycerol (Miller and Weinhold 1981). Using HPLC analysis of the molecular species of PC from whole lung microsomes an estimated 50% of DPPC was believed to be synthesised de novo with remodelling probably accounting for the rest (Rustow et al 1985). In two studies of isolated type II cells, de novo synthesis of DPPC accounted for 56% and 65% of DPPC respectively (Post et al 1983, Crecelius and Longmore 1984). This high incorporation of disaturated species appeared to reflect enrichment of dipalmitoylglycerol in type II cells rather than preferential incorporation (Crecelius and Longmore 1984).

The use of detergent and sonication has allowed separation of cholinephosphotransferase from the analogous ethanolaminephospho-transferase (EC 2.7.8.1) (Kanoh and Ohno 1976) with a 4 - 5 fold enrichment. In this form the enzyme was unstable during subsequent purification steps, although a recent report of the promotion of stability by diacylglycerol and glycerol offers a potential approach to further purification (Cornell and MaClennan 1985b). Cholinephosphotransferase and ethanolaminephosphotransferase appear to reside on the same enzyme in malaria infested primate erythrocytes (Vial et al 1984). Further purification will be necessary to establish unambiguously whether separate activities are present.

Mechanistic studies with the partially purified enzyme, using a variety of CDPcholine analogues have shown that the reaction proceeds via direct nucleophilic attack of diacylglycerol on CDPcholine (Pontroni et al 1985). Substitution of various groups has shown that at least three features, the amino group on the pyrimidine ring, the 2'-OH of ribose and the pyrophosphated bridge are critical sites for enzyme interaction.

1.3.4 Phosphatidylcholine saturation enrichment in lung

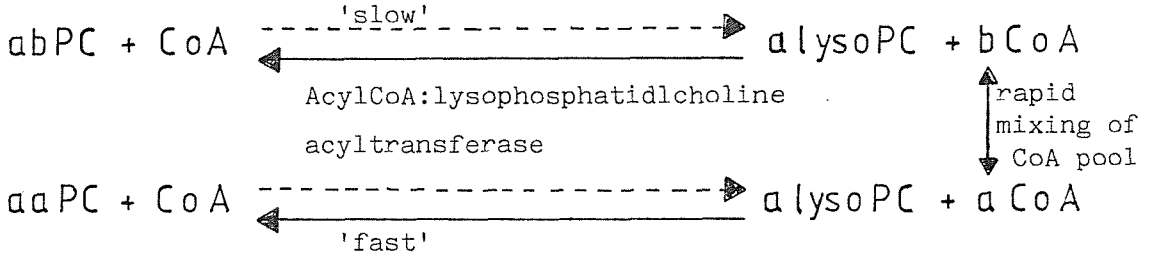
Potential pathways for lung PC saturation enrichment are shown in fig 1.6. Preferential incorporation of saturated diacylglycerols at the cholinephosphotransferase level, fig 1.6(A), cannot be shown (Crecelius and Longmore 1984). Several remodeling pathways, fig 1.6.(B - F) may

operate. Pathways (B), (C) and (D) are phospholipase A_2-dependant, while (E) and (F), which have only recently been described in the lung, (Nijssen and Van den Bosch 1986, Stymme and Stobart 1985) are phospholipase A_2-independant.

The importance of phospholipase A_2 in remodeling pathways of isolated type II cells has been shown by incubation with the specific phospholipase A_2 inhibitor 4-bromophenacyl bromide (Post et al 1983). In the presence of this inhibitor, PC unsaturation increases. Following phospholipase A_2 action the relative importance of the re acylation step (C) and the transacylation step (D) has been evaluated. Experiments with adult rat lung indicated that the reacylation step (C) predominated (van Heusden et al 1980) and developmental increases in this activity have been shown to accompany the maturation of surfactant synthesis in type II cells isolated from fetal and neonatal rats (De Vries et al 1985). In the latter case no change in transacylation step (D) was recorded.

Pathways (E) and (F) are ATP-independant/CoA-dependant mechanisms which bypass phospholipase A_2 action. Pathway (E) transfers the unsaturated acyl group of 1, palmitoyl, 2 (unsat), -sn-glycerophospho choline to one of a series of lysophospholipid acceptors in a CoA dependant fashion. An order preference is seen with lyso PE favoured over lyso-PG and lyso-PS to yield PE, PG and PS respectively. Acylation of the palmitoyl lyso-PC with Palmitoyl CoA then proceeds via step (C) (Nijssen and Van den Bosch 1986). Pathway (F) is CoA-dependant despite appearing to involve direct acyl exchange.

a = palmitoyl b = unsaturated acyl group



Overall reaction :

The reaction appears to work, as shown above, by the reversal and subsequent forward action of pathway (C). The reverse action favouring the CoA-dependant removal of arachidonate from the Sn-2 position and the 'forward' action selecting palmitoyl CoA (Stymme and Stobart 1985).

Quantitative contributions of each of these steps to remodeling have not been ascertained, with the exception that the transacylation step (D) does not appear significant in either fetal or adult rat lung (De Vries et al 1985, Van Heusden et al 1980). As was noted earlier remodeling merely alters the extent of saturation of existing PC, consequently any search for regulatory mechanisms in DPPC synthesis must concentrate on bulk PC synthesis rather than the fine tuning of saturation enrichment.

1.4 Regulation of Metabolic Pathways

Metabolic regulation in eukaryotes is effected by the coordination of many interelated biochemical reactions. Several types of control mechanism may operate to regulate the flux through a pathway involving complex metabolic sequences or where tissue differentation must be achieved. The integration of these fluxes between the various mammalian organs poses a complex problem. In part their regulation is brought about by the collaboration of the circulatory system, to supply substrates, and the endocrine system, which may augment flux under hormonal control.

Two models have been used to describe regulation at the enzyme level. The first involves a search for the controlling, key enzyme in a pathway, the so-called rate limiting enzyme (Krebs 1963). The second approach considers the flux through the whole pathway, apportioning control between enzyme, and changes in fractional control dependant on cellular conditions (Kacser and Burns 1973, Kacser and Porteous 1987).

At the molecular level enzyme activity can be controlled by the regulation of effective enzyme concentration. Long term control may involve increased enzyme synthesis or activation of an inactive proenzyme. Shorter term control may involve regulation of enzyme activity by the binding of allosteric effectors or covalent modification, for example by phosphorylation or dephosphorylation at hydroxyl groups on amino acid side chains. Alternatively control may be exercised in part by compartmentation of enzyme and substrates within the cell.

1.4.1 Rate limiting Enzymes

The concept of rate limiting reactions in complex biochemical pathways was advanced by Krebs and others in relationship to glycolysis and gluconeogenesis (Krebs 1963, Weber et al 1964). Classification of rate limiting enzymes is undertaken following the examination of the steady state concentrations of substrates and products, *in vivo*, in the ratio commonly known as the mass action ratio. The principle underlying the classification is the suggestion that earlier enzymes in a pathway produce the substrate of a rate limiting step faster than the enzyme can utilise it. In consequence, it is reasoned, reaction products are rapidly consumed by subsequent enzymes in the pathway and the reaction is not in equilibrium. A corollary of this analysis is the suggestion that non-rate limiting enzymes are close to equilibrium. Regulation of the activity of a rate limiting enzyme, by allosteric activation or inhibition or covalent modification, is envisaged as producing a proportionate change in pathway flux.

Some equivocal assumptions are made in this type of analysis which cannot be substantiated. In particular the assumption that the intracellular milieu is heterogenous, apart from membrane bound regions, and that substrate and product concentrations measured in broken cell preparations represent the concentrations to which the enzymes are exposed *in vivo*. The approach takes no account of compartmentation and the potential existance, *in vivo*, of multienzyme complexes with direct metabolite transfer via enzyme-enzyme complexes. The concentrations of glycolytic enzymes in muscle have recently been shown to exceed the concentrations of the intermediary metabolites (Srivastava and Bernhard 1986(a)(b)). This casts doubt on ideas of bottle necks in pathways. Experimental determinations of the effects of subcellular substrate pool sizes focuses on individual enzymes under conditions which are far from physiological. The relevance of parameters obtained for isolated single enzymes in dilute solutions *in vitro* has been questioned. In particular to what extent this data gives us insight into metabolism and its regulation in intact eukaryotic cells (Clegg 1984).

1.4.2 Control Analysis

The search for rate limiting steps has tended to obscure the lack of a sound, quantitative, theoretical basis for analysis of metabolic

control (Kacser and Burns 1973). Claims for regulatory steps often contradict one another while allowing no quantification of the measure of control or whether the quantitatively specified conditions operate in vivo. Consequently the use of such terms as rate limiting has been viewed by many as qualitatively evocative while quantitatively meaningless. A more rigourous theoretical framework of rate-control analysis has been developed and applied in recent years (Kacser and Burns 1973, Heinrich and Rappaport 1974, Porteous and others 1983, Kacser and Porteous 1987). Their analysis involves a coordinated evaluation of whole pathways, whether linear or branched, and assesses the contribution of each enzymic step to the overall flux of metabolites through a pathway under steady state conditions. A number of experimentally determined coefficients in intact systems for the overall pathway (global coefficients) or individual steps (local coefficients) can be obtained. These allow quantification of the amount of control exerted by an enzyme under defined conditions.

A small change (δJ) in flux (J) can be related to a small change (δE_1) in enzyme activity or amount (E_1) by the relationship:

$$\frac{\delta J}{J} \Big/ \frac{\delta E_1}{E_1} \longrightarrow \frac{dJ}{J} \Big/ \frac{dE_1}{E_1} = \frac{d\ln J}{d\ln E_1} = C^{J}_{E_1}$$

where C is designated the flux control coefficient (Burns et al 1985). It can be shown that for a pathway of n enzymic steps the sum of the individual flux control coefficients $\sum_{E_1}^{E_n} C = 1$ (Kacser and Burns 1973, 1979). Enzymes with flux control coefficients near unity are a rare case and variations in their activity will produce an almost proportional change in flux. A coefficient of 0.01, by contrast, will produce a flux change of only one hundredth of the enzyme fractional change. The flux control coefficient is a quantitative measure of any enzyme under a defined set of conditions describing, by its value, the amount of control which it exercises on the flux. It is a systemic property, dependant on all other enzymes in the pathway. Variation in one enzyme's activity will be reflected in a change in the coefficients and consequently of its share of control within the pathway. Other coefficients relate flux changes to the enzymes substrate sensitivity, and connections with other enzymes. Together these derived coefficients provide a quantitative description and understanding of control (Kacser and Burns 1979, Kacser

and Porteous 1987). Enzymes classified as rate limiting may well have flux control coefficients near unity under defined conditions. Alteration of effective enzyme concentration, however, by activation, inhibition, de novo synthesis or degradation will redistribute the share of control between the enzymes of the pathway.

1.4.3 Covalent modification of Enzyme Activity

Covalent modification of enzymes with allosteric modification (section 1.4.4) are the two principle means of modifying flux through metabolic pathways. Several types of reversible, covalent modification which are capable of producing alterations in prokaryotic and eukaryotic enzyme activities, have been described. The prokaryotic glutamine synthase, for example, has been shown to be inactivated by a reversible adenylation (Shapiro and Stadtman 1970). In mammalian tissue, protein phosphorylation is a major mechanism by which external physiological stimuli control intracellular processes(Cohen 1982). Recently the regulatory potential of reversible, NAD-dependant, ADP ribosylation has been demonstrated with the activation of acetyl CoA carboxylase (ACC) by this covalent modification (Witters and McDermott 1986).

Phosphorylation-dependant changes in the activities of a large number of enzymes have been described (Cohen 1982). Such phosphorylation occurs under the action of specific kinases at the hydroxy group of either serine, threonine or tyrosine amino acid residues, with dephosphorylations under the control of phosphatases, which are often specific. Reversible phosphorylation may occur at a single site but is more usually a multiple site phenomenon. An example of the former type is provided by phosphorylase b which is present in resting muscle and which, under the action of phosphorylase kinase (itself regulated by phosphorylation) is phosphorylated to phosphorylase a, an active form. Phosphorylation may also inactivate enzymes, as in for example the multiple site phosphorylation of ACC (Hardie and Guy 1981) but may also have no direct effect on enzyme activity, a so-called 'silent' phosphorylation. An example of 'silent' phosphorylation is provided by rabbit muscle phosphofuctokinase (PFK). Phosphorylation increases the affinity of the enzyme for F-actin, which in turn is a specific allosteric effector (section 1.4.4) of PFK (Luther and Lee 1986, Kuo et al 1986). PFK phosphorylation indirectly alters the kinetic behaviour of the enzyme but also serves to regulate the compartmentalization of the

enzyme in order to provide energy to the cellular component where it is needed.

1.4.4 Allosteric Effects

Allosteric control of enzymes is seen when effector molecules bind at sites other than the active site and in doing so alter the rate of the reaction. The effect may be to change the affinity of an enzyme for its substrate(s) or to stabilise an active or less active conformational state. Control may be homotropic, where the binding of a substrate molecule changes the affinity for subsequent substrate molecules, or heterotropic, where interaction with non-substrate molecules changes substrate affinity. An example of some potential complexities of allosteric control is provided by the glycolytic enzyme PFK, which catalyses the conversion of fructose-6-phosphate and ATP to fructose 1, 6-bisphosphate and ADP. The enzyme is regulated by a variety of metabolites (Bloxham and Lardy 1973, Kemp and Foe 1983), being activated by AMP, ADP, 3', 5'-cyclic AMP, glucose-1, 6-bisphosphate, fructose 1, 6-bisphosphate and the potent sugar activator fructose-2, 6-bisphosphate. Inhibition is bought about by ATP and by citrate, a subsequent product of glycolysis. The practical effect of this form of control of PFK activity is that the flux through the glycolytic pathway increases with a cellular requirement for energy and decreases when energy is not required. Allosteric changes in the kinetic properties of PFK, the response to effectors, can be modified by phosphorylation (Uyeda et al 1978) in addition to the increase of affinity for the allosteric activator F-actin (Kuo et al 1986, Luther and Lee 1986) described above.

1.4.5 Ambiquity and control by translocation

The 'ambiquitous enzyme' hypothesis has been proposed as a regulatory mechanism for enzymes which demonstrate *in vitro*, flux dependant variations in their subcellular location (Wilson 1978, 1980). Extrapolation of these changes for the glycolytic enzyme hexokinase (HK) between the cytosol and outer mitochondrial membrane (OMM) led to a definition of this prototype ambiquitous enzyme. "HK activity *in vivo* may be controlled by the relative distribution between soluble and particulate forms, the latter being active" (Wilson 1968). A rapid, reversible translocation between particulate and soluble forms has been

Table 1.3

Eukaryotic enzymes and proteins displaying "ambiquitous" behaviour

Enzyme/Protein	Distribution(in vitro)
Aldolase	cytosol and myofibrils
Hexokinase	cytosol and membranes
Phosphofructokinase	cytosol and myofibrils
Glyceraldehyde-3-phosphate dehydrogenase	cytosol and myofibrils
Phosphoglycerate kinase	cytosol and membrane
Lactate dehydrogenase	cytosol and particulate
Cytidylyltransferase	cytosol and microsomes
Phosphatidate phosphohydrolase	cytosol and microsomes
Hormone sensitive lipase	cytosol and membranes
Tyrosine hydroxylase	cytosol and membrane
Acetyl-CoA carboxylase	cytosol and mitochondria
Monoacylglycerol lipase	cytosol and particulate
ATP citrate lyase	cytosol and microsomes
Diacylglycerol kinase	cytosol and membrane
Ca^{++}/phospholipid dependant protein kinase C	cytosol,membrane and cytoskeletal components
Glucose-6-phosphate dehydrogenase	cytosol and particulate
Alcohol dehydrogenase	cytosol and F-actin
Adenylsuccinate synthase	soluble and particulate
Type II Ca^{++}/calmodulin dependant protein kinase	cytosol and cytoskeleton
RII subunit of cAMP dependant protein kinase	cytosol and MAP2
Amino acyl-tRNA synthetase complex Phenylalanyl-tRNA sythetase	cytosol,ribosomes, microsomes/cytoskeleton
Calmodulin	cytosol and membrane-associated cytoskeleton
PI-specific phospholipase C	cytosol and membrane

Factors altering distribution	Investigators
ionic strength,substrates	Arnold and Pette(1971)
ionic strength,substrates	Wilson(1980)
ionic strength,substrates	Choate et al(1985)
ionic strength,substrates	Arnold and Pette(1971)
ionic strength,substrates	De and Kirtley(1977)
ionic strength,substrates,pH	Hultin and Westoff(1966)
ionic strength,flux changes	Vance and Pelech(1984)
ionic strength,flux changes	Brindley(1985)
ionic strength,flux changes	Hirsch and Rosen(1984)
flux changes	Mandell et al(1972)
flux changes	Alred et al(1985)
-	Sakwala et al(1981)
ionic strength	Linn and Srere(1984)
-	Kanoh and Akeson(1978)
metabolic status of tissue	Kraft and Anderson(1983),Pelech et al(1986),Wolf and Sahyoun(1986)
ionic strength,substrates	Swezy and Epel(1986)
ionic strength	Taylor and Chan(1985)
ionic strength,substrates	Ogawa et al(1978)
ionic strength,Ca^{++},calmodulin	Ohta et al(1986)
-	Leiser et al(1986)
ionic strength	Mirande et al(1985)
metabolic status of tissue	Gazzotti et al(1985)
ionic strength	Wang et al(1986)

shown in the presence of glucose-6-phosphate, Pi or high ionic strength but dependant on specific ionic species (Wilson 1980). For HK the crux of the 'ambiquitous enzyme' hypothesis is that, under low energy flux conditions, subcellular glucose-6-phosphate concentration will be at their highest, disposing HK to exist in its most soluble, least active state.

Substrate-, ion- or flux -dependant distribution is generally characteristic of candidate ambiquitous enzymes (Nemat-Gorgani and Wilson 1980). A number of enzyme sharing in vitro properties potentially classifying them as ambiquitous are shown in table 1.3. The list includes enzymes of carbohydrate metabolism, enzymes involved in glycerolipid metabolism including cytidylyltransferase, enzymes involved in protein synthesis and regulatory kinases including the Ca^{++}, phospholipid dependant protein kinase C. Such a widespread involvement of different enzymes indicate the potential of such a mechanism in many metabolic pathways.

The physiological significance of the proposed HK translocation, accompanying flux changes, has been questioned (Kyriazi and Basford 1986). Rapid removal and fractionation techniques were used to minimise tissue ischaemia, previously shown to lower glucose-6-phosphate and which might yield distribution artefacts (Lowry et al 1964, Nordstrom and Siesjo 1978). HK distribution in the rat brain was determined during a 2-3 fold stimulation of glycolysis produced by the plant alkaloid varatridine or under anaesthesia, where the glycolytic rate falls. Unchanged distributions were observed and no net movement of HK on or off of the OMM occured, suggesting no physiological significance to glycolytic regulation (Kyriazi and Basford 1986).

1.4.6 Cell structure and regulation by compartmentalisation

Two opposite views of aqueous cytoplasm dominate the theoretical understanding of cell structure. In one it is considered that, apart from organelles, experimentally defined 'soluble' proteins, substrates and other solutes exist in homogenous, concentrated solution. Compartmentalisation of enzymes and substrates is confined to membranes, membrane surfaces or regions bound by membranes (e.g. Veech et al 1979). The cytosol "that portion of the cell which is found in the supernatant fraction after centrifugation of the homogenate at 105,000 x g x 60 min"

(Lardy 1965) is held by many to mirror the aqueous cytoplasm, despite the possible artefacts or alterations resulting from cell disruption and dilution. Randomly dispersed enzymes and metabolites are envisaged interacting by simple mass action mechanisms, according to the laws of solution chemistry, in a well mixed solution. Increasing evidence of a highly ordered structure within the aqueous cytoplasm argues against this view (Clegg 1984(a)). In many glycolytic enzyme pairs, moreover, the physiological concentrations of intermediates precludes random diffusion mechanisms and requires direct enzyme/enzyme transfer (Srivastava and Bernhard 1986(a)(b)). Evidence has been presented of an aqueous cytoplasm where few solutes exist in the aqueous component but are loosely associated with the cellular ultrastructure (Clegg 1984(a)).

The classically 'soluble' enzymes of the glycolytic pathway are physically separated from those of the glycogenolytic pathway (Lynch and Paul 1983, Clegg 1984(b)). The individual glycolytic enzymes in muscle appear to exist in close proximity providing, by direct metabolite transfer, a dynamic compartmentalisation (Srivastava and Bernhard 1986(a)(b)). Stratification of Euglena cells, in vivo, by centrifugation at 100,000 x g x 60 minutes, separated an aqueous compartment from nuclei, mitochondria and other organelles within the cells (Kempner and Miller 1968(a)(b)). Cytochemical analysis showed no aqueous macromolecules and enzymes described as 'soluble' in homogenates were found to be associated with cell ultrastructure in the living cells which remained viable.

An indication of the complexities of subcellular structure has been provided by electron microscopy. The aqueous cytoplasm has been shown to be gel-like with, in addition to organelles, microtubules, actin-rich microfilaments and other cytoskeletal elements incorporated within an apparently non-artefactual meshwork, the microtrabecular lattice (MTL) (Wolosewick and Porter 1979, Porter and Tucker 1981). Individual strands of the MTL (5-10nm x 50-100nm) possess polysomes at their junctions and are coextensive with the cortex of the cytoplasmic surfaces and the endoplasmic reticulum. The network is fragile and may be disrupted by cooling to 5°C with reassembly occuring at 37°C. Estimates of 'pore size' enclosed by MTL elements, from electron micrographs, give a diameter of 100nm, much larger than most globular 'cytoplasmic' proteins. Steric hinderence effects only occur where the diffusing species are close to mean pore diameter (Renkin 1955). A smaller average pore diameter (30 - 40nm) was shown in vivo by translational measurement of molecules of fluorescent dextrans in 3T3 cells, using a technique known

as fluorescent recovery after photobleaching (FRAP) (Luby-Phelps et al 1986). A size dependant diffusion barrier was shown, while such dextrans injected directly into the aqueous cytoplasm do not interact significantly with intracellular components (Luby-Phelps et al 1985). The smaller diameter may reflect water molecules bound to the MTL in vivo which are not seen in electron micrographs. The viscosity of the aqueous cytoplasm was approximately four fold higher than water, agreeing with a previous non-FRAP technique (Mastro et al 1984). The diffusion of fluorescein labelled proteins of molecular sizes below the MTL pore size, insulin (6,000 daltons, 3.2nm diameter), B.S.A. (67,000 dalton, 7.1nm diameter) and apoferritin (440,000 daltons, 12.2nm diameter) has been measured (Jacobsen and Wojcieszyn 1984, Gershon et al 1985). Diffusion rates were similar, size independant, and slower than viscosity measurements would predict. Caboxy fluorescein (400 daltons, 1.2nm) or labelled dextrans of similar size diffused much faster (Jacobsen and Wojciezyn 1984, Luby-Phelps et al 1986). Analysis suggested that 98% of 'soluble' protein in vivo was transiently bound to the cell ultrastructure at any one instant (Gershon et al 1985, Jacobson and Wojcieszyn 1984).

It appears that the majority of native cytoplasmic proteins either comprise the cytoskeletal matrix or are bound to these elements in vivo rather than existing in solution. The variations between 'soluble' and 'particulate' distribution of ambiquitous enzymes, seen under constant fractionation conditions, in vitro may simply reflect flux-dependant changes in their affinities for the ultrastructural element. The observed kinetic variation between soluble and particulate enzymes might result from conformational changes, concurrent with changes in cytoskeletal binding constants.

1.4.7 Enzyme-cytoskeletal interactions and multienzyme complexes

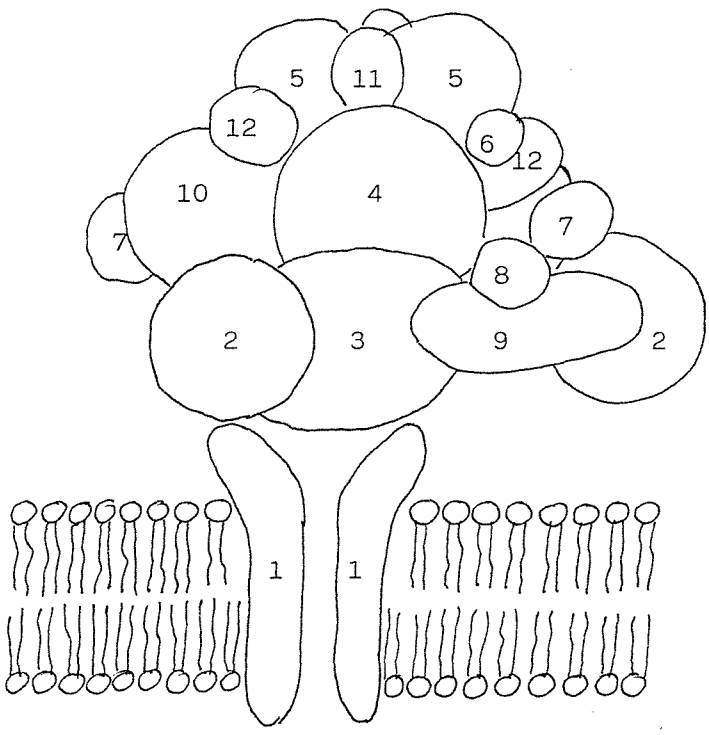
A number of enzymes have been shown to bind cytoskeletal elements in vitro, Table 1.4. This list includes enzymes of carbohydrate metabolism, regulatory kinases, the integral membrane protein 5'-nucleotidase and several others. Some enzymes classified as 'ambiquitous' also feature, with an ionic strength dependance generally seen where investigated. High ionic strengths promoted dissociation, while at low ionic strengths bound enzymes were often dissociated in the presence of their substrates. In several cases purified enzymes, particularly those of the glycolytic

Table 1.4 Eukaryotic enzymes, regulatory proteins and enzyme systems which show in vitro and/or in vivo associations with elements of the cytoskeleton

Enzymes of carbohydrate metabolism	Elements involved in binding	Investigators
Aldolase	F-actin	Clarke and Morton(1982)
Phosphofructokinase	F-actin	Luther and Lee(1986)
Glyceraldehyde-3-phosphate dehydrogenase	F-actin	Clarke and Morton(1982)
Glucose-6-phosphate dehydrogenase	structural, unidentified	Swezy and Epel(1986)
Pyruvate kinase	F-actin	Clarke and Morton(1982)
Cellulase	F-actin	Klysov et al(1985)
Lactate dehydrogenase	F-actin	Poglazov and Livanova (1986)
Phosphoglyceromutase	F-actin	''
Glucose phosphate isomerase	F-actin	''
Phosphorylase kinase	F-actin	''
Phosphoglycerate kinase	F-actin	''
Phosphorylase b	G-actin	Livanova and Poglazov (1982)
Regulatory kinases		
Ca^{++}, phospholipid dependant protein kinase C	110,000 & 115,000 peptides of the cytoskeleton	Wolf and Sahyoun(1986)
Myosin light chain kinase	Myosin and actin	Sellars and Pato(1984)
RII subunit of cAMP-dependant protein kinase	MAP-2	Leiser et al(1986)
Type II Ca^{++}, calmodulin-dependant protein kinase	F-actin	Ohta et al(1986)

Other enzymes and regulatory proteins		
Adenylosuccinate synthetase	F-actin	Ogawa et al(1978)
Alcohol dehydrogenase	F-actin	Taylor and Chan(1985)
HMG CoA reductase	Microtubules	Volpe(1979)
Nucleosidediphosphate kinase	Microtubules	Nickerson and Wells (1978)
DNA'se I	G-actin	Lazrides and Lindberg (1974)
RNA'se	F-actin	Simm et al(1987)
5'-nucleotidase	F-actin	Dieckhoff and Mannherz (1985)
RNA polymerase II	G-actin	Smith et al(1979)
AMP deaminase	Myosin	Byrnes and Suelter (1965)
5- and 15- lipoxygenase	Microtubules	Reitman et al(1986)
Neutrophil oxidase	F-actin	Al-Mohamma et al(1987)
Calmodulin	Cytoskeleton	Gazzotti et al(1985)
Enzyme systems		
Aminoacyl tRNA synthetase complex	Cytoskeleton	Mirande et al(1985)
CDPcholine and N-methylation enzymes	Microfilaments and microtubules	Pike et al(1980)
Enzymes of eicosanoid metabolism (phospholipase A_2?)	F-actin	Dubose et al(1987)

Fig 1.7
A proposed structure for the glycolytic enzyme complex of the erythrocyte membrane (after Kurganov 1986)



1. Band 3 protein
2. Glucose phosphate isomerase
3. PFK
4. Fructose bisphosphate aldolase
5. Glyceraldehyde-3-phosphate dehydrogenase
6. Phosphoglycerate kinase
7. Phosphoglyceromutase
8. Enolase
9. Pyruvate kinase
10. LDH
11. Triosephosphate isomerase
12. Glycerol-3-phosphate dehydrogenase

pathway, were observed to bind directly to the monomeric G-actin or the polymeric F-actin component of the cytoskeleton. Phosphorylated PFK, glyceraldehyde-3-phosphate dehydrogenase and cellulase increased V_{max} activities in vitro when bound to F-actin (Luther and Lee 1986, Poglazov and Livanova 1986) while lactate dehydrogenase, 5'-nucleotidase and alcohol dehydrogenase were inhibited (Poglazov and Livanova 1986, Dieckhoff and Mannherz 1985, Taylor and Chan 1985). G-actin stimulated muscle phosphorylase kinase b (Livanova and Poglazov 1982) but inhibits DNA'se I (Lazrides and Lindberg 1978). In addition some enzymes were complexed and bound to F-actin, as determined by ultrasedimentation, with no change in V_{max} activity in vitro (Poglazov and Livanova 1986).

Reversible association of 'soluble' glycolytic enzymes with sub-cellular structure has been proposed as a regulatory mechanism (Masters 1977, 1984). Myofibril binding of glycolytic enzymes is mimicked by the binding of erythrocyte enzymes to the cytoplasmic component of the membrane-spanning protein band 3 (Higashi et al 1979, Low 1986). Band 3 also binds actin and the band 4.1 protein serving to 'anchor' the cytoskeleton (Low 1986). Several glycolytic enzymes which have shown no direct cytoskeletal interaction have demonstrated a binding to immobilised PFK or glycolytic enzyme-actin complexes (Gerlach and Hofer 1986,Bronstein and Knull 1981) effectively bound cytoskeletally in 'piggy back' fashion.

Glycolytic multienzyme complexes have been suggested for myofibrils or band 3 (Masters 1984, Kurganov 1986). A schematic representation of the proposed erythrocyte complex is shown in fig 1.7 (Kurganov 1986). This is derived from published affinity data for individual components. The compartmentalisation of pathways previously proposed (Ureta 1978, Clegg 1984(b), Masters 1984) may be achieved with such complexes. A physical, functionally exclusive, compartmentalisation of glycolytic and glycogenolytic pathways in vascular smooth muscle has been demonstrated offering support to this concept (Lynch and Paul 1983). Enzymes such as those of glycolysis and horse liver alcohol dehydrogenase have been comprehensively investigated and it is likely that similar, unrecognised cytoskeletal interactions in vitro exist with other pathways. The observations of in vitro binding, if reflected in vivo, would be consistant with the picture of subcellular structure-associated 'soluble' proteins presented in section 1.4.6. The multienzyme complexes would provide for the direct metabolite transfer between glycolytic enzymes recently demonstrated (Srivastava and Bernhard 1986(a)(b)).

1.4.8 Hormonal Regulation and its effect on the cytoskeleton

Hormones are of two types, those with effects mediated through cell surface receptors and those which act via intracellular receptors. The former group includes the polypeptide hormones insulin and glucagon, and small aromatic molecules such as adrenaline. The latter group of lipid soluble molecules includes steroid hormones and the thyroid hormones T_3 and T_4. Those hormones binding at cell surface receptors initiate a chain of events, as for example with adrenaline where the hormone-receptor complex interacts with the GTP binding G protein, followed by adenyl cyclase to yield the 2nd messenger cAMP. The cAMP-dependant protein kinases then phosphorylate their substrates producing metabolic changes. Longer term effects of cAMP include modulation of DNA and protein synthesis. The hormones which interact with cytoplasmic receptors, e.g. progesterone, T_3 and T_4, are subsequently transported to the nucleus, where the exert their effects, for example by changing the rate of mRNA synthesis .

Recent evidence has shown that both types of hormone are able to regulate the state of cytoskeletal assembly, offering a potential mechanism for controlling the activity of cytoskeleton bound enzymes (Rao et al 1985, Kadowaki et al 1986). Rat hepatoma cells exposed to insulin, glucagon, T_3, T_4 and the adrenergic agonists phenylephrine and isoprenaline were all able to increase the F-actin/G-actin ratio (Rao et al 1985) by producing actin polymerisation. Insulin, insulin-like growth factors and epidermal growth factor all evoked a rapid cytoskeletal reorganisation in KB cells (Kadowaki et al 1986). The observation of similar polymerisations by hormones having opposing physiological functions i.e. insulin and glucagon, α- and β-adrenergic agents, suggested that the polymerisation was a common cellular event. Possibly their action stabilised the cytoskeletal framework for expression of different physiological effects under the action of cAMP-dependant protein kinases, C-Kinase and Ca^{++} fluxes. Actin is the most abundant nuclear protein, with equal distribution between nucleus and cytoplasm (De Robertis et al 1978, LeStourgen 1978) and T_3/T_4 mediated changes may have been the result of nuclear binding of the hormone. These results suggest that "actin polymerisation may be an important common and essential event accompanying hormone-mediated changes in cell metabolism and growth" (Rao et al 1985).

1.5 The Regulation of Mammalian PC biosynthesis

Evidence supplied from a variety of mammalian tissues has provided support for the suggestion that cytidylyltransferase has an important role in PC biosynthesis (Pelech and Vance 1984(a)). The properties which led to its classification as 'ambiquitous' have been interpreted as reflecting a membrane-associated enzyme which is more active than that present in a cytosolic reservoir (Vance and Pelech 1984). Consequently those factors regulating *in vitro* distribution have been investigated in attempts to define the regulatory process *in vivo*.

1.5.1 The identification of cytidylyltransferase as 'rate limiting'

Control analysis of mammalian PC synthesis has concentrated on the search for a 'rate limiting' enzyme and much of the available evidence has been extensively reviewed (Pelech and Vance 1984(a)). Measurements of steady state pool sizes has shown that the choline phosphate pool exceeds that of CDPcholine by 43 fold in rat liver (Pritchard and Vance 1981, Schneider 1971), 60 fold in Hela cells (Vance et al 1980), between 3 and 25 fold in adult and fetal rabbit lung (Possmayer et al 1981), 8 and 5 fold in adult rat lung and derived type II cells respectively (Post et al 1984(b), and 20 fold in fetal rat lung and derived type II cells (Post et al 1984(a)). The passage of [^{14}C] choline through intracellular pools showed an accumulation of radiolabel in the large choline phosphate pool of rat hepatocytes (Pritchard and Vance 1981), adult and fetal type II cells (Post et al 1982, 1984(a)) and many other tissues (Pelech and Vance 1984(a)). On the basis of these measurements cytidylyltransferase has been claimed as the 'slowest step' in the CDPcholine pathway and ascribed a regulatory function. The interpretation of such pool size measurements is complicated by the possibility of compartmentalisation of both enzyme and substrates within the aqueous cytoplasm. In addition a recent report of a potential 2nd messenger role for choline phosphate by Cuatrecasas and co-workers has shown rapid fluctuations in the choline phosphate pool under the action of a PC specific phospolipase C (Besterman et al 1986).

CK and cholinephosphotransferase have both been proposed as rate limiting in some circumstances. Analysis of rat liver pool sizes led Infante to suggest that CK was 49 times more rate limiting than

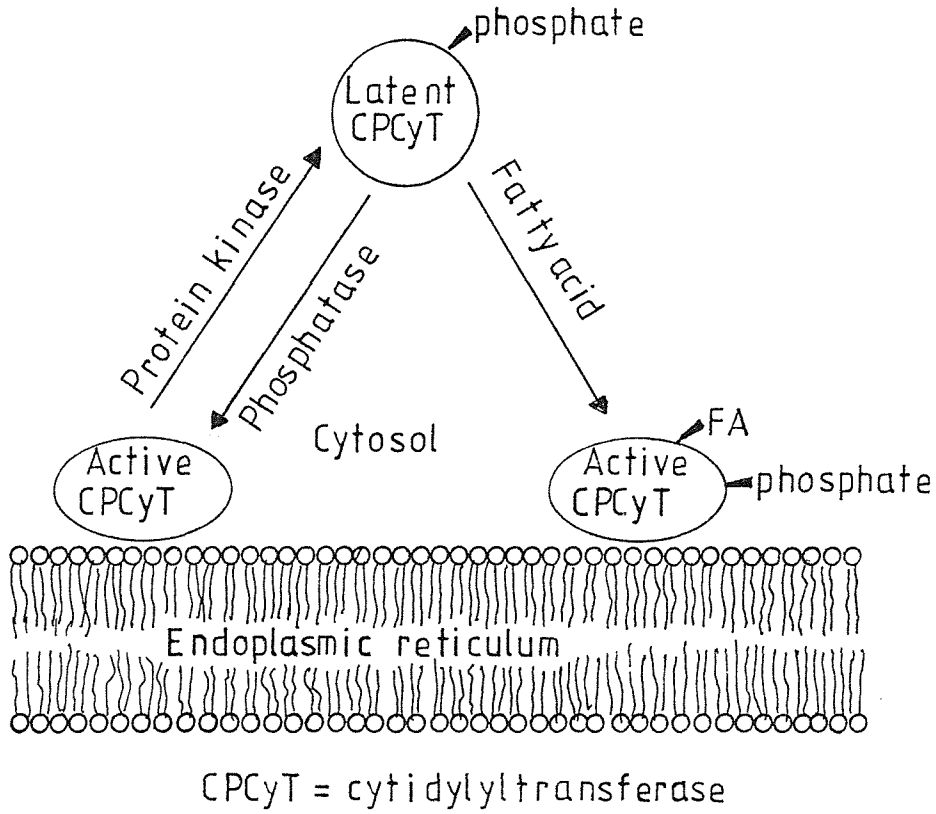
cytidylyltransferase (Infante 1977). Use of the specific CK inhibitor HC3 *in vivo* produced proportional inhibitions of PC synthesis, implying a regulatory function (Ulane 1982). In several studies, moreover, changes in CK V_{max} activities *in vitro* have been shown to correlate with PC flux changes. Increased CK activity in rooster liver coincided with a doubling of PC synthesis (Paddon et al 1982, Vigo and Vance 1981) while a 3.5 fold increase in rat liver CK was matched by a 3.8 fold increase in PC synthesis in rats deprived of essential fatty acids (Infante and Kinsella 1978). The cholinephosphotransferases of rat and rabbit lung (Farrell et al 1974, Farrell 1973) rat intestine (Mansbach and Parthasorathy 1979) and rat kidney (Havenna and Toback 1980) have also been postulated as rate limiting. Rejection of this idea has been justified by pool size measurements which suggest the enzyme is 'at or near equilibrium' (Infante 1977).

The conflicts surrounding what may or may not be a rate regulating enzyme may reflect the limitations of this type of analysis. Using the quantitative control analysis approach outlined in section 1.4.2, it is likely that under most conditions cytidylyltransferase has a large share of control, with a flux control coefficient near unity. Under appropriate conditions, however, a greater share of control may be transferred to CK or choline-phosphotransferase, resulting in their classification as 'rate limiting'.

1.5.2 The role of phosphorylation in cytidylyltransferase activity and translocation

Control of reversible phosphorylation has been implicated in the pathways of both fatty acid and cholesterol synthesis, where ACC (Acetyl CoA carboxylase) (Hardie 1980) and HMG-CoA reductase (Ingebritson and Gibson 1980) have been shown to be inhibited by phosphorylation. Many hormones stimulate protein phosphorylation via cAMP dependant mechanisms. These include adrenaline, thyroxine and glucagon. Glucagon, a polypeptide hormone, appeared to inhibit flux through the CDPcholine pathway in liver(Geelen et al 1979) suggesting that phosphorylation was involved in the regulation of PC synthesis. Direct phosphorylation of cytidylyltransferase has not been shown, but increasing evidence of a phosphorylation mediated regulation has been shown for liver and lung (Pelech and Vanse 1982, 1984(b) Radika and Possmayer 1985).

Fig 1.8 The Translocation model of cytidylyltransferase regulation



The translocation hypothesis postulates a regulatory shift between active, endoplasmic reticulum-bound cytidylyltransferase and a low activity or inactive 'cytosolic' enzyme. A phosphorylation/dephosphorylation cycle is believed to operate in this shift and fatty acids able to overide the phosphorylation 'inactivation'.

In rat hepatocytes stimulation of cAMP-dependant protein kinases by cAMP analogues produced an acute, 35% decrease in PC synthesis, accompanied by a 34% decline in microsome-associated enzyme (Pelech et al 1981). Longer term exposure, over 15 hours, by contrast, produced a 3 fold enhancement of PC synthesis and 6.4 fold increase in microsome-associated enzyme (Pelech et al 1982), which may reflect new enzyme synthesis. The time and temperature dependant increase of soluble liver enzyme in vitro was accompanied by an L to H transition (Choy et al). This effect was abolished in the presence of 14mMMg^{++}, 0.5mM ATP and accelerated in the presence of protein kinase inhibitors or alkaline phosphatase (Pelech and Vance 1982, 1984(b)). The cytosol-associated enzyme had a Km for CTP of 5mM in the presence of MgATP and 20mM NaF a phosphatase inhibitor, but 0.35mM under dephosphorylating conditions and in the presence of protein kinase inhibitors. Such inhibitors reduce the level of phosphorylation in vitro. The enzyme is neither appreciably activated nor aggregated in highly delipidated cytosols (Pelech and Vance 1984(b)) and consequently it has been suggested that membranes are required to express the phosphorylation effect, classifying it as of the 'silent type' (Cohen 1982).

These results, together with the observations of ambiquitous nature have been incorporated into a translocation model, fig 1.8, which envisages a phosphorylation cycle accompanying the transition between active, membrane - associated and inactive cytosol associated enzyme (Vance and Pelech 1984, Pelech and Vance 1984(b)). Certain lipids appear to be able to override this effect (section 1.5.3). Investigation of phosphorylation and translocation in fetal rabbit lung has shown a decline in cytosolic, PG stimulated, cytidylyltransferase with also a small fall in microsomal activity (Radika and Possmayer 1985). The presence of protein kinase inhibitors produced no cytidylyltransferase inhibition. The authors inferred that their observed translocations correlated with increased PC synthesis in late gestation and the early perinatal period.

The effect of phosphorylation by Ca^{++}, phospholipid dependant protein kinase C on PC biosynthesis has recently been investigated. Increases in both PC synthesis and membrane-associated cytidylyltransferase have been recorded in HeLa cells and myoblasts treated with 12-O-tetradecanoylphorbol13- acetate, a C-Kinase activator (Hill et al 1984, Pelech et al 1984(b), Cook and Vance 1984). An increase in Vmax was recorded in myoblasts with no change in the Km for

CTP (Hill et al 1984). In HeLa cells, cytosolic protein phosphorylation was seen with C-Kinase but not reproduced with partially purified enzyme (Cook and Vance 1984) and direct phosphorylation by C-Kinase appeared unlikely. This suggested that phosphorylation of a modulator protein might be involved, while such an indirect phosphorylation might also underlie the cAMP dependant mechanism (Pelech and Vance 1984(b)).

1.5.3 The effects of lipids on cytidylyltransferase activity and translocation

Soluble liver cytidylyltransferase has been shown to be activated and/or aggregated by several lipids. Activation was seen with whole liver lipid, lyso PE, PS, PI (Choy and Vance 1978) and PG (Choy et al 1979 (b)). Aggregation without activation occured with diacylglycerol (Choy et al 1979 (b)) but only where diacylglycerol was elevated *in situ*, since the exogenous lipid was ineffective. Soluble lung enzyme was found to be activated by total lung lipids (Stern et al 1976) and particularly PG (Feldman et al 1978). Delipidation of rat lung cytosols, followed by selective FA restoration showed a number of naturally occuring unsaturated FAs to be potent activators while saturated FAs were inactive (Feldman et al 1981). Exogenous FA promoted the recovery of lung membrane-associated enzyme *in vitro* from broken cell preparations (Weinhold et al 1984).

Incubation of liver slices and isolated heptocytes with FAs in the external medium at 1mM stimulated [^{3}H] glycerol incorporation into triacylglycerol over 15 fold and into PC by 1.4 fold (Rose et al 1963, Nelson and Shersten 1969, Sundler et al 1974). Hepatocytes from fasted rats, depleted of intracellular FA, showed preferential incorporation into phospholipid with low exogenous FA, at the expense of triacylglycerol synthesis (Ontko 1972). A coordinate regulation of triacylglycerol and phospholipid synthesis appears to operate in hepatocytes, with PC requirements being satisfied first. Incubation of hepatocytes with long chain FAs at 1mM gave a 1.9 fold increase in PC synthesis, while 4mM oleate yielded a 3 fold increase in PC flux (Pelech et al 1983(a)). A doubling of microsome-associated enzyme was seen in each case. Oleate activation lowered the apparent Km for CTP by 3.6 fold. Exogenous oleate at 3mM abolished the cAMP analogue mediated inhibition in hepatocytes and led to an increase in membrane-associated enzyme (Pelech et al 1983(b)). These results have been incorporated

into the translocation model, fig 1.8., where intracellular FAs are envisaged as able to overide the phosphorylation mediated dissociation.

The concept of the involvement of a regulatory translocation in lung PC synthesis has recently been rejected by some investigators able to demonstrate flux changes independant of redistribution and redistribution independant of flux changes (Chu and Rooney 1985, Tesan et al 1985, Rooney et al 1986). Rooney and colleagues have argued that a regulatory role exists for cytosol associated enzyme. They have shown a stimulatory effect of estrogen on cytosolic enzyme and PC flux with no redistribution (Chu and Rooney 1985) while only cytosolic enzyme activity was increased and this appeared to be mediated by endogenous phospholipids. In addition some investigation of rat hepatic enzyme have posed questions about their incorporation within the translocation theory. In particular flux dependant changes in microsome-associated enzyme were mirrored by a similar change in cytosol-associated enzyme (Lim et al 1983, Pelech et al 1983 (a), Pelech et al 1982).

1.5.4 Cytoskeletal involvement in the regulation of PC synthesis

A large number of enzymes have been recognized to interact with cytoskeletal elements *in vitro* and potentially *in vivo* (section 1.4.7) with a close relationship shown between some pathways of lipid metabolism and cytoskeletal integrity. In liver, lymphocytes and glial cells, the antimicrotubule agents colchicine and vinblastine reduce cholesterol synthesis (Ottery 1976, Chen and Kou 1980, Volpe 1979). A decrease in PI turnover has been recorded in lymphocytes treated with antimicrotubules (Schellenberg and Gillespie 1980). The reduction in cholesterol anabolism appeared to be due to a disruption of the active microtubule associated form of HMG-CoA reductase (Volpe 1979). Rat hepatocytes treated with anti-microtubules showed a diminished PC, PE and PI synthesis (Azhar et al 1985) suggesting a link for these pathways with cytoskeletal integrity. Changes in the phospholipid synthesis of phagocytic cells treated with anti microtubular agents were a reduction in the PE methylation pathway of up to 64%, with a two fold increase in synthesis via the CDP choline pathway (Pike et al 1980). In the same study the CDPcholine pathway was inhibited in the presence of antimicrofilamental agents by up to 45% while no effect was seen on the methylation pathway. It appeared that, in phagocytic cells at least, the biosynthesis of PC by the CDPcholine or N-methylation pathways is

regulated by the state of cytoskeletal assembly. Such an observation is consistant with a coordinate regulation of N-methylation and CDPcholine pathways, and may, for example, explain the concomitant inhibition of N-methylation and stimulation of CDPcholine routes in livers treated with 3-Deazaadenosine (Pritchard et al 1982).

1.5.5 Developmental changes in Lung PC Synthesis

Increased cytidylyltransferase activities *in vitro* have been found, in association with increased PC synthesis *in vivo*, in a number of pulmonary and non-pulmonary systems(Rooney 1985, Pelech and Vance 1984(a)). PC synthesis in lung slices from fetal rabbit, rat and rhesus monkey have all been shown to increase during the last 10-15% of gestation (Rooney et al 1979, Weinhold 1968, Epstein et al 1976). Concomitant increases in whole lung cytidylyltransferase activities, before or immediately after parturition, have been demonstrated in rat, mouse and rabbit (Stern et al 1976, Oldenburg and Van Golde 1977, Rooney et al 1977). Measurement of choline metabolite pool sizes in fetal rabbit lung have shown a gestational fall in both choline phosphate and CDPcholine pools, together with changes in pulse labelling patterns, interpreted as indicative of an increase in flux through the cytidylyltransferase step(Tokmakjian et al 1981). Some contradictory results from fetal mouse and rat lungs show peaks of choline phosphotransferase and CK activities respectively just prior to term (Oldenbury and Van Golde 1977, Farrell and Hamosh 1978). Such changes may, however, reflect changes in control distribution of the type discussed earlier.

The involvement of a developmental translocation of enzyme has been proposed from investigations from fetal rat lungs. Lung slices from 1-2 day premature rat fetuses, maintained in culture for 3 hours, showed increased PC synthesis, above that determined in freshly delivered fetal rat lung slices (Weinhold et al 1980, 1981). During this period no change in total CDPcholine pathway enzymes was recorded, while an apparent redistribution from soluble to particulate enzyme was noted. Hormonal control appears to underlie the developmental changes in PC synthesis, with glucocorticoids, estrogens and thyroid hormones increasing PC flux and measureable cytidylyltransferase following administration by maternal injection or to fetal explant culture (Rooney 1985. Rooney et al 1986). In a number of the experiments reviewed by

Rooney (Rooney 1985) no flux-dependant enzyme redistribution was seen and the mediators of such flux changes are uncertain. Fetal rabbit lung exposed to estrogen, for example, showed increased cytidylyltransferase with no translocation (Chu and Rooney 1985). Increases in total soluble activity with a reduction in magnitude of lipid stimulation suggested that mobilisation of intracellular PG or other phospholipids might be involved (Rooney 1985, Chu and Rooney 1985, Feldman et al 1978, Pelech and Vance 1984(a)). Glucocorticoid stimulation of rat pulmonary enzyme in organ culture, by contrast, seemed to require both RNA and undefined protein synthesis (Rooney et al 1986). In the presence of dexamethasome cytidylyltransferase activity increased, but no corresponding redistribution was shown, with approximately 70% of enzyme constantly present in 'soluble' form. When actinomycin D was added the stimulatory effect was abolished in dose-dependant fashion. New protein but not enzyme synthesis seemed to be involved in this effect (Rooney 1985, Rooney et al 1986), implying some activation of pre-existing enzyme.

PC flux changes without cytidylyltransferase translocation (Rooney et al 1986, Chu and Rooney 1985) coupled with flux-independant redistribution in type II cells cultured with exogenous surfactant (Tesan et al 1985) have questioned the physiological significance of a regulatory translocation in pulmonary tissue (Rooney 1985, Chu and Rooney 1985, Rooney et al 1986).

1.6. Aims of the Project

The literature reviewed in this chapter reveals that most of the reported properties of mammalian cytidylyltransferase have been obtained from investigation of the rat hepatic enzyme. Cytidylyltransferase purification attempts have been confined largely to this highly enriched source. This is in sharp contrast with the pulmonary enzyme, which has been characterised in crude preparations of many species, but which has not been successfully purified. The failure reflects a lower tissue concentration of enzyme coupled with detergent instability and low recoveries inherent in hepatic enzyme purification schemes. With the exception of one study of neonatal homogenate enzyme, nothing is known of human lung cytidylyltransferase or any human cytidylyltransferase and nothing of any variation between fetal and adult lung enzymes.

The work set out in this thesis is intended to characterise some of the properties of human lung cytidylyltransferase *in vitro* and how these might pertain to the situation *in vivo*. A primary objective therefore, was the characterisation of the enzyme from early gestation and adult lung tissue and that from fetal lung-derived fibroblasts. In particular, subcellular distribution *in vitro*, the presence or absence of H and L forms, effects of PG and storage at 37°C would be monitored, with the nature of any soluble/particulate transitions investigated. Rat lung enzyme behaviour, under similar conditions, would be studied, and the results compared with regard to a potential, putative translocation regulation *in vivo*.

The second aim involved incorporation of these observations within the framework of purification schemes for human lung-derived enzyme. A lack of stable, pure cytidylyltransferase has hindered detailed investigation of lung enzyme from any source, and is an essential pre-requisite for the understanding of control processes, structural and mechanistic studies. In addition purified enzyme may allow resolution between translocation or other control mechanisms by use of specific antibodies. Such antibodies would also allow assessment of phosphorylation phenomena. The application of both conventional purification techniques and a number of newer techniques, including Polyethylene glycol fractionation, affinity chromatography and the use of biocompatible detergents, alone or in combination would be assessed. These attempts would be directed towards maximising both the purification factor and yield of stable enzyme. The application of triazine dye-affinity chromatography, following encouraging preliminary observations, offered the prospect of both a rapid, high yield purification step and a potential active-site directed inhibitor, if suitable dye ligand and binding and elution conditions could be established.

CHAPTER 2

MATERIALS AND METHODS

2 Materials and Methods

Fractionation of human and rat lung tissue

Fetal human lung from therapeutic abortion at 15-16 weeks gestation was used fresh, or frozen at -70°C prior to fractionation. Portions of the same lung processed immediately, or after 2 weeks at -70°C, showed no difference in enzyme recovery or distribution between 'soluble' and particulate enzyme. Non-malignant adult human lung biopsies obtained at surgery were processed within one hour of removal. Fetal human lung-derived fibroblasts were maintained in monolayer culture, with RPMI 1640 tissue culture medium containing 10% fetal calf serum (Flow laboratories). Cells were harvested in logarithmic phase growth.

Adult rat lungs were removed under Sagatal anaesthesia, after blanching *in situ* with heparinised saline, via the right atrium. Pulmonary surfactant was removed by lavage with 20-30ml saline. Large airways were trimmed free of the lungs prior to processing. Dated pregnant rats provided fetal samples with the vaginal positive smear taken as day 0. Fetal rats were removed under Sagatal anaesthesia, their lungs removed and pooled. Postnatal rat lungs were collected individually after decapitation.

The fibrous human and rat tissues were effectively homogenised in buffer A: 150mM NaCl, 50mM TrisHCl, 1mM Ethylene (diaminetetraacetic) acid (EDTA), 1mM dithiothreitol (DTT), 1mM NaN_3, 200μM (Phenylmethylsulphonyl) fluoride (PMSF) at 4°C. Tissue disruption was achieved using an IKA-Ultra Turrax T18 homogeniser (Janke and Kunkel, Stanfen, FRG). Fibroblasts were washed x 3 with ice cold buffer A, scraped with a rubber policeman into buffer A (6ml). The cells were disrupted by sonication (3 x 20 seconds, 14 microns amplitude) using a Soniprep 150 (MSE Instruments), fitted with an exponantial probe.

Subcellular fractionation involved sequential centrifugation of: 10,000 x g x 10 minutes to yield pellet (P_{10}), 100,000 x g x 60 minutes to yield pellet (P_{100}) and supernatant (S_{100}). Supernatants were filtered through glass wool to remove the lipid-rich layer floating on the surface. Negligible cytidylyltransferase activity could be measured in P_{10} fractions, although the tar-like component of adult human P_{10} fractions precluded effective assay.

Cytidylyltransferase Assays

Methyl [^{14}C] choline phosphate conversion to methyl [^{14}C] CDPcholine was monitored by a selective charcoal binding (Stern et al 1976, Feldman et al 1978) modified for use with 1.5ml microfuge tubes. Enzyme (50µl) was mixed with assay buffer (30µl), comprising 100mM TrisHCl, 100mM NaCl, 2.5mM EDTA, 5mM DTT, 20mM $MgCl_2$, 12mM CTP pH 7.4 The pH was adjusted after the addition of the acidic CTP. PG, where included, was sonicated directly into the assay buffer (3 x 20 seconds, 14 microns.) to produce 0.25mM PG in the final mixture (90µl). Storage of DTT-containing buffers gave false apparent activities in samples and controls, and consequently buffers were prepared daily.

The reaction was started with methyl [^{14}C] choline phosphate (10ul, 16.6mM, 2µCi/µmole). After 20 minutes at 37°C the reaction was stopped by placing the tubes in boiling water for 2 minutes. The boiled samples were vigourously mixed with a 5.5% slurry of 2 x acid washed charcoal in 20mM choline phosphate (500µl) and allowed to stand for 10 minutes. Charcoal-bound CDPcholine was separated from the diluted, unbound choline phosphate by centrifugation at 12,000 x g x 1 minute, and the charcoal pellet washed by sequential mixing and centrifugation form 4 x 1ml distilled water, to remove non-specifically bound choline phosphate.

CDPcholine was then eluted by incubation with ethanol:water:0.880 ammonia (60:37:3) (1ml, 90°C for 20 minutes), and recovery completed by a 1ml wash with this eluant. The pooled supernatants were taken up in Cocktail T(BDH Chemicals) and radioactivity determined. Duplicate blank incubations typically contained less than 1,000 DPM and were subtracted prior to activity calculations.

Activities were calculated from the following equation:

$$\text{Cytidylyltransferase Activity (mU/ml)} = \frac{\text{DPM } (^{14}\text{C CDPcholine})}{\underset{\text{(Sp.Act choline phosphate)}}{4726}} \times \frac{1}{\underset{\text{(Time mins)}}{20}} \times \frac{1}{\underset{\text{(Vol. mls)}}{0.05}}$$

1mU = 1nmole CDPcholine synthesised/minute.

Protein determinations allowed specific activity measurements (mU/mg protein).

The charcoal eluant comigrated with an authentic [^{14}C] CDPcholine (Amersham) on LK5 TLC plates (Whatman) developed in methanol:0.6% NaCl:0.880 ammonia (50:50:5,v/v/v). Quadruplicate human lung cytidylyltransferase assay using the TLC method (Vance et al 1981) were within 5% of simultaneous charcoal determinations. Recoveries of known methyl [^{14}C] CDPcholine standards, corresponding to activities between 1 and 20mU/ml, in the presence of methyl [^{14}C] choline phosphate, gave figures between 96% and 103%. Assays in the presence of CHAPS did not include PG.

The reaction velocity was constant over 30 minutes and proportional to the amount of enzyme preparation added to the assay. Increasing CTP from 4mM to 8mM produced a slight inhibition of cytidylyltransferase, while doubling the choline phosphate concentration gave no apparent change. A five fold increase in PG concentration to 1.25mM increased soluble enzyme activities by less than 10%.

Protein determinations

The protein concentration of human and rat tissue samples was measured using a modification of the Folin-Coicalteau reagent (Lowry et al 1951). The method utilised a deoxycholate/trichloroacetic acid coprecipitation step (Peterson 1983) to remove uncharacterised interfering substances present in buffer A. The smaller amounts of protein present in fibroblast preparations were quantified by the coomassie dye binding method (Bradford 1976) with sensitivity enhancement as described (Peterson 1983). For both determinations bovine serum albumin (BSA 1mg/ml) was used as a reference standard.

Lipid Phosphorous

Phopholipid phosphorous was determined by the method of Bartlett (Bartlett 1959).

Ammonium sulphate fractionation

Solid ammonium sulphate (AnalaR grade BDH Chemicals) was added to S_{100} to give the appropriate fractional saturation at 4°C using the formula:

$$w = \frac{0.515 \times v \times (S_2 - S_1)}{1 - (0.272 \times S_2)}$$

Where w = weight of $(NH_4)_2SO_4$ added
v = volume S_{100}(ml)
S_2 = final fractional saturation
S_1 = initial fractional saturation

Polyethyleneglycol(PEG) fractionation

Stock PEG_{6000} (10% in buffer A) was mixed with the enzyme fraction to give the appropriate concentration according to the formula:

$$v = \frac{a \times y}{10 - y}$$

Where v = volume of 10% PEG added
a = initial volume
and y = required concentration.

Gel filtration of H and L form cytidylyltransferase

Gel filtration on Sepharose 6B (Pharmacia, Upsalla, Sweden) was used to resolve H and L form cytidylyltransferase. Column chromatography was performed in the presence of buffer A at 4°C. Calibration of the Sepharose 6B column (90cm x 2.5cm) was made using a Blue Dextran as a void volume marker, rat liver pyruvate kinase (approximately 200,000 dalton tetramer), BSA (67,000 daltons), soybean trypsin inhibitor (21,000 daltons) and tryptophan (206 daltons). Flow rate was approximately 40ml/hr and 15 minute/10ml fractions were collected.

Cytidylyltransferase assays of column fractions were made singly in the absence and presence of 0.25mM PG.

Coupling of triazine dyes to Sepharose CL-4B

Selected triazine dyes were coupled to Sepharose CL-4B as described (Lowe and Pearson 1984). Sepharose CL-4B (5g) washed with water (1L) on a glass sinter, was mixed with dye (50mg) in distilled water (5ml) and mixed for 30 minutes at room temperature. NaCl solution (22% w/v, 1ml) was added to give a final concentration of 2% NaCl and gently mixed for a further 30 minutes. Solid sodium carbonate was added to 1% (w/v) and gel/dye mixture tumbled on a spiramixer for five days to ensure efficient coupling of both mono- and dichlorotriazine dyes. Gels were washed with copious volumes of water, followed by 6M urea until the washings were free of colour. They were then stored in distilled water containing NaN_3(1mM) as a bacteriostat. The immobilised dye concentrations were determined by acid hydrolysis of a known weight of gel followed by dye quantitation at their absorption maximum from known molar absorbance coefficients (Lowe and Pearson 1984).

Synthesis of p-diazonium phenyl phosphoryl choline and its immobilisation to Sepharose 4B-bound glycyl tyrosine

Cyanogen bromide activated Sepharose 4B (10ml, Pharmacia, Upsalla, Sweden) was mixed with glycyl tyrosine (15mg) in 100mM $NaHCO_3$, 500mM NaCl pH 7.4 (10ml) end over end at 4°C for 3 days. Gel was washed alternately with 100mM Na acetate, 500mM NaCl pH 4 and coupling buffer.

Synthesis of the choline phosphate analogue was that previously described (Chesebro and Metzger 1972). Briefly, choline iodide (2 mmoles), p-nitrophenylphosphorodichloridate (2 mmoles) and dry quinoline (2 mmoles) were each dissolved in acetonitrile (0.5ml), mixed and stirred on ice for 4 hours in the dark. Pyridine (1ml) and H_2O (0.2ml) were added and the mixture allowed to stand at room temperature for 30 minutes. The mixture was rotary evaporated, dissolved in water and 'desalted' by passage through Amberlite MB3. The eluate was lyophilised and stored in a dark bottle at room temperature.

The p-nitrophenylphosphorylcholine (30mg, 100μmoles) was dissolved in methanol and quantitatively reduced with H_2 at one atmosphere in the presence of 10% palladium on charcoal (10mg). After filtration and rotary evaporation, the residue was dissolved in 1.5M HCl (1.5ml, 0°C). H_2O (500μl) containing $NaNO_2$ (700 μmoles) was added slowly, with mixing, on ice for 1 minute. After 7-8 minutes the solution was mixed with washed tyrosyl Sepharose 4B in 0.2M Na_2CO_3 on ice for 3 hours. Coloured gel (yellow/orange) was washed with distilled water and stored in 1M acetic acid at 4°C.

SDS polyacrylamide gel electrophoresis (SDS PAGE)

Discontinuous SDS PAGE (Laemlli 1970) was performed on a vertical Gel electrophoresis system GE-2/4 (Pharmacia, Upsalla, Sweden). Gels (80mm x 140mm x 2.7mm, or 180mm x 140mm x 0.7mm) were cast in Pharmacia Gel Slab Casting Apparatus, GSC-8 or GSC-2 respectively. Homogenous 10% gels had the following composition (each 25ml): Acrylamide (2.5g), bis-acrylamide (83mg), 1M TrisHCl pH 6.8 (3.1ml), SDS (25mg), ammonium persulphate (25mg), made up to 25ml. Polymerisation was initiated by the addition of TEMED (12.5μl). Polyacrylamide gels were polymerised 2-3cm from the top edge and overlaid with a 6% stacking gel (each 10ml): Acrylamide (0.6g), bis-acrylamide (63mg), 3M TrisHCl (1.25ml), SDS (10mg), ammonium persulphate (10mg), TEMED (5μl). Well formers were used to provide 6 wells/80mm x 140mm gel and 14 wells/180mm x 140mm gel.

The buffer composition of upper and lower reservoir was: 25mM Tris, 192mM glycine, 0.1% SDS pH 8.3. Samples were boiled prior to electrophoresis for 3 minutes with 8M urea, 5% SDS, 5% -mercaptoethanol, 62.5mM TrisHCl pH 6.8 (1:1, v/v). Molecular weight calibration was achieved with a mixed standard set: lysozyme (14,300 daltons), soybean trypsin inhibitor (20,100 daltons), carbonic anhydrase (29,000 daltons), ovalbumin (45,000 daltons), BSA (68,000 daltons), rabbit muscle phosphorylase a (92,500 daltons) (Sigma Chemical Company, Poole, Dorset).

After electrophoresis (25mA/80mm x 140mm gel or 40mA/180mm x 140mm gel), protein bands were visualised by staining with Coomassie blue R250 (0.25%) in water:methanol:glacial acetic acid (50:40:10, v/v/v) at 60°C for 1 hour. Excess stain was removed in water:methanol:glacial acetic acid (60:30:10) and acceleration was achieved with an electrophoretic destainer GD-4 II (Pharmacia, Upsalla, Sweden). Gels were equilibrated

in 3% glycerol, photographed and dried onto 3MM paper (Whatman Chemical Separations, Maidstone, Kent). Where semi-quantitative analysis was required, equilibrated gels were scanned at 570nm in an SP8-400 uv/vis spectrophotometer fitted with a densitometer attachment (Pye-Unican, Cambridge), followed by gravimetric integration.

Reagents and Chemicals

Chemicals were supplied by BDH Chemicals, Poole, Dorset, Sigma Chemical Company Ltd., Poole, Dorset or Aldrich Chemical Company Ltd., Gillingham, Dorset. Triazine dyes were purchased from Sigma or BDH except Procion dyes Turquoise HA and Turquoise MX-G a kind gift from Dr. Alan Batters, ICI Organics Division, Blakely, Manchester. Levafix Brilliant Green E-5BNA was kindly provided by Bayer UK Ltd., Manchester. Radiochemicals were obtained from Amersham, Buckinghamshire. Chromatography media were supplied by Pharmacia, Upsalla, Sweden. Sepharose 4B-immobilised Procion Blue MX-R was a kind gift from Dr. Mike Byford, Biochemistry Department, Southampton University.
PG (Sigma) was enzymically prepared from egg yolk PC.

CHAPTER 3

PROPERTIES AND BEHAVIOUR OF FETAL AND ADULT HUMAN LUNG CYTIDYLYLTRANSFERASE IN VITRO

3.1 Introduction

Cytidylyltransferase from rat liver and lung has been found in membrane associated (particulate) and membrane-free supernatant (soluble) fractions of tissue homogenates (Schneider 1963, Stern et al 1976). The authors recognised that the subcellular distribution *in vitro* was modulated by, and critically dependant on the ionic strength of homogenising media. Saline based buffers promoted recovery of soluble enzyme, while the use of sucrose or distilled water resulted in a greater recovery of a particulate form. Early investigations of flux changes in PC synthesis, and a potential regulatory role for cytidylyltransferase, concentrated on alterations in soluble enzyme activity and its activation by lipids (Stern et al 1976, Choy and Vance 1978, Feldman et al 1980). It was shown experimentally that rat lung and liver soluble enzyme existed as high (H) and low (L) molecular weight forms resolvable by gel filtration (Stern et al 1976, Choy et al 1977).

Storage (Ageing) of soluble liver enzyme at 4°C for 5 days or 20°C for 8 hours produced an increase in measureable activity with a concomitant shift from L to H form (Choy et al 1977, Vance et al 1981). The transition from H to L form *in vitro* has been effected by 0.05% SDS or 2M NaCl (Choy et al 1977) while antibody to partially purified L form enzyme cross-reacted with H form and microsome associated enzyme (Choy et al 1978). Liver H form from gel filtration was found in the pellet of a 100,000 x g x 60 min centrifugation. It has been suggested that H form may comprise L form enzyme bound to initially non-sedimentable membrane fragments (Choy et al 1977, Feldman et al 1978). The proposed L form binding to membrane fragments is envisaged as an artefactual observation *in vitro* which may mirror a soluble L form/particulate H form regulatory translocation *in vivo*.

Reports of increases in microsomal membrane-associated enzyme accompanying increased flux through the CDPcholine pathway, have been made in many tissues. In rat hepatocytes (Pelech et al 1983 (a), (b)) developing rat lung (Weinhold et al 1981), developing rabbit skeletal muscle (Cornell and Maclennan 1985(a)) and other cell and tissue types (Vance and Pelech 1984) an apparent flux-dependant redistribution has been described. Translocation (Vance and Pelech 1984) has been proposed as a potential mechanism to explain alterations in subcellular locations. It was suggested that soluble L form enzyme constitutes an inactive reservoir which may be activated and translocated to the endoplasmic

reticulum. In both liver and lung reversible phosphorylation (Pelech and Vance 1982, 1984(b), Radika and Possmayer 1986) and fatty acid binding (Pelech et al 1983(a), Weinhold et al 1984) have been implicated as mediators of this putative translocation.

The significance of translocation phenomena observed with brain hexokinase, the archetypal 'ambiquitous' enzyme (Wilson 1978, 1980), has been questioned recently. Rapid brain fractionation techniques in particular show most of the enzyme associated with the outer mitochondrial membrane under a variety of flux modifications (Kyriazi and Basford 1986). Similarly cytidylyltransferase translocation in lung has been questioned by demonstrations of flux changes through the CDPcholine pathway unaccompanied by measureable redistribution phenomena. A concommitant increase in both soluble fetal rabbit lung cytidylyltransferase and PC synthesis was seen upon estrogen treatment (Chu and Rooney 1985) but no redistribution was shown. Glucocorticoid treatment of fetal rat lung explants also activated PC synthesis and cytidylyltransferase with no redistribution (Rooney et al 1986). The picture was further complicated in rat type II cells, where choline depletion led to activation and redistribution (Anceschi et al 1984, Tesan et al 1985), but culture in the presence of added, purified, surfactant led to redistribution without increased synthesis (Tesan et al 1985). The ability to demonstrate independant activation and redistribution appeared to indicate that translocation was not an obligatory accompaniment of changes in PC synthesis in lung tissue.

The work presented in this chapter investigates human and rat lung cytidylyltransferase and some of their properties in vitro in an attempt to clarify the behaviour of the enzyme in lung tissue.

3.2 Results

3.2.1 Human lung cytidylyltransferase

A single report of the measurement of human lung enzyme exists (Thom and Zachman 1975) from post mortem neonatal lung homogenates. Fetal lung at 15-16 weeks gestation does not synthesise pulmonary surfactant and enzyme distribution might be expected to differ from the adult human pattern if a translocation process operates. Fetal and neonatal human lung around the time of delivery was not available and consequently

Table 3.1 Subcellular location of fetal and adult human lung cytidylyltransferase

activities = nmoles/minute/mg protein (mU/mg) ± SEM

		Activity w/o PG	Activity + 0.25mM PG	% Total Activity (with PG)
Fetal lung	S_{100}	0.36 ± 0.05	0.80 ± 0.06*(n = 25)	66.8% ° ± 4.8% (n = 16)
	P_{100}	1.65 ± 0.17	1.65 ± 0.29°(n = 16)	33.2%
Adult lung	S_{100}	0.23 ± 0.03	0.58 ± 0.07*(n = 10)	66.1% ° ± 7.5% (n = 5)
	P_{100}	1.58 ± 0.39	1.88 ± 0.44°(n = 5)	33.9%
Fibroblasts	S_{100}	1.17 ± 0.24	4.81 ± 0.51*(n = 9)	74.1% * ± 4.6% (n = 5)
	P_{100}	2.90 ± 0.40	2.42 ± 0.31°(n = 5)	25.9%

* $p < 0.005$ ° n.s.

Lung and cell homogenates in buffer A were separated into 100,000 x g x 60 minute supernatants (S_{100}) and 10,000 x g x 10 minute - 100,000 x g x 60 minute pellet fractions. Pellets were resuspended in volume equal to S_{100} and cytidylyltransferase assayed in each fraction ± 0.25mM PG.

changes in the subcellular distribution of the enzyme was investigated in the fetal and neonatal rat. This would allow any transient changes at or around birth to be monitored.

3.2.1.1 Subcellular distribution of Fetal and Adult Human Lung Cytidylyltransferase

In this section the subcellular distribution between soluble and particulate human enzyme was investigated in fetal lung, adult lung and fetal lung derived fibroblasts. Homogenates (20% w/v) of 14-16 week fetal lung and adult lung biopsies were made in buffer A, 0.145M NaCl, 50mM TrisHCl pH7.4, 1mM EDTA, 1mM DTT, 200μM PMSF. A black, tar-like, contamination present in all adult lung homogenates was removed by centrifugation (10,000 x g x 10 minutes). Failure to remove this material resulted in an inability to resuspend the 100,000 x g x 60 min particulate fraction. The corresponding fraction from the uncontaminated fetal lung showed negligible cytidylyltransferase activity. The post 10,000 x g x 10 min supernatants were centrifuged at 100,000 x g x 60 mins and the pellets were resuspended in an equal volume of buffer A. No membrane markers or cytosol markers were assayed as these have not been characterised in human lung. Fetal lung fibroblasts homogenates (3-4% w/v) were similarly fractionated.

There was no difference between adult and fetal lung in the percentages of enzyme present in the 100,000 x g x 60 min supernatant (S_{100}) and the 100,000 x g x 60 min pellet (P_{100}), Table 3.1. The specific activities of S_{100} and P_{100} enzyme in fetal and adult lungs were similar, while fibroblasts demonstrated higher specific activities than the fetal lungs from which they were derived. The stimulation of fetal and adult S_{100} enzyme by 0.25mM PG was also similar at 2.2 fold and 2.5 fold respectively, while fibroblast S_{100} was stimulated 4.1 fold. The greater specific activity of the fibroblast enzyme was probably the result of the absence of extracellular matrix proteins and the ability to remove serum proteins before homogenisation. The PG stimulation was lower than that previously recorded for lipid stimulations of rat liver and lung soluble enzymes (Choy et al 1979, Feldman et al 1980). Increasing PG fivefold to 1.25mM resulted in a less than 10% increase in fetal or adult soluble activity.

The specific activities of soluble enzyme in the absence of PG were higher than those previously recorded in neonatal lung homogenates (Thom and Zachman 1975). They quoted a value of 0.1mU/mg, possibly reflecting some degradation of activity during the 1-7 hour post mortem delay in processing. This interpretation was offered support by observations of post mortem adult human lung, where a delay of many hours before removal gave S_{100} activities of 0.03 - 0.05mU/mg in the absence of PG.

These determinations provided no support for a developmental translocation of cytidylyltransferase. While no significant variation in distribution was shown, data on flux through the CDPcholine pathway in these tissues was unavailable. It was possible that distribution changes specific to the surfactant synthesising type II cells may have been masked by a lack of overall tissue response. Type II cells contribute an estimated 16% of total lung parenchyma (Crapo et al 1982). Consequently if, from the results above, a 2.2 fold increase of 'soluble' enzyme activity upon binding whole lung membranes is assumed to represent the maximum variation achievable in vivo, then at least a 16 fold increase in type II cell PC synthesis would be necessary to double whole lung P_{100} activity. This may be a conservative estimate if human lung enzyme is stimulated to a greater extent by whole lung lipid as seen in rat lung cytidylyltransferase (Stern et al 1976, Feldman et al 1978).

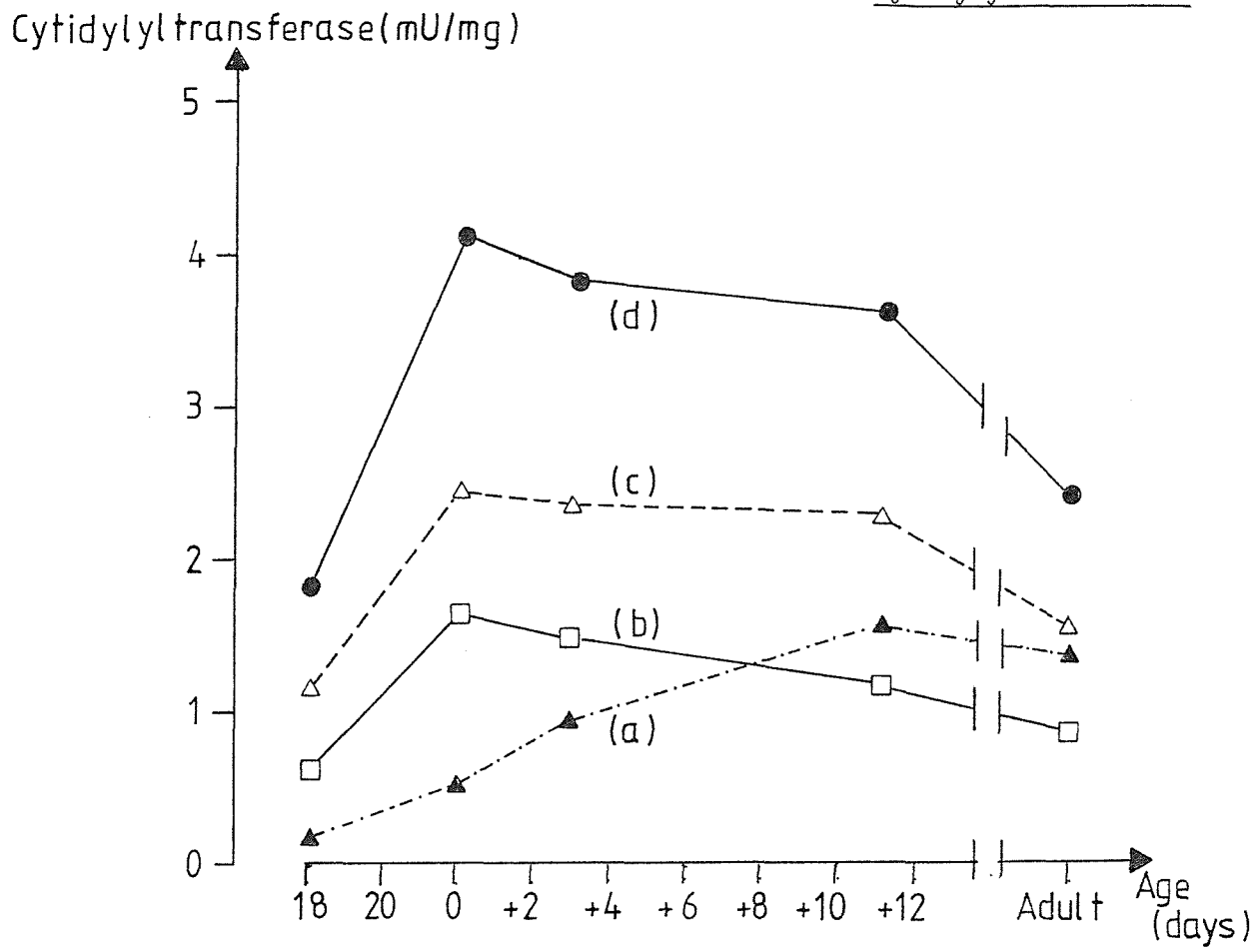
Results from two time points were clearly unable to exclude the possibility of increased cytidylyltransferase activities and possible translocation in the immediate pre- and post natal period. Human lung tissue covering this period was unavailable for confirmation, while the results of Thom and Zachman were unable to clarify the position due to their confinement to homogenate activities (Thom and Zachman 1975).

3.2.1.2 Developmental changes in rat lung enzyme subcellular distribution

Rat lung cytidylyltransferase was investigated in tissue from day 18 gestation (Term 22 days), birth, day 3 and day 11 postnatally and these results compared with adult values. Fetal and neonatal lung homogenates (7 - 8.5% w/v) and adult lung homogenates (20% w/v) were separated into S_{100} and P_{100} fractions.

The activities of the S_{100} and P_{100} fractions were reported as mU/mg protein of the 10,000 x g x 10 minute supernatant from which they were

Fig 3.1 Developmental changes in subcellular distribution of rat lung cytidylyltransferase



Rat lung cytidylyltransferase distribution in S_{100} and P_{100} fractions was measured between d18 gestation and adult. Results were calculated and expressed as mU/mg protein in the 10,000 x g x 10 minute supernatant.

(a) = S_{100} activity without PG.

(c) = S_{100} activity + 0.25mM PG.

(b) = P_{100} activity without PG.

(d) = (b) + (c).

derived, Fig 3.1. The maximum measureable activity per mg protein in the combined S_{100} and P_{100} was found at birth, falling on days 3 and 11 postnatally, toward adult values. In the absence of PC synthesis measurements it was not possible to assess the significance of the increased potential for CDPcholine synthesis represented by increased specific activity of total cytidylytransferase. A previous study has noted increased PC synthesis in lung slices from prematurely delivered 20 and 21 day rat fetuses (Weinhold et al 1981). This suggested that PC synthesis increased at or immediately following delivery.

Particulate enzyme comprised 31% of activity on day 18 of gestation and also 31% in adult rat which was remarkably similar to the lack of change recorded between fetal and adult human enzyme, in the previous section. At birth, day 3 and day 11 postnatally, however, 40%, 40% and 37% of activities respectively were particulate. Soluble enzyme activities compared favourably with observations with rabbit lung cytosols (Rooney et al 1976, Tsao and Zachman 1977) where highest values were recorded post natally. Soluble activities in the absence of PG were similar to those previously reported for rat (Maniscalco et al 1978) and followed the same trend observed in fetal mouse lung (Brehier and Rooney 1981).

The change in particulate enzyme appeared to offer some support for a translocation at or around birth, with the increase from 31% to 40% coinciding with a 2.3 fold increase in the specific activity of total measureable enzyme. The behaviour of soluble enzyme, however, seemed more consistent with the observations of Rooney and Colleagues (Chu and Rooney 1985, Rooney et al 1986) with increased soluble enzyme measured in the presence of PG. The S_{100} enzyme was most sensitive to PG stimulation at birth (4.9 fold) and progressively less sensitive on days 3 and 11 post natally reaching 1.28 fold in the adult. The increased specific activity of rat lung enzyme observed around birth may be the result of increased enzyme protein synthesis. Recent results from dexamethasome activation of cytidylyltransferase in fetal rat lung explants (Rooney et al 1986) suggested that de novo RNA and protein synthesis were involved with no observed translocation.

3.2.2 Cytidylyltransferase H and L forms, ageing effects and lipid stimulation

The molecular weight, whether H and L forms are present, and any aggregation of soluble human lung cytidylyltransferase have not previously been characterised. Gel filtration of soluble rat lung and liverenzymes had previously shown H and L forms (Stern et al 1976, Choy et al 1977), while ageing of liver soluble enzyme increased the proportion of H form on gel filtration (Choy et al 1977). Ageing of rat liver cytosols has also been used as a step in cytidylyltransferase purification (Choy et al 1977, Vance et al 1981). Storage of cytosols at 4°C for 5 days produced a six fold stimulation of activity. Gel filtration analysis of human soluble enzyme was undertaken and aspects of ageing were investigated for human and rat S_{100}s.

3.2.2.1 Ageing of human and rat lung S_{100} enzyme

Two samples of fetal and adult human S_{100}s were stored at 4°C for 5 days and assayed for changes in activity of cytidylyltransferase. The observed increases in fetal and adult S_{100} activities of 16% and 7% were small in comparison with the six fold increases with rat liver cytosol, Table 3.2. A consistant finding in each sample, however, was a copious precipitate of material rich in protein, as determind by Lowry analysis. The possibility of bacterial contamination or proteolysis was discounted due to the inclusion of azide and PMSF in all buffers. When the precipitates were analysed by solubilisation and SDS PAGE they were found to contain numerous proteins with an intensly stained band between 40,000 and 45,000 daltons.

Ageing of rat liver cytosols was faster at 20°C than at 4°C (8 hours vs 5 days) (Pelech and Vance 1982) suggesting a temperature - dependant mechanism. In an attempt to accelerate the process, to avoid long delays in purification schemes, ageing was investigated at 37°C. A greater activation was observed for fetal lung S_{100} at 37°C and maximum activation was seen after 2 hours. All S_{100}s became progressively cloudier on incubation. The particulate matter produced was effectively removed by a second centrifugation of 100,00 x g x 60 minutes to yield a clear supernatant (2nd S_{100}). The protein precipitated from aged S_{100}s typically represented between 5% and 10% of total soluble protein and was consequently insufficient alone to dramatically alter supernatant

Table 3.2 The Ageing of human lung S_{100} Cytidylyltransferase at 4°C

	Activity (mU/mg) w/o PG	Activity + 0.25mM PG
Fetal S_{100}	0.47	0.93
(cf fresh S_{100}	0.36 ± 0.05	0.80 ± 0.06)
Adult S_{100}	0.40	0.62
(cf fresh S_{100}	0.23 ± 0.03	0.58 ± 0.07)

Two samples of fetal and adult human lung S_{100} were stored at 4°C for 5 days prior to assay. Cytidylyltransferase activities were then determined and compared with activities in fresh S_{100}.

Table 3.3 The Ageing of lung S_{100} Cytidylyltransferase at 37°C

		Fresh activity	Aged at 37°C	2nd S_{100}
Fetal human S_{100}	-PG	0.36 ± 0.06	0.65* ± 0.08	0.68* ± 0.26
	+PG	0.80 ± 0.06	1.48* ± 0.26	1.46* ± 0.26
		(n = 25)	(n = 8)	(n = 6)
Adult human S_{100}	-PG	0.23 ± 0.03	0.48° ± 0.20	1.23* ± 0.30
	+PG	0.58 ± 0.07	0.67° ± 0.15	1.30* ± 0.28
		(n = 10)	(n = 3)	(n = 3)
Fibroblast S_{100}	-PG	1.17 ± 0.24	2.12° ± 0.34	0.91° ± 0.17
	+PG	4.81 ± 0.51	5.19° ± 0.50	4.40° ± 0.32
		(n = 9)	(n = 9)	(n = 9)
Adult rat S_{100}	-PG	1.56 ± 0.08	1.88° ± 0.17	0.29* ± 0.04
	+PG	2.01 ± 0.11	2.03° ± 0.21	0.34* ± 0.07
		(n = 8)	(n = 8)	(n = 6)

* significantly different $p < 0.005$ from fresh S_{100}, ° not significant $p > 0.01$

Cytidylyltransferase activities were determined in fresh S_{100} S_{100} aged at 37°C for 2 hours and 2nd S_{100} fractions.

specific activities. Enzyme activities were determined in fresh and aged S_{100}s and 2nd S_{100} fractions.

The results of ageing, table 3.3, showed no effect on rat enzyme although removal of the particulate fraction following ageing led to a loss of 83% of activity. The particulate fraction contained an estimated 30-50% of lost activity, but problems in effective resolubilisation prevented accurate duplicate analysis. The effects on human enzyme showed distinct differences in behaviour. In the absence of PG all specific activities increased, while PG stimulated activity was only increased in fetal lung S_{100}. Removal of particulate fractions doubled the adult PG stimulated activity in the 2nd S_{100}, while fetal and fibroblast activities were unchanged from aged S_{100} values. When particulate fractions were resuspended enzyme activity was present but imprecisely quantifiable. The principal conclusion from these results was that measurement of human lung S_{100} enzyme did not accurately reflect the total potential activity present. Aged rat lung enzyme differed from the human enzyme since a large proportion of enzyme became less soluble. By comparison only a small amount of aged human enzyme was apparently associated with the insoluble fractions. The removal of the particulate fraction from adult human lung aged S_{100} appeared to activate the remaining enzyme, while ageing of fetal enzyme at 37°C activated enzyme irrespective of particulate component.

3.2.2.2 Cytidylyltransferase H and L forms in adult human lung S_{100}, and ageing phenomena

Gel filtration analysis of human adult S_{100} and changes upon ageing were investigated. Fresh adult human lung S_{100} (15ml, 80mU, 0.3mU/mg) was applied to a Sepharose 6B column (2.5cm x 90cm) in buffer A at 4°C. Fractions (10ml) were collected and A280nm was determined. Cytidylyltransferase activities were determined in void volume (V_o) fractions and around 200,000 daltons as previously determined from standard molecular weight markers.

Lowry protein determinations of the eluted fractions, fig 3.2, agreed closely with A280 values with the exception of V_o fractions, where A280 values were on average 2.1 fold higher than corresponding measurements. The V_o fractions appeared milky or turbid suggesting that light scattering was responsible. The soluble enzyme was resolved into a

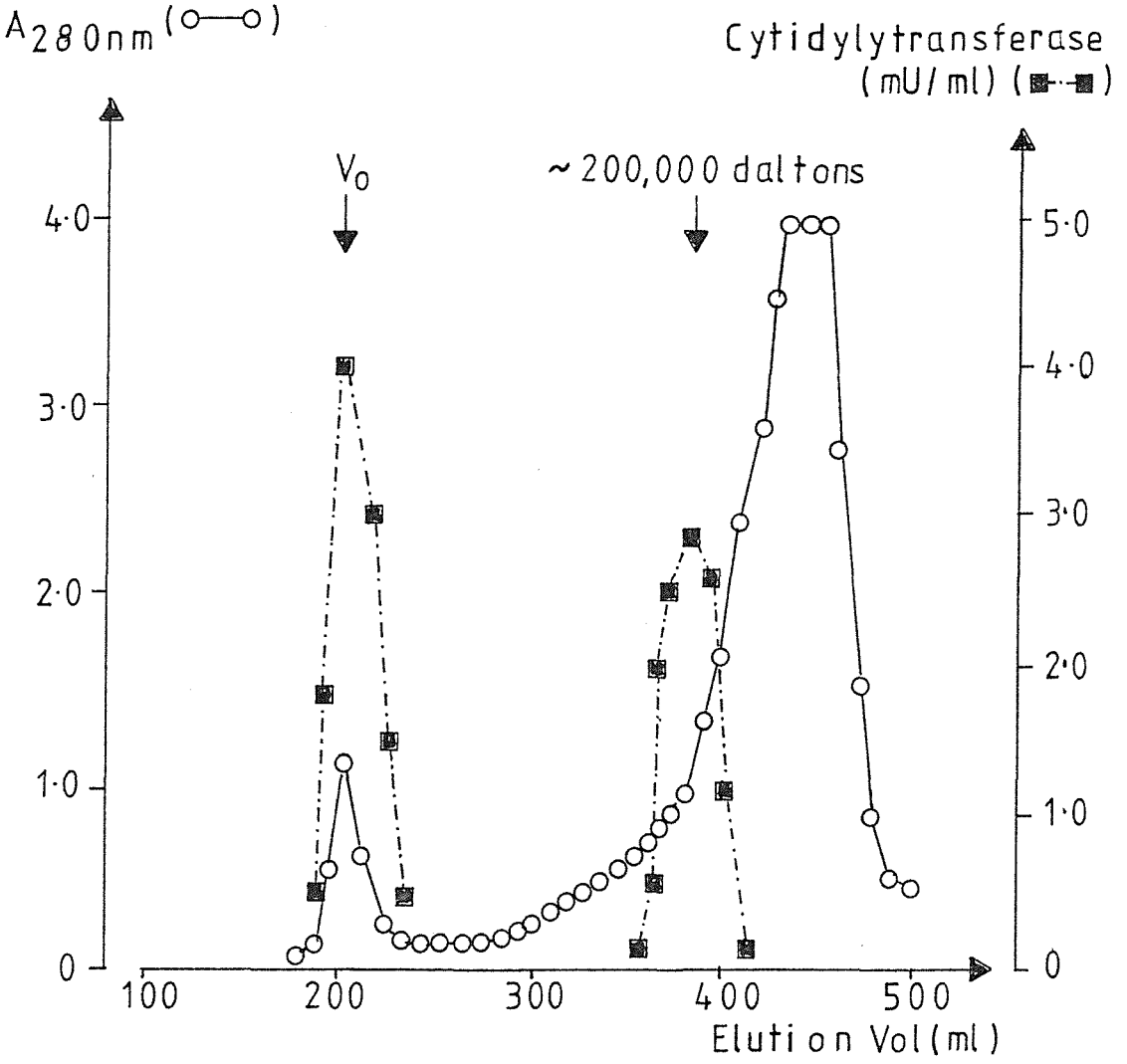
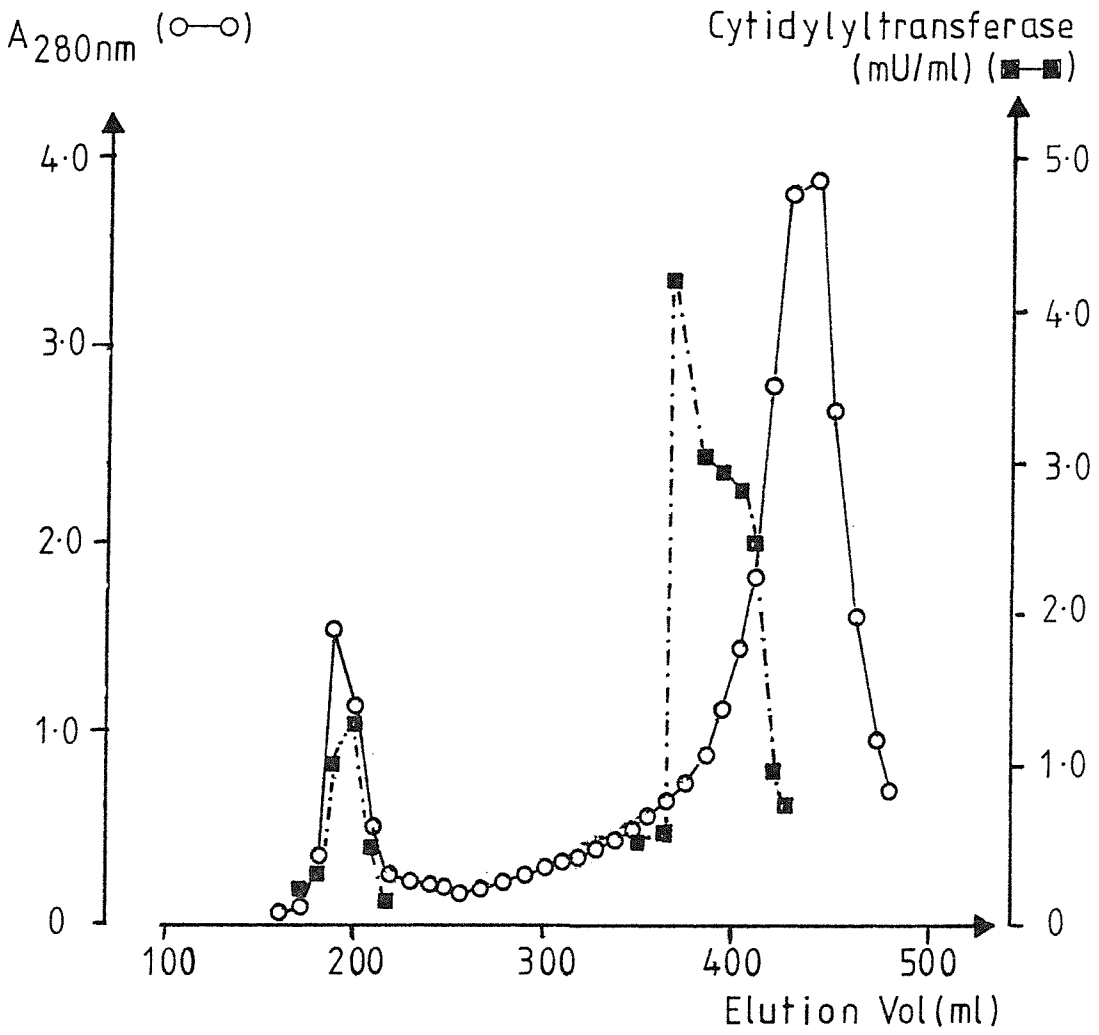


Fig 3.2 Gel filtration of fresh adult human lung S_{100}–

Fresh adult human lung S_{100} (15ml, 80mU, 0.3mU/mg) was applied to a Sepharose 6B column (2.5cm x 90cm) in buffer A at 4°C. Fractions (10ml) were collected and cytidylyltransferase assayed in V_o fractions and around 200,000 daltons. A total of 241 mU were recovered in these fractions, representing a three fold increase over applied activity.

Fig 3.3 Gel filtration of aged adult human lung S_{100}-



Aged human lung S_{100} (15ml, 95mU) was applied to Sepharose 6B (2.5cm x 90cm) in buffer A at 4°C. A total of 207mU were recovered, representing a 2.2 fold increase over applied activity.

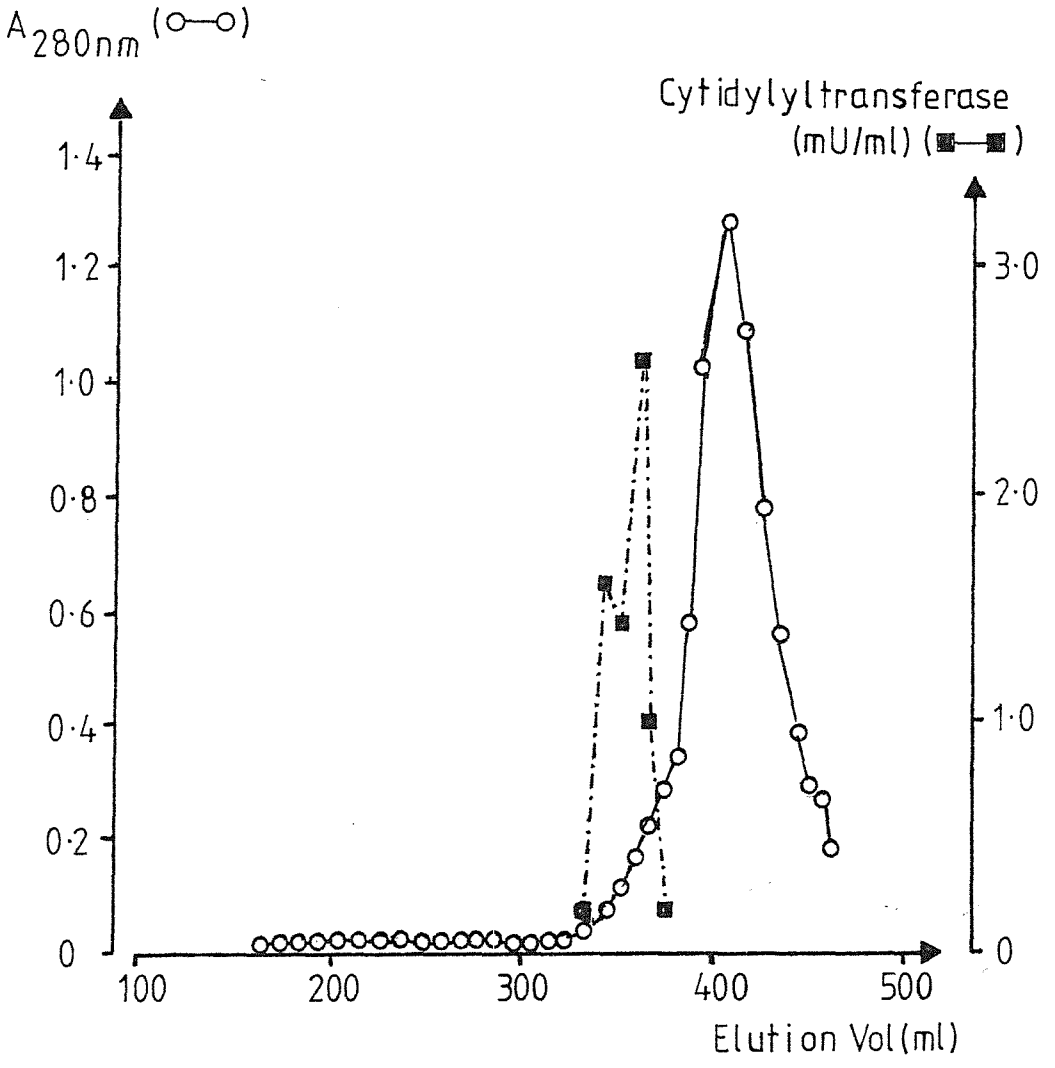


Fig 3.4 Gel filtration of adult human lung 2nd S_{100}

Adult human lung S_{100} (25mU) which gave a 2nd S_{100} containing 48mU cytidylyltransferase was applied to a Sepharose 6B column (2.5cm x 90cm) in buffer A at 4°C. A total of 60mU were recovered as L form activity and this represented a 1.25 fold increase over applied activity and and 2.4 fold over S_{100} activity.

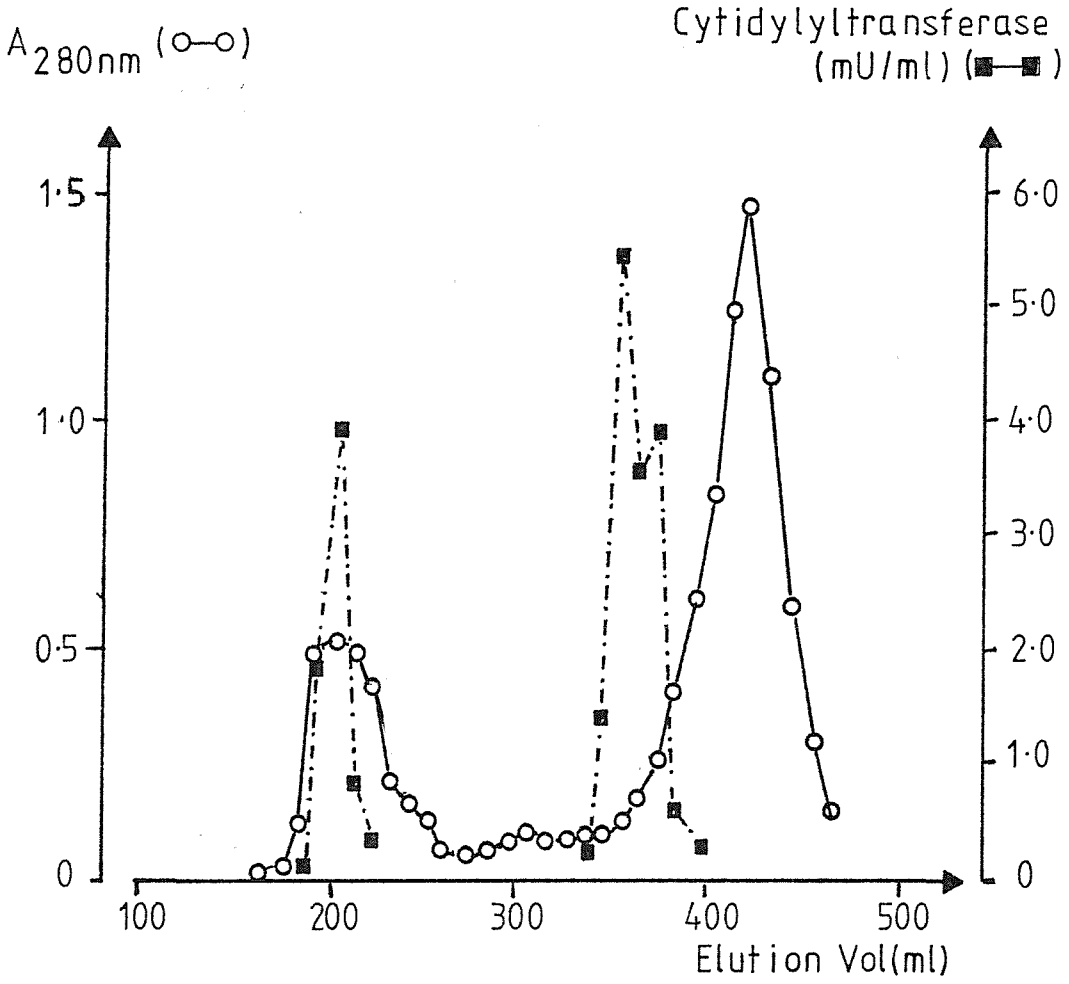
high molecular weight component eluting with V_o fractions and a lower molecular weight component of aroound 200,000 daltons, consistant with rat liver and lung H and L forms (Stern et al 1976, Choy et al 1977). The recovered activity, 241mU, represented an unexpected 3 fold increase with respect to applied activity. In addition the expression of L form activity did not require lipid and was partially inhibited by 0.25mM PG, in direct contrast with rat liver L form which required lipid for activity determination (Choy et al 1977). The increase in recovered activity may have been the result of separation from some inhibitory component.

The effect of ageing, for 2 hours at 37°C, on the gel filtration profile of adult human lung S_{100} is shown in fig 3.3. Both H and L forms were recovered with 207mU from 95mU applied ie a 2.2 fold increase in recovered activity. Once again L form activity did not require lipid activation. The increase in activity of 120% was comparable with the 94% increase observed in adult human lung 2nd S_{100} and consequently the elution profile of a 2nd S_{100} was investigated.

The elution profile of an adult human 2nd S_{100} (48mU) is shown in fig 3.4. The origional 100,000 x g x 60min supernatant contained 25mU cytidylyltransferase activity. All activity (60mU) was recovered as L form, with no detectable activity in the V_o fractions. Comparison with fig 3.3 suggested that H form activity in an aged S_{100} was associated with the particulate fraction, a result that agrees with previous observations of rat liver H form insolubility (Choy et al 1977). The inability to measure significant V_o protein in the 2nd S_{100} suggested that protein in the aged particulate fraction was present as large molecular weight aggregates.

The behaviour of adult lung S_{100}, aged S_{100} and 2nd S_{100} on gel filtration showed that H form activity was probably an artefact in S_{100}. Its removal by a second 100,000 x g x 60min centrifugation suggested that it was formed from L form enzyme present in fresh S_{100}. The finding of H form enzyme in fresh S_{100} was likely to be the result of aggregation occuring during the 5 hours between application to the Sepharose 6B column and elution of the V_o fractions.

Fig 3.5 Gel filtration of aged fetal human lung S_{100}



Aged fetal human lung S_{100} (15ml,108mU) was applied to a Sepharose 6B column (2.5cm x 90cm) and eluted in buffer A at 4°C. A total of 216mU was recovered and represented a two fold increas in activity applied.

3.2.2.3 Cytidylyltransferase H and L forms of fetal human lung S_{100} and ageing phenomena

Aged fetal S_{100} (108mU) was fractionated by gel filtration on Sepharose 6B. The elution profile, fig 3.5, gave an H form enzyme (60mU) and an L form enzyme (156mU), representing a doubling of applied activity. Fetal L form enzyme did not require lipid for activity and was partially inhibited by 0.25mM PG. Unlike the adult anzyme the increase in recovered activity did not correlate with changes between aged fetal S_{100} activity, table 3.3.

When fetal human lung 2nd S_{100} (167mU) was applied to a Sepharose 6B column, the profile, fig 3.6, was very different from that of the corresponding adult lung fraction. H form activity (39mU) and L form activity (71mU) were recovered but this represented only 67% of applied activity. The presence of protein and enzyme activity associated with the Vo fractions showed that the fetal lung H form enzyme was not totally associated with the particulate fraction sedimented by the second 100,000 x g x 60min centrifugation. The reduction in recovery of enzyme activity suggested that H and/or L form enzyme may be unstable in fetal preparations. When tested H form activity was unchanged after 24 hours at 4°C. but declined rapidly thereafter. By contrast 50% of L form activity was lost in the first 24 hours, which would be sufficient to explain the initial reduction in recovered activity.

Gel filtration analysis of adult and fetal human lung enzyme provided further evidence of the inadequacy of fresh S_{100} enzyme measurements from human samples. The results showed that potentially as little as one third of V_{max} activity might be disclosed.

3.2.2.4 Cytidylyltransferase H and L forms from human lung S_{100} aged in the presence of PG

The addition of lipids has previously been shown to produce an L/H transition with both rat lung and liver enzyme *in vitro* (Feldman et al 1978, Choy et al 1979). The effect of 0.25mM PG on fetal and adult human lung cytidylyltransferase ageing phenomena was investigated. The S_{100}s were aged in the presence of 0.25mM PG and then eluted on Sepharose 6B.

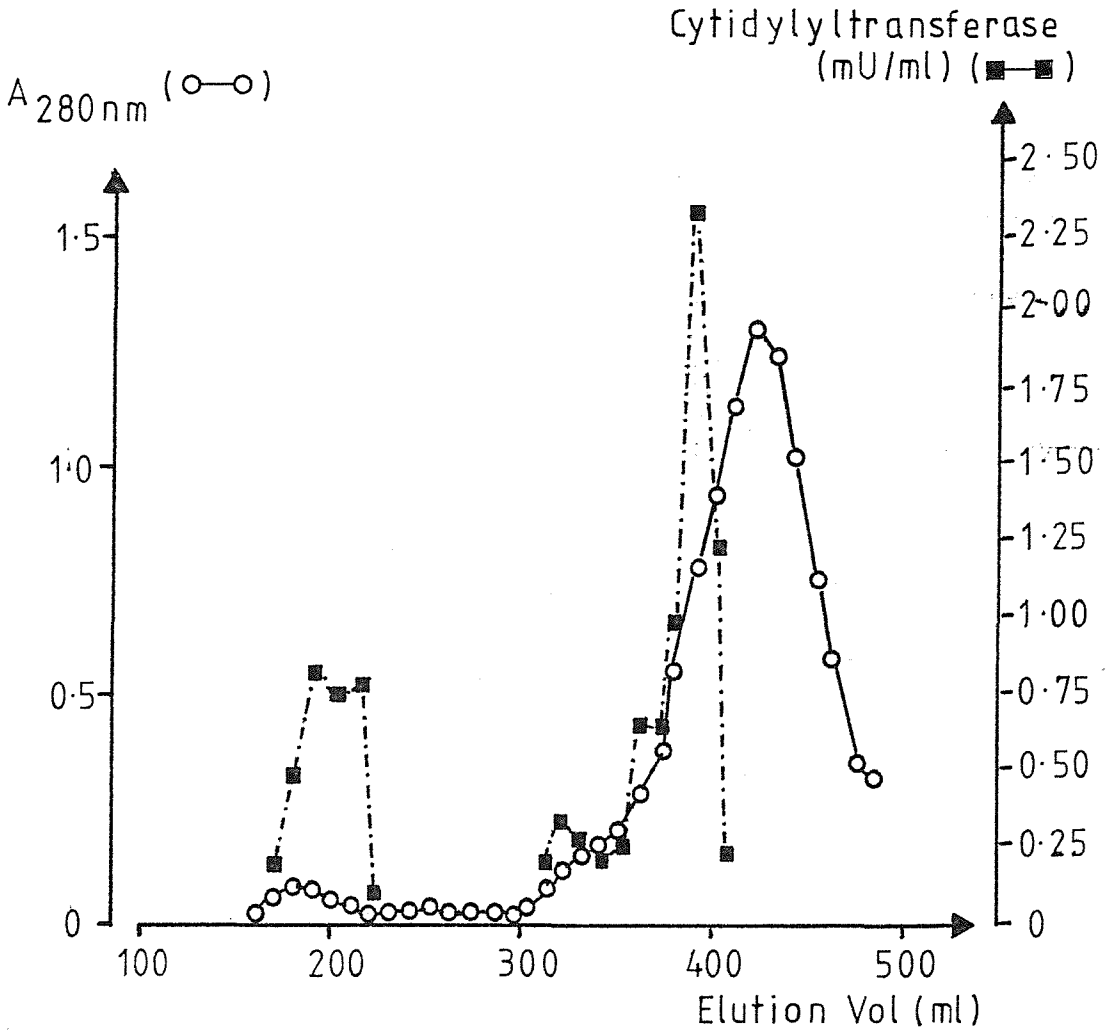
Table 3.4 The effect of ageing human adult and fetal lung S_{100} at 37°C in the presence of 0.25mM PG

	Cytidylyltransferase in S_{100} (+ 0.25mM PG)	Cytidylyltransferase in aged S_{100} (+ PG)	H form enzyme	L form enzyme
Fetal human lung S_{100}	140mU	108mU (23%)	113mU	176mU
Adult human lung S_{100}	79mU	106mU	217mU	100mU

Adult and fetal human lung S_{100} fractions (15ml) were assayed in the presence of 0.25mM PG, before incubation at 37°C for 2 hours. After the incubation the cytidylyltransferase was assayed again with assay mix adjusted to contain 0.25mM PG. The aged S_{100}s were then applied to Sepharose 6B and eluted with buffer A at 4°C. H and L forms of cytidylyltransferase were then assayed in the absence of PG, since H form did not require PG and L form was partially inhibited by it.

Activity recovered in H and L forms of fetal lung (289mU) represented 206% of S_{100} enzyme, while that in adult H and L forms (317mU) was 401% of fresh S_{100} cytidylyltransferase. The distribution between H and L forms was of 39% fetal H form and 69% adult H form.

Fig 3.6 Gel filtration of fetal human lung 2nd S_{100}-



Fetal human lung 2nd S_{100} (15ml,167mU) was applied to a Sepharose 6B column (2.5cm x 90cm) in buffer A at 4°C. The activity recovered (110mU) represented a decrease from applied activity to 67%.

A tabulation of applied and recovered activities is given in table 3.4. Fetal S_{100}, aged in the presence of PG, showed a reduction of 23% from fresh S_{100} activity measured with PG. The corresponding adult fraction was increased 34% above the fresh S_{100} activity measured with PG. Recovery of the total activity in H and L forms from fetal S_{100} aged with PG was 289mU, an increase of 2.06 fold over origional S_{100} activity. Adult S_{100} yielded 317mU distributed between H and L forms representing a 4 fold increase above origional S_{100} activity. The proportions of H form activity from samples aged with PG, 68% for adult and 39% for fetal, were increased above those from ageing without lipid of 11% and 28% respectively (figs 3.3 and 3.5). The presence of PG produced a promotion of the L to H transition, which was greatest with the adult S_{100} enzyme. This shift to H form enzyme was also reflected in a shift of total protein to the high molecular weight V_o fractions. Lowry protein determinations of Sepharose 6B elution profiles from duplicate adult S_{100} sample aged in the presence or absence of 0.25mM PG, fig 3.7, show the magnitude of total protein movement. The redistribution of protein, of several mgs, was consistant with an aggregation phenomenon.

3.2.3 Characterisation of the Principle H region protein from adult human lung

The most abundant protein present in the particulate fractions from aged rat and human lung S_{100}s had a molecular weight of between 40,000 and 45,000 daltons on SDS PAGE. In fresh S_{100} preparations it was present in lower molecular weight forms(s) and was converted to higher molecular weight aggregates during ageing proceedures. Its behaviour *in vitro* mirrored that of cytidylyltransferase and consequently its purification was undertaken.

3.2.3.1 Gel filtration in the presence of SDS

Rat liver H form enzyme has previously been shown to be converted to L form in the presence of SDS. (Choy et al 1977, Vance et al 1981), although with a large reduction in activity. The possibility that gel filtration in the presence of SDS might dissociated the high molecular weight aggregates, which co-eluted with human adult H form enzyme, was evaluated. The passage of cytidylyltransferase through these proceedures could not be monitored since SDS inhibited the enzyme irreversibly,

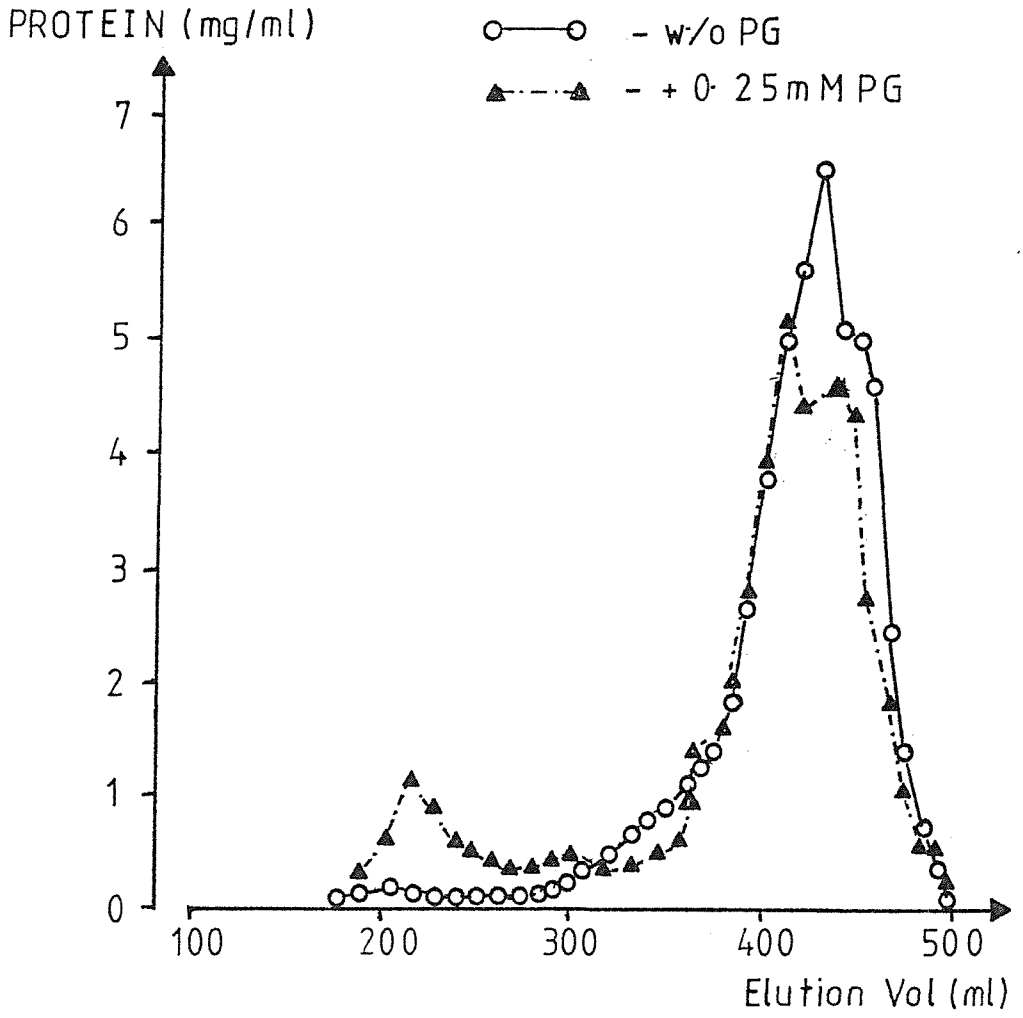
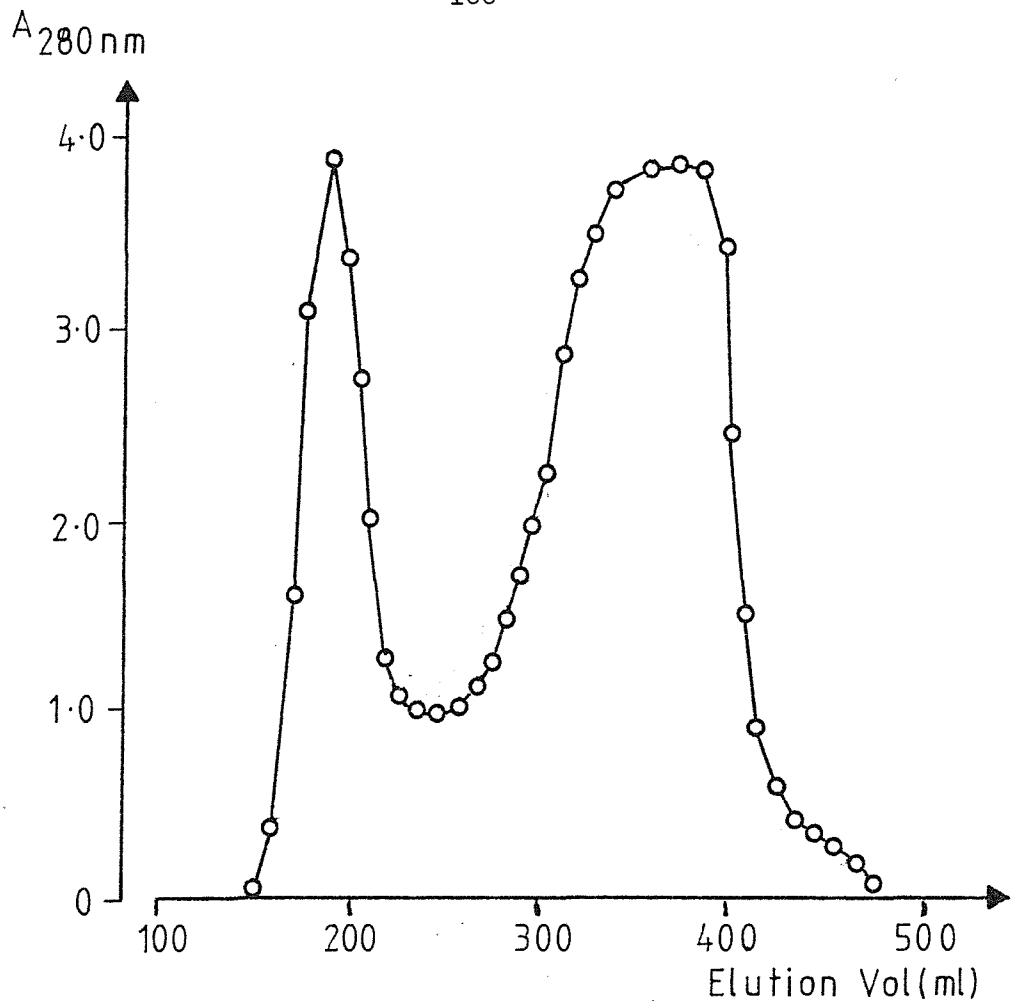


Fig 3.7 Gel filtration of duplicate fractions of adult human lung S_{100} aged in the presence or absence of 0.25 mM PG

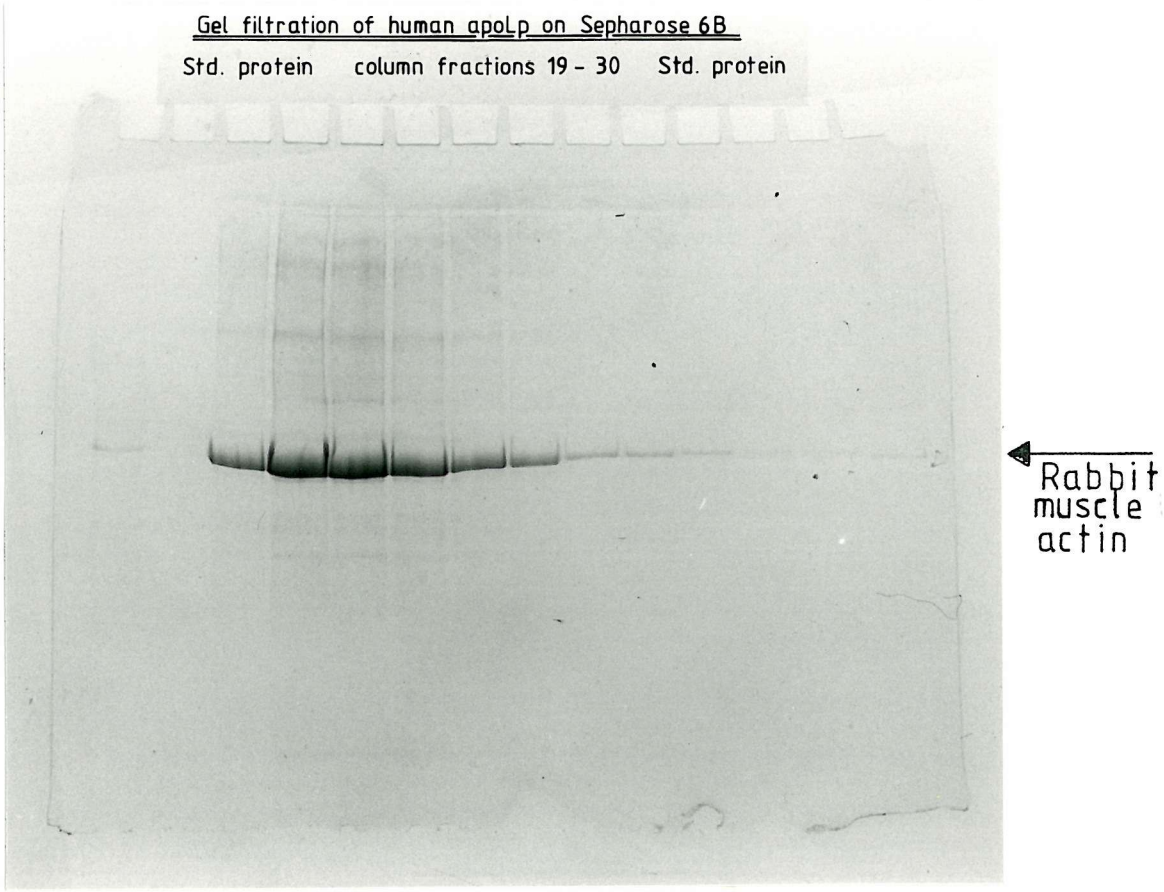
Duplicate samples of adult human lung S_{100} (15ml) were incubated in the presence or absence of 0.25 mM PG and eluted on Sepharose 6B (2.5cm x 90cm) in buffer A at 4°C. The eluted fractions (10ml) were assayed for protein and elution profiles superimposed to compare for significant differences.

Fig 3.8 Gel filtration of 0 - 40% ammonium sulphate fraction from adult human lung S_{100}-



0 - 40% ammonium sulphate fraction (dialysed volume 25.4ml, 589mg protein) was applied to a Sepharose 6B column (2.5cm x 90cm) in buffer A at 4°C and 10ml fractions collected. V_o fractions were pooled prior to application to DE 23.

Fig 3.9 SDS PAGE Analysis of Sepharose 6B V_o fractions from 0 - 40% ammonium sulphate fraction of human lung S_{100}



The 6B V_o fraction from fig 3.8, contained between tubes 19 and 30 were analysed by 10% SDS PAGE and rabbit muscle actin included in the flanking tracks. The gel was stained with Coomassie brilliant blue.

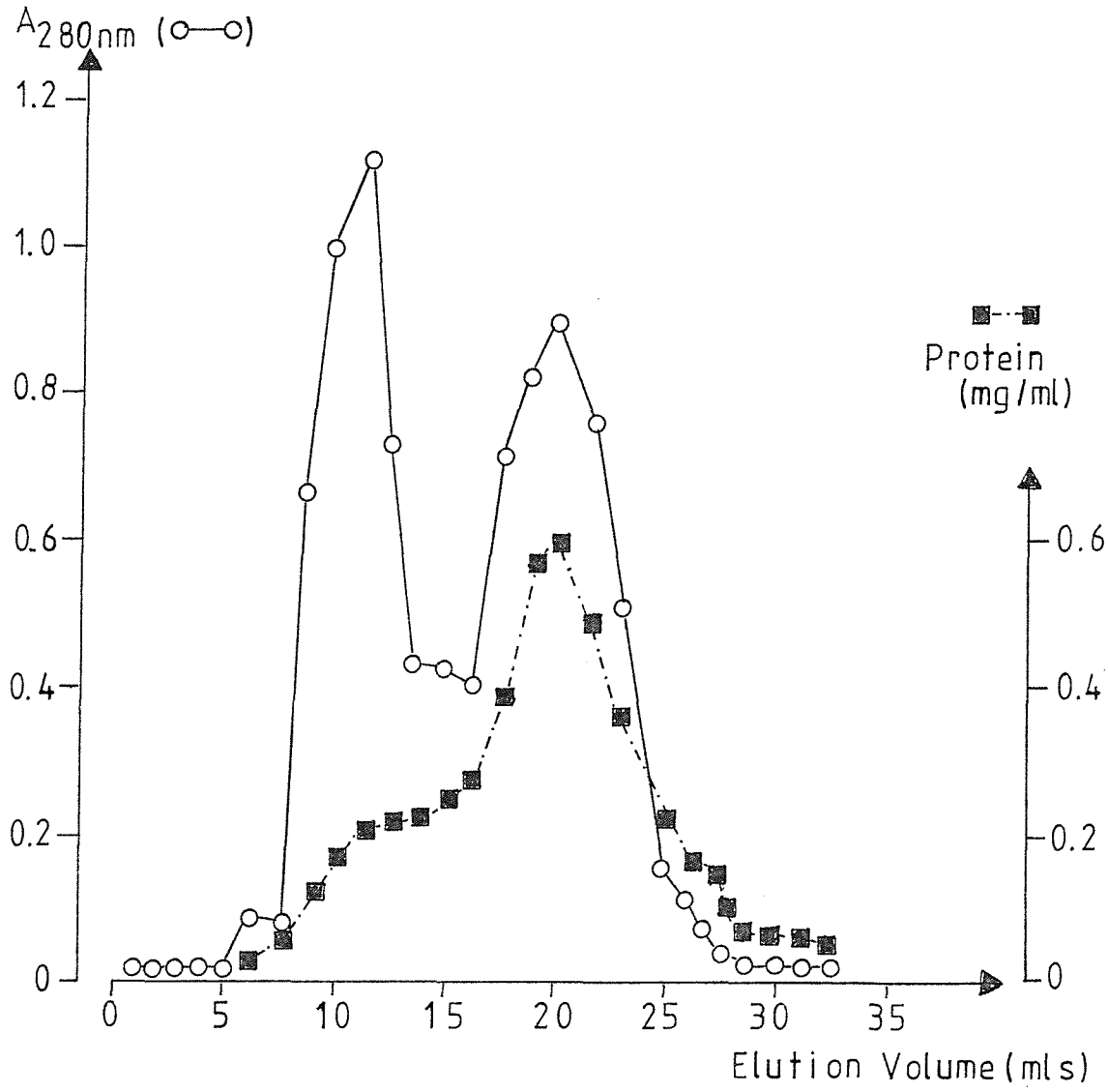
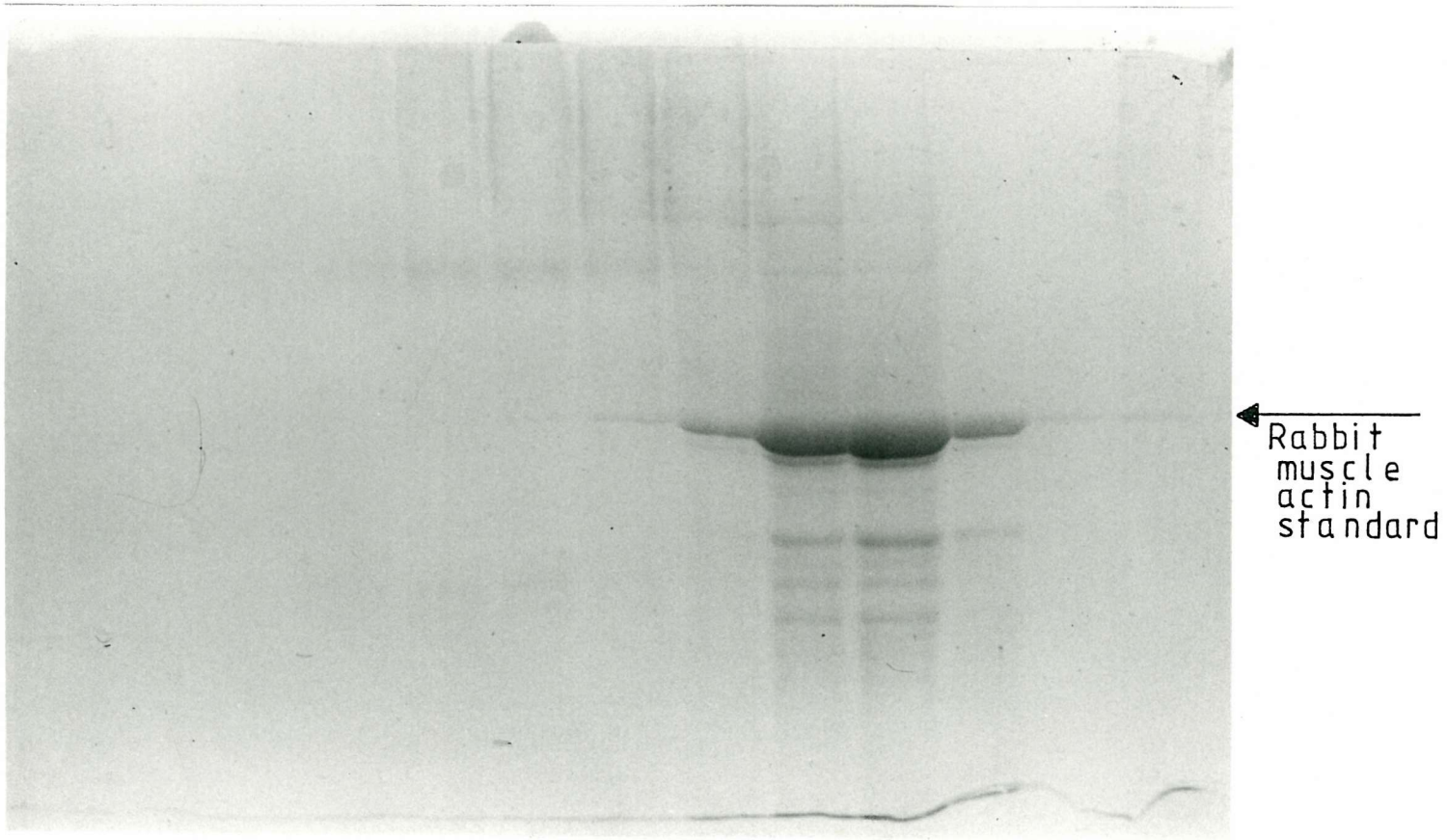


Fig 3.10 Gel filtration of Sepharose 6B V_o protein in the presence of 0.1% SDS

Pooled 6B V_o (2ml) from the elution of fig 3.8 was warmed in the presence of 0.1% SDS at 37°C for 2 hours. Elution was then made from a small Sepharose 6B column (1cm x 30cm) in buffer A containing 0.1% SDS.

Fig 3.11 SDS PAGE Analysis of fractions from 0.1% SDS/Sepharose 6B separation of pooled 6B V_o of fig 3.8



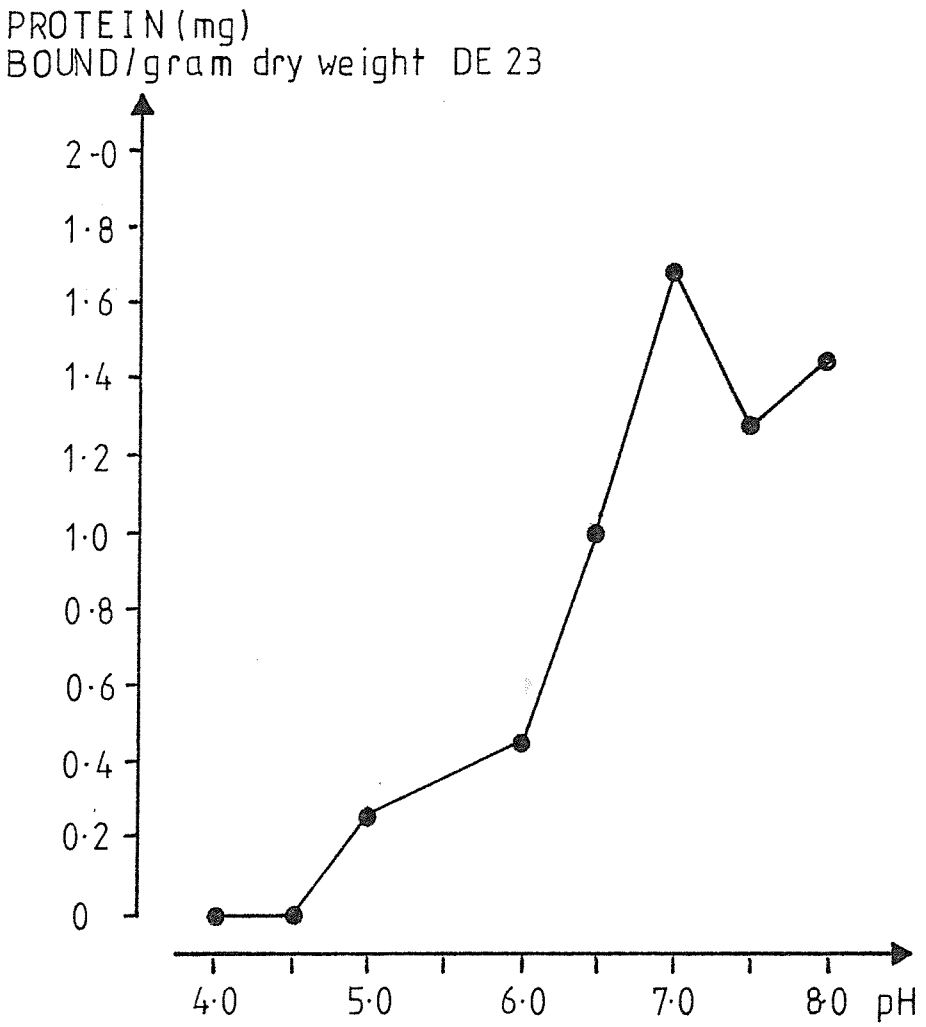
Pooled 6B V_o from fig 3.8 (2ml), warmed for 2hr at 37°C in the presence of 0.1% SDS and eluted on Sepharose 6B in the presence of 0.1% SDS was analysed on 10% SDS PAGE. Rabbit muscle actin was included as a standard and gels were stained in Coomassie blue.

consistant with previous observation of rat lung enzyme (Stern et al 1976).

A 0-40% ammonium sulphate pellet from adult S_{100} was dialysed against buffer A and insoluble material removed by a 10,000 x g x 10min centrifugation. The ammonium sulphate served to concentrate and selectively enrich a substantial portion of the soluble human enzyme (Section 4.2.1). The dialysed sample was applied to a Sepharose 6B column and gave the elution profile, fig 3.8. The V_o fractions contained between tubes 19 and 30 were analysed by SDS PAGE, in 10% gels, and the major protein, fig 3.9 was shown to comigrate with a rabbit muscle actin standard. Numerous other minor proteins were also evident. A portion of pooled V_o fraction (2mls) was warmed at 37°C for 2 hours in the presence of 0.1% SDS. During incubation an apparent decrease in turbidity was observed. The sample was applied to a small Sepharose 6B column (30cm x 1cm) and eluted in the presence of 0.1% SDS. SDS PAGE analysis in 10% gels, of the eluted fractions, fig 3.11, showed that the majority of protein now eluted in lower molecular weight fractions, following V_o region. These fractions were pooled and the SDS reduced to below 0.002% with the detergent binding matrix Extractigel D (Pierce Chemical Co.). The SDS removal was monitored by acridine orange dye binding (Sokoloff and Frigon 1981). The detergent-free protein was once more turbid and eluted with 6B V_o fractions in buffer A.

The assembly, dissociation and subsequent reassociation behaviour of the principal human lung H region protein was reminiscent of the polymerisation/depolymerisation cycle of the family of actins. Comigration on SDS PAGE with rabbit muscle actin provided more evidence that this protein might be a non-muscle actin. The properties of non-muscle actins are well documented (Bray and Thomas 1975, 1976, Pollard 1981) and they represent a major component of all cells, comprising as much as 16% of soluble protein in some instances (Pollard 1981). Actin has been shown to exist as both a soluble monomeric form (G-actin) and a polymeric form (F-actin), which is precipitated by ultracentrifugation. Ultracentrifugation has been used to separate and quantify F-actin in mixed samples (Bray and Thomas 1975). The proportions of G and F-actin recovered from fractionated fibroblasts was dependant on ionic strength, composition of homogenising buffers and the presence or absence of MgATP (Bray and Thomas 1976). Purification of the protein was necessary to identify the 43,000 dalton protein unambiguously as actin.

Fig 3.12 The binding of Sepharose 6B V_o protein to DE 23 at different pH



Pooled V_o fraction from Sepharose 6B elution of 0 - 40% ammonium sulphate fraction of adult human lung S_{100}, fig 3.8, was examined for its ability to bind DE 23 between pH 4 and 8. DE 23 (200mg moist weight) was mixed with pooled V_o (500ul) After 30 minutes at room temperature the unbound protein was determined in 12,000 x g x 2 minute supernatants.

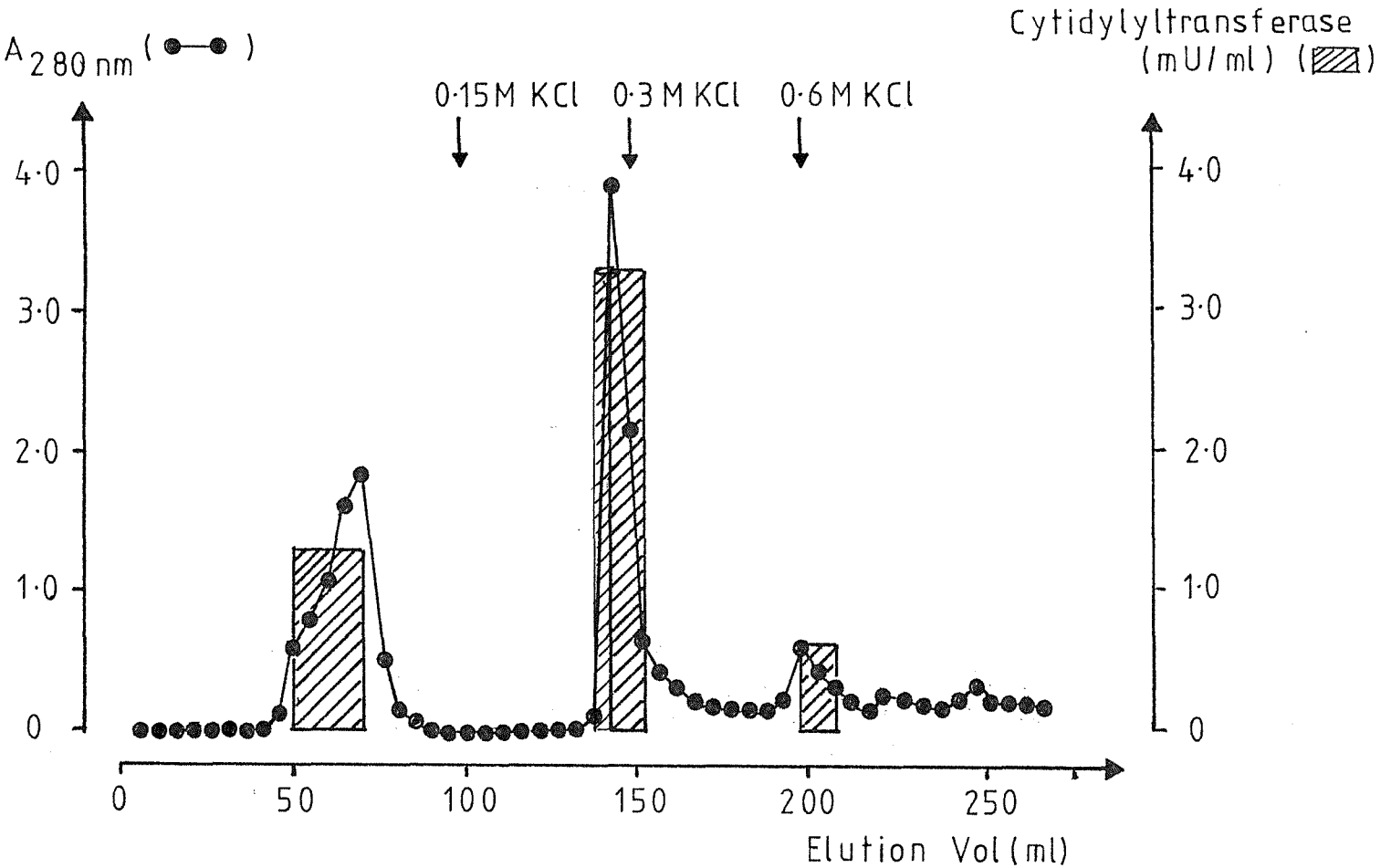


Fig 3.13 DE 23 ion exchange chromatography of pooled Sepharose 6B V_o protein

Pooled Sepharose 6B V_o (36ml) was adjusted to pH 8 with 0.2M NaOH and applied to a DE 23 column (1cm x 30cm) in buffer A. Elution was made with 3 x 50ml fractions of 0.15m, 0.3M and 0.6M KCl respectively. Cytidylyltransferase was assayed in pooled peaks.

3.2.3.2 Ion exchange Chromatography

Pooled V_o fractions from Sepharose 6B gel filtration of the 0-40% ammonium sulphate fraction of adult human lung was examined for its ability to bind to DE23 ion exchange resin over a pH range of 4 to 8. Aliquots of DE23 (200mg moist weight) were mixed with pooled V_o fractions (500ml) in buffers between pH 4 and 8. Tris buffers were employed between pH 6.5 and 8.0 while acetate buffers were used at pH 6.5 and below. Binding at 6.5 was assessed in both buffers. The protein concentration in supernatants of centrifugation at 12,000 x g x 2 mins, following 30 min incubation at room temperature, represented unbound protein. The maximum binding, fig 3.12, occured between pH 7 and 8.

The pooled 6B V_o fractions (35mls), described above were adjusted to pH 8.0 and applied to a DE23 column (30cm x 1cm) equilibrated in buffer A adjusted to pH 8.0. Elution, fig 3.13, waswith 3 x 50 mls KCl pulses (successively 0.15M, 0.3M and 0.6M KCl). The majority of bound protein eluted with 0.15M KCl. Cytidylyltransferase assays showed 32.5% of recovered activity was unbound, and 59.5% was associated with the 0.15M KCl fraction with the remaining 8% associated with the 0.3M KCl fraction. The specific activity of cytidylyltransferase in the 0.15M KCl fraction was 3.54mU/mg protein (3.22mU/mg with PG) purification could not be quantified since the pH dependance of the enzyme was not characterised. The enzyme was very unstable and unmeasureable after 24 hours. The pooled 0.15M KCl fraction was concentrated by ultrafiltration, using an immersible CX cartridge (Whatman Chemical Separations). When the concentrated sample was applied to a sepharose 6B column the protein eluted with the V_o fractions. A single band of protein of 43,000 daltons was observed on 10% SDS PAGE. A summary of the protein purification is shown in table 3.6. Recovery of the 43,000 dalton protein was monitored by binding and co precipitation with an emulsion of 1mM DPPC (Section 3.2.3.5) and polyethylene glycol precipitation (3.2.3.6).

3.2.3.3 Amino acid composition of purified 43,000 dalton protein

The amino acid composition of the purified protein from the 0.15M KCl fraction of the DE23 column was determined following hydrolysis in 6M HCl. The striking feature of this analysis, Table 3.7, was the presence of 3-methyl histidine, an amino acid produced as a post-translational modification. Its uniqueness lies in its confinement to the muscle and

Table 3.6 The purification of the 43,000 dalton protein from adult human lung

Purification stage	Volume (ml)	Protein (mg)	43,000 dalton protein(mg)	Recovery of 43,000 dalton protein(%)	Phospholipid (mg)
100,000 x g supernatant	227	4131	31.8	100	57
40% ammonium sulphate precipitate	25.4	589	24.1	76	21
V_0,Sepharose 6B	72	30	19.4	61	1.9
DE-23 peak	40	15.6	13.2	42	1.6

The purification of this protein from the 100,000 x g supernatant of human lung is sumarised above. Gel filtration on Sepharose 6B achieved the greatest degree of purification, with the protein eluted as a high molecular weight complex in the void volume(V_0) fractions. The aggregated state was maintained during ion exchange chromatography and elution with 300mM KCl. The recovery of specific protein was monitored by binding to and coprecipitation with an emulsion of 1mM DPPC,confirmed by SDS PAGE. The purified protein contained little lipid phosphorus,especially when compared with origional supernatant.

Table3.7

The amino acid composition of the purified 43,000 dalton protein

Amino acid	Residues/1000 amino acids
Asp	104
Thr	38
Ser	35
Glu	132
Pro	53
Gly	129
Ala	88
Val	72
Met	25
Iso	50
Leu	78
Tyr	30
Phe	35
His	33
3 methyl-His	4
Lys	64
Arg	56

Purified 43,000 dalton protein from the 0.15M KCl peak of DE-23 ion exchange chromatography was lyophylised then hydrolysed in 6M HCl. Component amino acids were separated by HPLC on reverse phase C18 columns and quantified by post-column fluorescent detection using OPA.

cytoskeletal proteins actin and myosin (Elzinga and Alonzo 1983). This provided further evidence that the 43,000 dalton protein was actin.

3.2.3.4 Western Blot analysis of purified 43,000 dalton protein

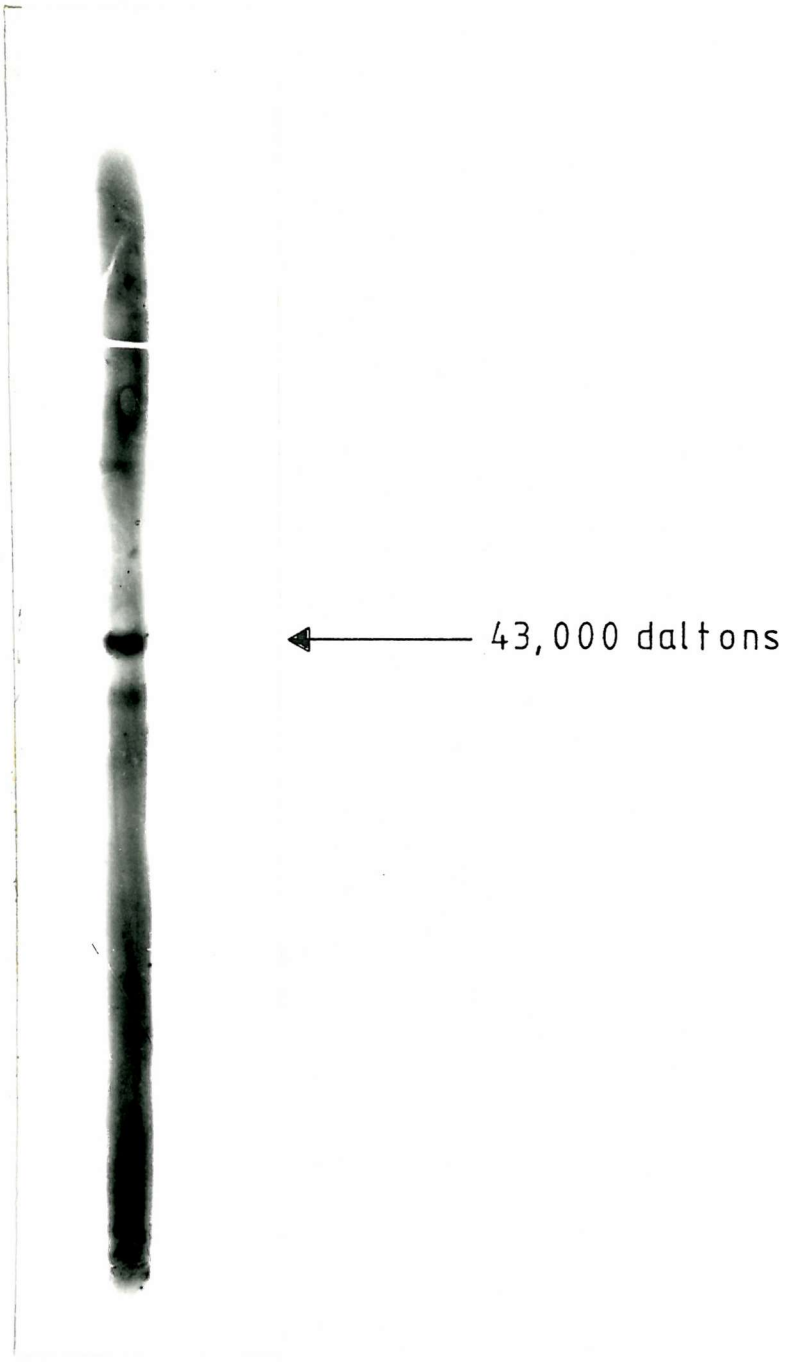
A immunological assay was used to confirm the identity of the putative actin. Antibody against chicken muscle actin was employed since it has a wide cross reactivity across species and between isomeric forms of actin. Purified 43,000 dalton protein was separated by SDS PAGE in a 10% gel and transferred electrophoretically to a nitrocellulose sheet. The nitro cellulose sheet was exposed to anti actin followed by anti-IgG linked to horse radish peroxidase. The actin/antibody conjugates were then visualised by blue colour formation after incubation with the substrates hydrogen peroxidase and 4-chloro-1-napthol. The immunoblot, fig 3.14, showed that antiactin labelled the 43,000 dalton band of purified protein, confirming its identity as a cytoplasmic actin. The H region actin probably represented F-actin, while the dissociated form comprised G-actin or small actin oligomers.

3.2.3.5 Phospholipid binding properties of purified F-actin

The activation of soluble rat liver cytidylyltransferase by ageing or under dephosphorylating conditions has been shown to be diminished or abolished when scrupulous efforts were made to avoid mixing cytosols with the lipid rich material floating at the air liquid interface (Pelech and Vance 1984(b)). Fetal and adult human lung S_{100}s had a similar lipid rich surface material. Filtration through glass wool, the usual removal step, may have resulted in some of this material mixing with S_{100}. Interactions between lung S_{100} actin and phospholipid might consequently have an important bearing on actin polymerisation and the postulated interaction with soluble enzyme. The H to L ratio in rat lung cytosols has been shown to be lower when pulmonary surfactant was removed by lavage before homogenisation (Feldman et al 1978). The interactions with DPPC were investigated, due to its major contribution to surfactant lipid content.

Purified lung actin (100mg/ml) was incubated with 1mM DP[^{14}C]-sn-glycerophosphocholine (25μCi/m mole) at room temperature for 2 hours. A control incubation was performed with BSA. Incubation mixtures

Fig 3.14 Western Blot analysis of purified 43,000 dalton protein



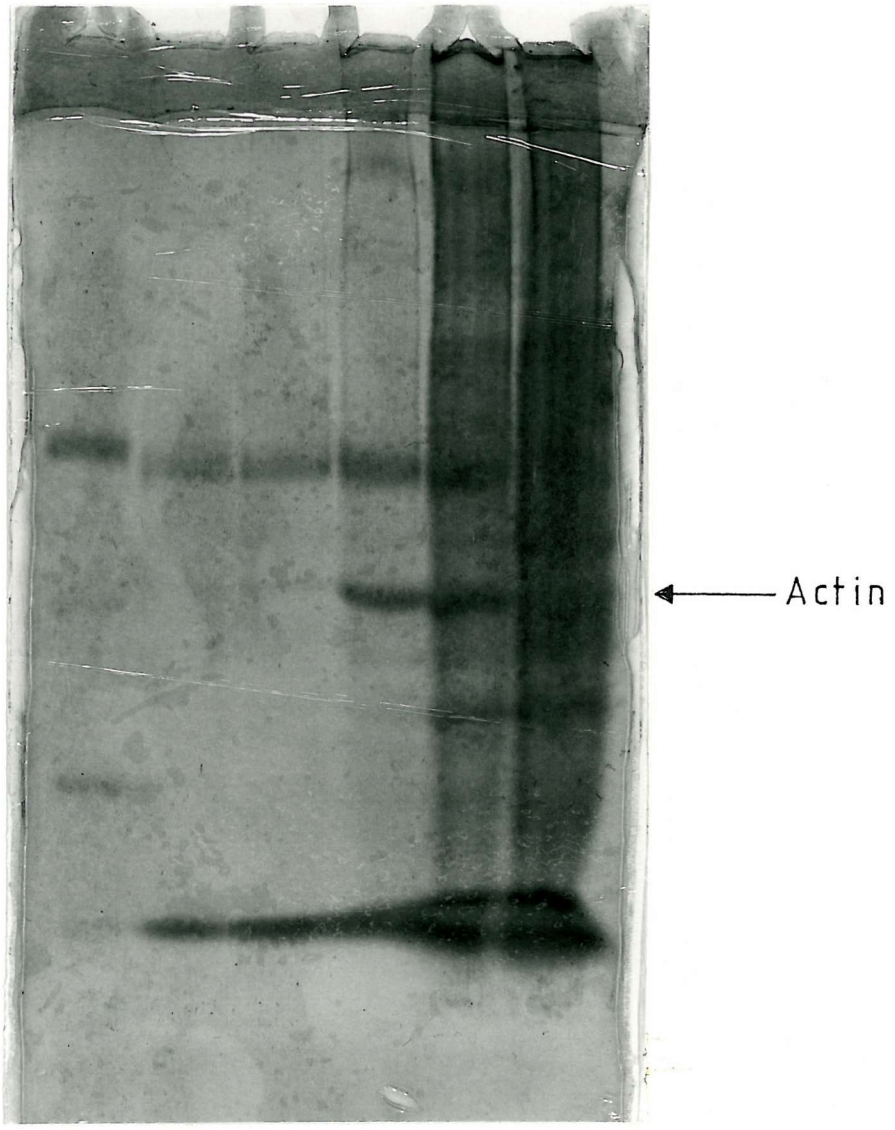
Purified 43,000 dalton protein, separated on 10% SDS PAGE was transferred electrophoretically to a nitrocellulose sheet. The single strip of nitrocellulose was then exposed to anti chicken muscle actin, followed by anti-IgG linked to horseradish peroxidase. The actin/ antibody conjugate was visualised by the blue colour produced from the substrates, hydrogen peroxide and 4-chloro-1-napthol.

(2ml) were layered over a continuous gradient from 16-19% (w/v) (10ml) and centrifuged at 150,000 x g x 20 hours. Sequential aliquots of gradients were analysed for phospholipid radioactivity and protein. In test and control virtually all the phospholipid was recovered in the upper 2mls of gradient. The majority of albumin (63%) was recovered in the lower 2mls of the control gradient while no detectable protein was recovered in the lower 2mls of the sample gradient. 73% of actin was found associated with phospholipid while less than 30% of albumin was phospholipid bound. Actin not preincubated with phospholipid was recovered at the bottom of the gradient. These results provided good evidence that lung actin binds phospholipid, which agrees with previous observations of DPPC/cytoskeleton aggregations (Kumar et al 1982) and the recent report of actin paracrystal formation on phospholipid liposomes (Rioux and Gicquand 1985). Lipid induced actin polymerisation, whether to F-actin or to actin paracrystals, coupled with the colocation of some cytidylyltransferase shown above, may provide an alternative explanation for the lipid dependant ageing or dephosphorylation data provided by Pelech and Vance (Pelech and Vance 1982, 1984(6)).

3.2.3.6 Polyethyleneglycol precipitation of lung actin

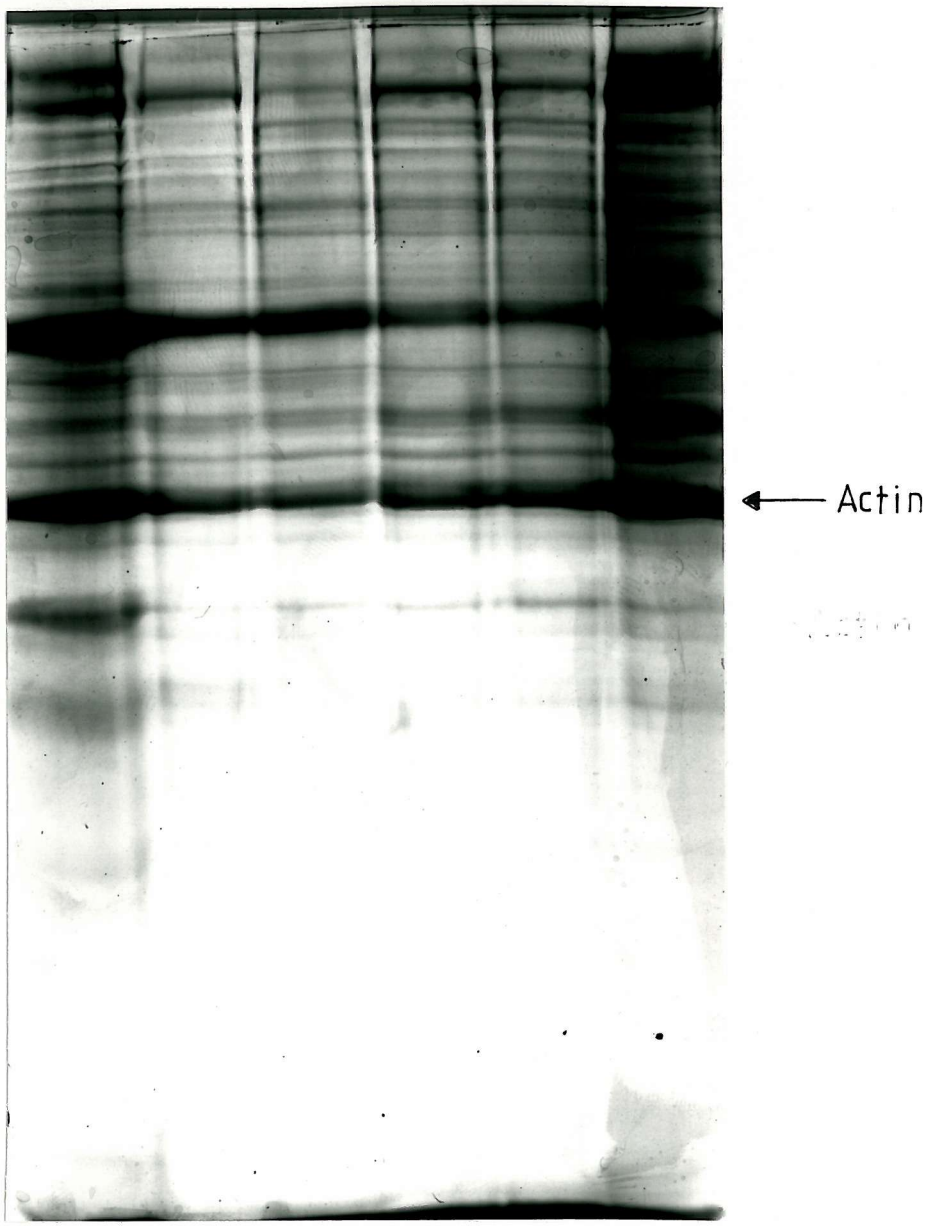
A method for the promotion of soluble lung actin polymerisation was suggested by the rapid, selective, polymerisation of skeletal muscle actin in the presence of the space filling polymer, polyethyleneglycol 6,000 (PEG_{6000}) (Tellam et al 1983). The authors postulated a mechanism which increased the exclusion volume, effectively concentrating G-actin and altering the equilibrium to favour F-actin. PEGs of various average molecular weights have been used for the fractional precipitation of enzymes (Polson et al 1964, Ingham 1984). Their actions were apparently the result of steric exclusion or crowding mechanisms in which the long chains of PEG confined the protein to relatively small pockets of solvent (Reinhart 1980, Bosca et al 1985). PEG_{6000} interactions with cytidylyltransferase are considered separately in Chapter 4, section 4.2.3. The effect of PEG_{6000} on adult lung S_{100} was monitored by the incubation of 1ml aliquots with PEG_{6000} between 0 and 10%. After incubation at 4°C for 2 hours and centrifugation at 12,000 x g x 2mins the purity of protein precipitates was analysed by SDS PAGE in 10% gels. The results, figs 3.15 and 3.16, showed that PEG_{6000} at 3% gave the largest actin pellet and was a useful method for total actin quantitation. Unpolymerised actin was not determined, but the results

Fig. 3.15. PEG Precipitation of adult human lung actin



Adult human lung S_{100}(1ml) was incubated with PEG_{6000} at 0.5%, 1%, 2.5%, 5% and 10% at 4°C for 2 hours. Precipitated protein was collected by centrifugation at 12,000 x g x 2 minutes and applied to a 10% SDS polyacrylamide gel. PEG concentrations above 2.5% appeared to precipitate most actin.

Fig. 3.16. PEG Precipitation of fetal human lung actin



Fetal human lung S_{100}s (6 x 1ml) were incubated with PEG_{6000} at 3% for 2 hours at 4°C. The 12,000 x g x 2 minute pellets were separated on 10% SDS PAGE.

from rabbit skeletal muscle actin, suggested that over 95% of actin was PEG precipitatable (Tellam et al 1983).

3.2.4 Rat Liver and Lung cytidylyltransferase distribution under cytoskeleton modulating conditions

In the preceeding sections changes in the state of aggregation of human and rat lung cytidylyltransferase *in vitro* were shown to accompany alterations in the state of actin polymerisation. Actin is the main component of microfilaments and part of the cytoskeletal network of non-muscle cells (Bray and Thomas 1976). A large number of enzymes and proteins have been found associated with cytoskeletal elements, section 1.4.7, including some whose variable subcellular distribution has led to their classification as ambiquitous (Wilson 1978, 1980). Observations of PC synthesis in phagocytic cells has previously shown that the state of cytoskeletal organisation in these cells regulated the flux through synthetic pathways (Pike et al 1981). The presence of antitubulins reduced flux through the N-methylation pathway by as much as 64% while anti microfilament agents did not alter N-methylation activity. The affect of antitubulins on CDPcholine flux was a stimulation by as much as two fold while the antimicrofilament agent cytochalasin B reduced flux by as much as 45% at 10μM. In each case the turnover of prelabelled PC was not altered.

The results described above suggested that reports of alterations in cytidylyltransferase distribution might be closely linked to variations in the assembly of cytoskeletal components and that this might have important bearing on the putative regulatory translocation. The use of MgATP and NaF to promote "phosphorylating" conditions, for example, may well alter the G-actin/F-actin ratio and possibly the cytidylyltransferase distribution observed *in vitro*. Consequently the proportions of soluble actin present under several homogenising protocols were investigated. In addition the cytidylyltransferase distribution and ageing effects under phosphorylation preserving conditions for rat lung were examined.

3.2.4.1 Homogenising protocol effects on soluble actin recovery from rat liver

The effect of several protocols on the recovery of soluble rat liver actin was investigated. A single rat liver was chosen in preference to rat lung simply because its size enabled 5 x 2 gram portions from the same liver to be studied in an attempt to avoid variations between animals. In addition similar variations in cytidylyltransferase subcellular distribution under different conditions have been recorded for liver (Schneider 1963) and lung samples (Stern et al 1976).

The compositions of buffers A - E are given in table 3.8. In addition to the standard buffer A and buffer B, the low ionic strength ethylene glycol-containing buffer, buffer C was based on the sucrose medium used by by both Schneider and Stern and colleagues (Schneider 1963 Stern et al 1976). Buffer D was a low ionic strength medium while E was a buffer previously noted to recover fibroblast actin in the G form (Bray and Thomas 1976). The latter was very similar in composition to the phosphorylation maintainance buffer used to demonstrate 'phosphorylation-mediated' increases in soluble cytidylyltransferase recovery (Pelech and Vance 1982, 1984(b), Radika and Possmayer 1986). The principle difference was a lower MgATP and ommision of NaF. All buffers contained azide as a bacteriostat and PMSF as a protease inhibitor.

S_{100} fractions were prepared from all five liver homogenates (20%), protein concentrations determined and fraction volumes adjusted to give equal protein concentrations. Aliquots of each (5ul) were separated by SDS PAGE, on a single 10% gel, into components bands, while rabbit muscle actin was included as a standard. Tracks, stained with Coomassie brilliant blue, were cut and scanned densitometrically at 570nm. The percentages of soluble protein comigrating with the actin standard were determined as the area under the peak, integrated gravimetrically.

A general correlation could be seen between low percentages of 43,000 dalton protein, table 3.9, and reports of low cytidylyltransferase in liver and lung cytosols(Schneider 1963, Stern et al 1976). The range of percentages, 10.9% - 14%, was small although absolute values and variations are likely to have been masked by some non-actin protein in the 43,000 dalton band. A two dimensional SDS PAGE system capable of the effective separation of actin would be able to provide much more accurate information if a reliable method of quantifying the protein

Table 3.9 Soluble actin as a percentage of total soluble protein in S_{100} fractions of rat livers using different buffers

Homogenising medium	Soluble protein which comigrates with actin
Buffer A	13.3%
Buffer B	12.2%
Buffer C	11.0%
Buffer D	10.9%
Buffer E	14.0%

Equal amounts of soluble protein from each protocol were simultaneously separated on 10% SDS PAGE,with a rabbit muscle actin standard. After staining with Coomassie brilliant blue the gel was cut into individual tracks and scanned densiometrically at 570nM. The area under the peak corresponding to actin was integrated gravimetrically and expressed as a percentage of total soluble protein.

Buffer compositions were as follows:- A: 150mM NaCl, 100mM TrisHCl pH 7.4, 1mM EDTA, 1mM DTT, 1mM Na azide, 200uM PMSF.

B: 10mM TrisHCl pH 7.4, 1mM EDTA, 1mM DTT, 1mM Na azide, 200uM PMSF, 20% Ethylene glycol.

C: Distilled water with 1mM EDTA, 1mM DTT, 1mM Na azide, 200uM PMSF.

D: 30% Sucrose, 1mM EDTA, 1mM DTT, 1mM Na azide, 200uM PMSF.

E: 150mM NaCl, 100mM TrisHCl pH 7.4, 1mM EDTA, 5mM DTT, 2mM ATP, 10mM $MgCl_2$, 20mM NaF.

spots could be devised.

3.2.4.2 The Recovery of rat lung cytidylyltransferase under phosphorylating conditions

The reported effect of lung cytidylyltransferase phosphorylation, determined under conditions favouring phosphorylation is confined to a study of fetal rabbit lung enzyme (Radika and Possmayer 1986). The authors observed an inhibition of both PG activated soluble enzyme (78%-89%) and microsomal enzyme (66%-72%) but were unable to comment on the possibility of redistribution because both forms of enzyme were inhibited. The subcellular distribution of enzyme in hepatocytes pretreated with cAMP analogues (Pelech and Vance 1984(b)) favoured cytosolic enzyme, a redistribution from membrane compartment to cytosolic fraction was suggested. No reports of the effect of homogenising rat lung under phosphorylating conditions have been made.

The subcellular distribution, between S_{100} and P_{100}, and specific activities of rat lung cytidylyltransferase were determined in buffer A and buffer A with 2mM ATP, 10mM $MgCl_2$, 5mMDTT, 20mM NaF (Phosphorylating conditions). The phosphorylating conditions caused a small but reproducible increase, from 68.5% to 74.7%, in soluble cytidylyltransferase, table 3.10. This was statistically significant p=0.01. When activities were expressed as mU/gram wet weight no significant differences were noted in recovery from either treatment, 118mU±20mU/g vs 100mU±11mU/gram for phosphorylating conditions. The change in distribution was not apparently the result of P_{100} enzyme inhibition. The specific activities of S_{100} and P_{100} enzymes were similar with the exception that P_{100} enzyme was PG stimulated under phosphorylating conditions.

When aged, S_{100}s from phosphorylating experiments behaved in a similar fashion to those in buffer A. Activities with PG, 2.03±0.20mU/mg (n=4) fell to 0.28±0.03mU/mg in 2nd S_{100} fractions. This 86% fall was comparable to the 83% seen with controls (section 3.2.2.1). This result appeared to differ from the reported reduction in liver cytosolic enzyme aggregation in the presence of NaF (Pelech and Vance 1984(b)). It is unclear why ageing of lung S_{100} under phosphorylating conditions should be unchanged while liver enzyme aggregation is reported to diminish, but phosphorylation phenomena have been shown to be overridden by fatty acids

Table 3.10 The distribution rat rat lung cytidylyltransferase under phosphorylating and non-phosphorylating conditions

Non - Phosphorylating conditions

	Cytidylyltransferase activity(w/o PG mU/mg)	Cytidylyltransferase activity(+ 0.25mM PG mU/mg)	% Total enzyme
S_{100}	1.56 ± 0.08	2.01 ± 0.11	68.5% ± 3.3%
P_{100}	3.21 ± 0.92	3.05 ± 0.61	31.5% ± 3.3%

(n = 4)

Phosphorylating conditions

S_{100}	1.85 ± 0.14	2.08 ± 0.18	**74.7% ± 0.8%
P_{100}	3.27 ± 0.34	4.04 ± 0.49	25.3% ± 0.8%

(n = 4)

** significant at p = 0.01

S_{100} and P_{100} fractions from blanched rat lungs homogenised under non - phosphorylating conditions (buffer A) and phosphorylating conditions (buffer A with 5mM DTT, 2mM ATP, 10mM $MgCl_2$, 20mM NaF.) were assayed for specific cytidylyltransferase activity and percentage distribution between fractions. Total lung activities (mU/gram wet weight) were 118mU ± 20mU vs 100mU ± 11mU respectively representing no significant difference.

and modified in the presence or absence of lipid in the cytosol (Vance and Pelech 1984, Pelech and Vance 1984(b)). One possible explanation involves the presence of surfactant phospholipids. Rat lungs, from which pulmonary surfactant has been removed by lavage, show a greater recovery of L form activity in soluble fractions (Feldman et al 1978). The presence of lipid alone, however, would not account for the difference since liver lipid is more abundant than lung. The particular species may be important and if, for example, dissociated actin subunits aggregated faster in the presence of pulmonary surfactant phospholipids then concomitant cytidylyltransferase aggregation might ensue.

3.3 Discussion

The results presented in this chapter establish that human lung cytidylyltransferase may be separated into soluble and particulate fractions from tissue homogenates. Soluble activity was stimulated in the presence of 0.25mM PG, while incubation at 37°C for 2 hours converted a portion of the enzyme to a less soluble, particulate form. The magnitude of this conversion was increased in the presence of 0.25mM PG. Soluble and particulate forms of cytidylyltransferase are a common finding in mammalian tissue homogenates (Pelech and Vance 1984(a)). The results from human lung enzyme show that its subcellular location as determined *in vitro*, could be classified as ambiquitous (Wilson 1978). The characteristic of two locations *in vitro* is shared with many enzymes including hexokinase (Wilson 1980), PAP (Moller and Hough 1892), PFK (Choate et al 1985), calcium and phospholipid-stimulated protein kinase C (C-Kinase) (Kraft and Anderson 1983, Pelech et al 1986) and the protein Calmodulin (Gazzotti et al 1985). Reversible translocation between soluble and membrane bound forms of enzymes has been proposed as a regulatory mechanism for hexokinase and other ambiquitous enzymes (Wilson 1980). Many of the experimental observations of flux-dependant variations in subcellular location, upon which the regulatory role of hexokinase translocation was origionally formulated have recently been questioned (Kyriazi and Basford 1986). The authors suggested that artefactual differences were responsible for *in vitro* changes, since rapid fractionation techniques showed no net movement between soluble and particulate enzyme irrespective of flux changes.

The aggregation of soluble human lung enzyme, aged at 37°C, was accompanied by the polymerisation of cytoskeletal, actin-rich components. Partial binding to these elements was seen with aged adult and fetal enzymes, but inhibition was only seen with adult enzyme. The identification of actin occured towards the end of these investigations and was critical in the interpretation and understanding of earlier observations. Once identified a subsequent literature search established a report of remarkably similar observations with fetal calf brain aldolase, glyceraldehyde-3-phosphate dehydrogenase and pyruvate kinase (Clarke and Morton 1982). The authors recorded a binding to, and coprecipitation of these enzymes with F-actin at high ionic strength. Their sedimentation was dependant on the presence of F-actin and was greater at 37°C than at 0°C. Incubation at 37°C increased aldolase binding four fold with respect to that at 0°C, while binding of pyruvate kinase and glyceraldehyde-3-phosphate dehydrogenase was doubled. Actin depleted extracts gave little enzyme sedimentation after incubation at 37°C, but addition of purified brain actin resulted in further enzyme precipitation. The antitubulin colchicine did not alter precipitation but the presence of Ca^{++}, which disrupts F-actin, abolished the coprecipitation. A number of enzymes which have demonstrated ambiquitous properties have also been shown to interact with cytoskeletal elements (section 1.4.7) as has the regulatory protein calmodulin (Gazzotti et al 1985). Calmodulin has been reported to be associated with cytosolic and mitochondrial fractions of fractionated rat liver (Harper 1980). Closer examination of the mitochondrial fraction by density gradient separations has shown negligible mitochondrial binding (Gazzotti et al 1985). The 'mitochondrial' calmodulin was found to be bound to a mixed membranous fraction, rich in cytoskeletal proteins. EGTA treatment could not release the protein, consistant with previous observations of cytoskeleton/calmodulin interactions (Deery et al 1984). The intermediate filaments, previously shown to be associated with rat liver plasma membrane (Chan et al 1970), were suggested as the binding site.

C-Kinase has been described as ambiquitous (Pelech et al 1986) and its interaction with erythrocyte and rat brain membranes has recently been shown to be of two types (Wolf and Sahyoun 1986). The enzyme was found linked by two cytoskeletal, peripheral membrane proteins of 110,000 and 115,000 daltons with phosphatidylserine which binds all three proteins in an undefined ternary complex. These polypeptides may also be phosphorylated by C-Kinase. A second, direct binding to the inner aspect

of the lipid bilayer was also identified, indicating that C-Kinase binds to membranes in both cytoskeletal dependant and independant fashions.

The ambiquitous distribution of sheep heart muscle PFK between cytosol and microfilaments has been shown to be modified in vitro by ionic strength and the presence or absence of substrates (Choate et al 1985). Rabbit skeletal muscle PFK can be activated by F-actin in vitro (Kno et al, Luther and Lee 1986). The affinity of PFK for F-actin varied according to its phosphorylation state (Luther and Lee 1986) with F-actin acting as a positive effector of the phosphorylated but not the unphosphorylated enzyme. PFK also has F-actin severing properties, sharing common immunological determinant with brevin (gelsolin), the Ca++ regulated actin severing protein (Fuchtbauer et al 1986). The regulatory characteristics of liver and muscle PFK have been modified by the use of PEG_{6000}, which also promotes muscle actin polymerisation (Tellam et al 1983). PEG_{6000} aggregated and 'crowded' the enzyme to predicted intracellular concentration ranges (Reinhart 1980, Bosca et al 1985). It is possible that PEG_{6000} may also have enhanced G-actin polymerisation, especially since G-actin has a strong affinity for PFK, with which it ends to copurify (Kuo et al 1986).

Clear precedents exist in the literature for cytoskeletal interactions with many enzymes and proteins (section 1.4.7). HMG CoA reductase, long regarded as an extrinsic microsomal enzyme, has been found associated with microtubules (Volpe 1979) being most active when microtubule bound. The microtubule - associated protein MAP-2 binds RII, the regulatory subunit of cAMP dependant protein Kinase (Leiser et al 1986), while myosin light chain kinase binds both myosin and actin (Sellers and Pato 1984). Calmodulin dependant protein kinase type II constitutes a major part of the neuronal microsome-associated cytoskeleton (Sahyoun et al 1985). The suggestion, from results presented in this chapter, that cytidylyltransferase interacts with cytoskeletal elements, at least in vitro, in addition to examples quoted above provide convincing evidence in favour of the emerging concept of a highly ordered aqueous cytoplasm built around a cytoskeletal network (Clegg 1984(a)).

If cytidylyltransferase were associated with microsome - associated cytoskeletal elements in vivo, then subcellular variation in vitro might reflect variable disruption of the cytoskeleton. Some support fot this idea was offered by variations in soluble actin recovery from rat liver

(section 3.2.4.1), while conditions favouring G or F-actin (Bray and Thomas 1976) also favour soluble or particulate enzyme respectively (Schneider 1963, Stern et al 1976). Alterations in the affinity for cytoskeletal elements, perhaps analogous to PFK phosphorylation effects (Kuo et al 1986, Luther and Lee 1986), may underlie some of the observed cytidylyltransferase translocation phenomena. Differences in the interactions between aged fetal and adult human lung enzyme and actin-rich elements in vitro were apparent (section 3.2.2), with these elements partially inhibitory for aged adult S_{100} enzyme. In contrast, the fetal enzyme became more active as the actin-rich aggregates formed.

Results from the digitonin permeabilisation of cells have been used to support translocation with both cytidylyltransferase and, more recently, C-kinase (Pelech et al 1984(a), Pelech and Vance 1984(c), Pelech et al 1984(b), Pelech et al 1986). The method analyses release of enzyme into the medium as a measure of soluble enzyme, though it is possible that the 'soluble' enzyme released results from cytoskeletal disruption. Digitonin acts by removing cholesterol from membranes, effectively 'punching' large holes (Gegelein and Huby 1984) and making membranes leaky. This will undoubtably alter the ionic millieu of cells, breaking down ionic gradients across the plasma membrane, and will be likely to disrupt cytoskeletal structures releasing enzyme into medium. The demonstrations of variations in rate of enzyme release may reflect changes in the strength of cytoskeletal interaction. In the case of changes in PC flux, for example, correlation between cytidylyltransferase release and flux may not be due to translocation but still reflect a regulatory change.

An alternative mechanism for some of the recorded variations in enzyme activity has been provided by the role of the cytoskeleton in hormone action. Alterations in actin polymerisation within rat hepatoma cells and human leucocytes have been recorded with T_3, T_4 and adrenergic agonists (Rao et al 1985). This may provide a sound basis and potential for translation of hormone stimulus to cytidylyltransferase activity. Association of cytidylyltransferase with the cytoskeleton in close proximity to the endoplasmic reticulum potentially allows for a close interaction with the membrane bound cholinephosphotransferase. If choline kinase interacted with the components, then a rapid substrate tunneling would be possible. Disruption of the cytoskeleton, under hormonal action, might then break the close proximity, reducing pathway flux. Changes in PC flux, under phosphorylating conditions might involve

phosphorylation of the enzyme, moving it away from cholinephosphotransferase, perhaps by reducing the affinity for F-actin - rich components. Alternatively phophorylation of actin itself mat occur and effect regulation. Such an explanation would account for the apparent 'silent phosphorylation' of cytidylyltransferase (Pelech and Vance 1984(b)).

In order to assess these possibilities a supply of pure cytidylyltransferase is necessary. With pure enzyme combinations of individual cytoskeletal elements, choline kinase and cholinephosphotransferase could be investigated. In conjunction with space filling polymers to more closely approximate in vivo protein concentrations this would allow the potential regulatory aspects of the in vitro observations to the situation in vivo to be evaluated. Chapter 4 and 5 concentrate on approaches to the purification of human lung cytidylyltransferase.

CHAPTER 4

APPROACHES TO THE PURIFICATION OF LUNG CYTIDYLYLTRANSFERASE

4.1 Introduction

The investigation of the properties and metabolic regulation of cytidylyltransferase has been hindered by the lack of reliable techniques for the purification of enzyme protein. Reported methods, confined to rat or guinea pig liver enzyme, were limited by both low yields and lability during purificaton (Borkenhagen and Kennedy 1957, Fiscus and Schneider 1963, Choy and Vance 1976, Choy et al 1977, Vance et al 1981, Weinhold et al 1986). A common feature of many of these descriptions was the use of an interconversion of liver H and L forms, followed by centrifugation or gel filtration. The original method involved preparation of particulate liver enzyme by heat treatment at 55°C for 20 minutes and pH reduction from 7.4 to 5.2 (Borkenhagen and Kennedy 1957). The particulate enzyme was then recovered by centrifugation. Vance and colleagues incorporated a soluble/particulate enzyme conversion followed by a solubilisation back to L form with gel filtration in the presence of S.D.S. (Choy et al 1977, Vance et al 1981).

Separation of rat lung H and L forms has been reported (Stern et al 1976) but further purification was prevented by at least 90% inhibition of H form by a wide range of detergents including S.D.S., Triton X100, NP_{40} and Tween 20 at 0.5%. The problems of low yield were evident even in the recent rat liver enzyme purification of Weinhold and co-workers (Weinhold et al 1986). A soluble-particulate enzyme transition was accomplished by incubation with phospholipid vesicles and subsequent pH reduction. Problems of detergent inhibition were apparantly overcome by use of the non-ionic detergent octyl glucoside, which had also been used to effectively solubilise skeletal muscle membrane-associated enzyme (Cornell and Maclennan 1985(a)). Octyl glucoside permitted the use of both ion exchange and hydroxyapatite chromatography with good recovery. Application of the authors quoted 6.4% overall yield to rat lung enzyme, coupled with the lower pulmonary activity, might require 200 lungs (of 1-1.5 grams) for a similar total and specific activity recovery.

Human lung enzyme has not been purified beyond neonatal lung homogenates (Thom and Zachman 1975) while reports of its properties are also confined to this single study. Experiments in this chapter consequently involved characterising some properties of human and rat lung enzyme. In the light of information obtained several potential approaches to the purification of lung enzyme were evaluated.

4.2 Results

4.2.1 Ammonium sulphate fractionation

Ammonium sulphate fractionation, by a 'salting out' mechanism, provides a convenient method for enrichment of many proteins. The introduction of a neutral salt at high ionic strength can often reduce the solubility of proteins without destroying their native conformation. This property allows for some measure of selective precipitation and concentration. The technique has been used as the second step in the liver enzyme purification protocol of Vance and colleagues following an ageing step (Choy et al 1977, Vance et al 1981). Ammonium sulphate partially inhibited liver enzyme and, although recovery of H form enzyme was greater than that applied, only 66% of activity from unfractionated, aged cytosol remained (Choy et al 1977).

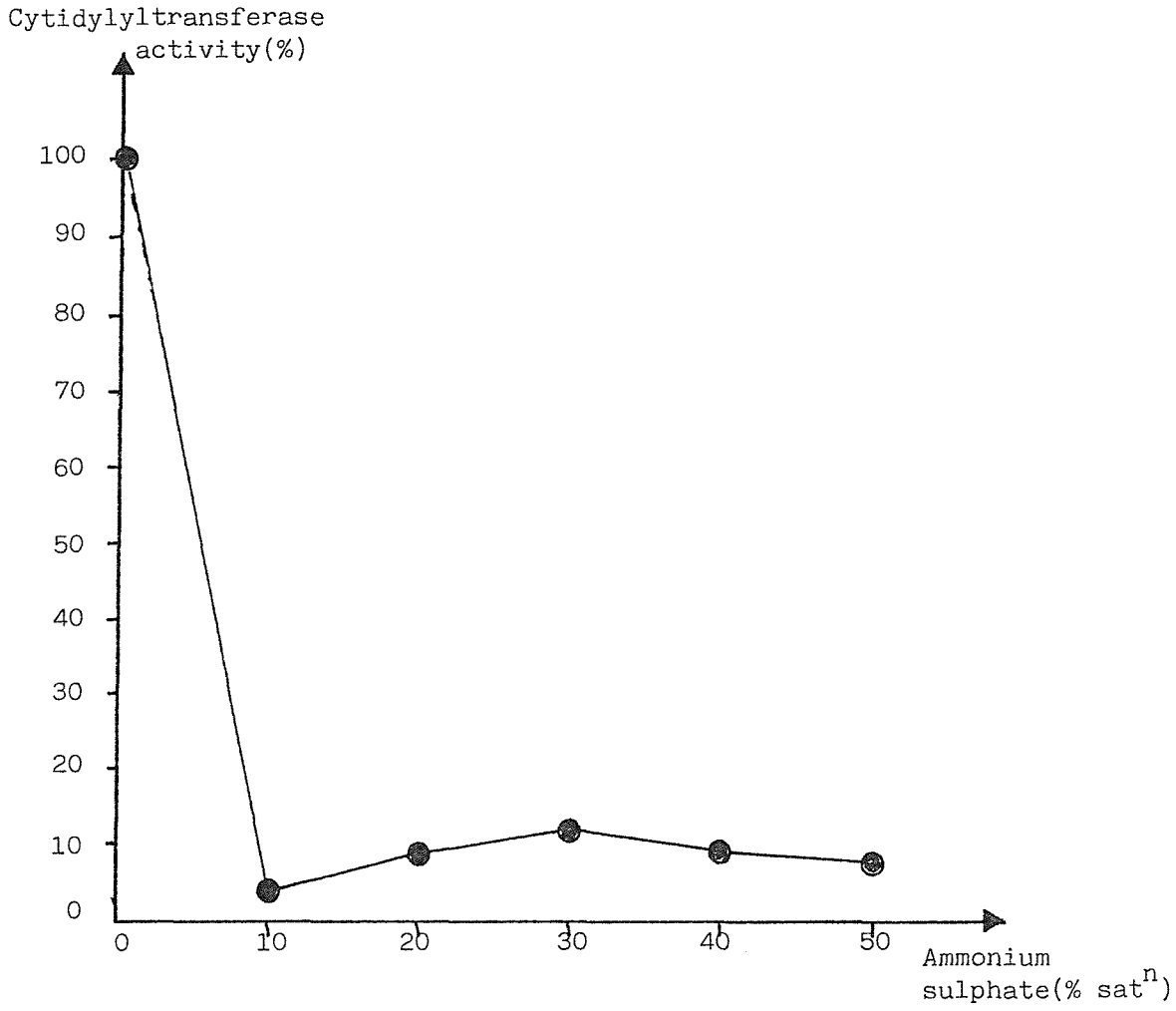
4.2.1.1 Ammonium Sulphate fractionation of soluble human and rat enzyme

Since rat liver enzyme was partially inhibited in the presence of ammonium sulphate, adult human S_{100} enzyme was assayed in the presence of ammonium sulphate between 10 and 50% saturation.

Measurable activities, fig. 4.1, in the presence of ammonium sulphate were expressed as a percentage of those in ammonium sulphate-free control. Inhibition was evident at all tested concentrations and indicated that dialysis, following ammonium sulphate fractionation was essential prior to evaluation of enzyme recovery.

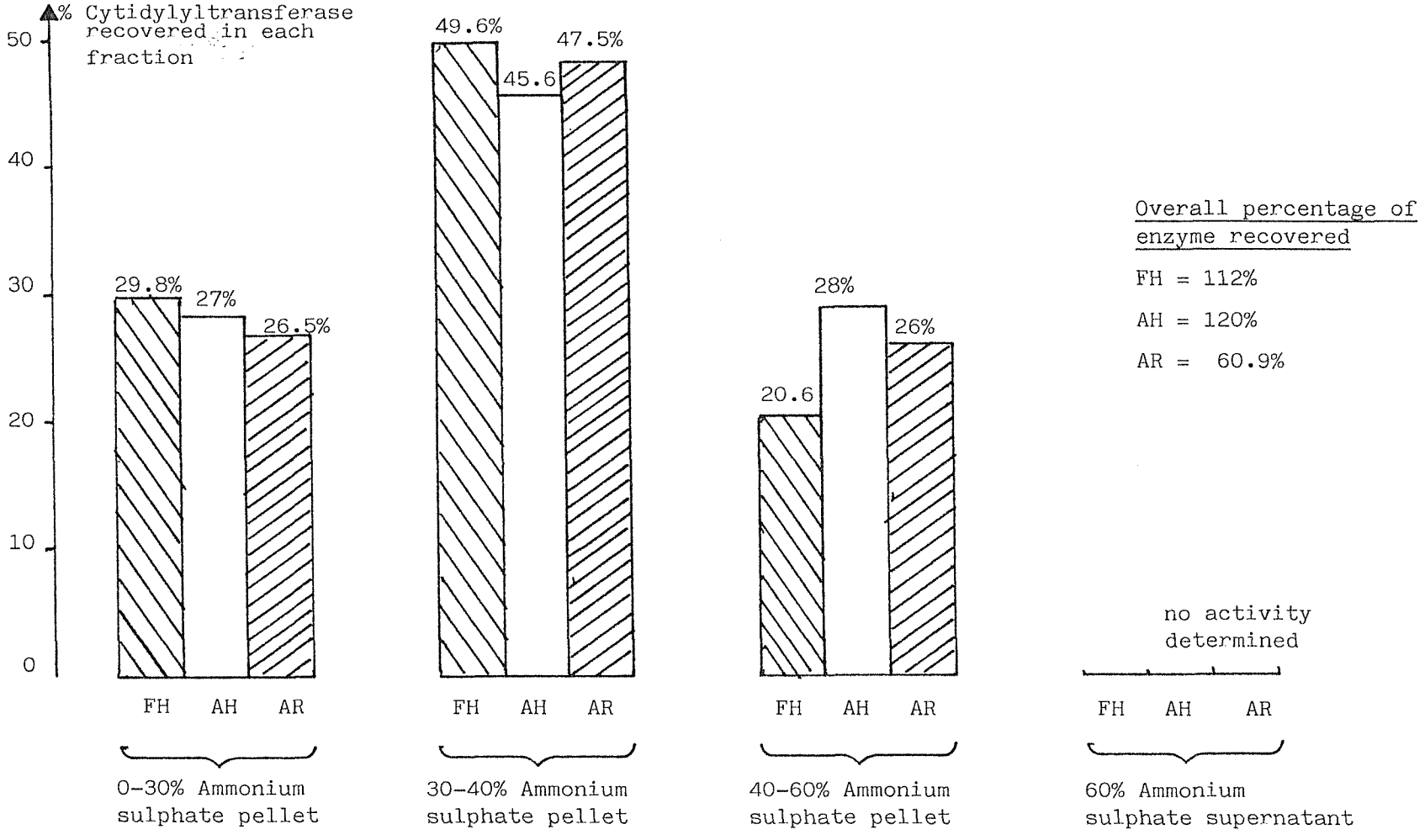
Sequential ammonium sulphate fractionation of fresh adult and fetal human lung S_{100} and adult rat lung S_{100} was undertaken to assess the most appropriate fraction for purification purposes. The protein precipitated between 0-30% saturation, 30-40% saturation and 40-60% saturation of fresh S_{100}s (20ml), at 4°C for 15 minutes, was collected by centrifugation (10,000 x g x 10 minutes). Pelleted protein was resuspended to 20ml in buffer A (150mM NaCl, 50mM TrisHCl pH 7.4, 1mM EDTA, 1mM DTT, 200μM PMSF) and dialysed against 3 x 1 litre changes of buffer A. Particulate matter remaining after dialysis was removed by centrifugation (10,000 x g x 10 minutes). Negligible enzyme activity was monitored in this fraction from human lung S_{100}s but activity was associated with the particulate fraction from rat lung. This particulate

Fig 4.1 Ammonium sulphate inhibition of adult human lung S_{100}-cytidylyltransferase



Aliquots of fresh adult human S_{100} cytidylyltransferase (200ul) were mixed with water and saturated ammonium sulphate to give final concentrations between 10% and 50%,in a total volume of 400ul. Cytidylyltransferase activity was assayed after incubation at 4°C for 30 minutes,and expressed as a percentage of ammonium sulphate free control.

Fig 4.2 Sequential ammonium sulphate fractionation of soluble lung cytidylyltransferases



FH = fetal human lung S_{100} , AH = adult human lung S_{100} , AR = adult rat lung S_{100}

Table 4.1 Ammonium sulphate fractionation of adult human lung 2nd S_{100} cytidylyltransferase

Ammonium sulphate(%)	Activity in pellet(%)	Specific activity(mU/mg)	Activity in supernatant(%)	Specific activity(mU/mg)	Overall recovery of cytidylyltransferase(%)
0	-	-	100	0.34	100
10	2	0.34	130	0.44	132
20	14	0.52	129	0.45	143
30	47	1.66	162	0.57	209
40	67	1.16	141	0.73	208
50	57	0.70	130	0.65	187
60	78	0.67	63	0.46	141

Aliquots of human lung 2nd S_{100} (5ml,0.35mU/mg) were adjusted to between 10% and 60% saturation by the addition of saturated ammonium sulphate in buffer A. After 15 minutes at 4°C the 10,000 x g x 10 minute pellet,resuspended in buffer A,was dialysed against 4 x 1L changes. Supernatants were also dialysed. The particulate component present after dialysis contained no cytidylyltransferase and was removed by centrifugation. Volume changes accompanying dialysis were noted and the protein and the cytidylyltransferase activities were determined.

activity could not be accurately assayed due to inability to satisfactorily resuspend precipitates. Dialysed 60% ammonium sulphate supernatants contained no measurable activity from any source.

The figures for overall recovery, fig. 4.2, show a difference between human and rat lung enzyme. Human fetal enzyme recovery of 112% and adult recovery of 120% contrasted sharply with the 60.9% obtained with adult rat lung enzyme. The lower activity recovered from rat lung may be a consequence of the particulate enzyme present after dialysis of ammonium sulphate fractions. The percentages of overall recovery in each fraction, fig. 4.2, were broadly similar and between 72% and 79% of total recovered enzyme was precipitated between 0 and 40% ammonium sulphate saturation.

The recovery of over 100% enzyme activity in combined human lung ammonium sulphate fractions was consistent with demonstrations of activity increases in human soluble enzyme following ageing and removal of particulate components (chapter 3). The particulate associations of some rat lung enzyme following ammonium sulphate precipitation were also consistent with its largely particulate location, after ageing.

Cytoplasmic actins are precipitated at concentrations of ammonium sulphate above 35% (Kuroda and Maruyami 1976). The potential cytoskeletal/cytidylyltransferase interactions postulated in chapter 3 might consequently alter ammonium sulphate fractionation profile in fresh adult S_{100} when compared with 2nd S_{100} from which actin rich aggregates have been removed. The coelution of a portion of enzyme with aggregated actin-rich particles from gel filtration (section 3.2) might be reflected in more enzyme co-precipitated by the ammonium sulphate induced salting out of fresh S_{100} actin. This possibility was investigated for adult human 2nd S_{100} but not for rat since aged rat S_{100} contained predominantly particulate enzyme.

Several differences were apparent in the behaviour of 2nd S_{100} enzyme when compared with fresh enzyme (Table 4.1). The principal changes were greater recovery of applied enzyme, which ranged from 132% to 209%, and the presence of enzyme in the 60% ammonium sulphate supernatant. The total recovered activities were greatest between 30% and 40% saturation and were of comparable magnitude to those seen with gel filtration of 2nd S_{100} samples (Section 3.2.2). The results appeared to suggest that, between 0 and 40% ammonium sulphate, increased recovery

of enzyme was made possible. By analogy with the suggestion made in chapter 3, this might be the result of salting out of some undefined factor. It is tempting to speculate that this may be related to the small quantities of G-actin and/or other cytoskeletal components present being increasingly salted out by ammonium sulphate to 35% saturation. The major difference between fresh S_{100} and 2nd S_{100} is the removal of substantial quantities of actin in the form of the actin-rich aggregates produced by ageing. If cytidylyltransferase has a weak interaction with aggregated actin-rich components then the presence of activity in the 60% 2nd S_{100} supernatant (45%) may reflect a reduction in cytoskeletal components with which to coprecipitate.

An additional important difference between fresh and 2nd S_{100} ammonium sulphate fractions was shown in a reduction in stability. The 0-40% ammonium sulphate fraction of fresh adult human lung S_{100} retained greater than 90% of activity after 24 hours at 4°C. Similar fractions from 2nd S_{100} lost 68% of activity in 24 hours and after 48 hours no activity could be determined.

The results clearly demonstrated that greater activity could be recovered from 2nd S_{100} than was able to be determined in fresh S_{100}. The stability of the resulting fractions was, however, compromised. Indeed, the rapid decay in activity during the first 24 hours may indicate that still greater activity was initially present in dialysed fractions but was lost during dialysis. Additional support for the concept of a proportion of cytidylyltransferase activity in human lung S_{100} being latent was provided by these observations.

4.2.1.2 Fractionation of Human L form

The suggestion from the previous section that the low stability of cytidylyltransferase from ammonium sulphate treated 2nd S_{100} was potentially related to the removal of actin-rich aggregates after ageing merited further investigation. Human lung L form enzyme, separated by gel filtration, is stable and, with a molecular weight around 200,000 dalton,was also F-actin free, although comigration with actin oligomers of up to 5 monomers long is possible. The stability of this human L form suggested that ammonium sulphate fractionation might yield more information about stability and actin effects.

Pooled fetal human L form enzyme from gel filtration (20ml, 71.4mU) was brought to 40% saturation with solid ammonium sulphate. After 15 minutes at 4°C, the pellets and supernatants were separated by centrifugation, dialysed against buffer A (3 x 1 litre) and assayed for cytidylyltransferase activity.

Of the cytidylyltransferase recovered, 30mU (42%) was precipitated while 12.9mU (18%) remained in the supernatant. The enzyme was very unstable and after 48 hours no activity could be measured in either fraction, consistant with the loss of 2nd S_{100} cytidylyltransferase activity under similar conditions.

Adult human L form enzyme (38ml, 48mU) was fractionated in similar fashion. The pellet contained 10 mU (21%) with no activity present in the supernatant. Ater 24 hours less than 5mU remained and even this small activity was unmeasurable after 48 hours. Ammonium sulphate treatment apparently destabilised L form cytidylyltransferase and consequently the enrichment advantage was outweighed by a yield reduction. Recombination experiments with the mixing of pellet and supernatant samples, post dialysis, gave no greater recovery of the enzyme ruling out the separation of cytidylyltransferase into two components. It seemed likely that ammonium sulphate treatment was causing a physical change in cytidylyltransferase which in turn reduced its stability.

The results of investigations with ammonium sulphate fractionation seemed to indicate that successful fractionation in buffer A required the presence of cytoskeletal elements or actin-rich aggregates. In the absence of such components the stability of cytidylyltransferase was apparently reduced. At the same time, however, the presence of these aggregatable elements prevented measurement of full activity making interpretation of activity measurements difficult. The 4.7 fold increase in human 2nd S_{100} enzyme specific activity following 30% ammonium sulphate precipitation, for example, was achieved at the expense of stability, making its use inappropriate.

4.2.2 Ionic Strength Effects

The distribution of cytidylyltransferase *in vitro* between soluble and particulate enzyme can be modulated by variations in ionic strength

(Schneider 1963, Stern et al 1976). Conditions of high ionic strength yielded a largely soluble enzyme, while a lower ionic strength led to increased recovery of a particulate form. Recent observations with partially purified rat liver enzyme have shown it to be inhibited by ionic strengths above 120mM and below 75mM and/or the presence of phosphate buffer (Weinhold et al 1986). The enzyme recovered from ammonium sulphate fractionation of rat lung S_{100} might consequently be more stable in lower ionic strength buffers or with different buffering species. A screening of the effect of low ionic strength buffers on partially purified rat H form enzyme activity was made before assessment of its efficacy for 0-40's ammonium sulphate pellet reconstitution.

The low ionic strength buffer B, 10mM Tris HCL, 1mM EDTA, 1mM DTT pH7.4, both with and without 20% ethylene glycol, was assessed. The instability of some enzymes in low ionic strength buffers or at low temperature has in some cases been overcome by the incorporation of ethylene glycol or glycerol into buffers (Schryver and Weiner 1981).

Buffer exchange of rat lung H form enzyme was achieved by overnight dialysis at 4°C. In buffer B without ethylene glycol only 56.3% cytidylyltransferase activity remained after overnight dialysis and after 48 hours no activity was measurable. Cytidylyltransferase activity in buffer B with ethylene glycol was unchanged overnight at 103%. Full activity was retained after one week at 4°C. When 10mM phosphate replaced Tris in buffer B with ethylene glycol, however, rapid inactivation occured.

The apparent phosphate inactivation agreed with observations of partially purified rat liver enzyme (Weinhold et al 1986) although these authors recorded their inhibitions at higher ionic strength.

The above observations were extended to resolubilisation of ammonium sulphate precipitated enzyme. Duplicate 0-40% ammonium sulphate pellets of fresh rat lung S_{100} were solubilised in and dialysed against Tris or phosphate containing buffer B with ethylene glycol. When fully dialysed, both samples contained particulate matter, which was removed by centrifugation (10,000 x g x 10 minutes). The particulate fraction found in Tris buffer reconstitution contained 13% of S_{100} cytidylyltransferase, while no activity was measurable in the phosphate buffer. The Tris buffered particle-free supernatant contained 59% S_{100} activity (cf 60.9% with buffer A) while only 5% of activity was recorded in the phosphate

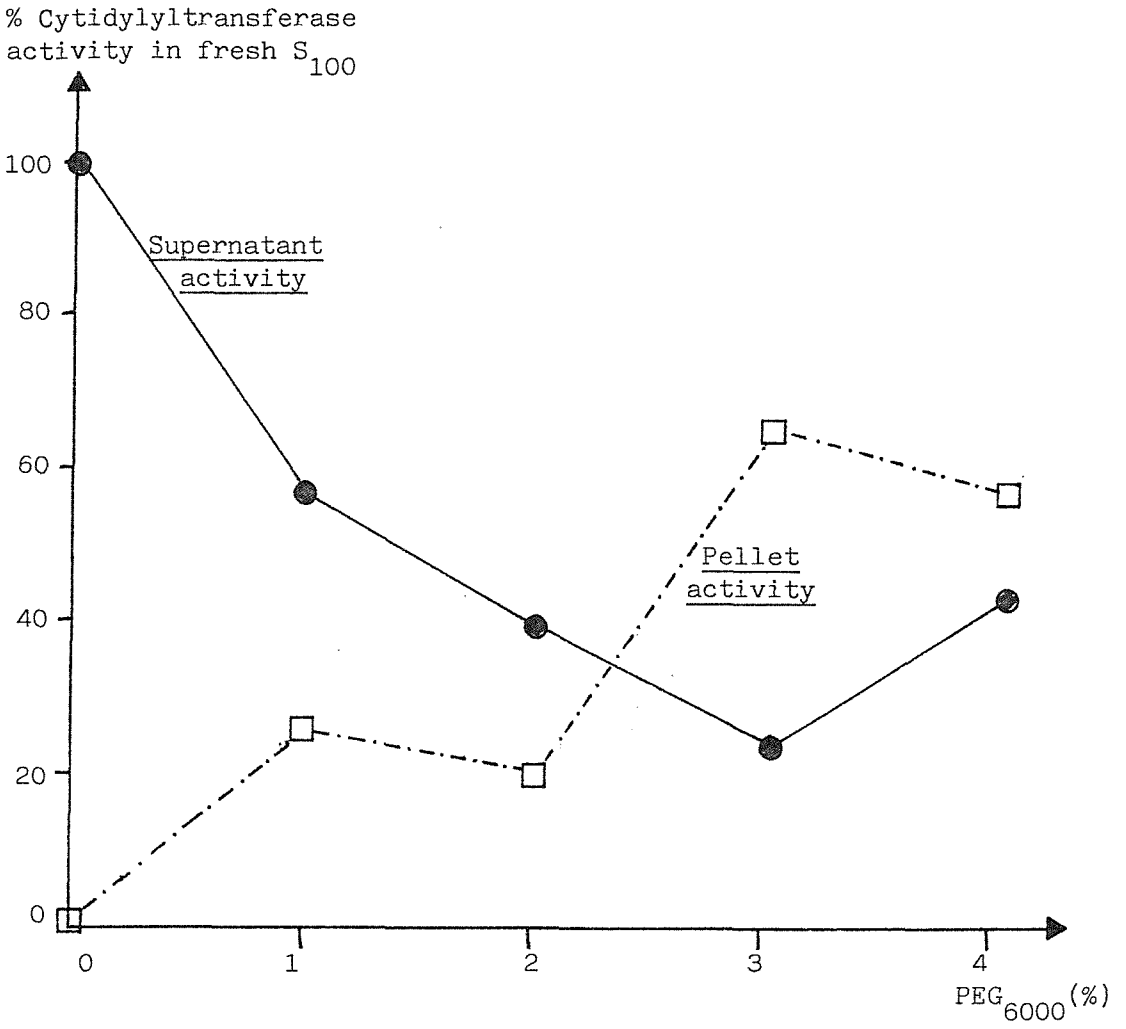
buffered supernatant. Sepharose 6B Gel filtration of these Tris and phosphate supernatants showed that only H form from Tris buffered reconstitution retained full activity after 24 hours. L form activity was highly unstable in both buffers. The stability of rat lung H form enzyme was clearly enhanced in the presence of low ionic strength buffer B while neither high or low ionic strength helped L form stability.

4.2.3 Polyethyleneglycol precipitation

The use of the nonionic, water soluble polymer polyethyleneglycol (PEG) for fractionation of proteins was introduced by Polson and colleagues (Polson et al 1964). PEG fractionation is a particularly useful technique for separation of proteins from complex mixtures (Ingham 1984). The polymer's action appears to be one of reducing solubility, possibly effecting a concentration of protein by an excluded volume mechanism. The unique advantages of its use lie both in its low tendancy to denature or interact with protein, even at high concentration, and its ease of removal following fractionation (Ingham 1984). A further advantage is its speed of action with protein precipitations being usually complete between 30 minutes and one hour (Ingham 1984). Empirical observation has shown that PEG of nominal average molecular weight 4,000 - 6,000 daltons provides optimum precipitation (Polson et al 1964). Above 6,000 daltons the derived PEG precipitation curves do not change, while viscosity increases dramatically. Below 4,000 daltons the spread of precipitations and the very shallow slopes obtained for individual protein precipitation with increasing PEG concentration, make their use undesirable (Ingham 1984).

PEG_{6000} has been shown to promote muscle G-actin polymerisation *in vitro* (Tellam et al 1983), while results presented in section 3.2.3.6 showed that PEG_{6000} at 3% precipitated the majority of soluble adult human lung S_{100} actin. Heteroassociation of PEG precipitatable protein with other proteins has been shown to reduce their solubilities in the presence of PEG (Ingham 1984). Removal of the hetero-associating species increases the amount of PEG required to initiate precipitation. An example of this phenomenon was provided by the functional interaction between human plasma fibronectin and denatured collagen (gelatin) (Ingham et al 1983). In phosphate buffered saline alone fibronectin required 11% PEG for precipitation. When gelatin was added, however, the complex was precipitated by PEG concentrations below 3%.

Fig 4.3 PEG_{6000} precipitation of adult rat S_{100} cytidylyltransferase



Aliquots of rat lung S_{100} (500ul) were mixed with 10% PEG_{6000} to final concentrations of 1,2,3,and 4%. After 2 hours at 4°C the pellets and supernatants were separated by centrifugation at 12,000 x g x 2 minutes.

The evidence presented in chapter 3 suggested an in vitro association of lung cytidylyltransferase with actin-rich particles. Ageing experiments indicated that a large proportion of rat enzyme was found in conjunction with aggregated cytoskeletal components. The efficacy of PEG_{6000} for fractionation of both rat and human soluble lung cytidylyltransferase was evaluated.

4.2.3.1 PEG precipitation of rat enzyme

The apparent strong associations of soluble rat lung enzyme with actin-rich aggregates was further investigated by incubations of S_{100}, H form and the unstable L form enzymes with PEG_{6000} at 4°C.

The results, fig 4.3, showed that less than 100% of enzyme was recovered between 1% and 4% PEG. Between 1% and 3% PEG an increased recovery of enzyme in pellets was noted and, at 3% PEG, 64% could be recovered in the pellet. The supernatant from 3% PEG fractionation contained 22.5% activity, but when this was adjusted to 6% PEG, 54% of initial activity was recovered, with no supernatant activity. The enzyme precipitated with 3% PEG coeluted with void volume fractions from sepharose 6B gel filtration indicating the PEG promoted aggregation of soluble enzyme. The latent cytidylyltransferase in the 3% PEG supernatant which could not be determined before addition of PEG to 6% suggests that the 3% PEG supernatant enzyme is more active when aggregated in the presence of 6% PEG. Alternatively the presence of 3% PEG might inhibit supernatant activity although this is unlikely since unfractionated S_{100}/3% PEG mixtures showed no inhibition.

The effect of 3% PEG on rat H and L form enzyme was examined by a similar protocol. In buffer A 79% of H form activity was precipitated with 3% PEG while no activity could be determined in the 3% supernatant. With the low ionic strength buffer B, however, no H region enzyme activity could be precipitated even when PEG concentration was elevated to 6%. This observation was unexpected since protein solubility differences in the presence of PEG are not usually encountered with variations in ionic strength (Ingham 1984). A possible explanation of this difference was that the ionic strength-dependant precipitation of cytidylyltransferase resulted from a secondary hetero-interaction with a PEG precipitated protein, perhaps F-actin. An ionic interaction with a

Table 4.2 PEG_{6000} precipitation of human lung cytidylyltransferase

		S_{100}		0-40% Ammonium sulphate fraction		40-60% Ammonium sulphate fraction		H form enzyme in buffer A		L form enzyme in buffer A	
		3% PEG	3-6% PEG	3% PEG	6% PEG	3% PEG	6% PEG	3% PEG	6% PEG	3% PEG	6% PEG
Adult lung enzyme	P	3.3	80	0	55	0	18	<1	93	0	0
	S	70	0	100	0	100	0	100	nd	100	100
Fetal lung enzyme	P	38	153	nd	nd	nd	nd	<1	75	0	0
	S	0	0	nd	nd	nd	nd	100	nd	100	100

P = precipitated enzyme, S = supernatant enzyme, nd = not determined

Aliquots of fetal or adult lung fractions(500ul) were adjusted to 3 or 6% PEG by the addition of 10% PEG stock solution. After 2 hours incubation at 4°C, pellets and supernatants were separated by centrifugation at 12,000 x g x 2 minutes. Cytidylyltransferase activities were all determined in the presence of 0.25mM PG, and expressed as a fraction of PEG-free controls.

hetero-precipitated protein might produce the apparent ionic strength dependant precipitation. Evidence in favour of this suggestion was provided by investigation of PEG/L form interaction. At 6%, PEG was unable to precipitate any of the very unstable L region enzyme from gel filtration in buffer A or B. In addition SDS PAGE analysis showed no actin bands from these regions. It is likely that any actin present as small oligomers of approximately 200,000 daltons in the pooled L region from gel filtration was below the critical actin monomer concentration. This is the concentration below which the F-actin/G-actin equilibrium does not favour F-actin formation (Bray and Thomas 1976). If the aggregation of cytidylyltransferase and subsequent precipitation were in part dependent on the transformation of actin monomers and small oligomers into F-actin enriched particles, then PEG, between 0 and 6°C might not precipitate enzyme in some cases. The evident failure to precipitate L form enzyme by PEG may, under this interpretation be the result of inability to reach the critical actin monomer concentration, even by an excluded volume mechanism, at 6% PEG.

4.2.3.2 PEG precipitation of human lung enzyme

The results presented in chapter 3 indicated that human lung and rat lung cytidylyltransferase have different properties on ageing. The aged rat lung S_{100} enzyme was aggregated and largely coprecipitated with actin-rich pellets. Human enzyme, by contrast, remained largely soluble with less aggregated enzyme associated with F-actin pellet. Any PEG promoted precipitations of human lung cytidylyltransferase might consequently be expected to be quantitatively different to that of rat enzyme if dependent on enzyme/F-actin interactions. The effect of PEG at two concentrations, 3% and 6%, was investigated with human lung cytidylyltransferase in S_{100} and partially purified preparations.

The results of fractionations, table 4.2, showed that both fetal and adult human lung enzyme could be precipitated by PEG, but at higher concentrations than those required for rat samples. 3% PEG, for example, precipitated only 3.3% adult lung S_{100} and 38% fetal lung S_{100} enzyme (c.f. 51% rat S_{100} enzyme). 3% PEG precipitation of fetal S_{100} enzyme was high and closer in behaviour to rat than adult human enzyme, but must be viewed in the light of an overall fetal S_{100} enzyme recovery of almost double fresh S_{100} enzyme determination. PEG concentrations of 6% were necessary to precipitate the majority of enzyme from S_{100}, 0-40% ammonium

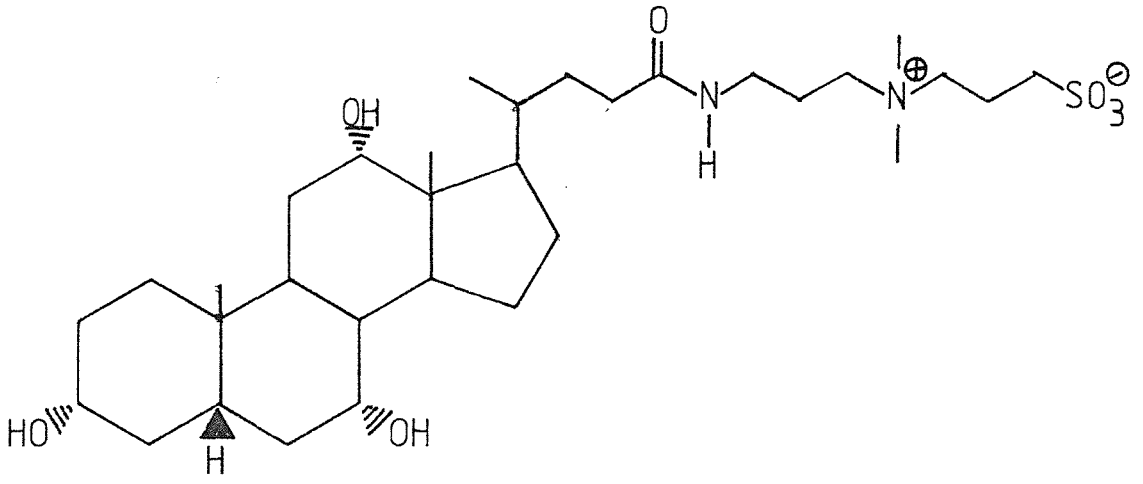
sulphate fraction and H region fraction. The bulk of enzyme activity from both the 40%-60% ammonium sulphate fraction and the L region fraction could not be precipitated by 6% PEG. PEG precipitated cytidylyltransferase from human S_{100} preparations coeluted with H region on gel filtration, and no L region activity was measurable.

The PEG precipitatable enzyme fractions characteristically contained actin, while those in which cytidylyltransferase remained soluble with PEG did not. SDS PAGE analysis of L form enzyme showed multiple bands but no quantifiable protein at 43,000 daltons. PEG appeared to offer a convenient method for fractionation of cytidylyltransferase in preparations containing actin. It is possible that the failure of precipitation at 6% PEG in L region and 40%-60% ammonium sulphate is the result of a reduced actin content in these fractions. PEG precipitation offers an advantage over ageing procedures in the preparation of particulate enzyme from soluble enzyme. The aggregation can be performed rapidly at 4°C rather than 37°C and more enzyme is associated with the aggregated actin-rich pellets.

4.2.4 Detergent solubilisation with CHAPS

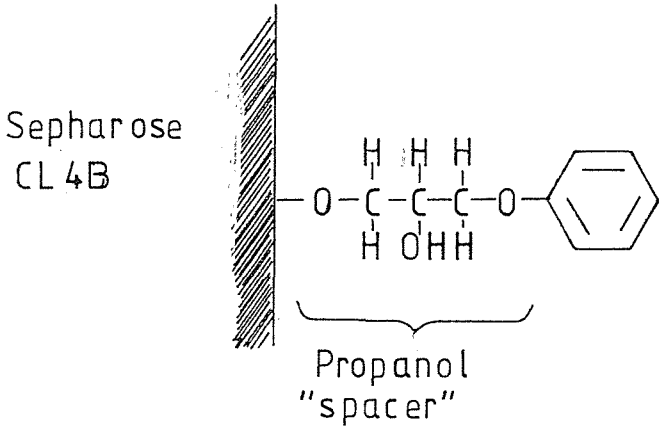
Both ageing of rat cytidylyltransferase and PEG precipitation of human cytidylyltransferase produced substantial quantities of enzyme associated with F-actin rich aggregates. Further purification required a separation of these components and detergent solubilisation seemed to offer a suitable method. In chapter 3 SDS was shown to solubilise lung F-actin which could then reassemble when SDS was removed. SDS solubilisation at 0.05% has been used to convert rat liver enzyme H form to L form (Choy et al 1977, Vance et al 1981). A disadvantage of this approach was the loss of as much as 90% of the activity applied to sepharose 6B/SDS columns (Choy et al 1977). Detergent solubilisation of cytidylyltransferase from actin-rich pellets required a detergent which ideally did not solubilise F-actin. The most well characterised detergent possessing this property is Triton X_{100} which is conventionally used for isolation of intact cytoskeletal structures being one of the few detergents in which F-actin is totally insoluble. Unfortunately Triton X_{100} has been shown to inhibit rat lung cytosolic enzyme (Stern et al 1976) in common with a number of other detergents, including SDS, deoxycholate and NP_{40}.

Fig 4.4 The structure of the detergent CHAPS(3 - (3 - Cholamido-propyl) dimethylamino - 1 - propane sulphonate



CHAPS is a zwitterionic detergent particularly useful in the purification of membrane receptors and sensitive proteins (Hjelmeland and Chrambach 1984).

Fig 4.12 The partial structure of Phenyl Sepharose CL4B



PhenylSepharose CL4B is one of two matrices commonly employed in hydrphobic interaction chromatography. The phenyl group is chemically linked to the monosaccharide subunits of Sepharose by a stable ether linkage.

Table 4.3 The effect of 1% CHAPS on soluble human and rat cytidylyltransferase

Source of cytidylyltransferase	Cytidylyltransferase activity in the presence of 1% CHAPS(% of control)
Adult human lung S_{100}	116
Adult human lung H form	103
Adult rat lung S_{100}	12
Adult rat lung H form in buffer A	27.6
Adult rat lung H form in low ionic strength buffer B	123

Fractions of soluble human and rat lung cytidylyltransferase preparations(1ml) were incubated for 2 hours at 37°C in the presence or absence of 1% CHAPS. Rat lung H form cytidylyltransferase was examined in both buffer A and low ionic strength buffer B.

Many newer detergents, specifically designed to aid the purification of membrane receptors and other sensitive proteins (Hjelmeland and Chrambach 1984) have yet to be evaluated. The zwitterionic CHAPS (3-[(3-Cholamidopropyl) dimethylamino-1-propane sulphonate]) is one such detergent, fig 4.4. CHAPS has proven useful in solubilising membrane-associated proteins. Microsomal cytochrome P450, a highly aggregated protein (Hjelmeland 1980) and functional receptors (Simonds et al 1980) have been successfully solubilised using CHAPS, without denaturation. The effect of CHAPS above and below its critical micelle concentration (CMC) of 0.55% (Pierce Chemical Company literature) was investigated with human and rat soluble, particulate and membrane-associated cytidylyltransferase.

4.2.4.1 The effect of CHAPS on soluble enzyme

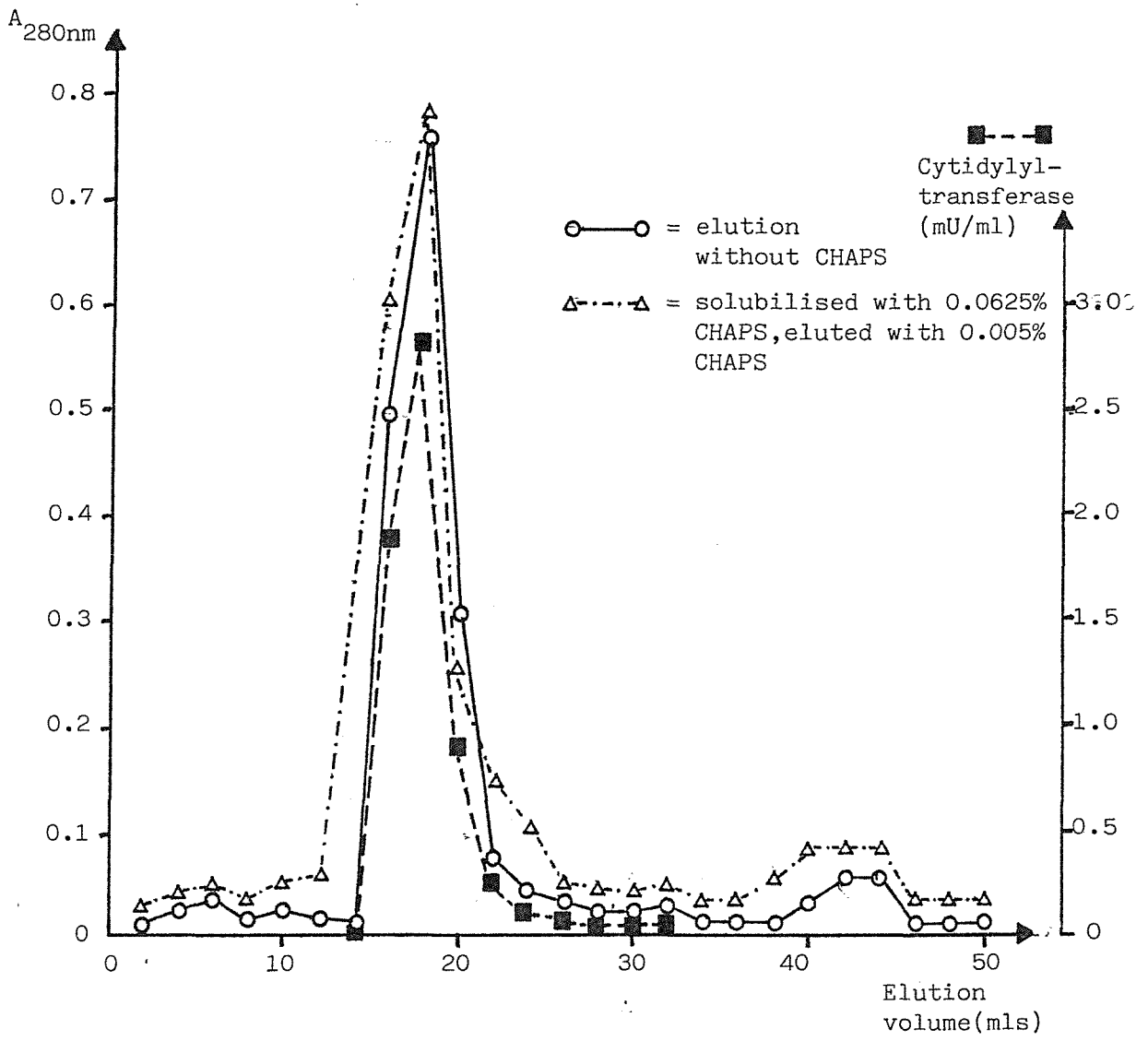
Soluble cytidylyltransferase preparations from human and rat lung were incubated in the presence or absence of 1% CHAPS to assess any inhibitory effect. Rat H form enzyme was assayed in both buffer A and low ionic strength buffer B.

The results, table 4.3, indicated that CHAPS at 1% did not inhibit cytidylyltransferase activity in human S_{100} or partially purified H form. Rat S_{100} enzyme, however, was inhibited by CHAPS at 1% while a separate time course investigation showed this inhibition was complete within 2 minutes of CHAPS addition. Rat H form enzyme was inhibited by 72.4% with 1% CHAPS when in buffer A but showed slight activation when in low ionic strength buffer B. It was not readily apparent how this detergent-mediated susceptibility to ionic strength operated. Solubilisation with CHAPS might have revealed an ionic strength sensitive region of cytidylyltransferase which in turn inhibited the enzyme.

The stability of rat H region enzyme in low ionic strength/CHAPS incubations was further investigated by gel filtration in the presence of CHAPS at concentrations below and above its CMC. An experiment combining incubation with CHAPS followed by gel filtration was devised to assess whether enzyme activity was associated with aggregated V_o region or smaller molecular weight regions.

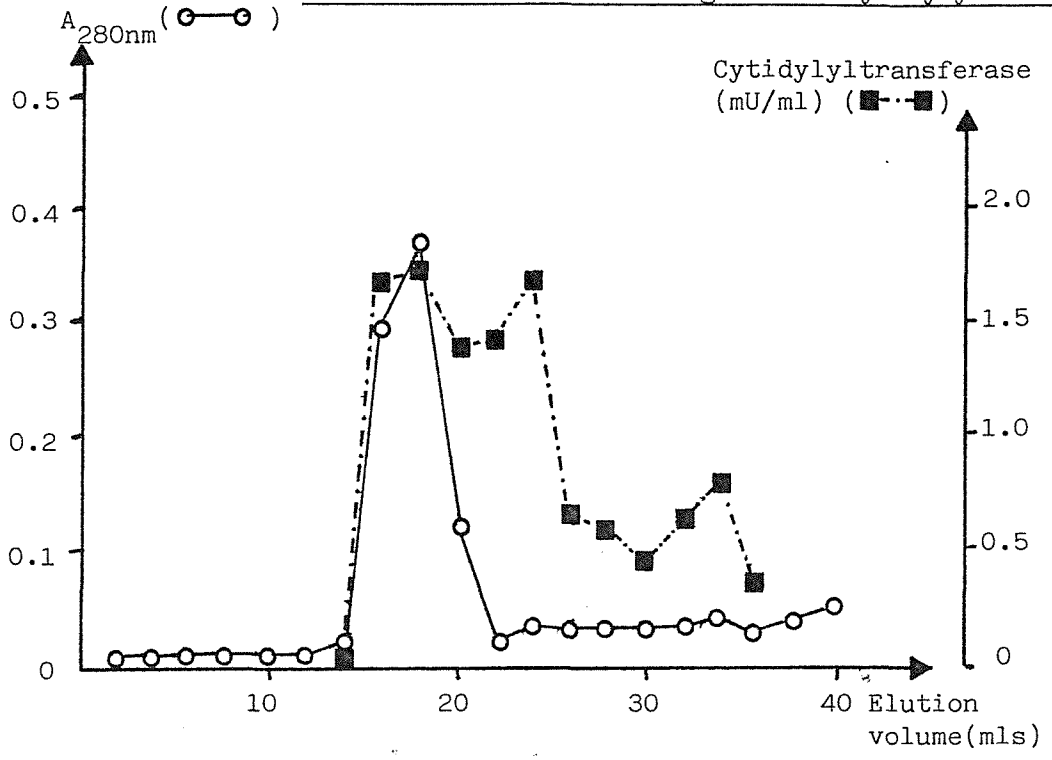
The results are shown in figs 4.5, 4.6 and 4.7. At 0.0625% CHAPS, below CMC, no solubilization in the form of a high to low molecular

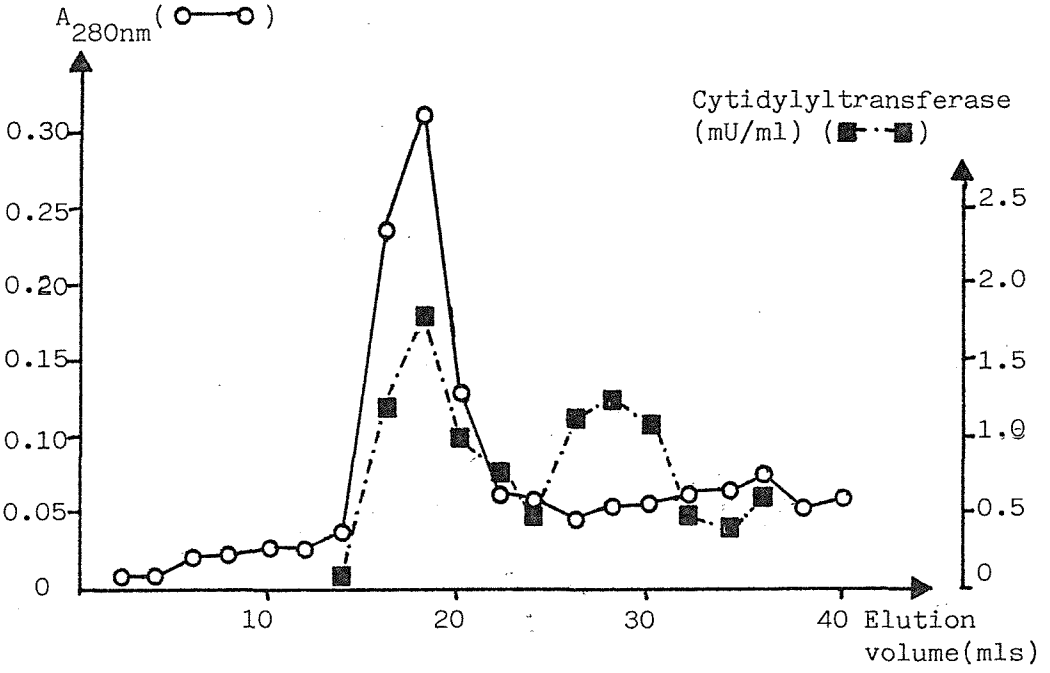
Fig 4.5 Sepharose 6B elution profile of rat H form cytidylyltransferase before and after solubilisation with 0.0625% CHAPS



The elution profile of rat lung H form cytidylyltransferase in low ionic strength buffer B was investigated before and after solubilisation with 0.0625% CHAPS. Duplicate portions of a pooled H form fraction(4ml,3.3mU/ml) were incubated for 2 hours at 37°C,with and without 0.0625% CHAPS. The elution profile of the CHAPS-free incubation was monitored from Sepharose 6B gel filtration column (60cm x 0.9cm) in the presence of low ionic strength buffer B. The column was then re-equilibrated with buffer B containing 0.005% CHAPS,and the profile of the CHAPS treated sample determined. Cytidylyltransferase activities from each 2ml fraction were measured and found to be similar from each column

Figs 4.6, 4.7 The effect of increasing CHAPS concentration on the solubilisation of rat lung H form cytidylyltransferase

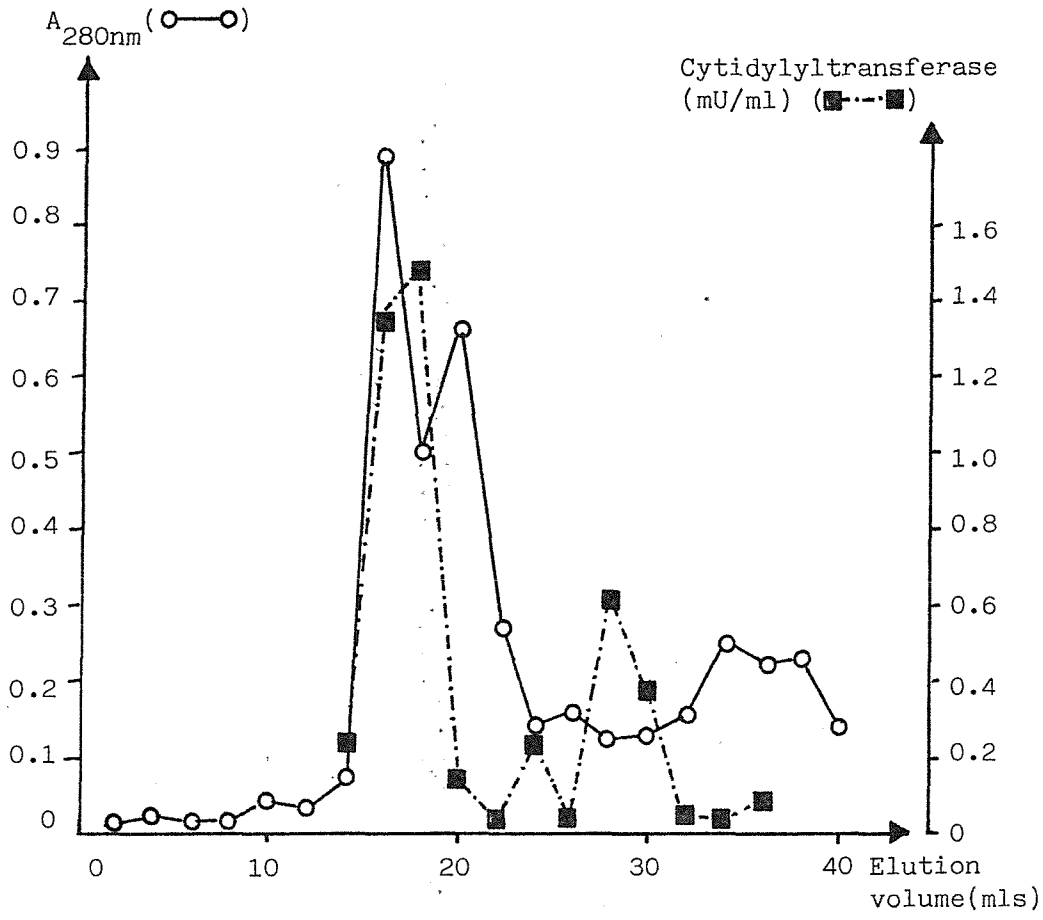




Fractions 8 and 9(between 14 and 18mls) from fig 4. were pooled and adjusted to 0.625% CHAPS,ie above CMC,and incubated at 37°C for 30 minutes. The sample(4ml,9.26mU) was applied to a Sepharose 6B column equilibrated in buffer B with 0.005% CHAPS and eluted,fig 4.6.

Fractions 8 and 9 from column 4.6. were similarly adjusted to 1.25% CHAPS(4ml,7.7mU) and rechromatographed,fig 4.7.

Fig 4.8. Sepharose 6B elution profile of cytidylyltransferase solubilised from rat lung S_{100} actin-rich pellet



Aged rat lung S_{100}(6ml) was centrifuged at 100,000 x g x 60 minutes and the actin-rich pellet resuspended in buffer B containing 1% CHAPS. After 2 hours at 37°C, the remaining non-solubilised particulate matter was removed by centrifugation at 12,000 x g x 2 minutes. The clear supernatant(1.9ml,7.7mU) was applied to the CHAPS/Sepharose 6B column. Eluted fractions(2ml) were assayed for cytidylyltransferase.

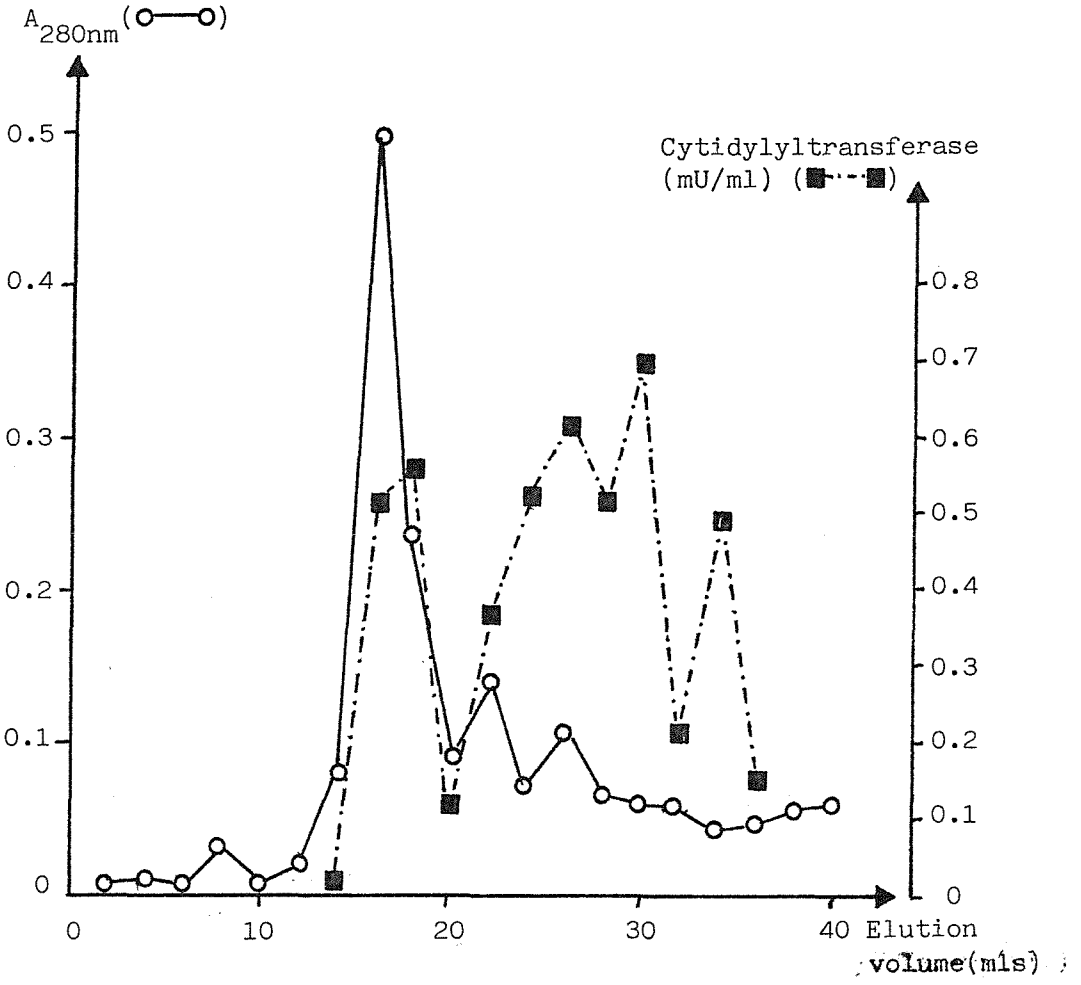
weight transition was observed with total protein and 97% of applied activity co-eluted with V_o fractions. This V_o peak (4ml, 9.26mU) was solubilised with 0.625% CHAPS, above CMC, and rechromatographed into 3 peaks of activity, fig 4.6. 10.9mU(117%) was recovered in V_o while 14.9mU (160%) recovered with lower molecular weight fractions contributed to a 227% recovery of applied activity. This 2nd V_o (4ml,7.7mU) adjusted to 1.25% CHAPS and chromatographed again, fig 4.7., gave a V_o of 4.2mU(54.8%) and 5.3mU (68.3%) in lower fractions. A total of 123.1% of applied activity was recovered from this column. In each case the bulk of protein, determined as A_{280nm}, was found in V_o fractions. CHAPS does not absorb at 280nm and these results suggested that it did not dissociate substantial amounts of the F-actin-rich aggregate. It was concluded that CHAPS, above its CMC, was dissociating cytidylyltransferase and/or releasing the enzyme from its tight association with the F-actin-rich complex present with rat H form enzyme.

4.2.4.2 CHAPS solubilisation of particulate cytidylyltransferase

The possibility that CHAPS might solubilise cytidylyltransferase from the particulate fractions produced during ageing of S_{100}s was tested with aged rat lung S_{100}. Rat enzyme was chosen since the majority of aged, soluble enzyme becomes particulate upon incubation at 37°C for 2 hours.

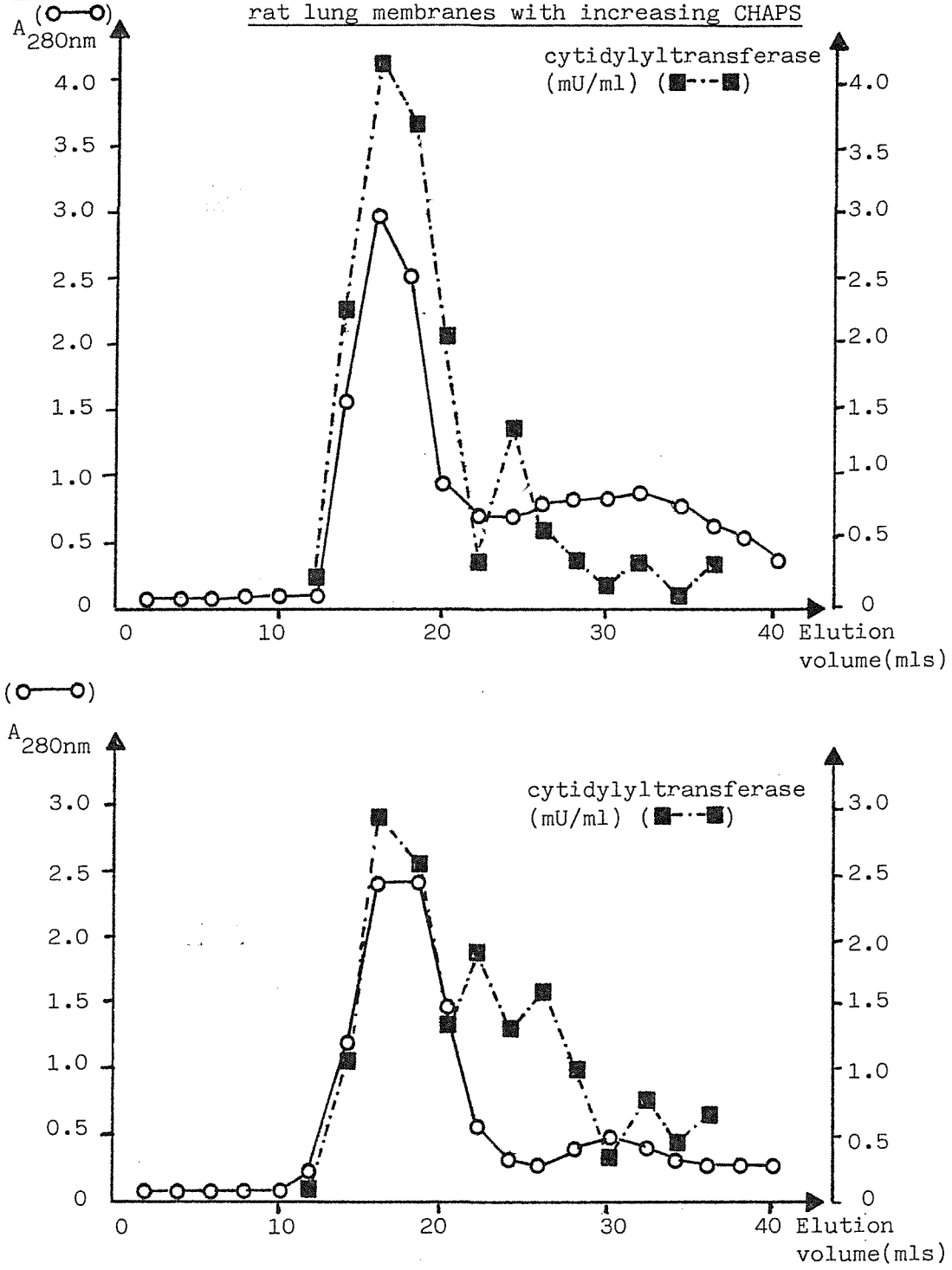
Aged rat lung S_{100} (6ml) was centrifuged at 100,000 x g x 45 minutes and the particulate, enzyme-rich fraction resuspended in buffer B (2ml) in the presence of 1% CHAPS. A particulate fraction persisted in suspension after 2 hours at 37°C although there was a very apparent decrease in turbidity. The light-scattering effect at 280nm made quantitation difficult, but at 340nm greater than 50% reduction in turbidity was noted. The particulate fraction was removed by centrifugation (12,000 x g x 2 minutes) and some unquantified activity persisted in this fraction. The magnitude of cytidylyltransferase remaining could not be accurately assessed due to resuspension problems and poor duplicates. Variations of 4 - 5 fold in duplicates reflected incomplete particle dispersion. The clear, pale yellow-green supernatant (about1.9ml) gave the CHAPS/6B elution profile shown in fig 4.8.

Fig 4.9 CHAPS solubilisation of cytidylyltransferase from washed rat lung membrane-rich fraction



The membranous fraction from a rat lung homogenate which was pelleted between 10,000 x g x 10 minutes and 100,000 x g x 60minutes,was washed with 10mM Na phosphate/150mM KCl/10mM EDTA pH 7.4 to remove extrinsic membrane proteins. The washed fraction was resuspended in buffer A with 1.25% CHAPS and incubated at 37°C for 2 hours. The 12,000 x g x 2 minute supernatant,which contained all measureable cytidylyltransferase,was applied to the CHAPS/Sepharose 6B column(3.5ml,10mU)

Fig 4.10, 4.11 Solubilisation of cytidylyltransferase from total rat lung membranes with increasing CHAPS



A rat lung membrane fraction P_{100}, washed with the extrinsic membrane removing, high EDTA buffer, was resuspended in low ionic strength buffer B with 0.1% CHAPS, ie below CMC. After 2 hours at 37°C the sample (4ml, 15.2mU) was applied to a CHAPS/ Sepharose 6B column, fig 4.10. The pooled V_o from this column (4ml, 17.4mU) was adjusted to 1.25% CHAPS and rechromatographed, fig 4.11

The V_o fractions contained only 77.4% of applied activity while 42.6% was found with lower molecular weight fractions. At 1% CHAPS was apparently partially dissociating the aged, aggregated rat lung cytidylyltransferase from the bulk of V_o-associated protein.

4.2.4.3 CHAPS solubilisation of rat lung membrane-associated enzyme

The CHAPS-mediated solubilisation of particulate rat lung cytidylyltransferase suggested that a similar approach might usefully be applied to attempt release of rat lung P_{100} enzyme. Solubilisation in both buffers A and B were performed since rat lung H form enzyme had been shown to be more stable in buffer B.

Rat lung P_{100}, washed free of extrinsic protein with 10mM EDTA, was solubilised in buffer A and CHAPS at 1.25%. The 12,000 x g x 2 minute supernatant (3.5ml, 10mU) eluted on CHAPS/6B column, fig 4.9, produced only 9.44mU recovery with 24% associated with V_o. When solubilised in buffer B and CHAPS at0.1% (below CMC) however, recovery was enhanced. CHAPS gel filtration, fig 4.10, showed the majority of recovered enzyme associated with V_o fractions, and a doubling of applied activity (15.2mU - 30.3mU). Pooled V_o from column 4.10 (17.4mU) and adjusted to 1.25% CHAPS gave the elution profile fig 4.11. Of the 225.7% activity recovered only 49.3% was V_o-associated. The specific activity of the low molecular weight, CHAPS solubilised region (13.5mU/mg) represented a 6.2 fold purification of fresh membrane-associated enzyme.

4.2.4.4 CHAPS solubilisation of human lung membrane-associated enzyme

The efficacy of CHAPS in solubilising fetal human lung membrane-associated cytidylyltransferase was investigated. Fetal human lung membranes were chosen because they could be obtained free of the tar-like components often associated with adult lung membranes.

Fetal lung membrane-rich P_{100} fractions were washed free of extrinsic proteins with 10mM EDTA buffer as described for rat P_{100}s. Fresh P_{100} (5ml, 83mU) in buffer A centrifuged at 100,000 x g x 60 minutes released 17mU (20.5%) into the supernatant. The pellet, subsequently washed in 10mM EDTA buffer produced a supernatant with 30mU(36%). When the washed

pellet was resuspended in buffer A (5ml), 125.5mU (151%) activity was recovered. Combined activities recovered represented 207% of freshly determined activity.

Washed membranes were adjusted to 0.625% CHAPS and incubated at 37°C for 2 hours. After incubation, activity remaining was 67mU (53%) and the supernatant from 100,000 x g x 60 minute centrifugation contained 33.4mU while the pellet contained 38.5mU. The cytidylyltransferase activity declined to less than 10% of these values overnight at 4°C.

Human membrane-associated enzyme clearly behaved differently to rat lung P_{100} enzyme. The human enzyme was more susceptible to removal with high EDTA and unexpected latent activity was revealed following washing with this extrinsic protein removing buffer. CHAPS solubilisation of membranes led to a loss of enzyme activity, unlike the increase observed with rat lung membranes. The results indicated that CHAPS solubilisation was not a suitable technique for application to the purification of human lung membrane-associated enzyme. The action of CHAPS in buffer B was not investigated and consequently the possibility of high ionic strength-mediated inhibition of solubilised enzyme could not be discounted. This explanation seemed unlikely since CHAPS did not inhibit the soluble enzyme of S_{100} preparations in buffer A.

4.2.5 Hydrophobic and affinity chromatography of cytidylyltransferase

Protein separations making use of differences in biological function or individual chemical composition have been favoured and have found wide application in recent years. Two commonly used techniques are those of hydrophobic interaction chromatography and affinity chromatography. Observations of the hydrophobic properties of rat liver cytidylyltransferase (Weinhold et al 1986) and its associations with membranous structures (Pelech and Vance 1984(a)) suggested that hydrophobic interaction chromatography might offer a potentially useful enrichment step in purification schemes. High ionic strengths are able to promote hydrophobic interactions. Proteins possessing hydrophobic sites on their surfaces which have greatest affinity for hydrophobic environments may be absorbed to the hydrophobic matrix of agarose derivatives such as Phenyl Sepharose CL4B. Elution may subsequently be effected by a lowering of ionic strength, polarity, the use of detergents or raising pH.

Affinity chromatography utilises an immobilised ligand or ligand analogue as a matrix or which to selectively adsorb proteins of interest. Affinity chromatography of rat liver cytidylyltransferase on a choline phosphate analogue, glycerophosphocholine, has been reported (Choy and Vana 1976). The enzyme was highly purified (specific activity 612mU/mg) but unstable with activity decaying by 80% in 6 days. PhenylSepharose Ch-4B was chosen as a hydrophobic matrix. The potential of affinity chromatography was assessed by using two choline phosphate analogues and an immobilised CTP as affinity ligands.

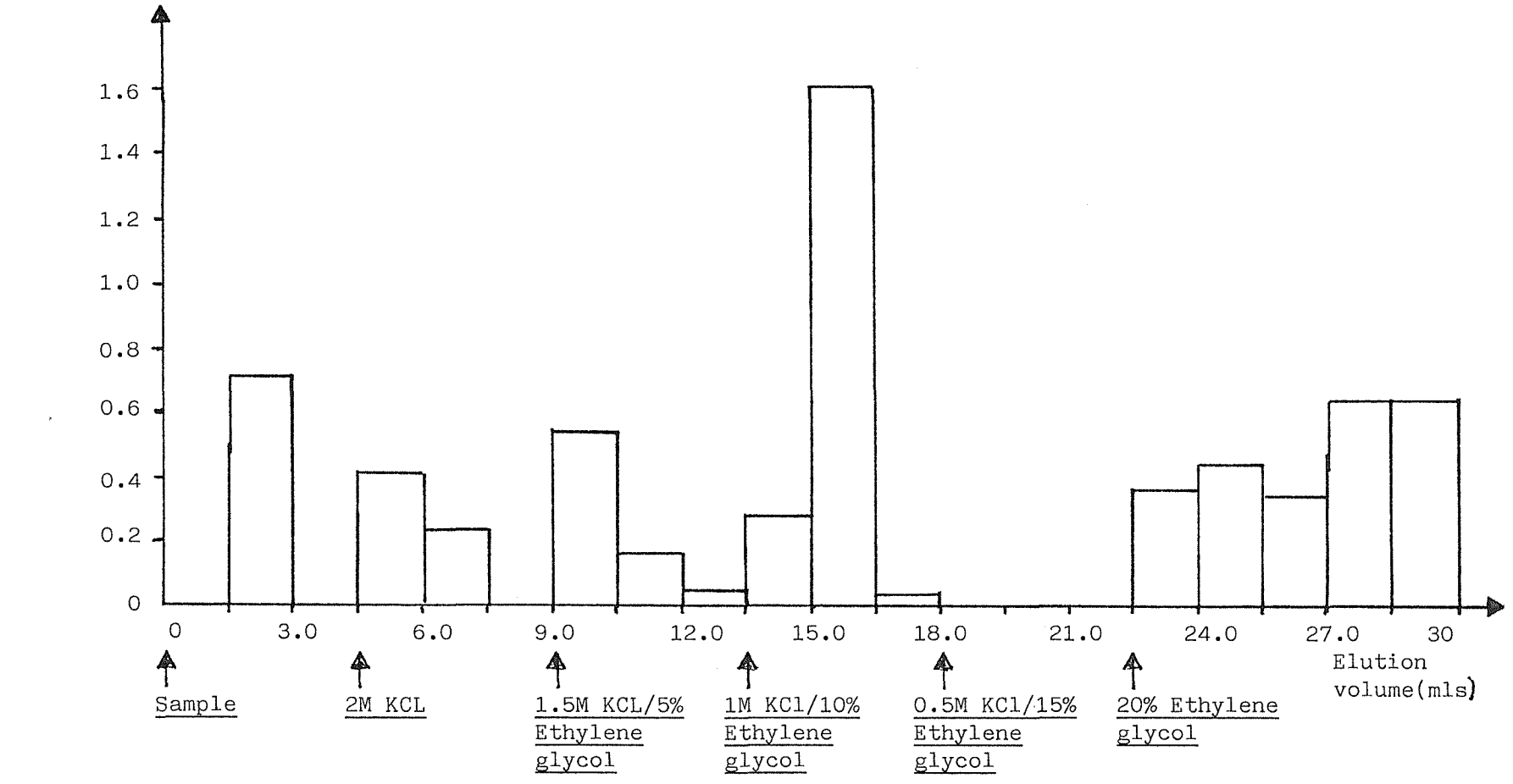
4.2.5.1 Hydrophobic interactions with Phenyl Sepharose CL-4B

Phenyl Sepharose CL-4B is one of two commonly used matrices for hydrophobic interaction chromatography of hydrophobic proteins. The structure of phenyl sepharose is shown in fig 4.12, phenyl groups are attached to the monosaccharide units of the agarose matrix via uncharged, chemically stable other linkages. An initial screening of rat and human lung enzymes was performed to assess the suitability of Phenyl Sepharose CL-4B as a hydrophobic matrix. Rat lung H form and fetal human lung L form enzyme from gel filtration were investigated. The rat H form was chosen due to the instability of L form.

In separate experiments rat H form enzyme (1ml, 3.18mU) and fetal human lung L form enzyme (1ml, 1.4mU) both in buffer A were mixed with equilibrated Phenyl Sepharose CL-4B (300mg moist weight). After 30 minutes incubation at 4°C with control incubations using Sepharose CL-4B, cytidylyltransferase activities were determined in 12,000 x g x 2 minute supernatants.

Only 30% of rat H form enzyme was bound to phenyl Sepharose CL-4B, while the corresponding figure for fetal L form enzyme was 61%. The lower affinity of rat enzyme may reflect a difference due to L/H transition, possibly the presence of F-actin or membrane fragments. The instability of rat L form enzyme prevented further comparison. Phenyl Sepharose CL-4B apppeared unsuitable for use with rat lung enzyme, although its lower affinity might be enhanced by replacement with OctylSepharose CL-4B matrix which tends to bind hydrophobic molecules more tightly.

Fig 4.13. Phenyl Sepharose chromatography of fibroblast 2nd S_{100} cytidylyltransferase



Fibroblast 2nd S_{100}(4.5ml,18.3mU without KCl,10.1mU with 2M KCl),was applied to a small Phenyl Sepharose CL4B column(0.5cm x 1.5cm) equilibrated in buffer A containing 2M KCl. Elution was effected by combination of decreasing KCL and increasing ethylene glycol in the irrigation buffer. 1.5 ml fractions were collected and assayed for cytidylyltransferase.

The binding of human enzyme was promising and was consequently investigated further. Fetal lung fibroblast 2nd S_{100} was used as the enzyme source because of its easy availability, high specific activity and the lack of serum proteins, which can be washed free prior to homogenisation. Cytidylyltransferase interaction, with a small Phenyl Sepharose CL-4B column, was investigated in the presence of 2M KCl to promote hydrophobic interaction. Elution was attempted by a combination of reduction of KCl and increase of ethylene glycol concentration.

Unretarded activity, fig 4.13, represented 21% (11.6% without KCl) of applied activity. A further 10.5% (5.8%) was eluted with 1.5M KCl/5% ethylene glycol, 28.5% (15.7%) with 1M KCl/10% ethylene glycol and 35.6% (19.6%) with remaining washes. A total of 95.6% (52.6% without KCl) of applied activity was recovered over the wide range of elution conditions examined.

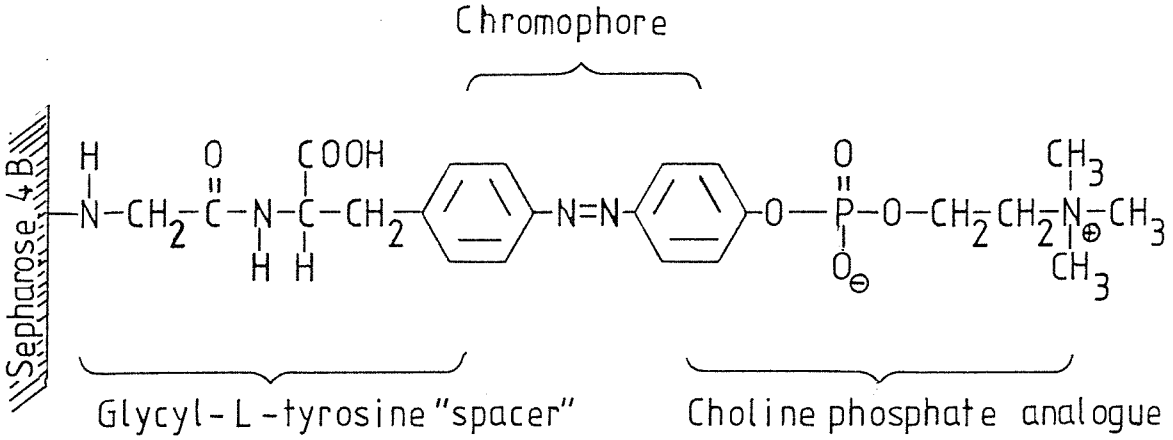
The binding of 79% of activity provided a potentially useful enrichment step. The 45% inhibition of soluble enzyme when mixed with 2M KCl, however, coupled with the broad range of ionic strengths necessary to effect complete elution made Phenyl Sepharose CL-4B chromatography an unsatisfactory choice under the conditions investigated.

4.2.5.2 Affinity chromatography using choline phosphate analogues

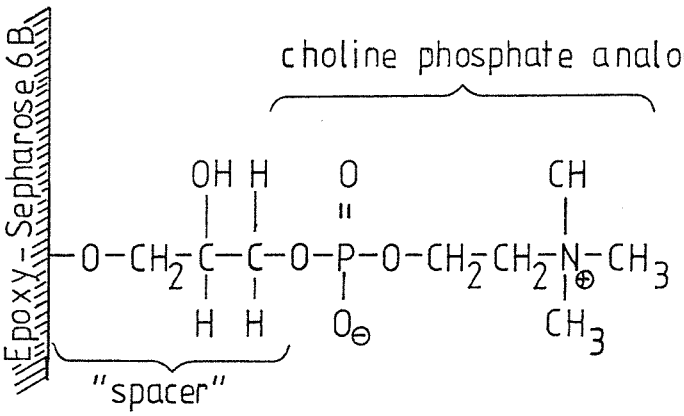
The use of affinity chromatography to selectively bind proteins of interest to an immobilised ligand or ligand analogue can offer a powerful tool for specific purification. Rapid separations can be achieved due to small bed volumes required in most circumstances. The separations are effected according to specific biological function rather than general physical or chemical properties of the protein. Elutions may be effected by specific or non-specific conditions. Specific eluting species can include addition of excess ligand or omission of cofactors. Non specific elnants may include pH shifts or ionic strength variation.

Two choline phosphate analogues were investigated for their ability to act as affinity ligands when immobilised. The first was the Sepharose 6B-linked glycerophosphocholine described by Choy and Vance (Choy and Vance 1976) and used to purify rat liver cytidylyltransferase. An alternative choline phosphate analogue was suggested by investigations of

Fig 4.14 The proposed structures of two immobilised choline phosphate analogues



(a) Sepharose 4B-immobilised p-diazoniumphenyl phosphoryl choline



(b) Epoxy-Sepharose 6B-immobilised glycerophosphocholine

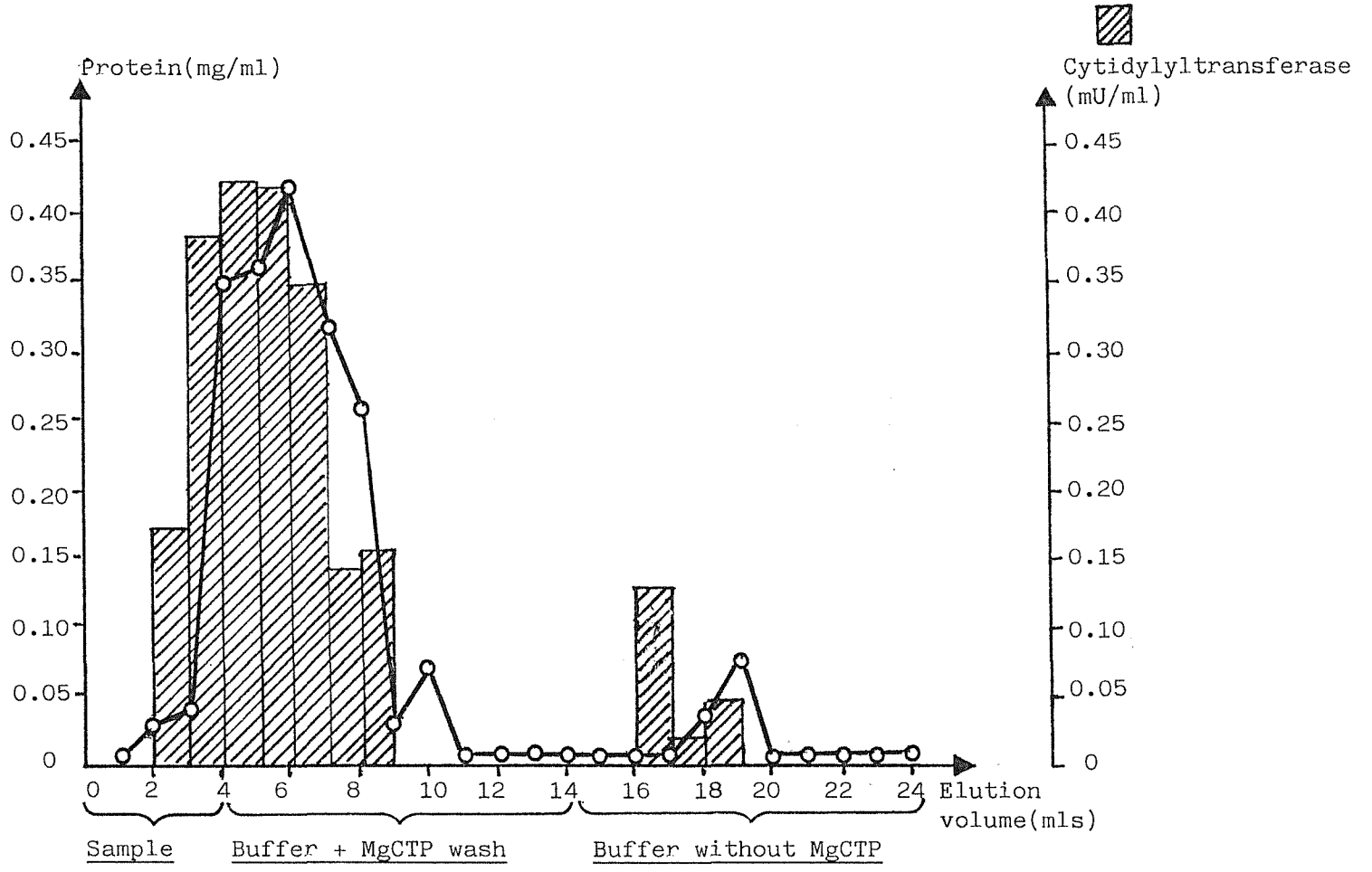
choline phosphate binding mouse myeloma proteins (Chesebro and Metzgler 1972). A number of mouse and human myeloma proteins have been identified as possessing definable binding activities. Some possess choline phosphate binding sites. Homogenous preparations of both mouse and human immunoglobulins, possessing choline phosphate binding sites, have been achieved by the use of affinity chromatography. The immobilised species was a choline phosphate analogue, p-diazonium phenyl phosphorylcholine (Ches ebro and Metzgler 1972, Riosen et al 1975) , fig 4.14.

The Sepharose 6B-glycerophosphocholine affinity matrix was prepared from epoxy activated Sepharose 6B and glycerophosphocholine as previously described (Choy and Vance 1976). Fibroblast 2nd S_{100} (3.7ml, 18.2mU) was applied to a small column of affinity matrix (0.5cm x 2cm) in the presence of MgCTP. Fractions (1ml) were collected and assayed for activity. After 10ml elution with MgCTP containing buffer, the MgCTP was omitted. The rationale behind this approach was that the removal of MgCTP might lower any affinity of cytidylyltransferase for the immobilised cholinephosphate analogue. This in turn might produce the elution previously demonstrated for rat liver enzyme (Choy and Vance 1976).

The elution profile, fig 4.14(a) shows a total of 22.14mU (122%) of applied activity recovered. The majority of activity, 113%, was unretarded by the glycerophosphocholine affinity matrix. An apparent 8.7% of activity was bound and subsequently eluted upon Mg CTP withdrawal.

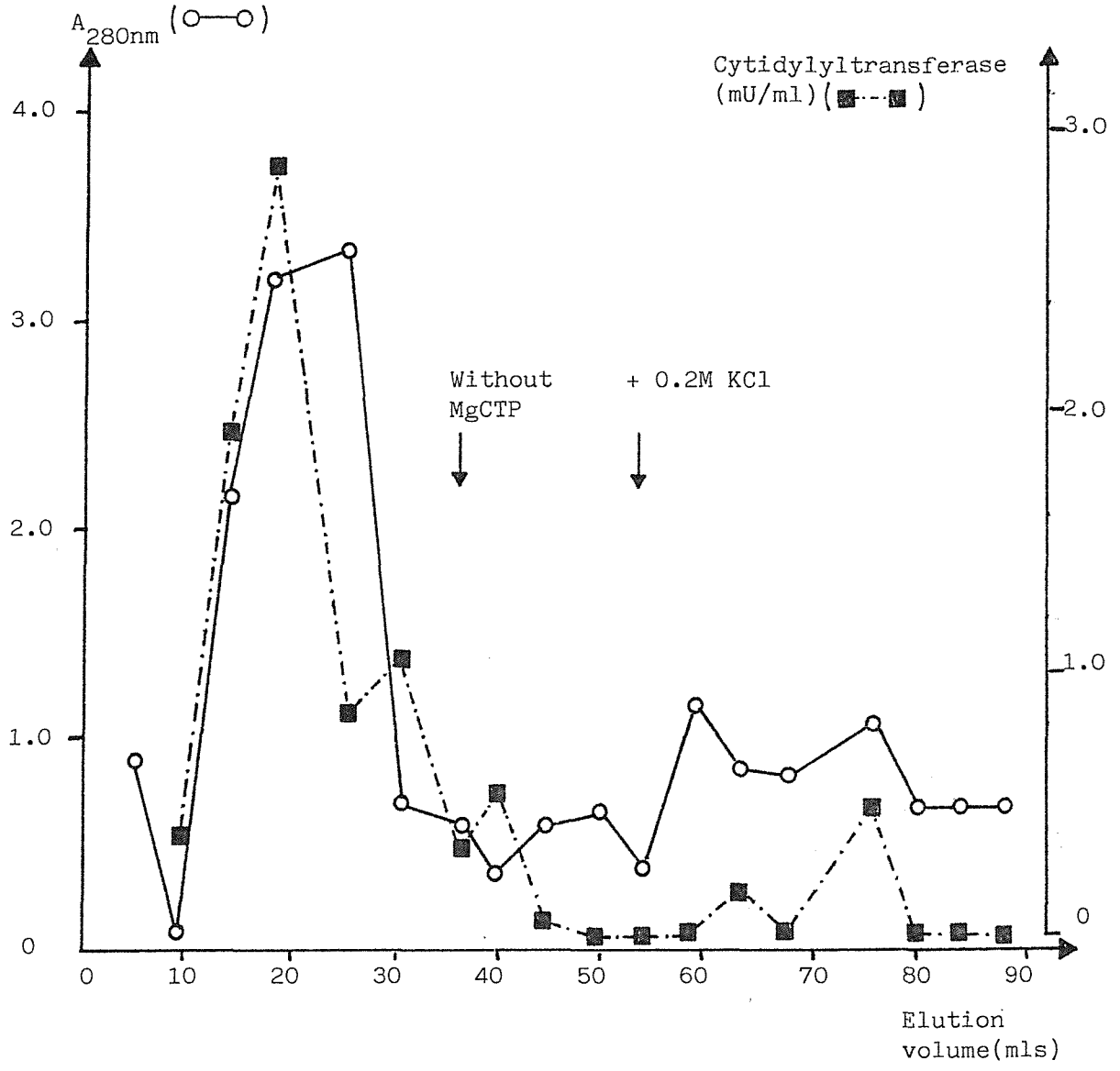
The total proportion of human enzyme bound contrasted sharply with rat liver-derived enzyme (Choy and Vance 1976). In the latter report soluble enzyme, in the presence of Mg CTP, showed more than 60% of enzyme bound to the affinity matrix and subsequently eluted with 2M NaCl. When the 25%-50% ammonium sulphate precipitate of rat liver cytosol was similarly chromatographed, 74.6% of enzyme bound. A later investigation with partially purified H and L forms of rat liver enzyme (Choy et al 1977), suggested that only L form enzyme binds to the matrix with H form unretarded. Gel filtration analysis of adult and fetal human lung 2nd S_{100}s had previously shown that more than 8.7% of activity existed as L form (Section 3.2.2) and it is likely that fibroblast 2nd S_{100} also contains a substantial proportion of L form enzyme. It is probable that glycerophosphocholine was not binding just L form enzyme or just H form enzyme. The low binding may represent a non-specific binding, perhaps a

Fig 4.14(a) Glycerophosphocholine affinity chromatography of fibroblast 2nd S_{100} cytidylyltransferase



Fibroblast 2nd S_{100}(4ml,18.2mU),was applied to a glycerophosphocholine affinity column,in the presence of 6mM $MgCl_2$/5mM CTP in buffer A. The column was then washed with 10ml of this equilibration buffer. Elution was then continued by the removal of MgCTP. Cytidylyltransferase and Lowry protein determinations were made on each 1ml fraction.

Fig 4.15 p - diazoniumphenylphosphorylcholine affinity chromatography of rat lung H form cytidylyltransferase



Rat lung H form cytidylyltransferase(15mls,68.7mU),was applied to a Sepharose-immobilised p-diazonium phenylphosphoryl choline column(15ml bed volume) in low ionic strength buffer B, containing 6mM $MgCl_2$/5mM CTP. Elution was attempted with ommision of MgCTP,followed by inclusion of 0.2M KCl in the irrigation buffer.

slight hydrophobic interaction. Consequently glycerophosphocholine appeared unsuitable as an affinity ligand for human lung cytidylyltransferase.

The second immobilised choline phosphate analogue was also synthesised according to a previously described method (Chesebro and Metzgler 1972) - see materials and methods. The structure of the matrix is unknown but a proposed structure for the gel coupled analogue is shown in fig 4.15 and the glycerophosphocholine matrix is shown for comparison. It can be seen that the 'spacer arm', which separates the choline phosphate analogue from the matrix is longer for the p-diazonium phenyl phosphorylcholine analogue. This property may make it more accessible to the enzyme. The use of Sepharose 4B also permits a larger exclusion limit for the gel matrix, allowing larger molecular weight aggregates to be exposed to the affinity ligand. The diazonium chromophore appeared to be responsible for the yellow colouration of the affinity matrix.

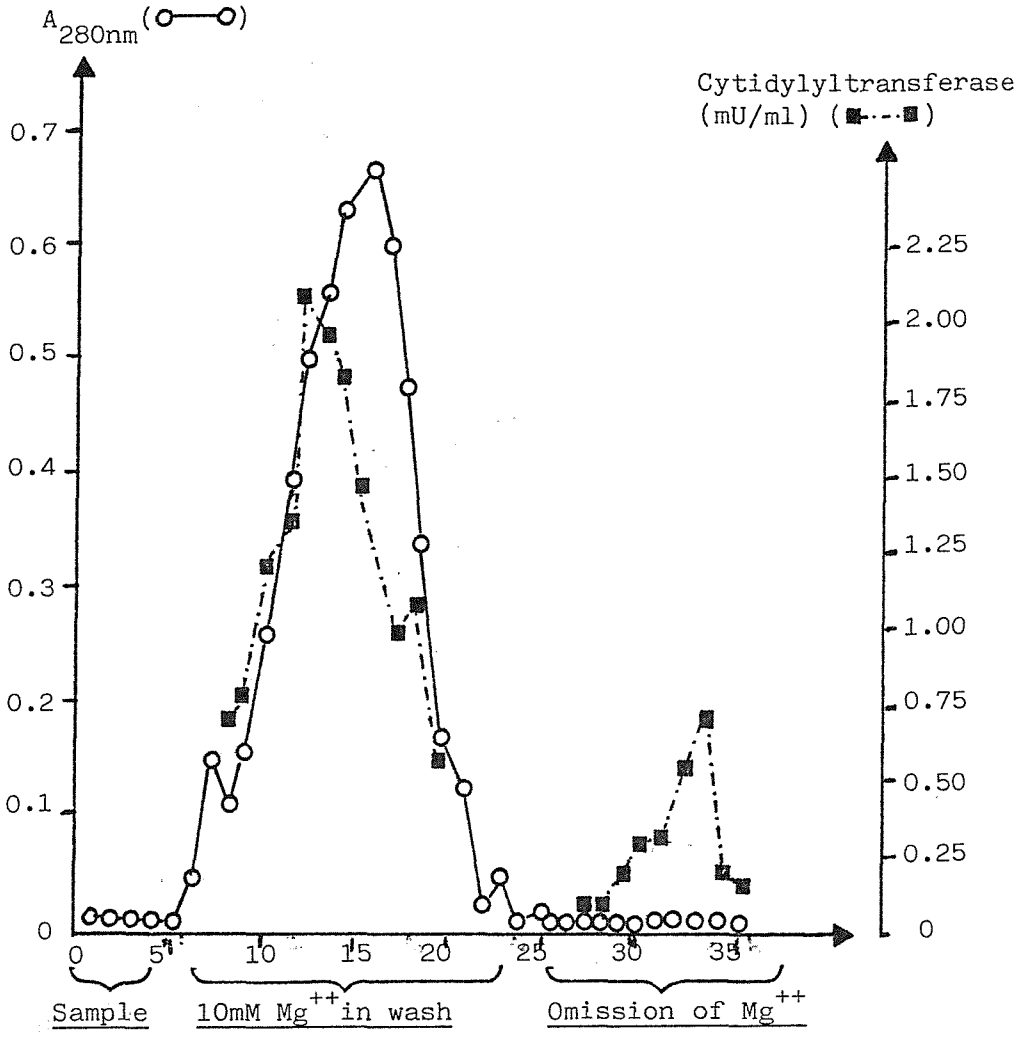
Rat H form enzyme from gel filtration was examined for its binding affinity for the p-diazonium phenylphosphorylcholine matrix in the presence of MgCTP. The elution profile, fig 4.16, showed 87.3mU (127%) recovery with only 13% bound and eluted following MgCTP withdrawal. The majority of applied activity was unretarded. Clearly the bulk of rat lung H form enzyme did not interact with the p-diazonium phenylphosphorylcholine. The bound enzyme was not eluted by MgCTP removal suggesting that the binding was not MgCTP dependant. Enzyme was eluted with 0.1M KCl. This suggested that the observed binding might be a non-specific electrostatic interaction as opposed to a specific binding to the affinity ligand.

Human adult S_{100} cytidylyltransferase was totally unretarded on the p-diazonium phenylphosphorylcholine matrix. The low capacity for adsorption of cytidylyltransferase to both immobilised choline phosphate analogue indicated that they were inappropriate for use in rat or human lung enzyme purification.

4.2.5.3 Human cytidylyltransferase interaction with immobilised CTP

The commercial availability of an agarose immobilised CTP provided a second potential route for the affinity purification of human cytidylyltransferase. CTP agarose (Sigma Chemical Company) was screened

Fig 4.16 Dye-affinity chromatography of fibroblast 2nd S_{100} on Sepharose-immobilised Procion Blue MX-R



Fibroblast 2nd S_{100} (4.5ml, 45.2mU), was applied to a Sepharose/Blue MX-R column (0.9cm x 15cm) equilibrated in buffer A with 6mM Mg^{++}. After washing with 21.5ml equilibration buffer, elution was continued with omission of Mg^{++}. 1ml fraction were collected and assayed for cytidylyltransferase binding and recovery.

for its ability to bind cytidylyltransferase in the presence or absence of Mg^{++}. The rationale for this approach lay in the early demonstration of an absolute requirement of Mg^{++} or Mn^{++} for cytidylyltransferase action (Borkenhagen and Kenndy 1957). It was postulated that an immobilised CTP/cytidylyltransferase interaction would require Mg^{++} and that its removal would lead to dissociation.

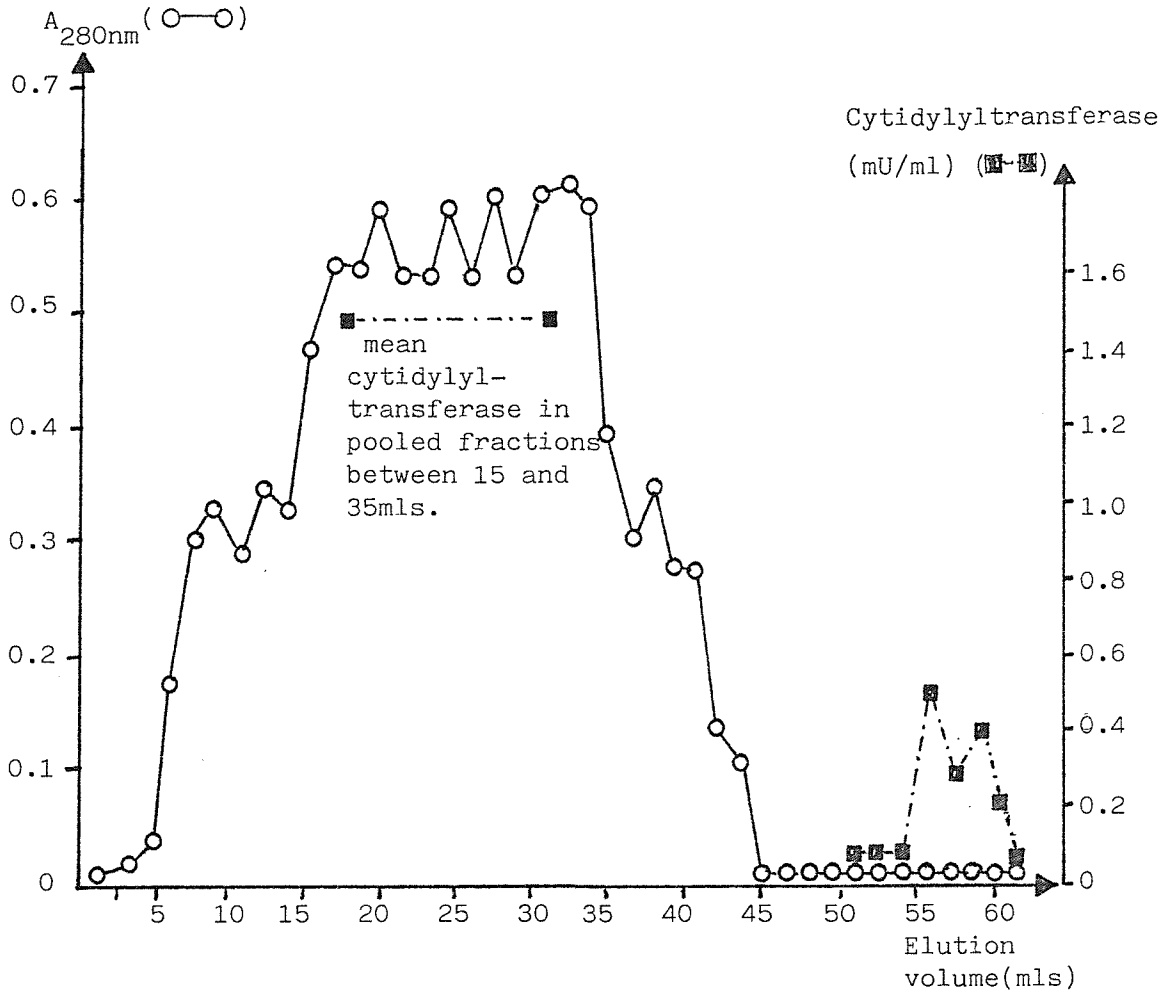
Aliquots of CTP agarose equilibrated in buffer B (100mg moist weight) were mixed with adult human lung 2nd S_{100} previously dialysed into buffer B (1ml, 248mU/ml) in the presence or absence of 10mM Mg^{++} for 30 minutes at 4°C. The activity of cytidylyltransferase in the supernatant was determind following centriguation at 12,000 x g x 2 minutes. Control incubations were performed with sepharose 6B.

The activity of 2nd S_{100} in low ionic strength buffer B was 110% of pre dialysis value in buffer A. The binding of cytidylyltransferase to CTP - agarose was only 27.2% of control value, in the presence of Mg^{++} (10mU) but only 9.7% in the absence of Mg^{++}. This shows that only 17.5% of total measured activity was bound in a Mg^{++} dependant fashion. The order of substrate binding for cytidylyltransferase from human lung is not clear and it is possible that the inclusion and subsequent withdrawal of choline phosphate/eluting buffer might have promoted binding but would have diluted radiolabelled substrate in the assay, necessitating the use of a radiolabelled CTP assay. In the absence of such an assay, or the prior removal of choline phosphate the assay values would be invalid making enzyme activity unquantifiable. Some of the observed binding may have been due to the presence of endogenous choline phosphate within 2nd S_{100} samples perhaps generated by a PC specific Phospholipase C during 37°C incubation. Under the conditions examined, however, CTP agarose was unsuitable as an immobilised affinity ligand for cytidylyltransferase purification.

4.2.6 Chromatography or Immobilised Procion Blue MXR

Pseudoaffinity chromatography of nucleotide dependant enzymes on immobilised dyes is now an established technique (Lowe and Pearson 1984). A number of immobilised reactive dyes, for example Cibacron Blue F3G-A, possess an ability to bind a range of NAD- and NADP- dependant enzymes, possibly at the dinucleotide fold (Thompson et al 1975). Other dye/enzyme interactions have been determined empirically by screening a number of dyes (Lowe and Pearson 1984). Immobilised Procion Blue MX-R

Fig 4.17 Dye-affinity chromatography of adult human lung H form cytidylyltransferase on Sepharose/Blue MX-R



Adult human lung cytidylyltransferase(28ml,36mU)was applied to a Sepharose/Blue MX-R column(0.9cm x 15cm),equilibrated in buffer A containing 10mM Mg^{++}. After washing with equilibration buffer,elution was accomplished by omission of Mg^{++}.

is one such dye which has been used recently for the purification of rat liver pyruvate kinase (Byford and Bloxham 1984). A sample of immobilised Procion Blue MX-R was a kind gift provided by Dr. Michael Byford (Biochemistry Department, Southampton University) for screening in cytidylyltransferase purification. Experience with other nucleotide dependent enzymes had shown that potential interactions might be Mg^{++} dependent (Lowe and Pearson 1984) and consequently binding and elution was investigated in the presence or absence of Mg^{++}.

Fibroblast 2nd S_{100} (4.5ml, 45.2mU) was applied to a Procion Blue MX-R column (0.9cm x 15cm) in buffer A with 6mM Mg^{++}. After 21.5ml elution Mg^{++} was omitted from the elutant. The elution profile, fig. 4.16, showed that 21.6% of applied activity was bound and subsequently eluted when Mg^{++} was omitted.

The above experiment was repeated with adult H-form enzyme from gel filtration (28ml, 36mU) and elution performed as with fibroblast enzyme. The elution profile, fig. 4.17, showed that 2.4mU (6.7%) was bound and eluted upon Mg^{++} withdrawal.

Procion Blue MX-R was not a good pseudo-affinity ligand but a small Mg^{++} dependent binding did show the potential for this type of chromatography. A large number of reactive dyes are available for immobilisation and use as dye-affinity ligands. They offer distinct advantages over conventional affinity ligands in capacity for protein binding and cheapness (Lowe and Pearson 1984). The use of immobilised triazine dyes in cytidylyltransferase is investigated further in Chapter five.

4.3 Discussion

The liver cytidylyltransferase purification schemes of both Vance's and Weinhold's groups produced overall yields of 6.6% (without added lipid) (Choy et al 1977), 2.1% (with added lipid) (Vance et al 1981) and 6.4% (with added lipid) (Weinhold et al 1986), respectively. The latter protocol provided acceptable quantities of rat liver enzyme, from 90-100g rat livers, for further detailed investigation. Lung tissue, however, contains lower total cytidylyltransferase activities and the consequently necessary scaling up of Weinhold's scheme make their approach difficult and expensive. In an effort to overcome this limitation, a protocol was

sought which would require few steps, providing a substantial enrichment, coupled with high recovery. In this chapter a number of approaches were pursued in an attempt to optimise both recovery and purification of human and rat lung enzyme.

An initial appraisal of ammonium sulphate fractionation appeared to offer a good recovery for both rat and human lung S_{100} enzyme. The yields, however, were lower than the increased recoveries observed with gel filtration. (Section 3.2.2) One possible explanation for the difference centres on the salting out of fresh S_{100} actin which occurs at ammonium sulphate concentrations up to 40%. Support for this suggestion was provided by fractionation of human lung 2nd S_{100}. The fractionation pattern for human 2nd S_{100} enzyme was quite different. In particular a greater total enzyme activity was recovered at 30% and 40% consistant with the increases observed with gel filtration. The advantage of this increased recovery was lessened by the observation of smaller percentages of enzyme precipitated. When the ammonium sulphate concentration was raised still further, in an attempt to precipitate more cytidylyltransferase, inhibition of total recovered enzyme was noted. The greatest purification of adult 2nd S_{100} enzyme, 4.7 fold, was achieved with the 30% pellet, but this represented only a 22.5% total recovery. In addition enzyme activities in the fractions produced were unstable, decaying rapidly. The results of these studies showed that ammonium sulphate precipitation of soluble human lung enzyme was not applicable in general purification schemes unless precipitatable cytoskeletal elements were present. In the presence of these components, however, total recovery was compromised.

A similar apparent dependance on actin aggregation was noted with PEG fractionation of rat and human lung enzyme. PEG precipitation involved aggregation of cytidylyltransferase, with all precipitated enzyme activity as H form judged by gel filtration. The aggregation seemed to accompany the PEG induced actin polymerisation detailed in chapter 3. L form activities were not precipitated in the PEG concentration ranges examined. This may reflect a lack of polymerisable actin in these fractions with which to form heteroassociations (Ingham 1984). The increased yield of enzyme activity from PEG treated S_{100} apeared to indicate an activation which was concomitant with the G-actin/F-actin transition. This may show that G-actin is inhibitory, possibly in a fashion analogous to the G-actin binding and inhibition of DNA'se I (Lazarides and Lindberg 1974).

These results showed that PEG precipitation of cytidylyltransferase was a suitable technique for application in purification schemes provided it was an early step when actin containing components were present. Its advantage over ammonium sulphate fractionation lies in its lack of enzyme inhibition and the rapid aggregation at 4°C. The use of this technique, however, demanded a subsequent method for the separation of aggregated cytoskeletal components from cytidylyltransferase.

Both rat and human lung cytidylyltransferase were found to be solubilised effectively by CHAPS. CHAPS treated rat enzyme required low ionic strength buffer B to maintain activity while CHAPS treated human enzyme appeared stable in buffer A. Rat H form, actin-associated enzyme was partially resolved from the bulk of protein and dissociated to a lower molecular weight form in buffer B. At the same time a greater cytidylyltransferase activity was revealed. CHAPS was also useful in solubilising rat and human lung membrane-associated enzyme, liberating increased activity with rat but not human membranes.

Extrapolation of these recoveries suggested that as much as 320% of initially recorded rat lung membrane-associated activity was potentially measureable after 1.25% CHAPS treatment. At this concentration CHAPS was able to solubilise 50% of total membrane associated cytidylyltransferase. The results demonstrated the inadequacy of fresh membrane-associated enzyme measurements in rat lung preparations, which clearly do not reflect V_{max} activities. It is difficult to reconcile this observation with a translocation theory which postulates an activated membrane-associated enzyme. At the very least, measurements of fresh membrane-associated enzyme activities in vitro cannot be used to support translocation hypotheses. A recent report of octyl glucoside treatment of rabbit skeletal muscle microsomes (Cornell and Maclennan 1985) showed muscle cytidylyltransferase was released in direct proportion to solubilised microsomal protein. Their results did not show increased enzyme recovery but were obtained in the presence of 1M KCl.

The KCl inhibition of rat lung cytidylyltransferase (Chapter 5, section 5.2) may indicate that their estimates of solubilised microsomal enzyme were below the maximum measurable. CHAPS is consequently a detergent which may be productively employed in purification protocols under controlled ionic strength conditions.

The interpretation of the above results provides an attractive explanation for the success of the cytidylyltransferase purification scheme of Weinhold and colleagues (Weinhold et al 1986). Their first step, an incubation of liver cytosolic enzyme at room temperature with PC/oleate vesicles, followed by pH5.0 precipitation, may be the result of lipid vesicle surface-induced formation of actin paracrystals (Rioux and Giquand 1985). This would be analogous to the 37°C ageing of rat lung enzyme detailed in chapter 3. Their quoted yield from this step (53%) indicates that the particulate activity from the pellet does not represent the total recovered activity. This is shown by the activity present in the octyl glucoside extract of the insoluble component (72%). Octyl glucoside has also recently found application for the solubilisation of rabbit skeletal muscle membrane-associated cytidylyltransferase (Cornell and Maclennan 1985a). Weinhold's group claimed that their use of both octyl glucoside and Triton X_{100} to solubilise and elute cytidylyltransferase, from the subsequent DEAE or hydroxyapatite columns, was critical in the scheme (Weinhold et al 1986). It is tempting to speculate that their successful employment of Triton X_{100} may have stemmed from its well documented inability to solubilise F-actin. Their success in separating cytidylytransferase from any actin present is evident from the polyacrylamide gel of their purified preparation which showed only a small band comigrating with a molecular weight of 43K daltons (Weinhold et al 1986).

Ion exchange was not investigated in this study due to the observations of salt inhibition of partially purified human enzyme, section 5.2.2. In chapter 3, in the course of human lung actin purification, however, a portion of H form cytidylyltransferase activity, from gel filtration, was observed to co-purify from DE23 ion exchange resin at pH8.0 (section 3.2.3.2). It is possible that a combination of ion exchange with low salt and CHAPS combinations might prove a suitable purification step, but this possibility was not tested.

Hydrophobic interaction chromatography on Phenyl Sepharose CL-4B was investigated and rejected as a purification step for both rat and human enzyme. Rat lung enzyme was not strongly retained by the matrix. Phenyl Sepharose CL-4B was effective in the binding of human fibroblast 2nd S_{100} enzyme, although the high salt concentrations necessary to promote binding also inhibited the enzyme.

Effective incorporation of individual steps into a purification scheme was unsuccessful due to the instability of the enzyme at all stages beyond gel filtration. Instead a more selective approach sought to find a specific affinity step which might rapidly purify H form enzyme.

Affinity chromatography on an immobilised choline phosphate analogue has previously been used as a purification step for rat liver enzyme (Choy and Vance 1976). Two immobilised choline phosphate analogues and an immobilised CTP were investigated and found to be unsuitable as affinity ligands. Both human and rat enzymes produced low yields of enzyme from affinity matrices. Pseudoaffinity chromatography, on the immobilised triazine dye Procion Blue MX-R, by contrast showed great potential for this type of purification. A binding of 21.6% of fibroblast 2nd S_{100} enzyme showed great promise, especially with the many dyes which are available for investigation and possible application (Lowe and Pearson 1984). Greater investigation of dye-affinity chromatography was undertaken in Chapter 5.

CHAPTER 5

DYE - AFFINITY CHROMATOGRAPHY OF HUMAN LUNG CYTIDYLYLTRANSFERASE

5.1 Introduction

In the 1950's Imperial Chemical Industries (ICI) developed a series of reactive, triazine textile dyes. These comprised various polysulphonated chromophores linked to reactive dichlorotriazinyl functional groups by an aminoether bridge (Procion MX dyes) or to the less reactive monochlorotriazinyl group (Procion H, HG or P dyes). Chlorotriazine dyes encompass a comprehensive range of shades derived from three principle chromophores; azo, anthroquinone and phthalocyanine. Single or mixed chromophores span the colour spectrum from red to violet. The reactive part of the dye is the chlorotriazinyl ring, which forms a stable, covalent link with textiles under mildly basic conditions at room temperature.

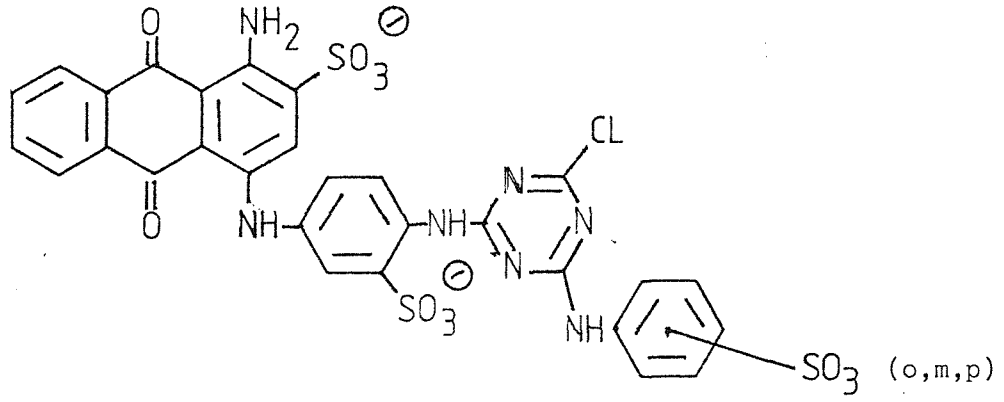
Other manufacturers produce similar dyes with modified chromophores, different co-ordinated metal ions or reactive groups. The phthalocyanine chromophore, for example, is also exploited by BAYER in their textile dye Levafix Brilliant Green E-5BNA, whose reactive group is based on 2,3-dichloroquinoxalone. The chemical structure of the majority of commercial dyes is not disclosed, while some dyes have only partial structures within the public domain. Some structures or partial structures of the dyes investigated in this chapter are shown in fig 5.1.

Cibacron F3G-A is a monochlorotriazine dye manufactured by CIBA and similar in structure to Procion Blue HB, fig 5.1. It has been used to colour the high molecular weight dextran, 'Blue Dextran', often used as a void volume marker in gel filtration. In the early 1970's several investigators noted that some proteins chromatographed anomalously when blue dextron was included in gel filtration experiments. Enzymes, including PFK (Kopperschlager et al, 1971) and LDH (Ryan and Vestling 1974) coeluted with blue dextran in the void volume of gel filtration columns. This behaviour was ascribed to a reversible binding of the dextran-immobilised aromatic dye molecule to the enzyme. Many different proteins have subsequently been reported to interact with a range of reactive dyes (Lowe and Pearson 1984). When immobilised to a supporting matrix, for example agarose, the dyes have found application as pseudoaffinity ligands.

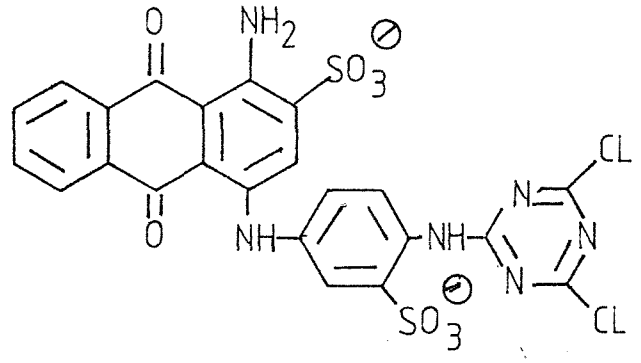
The Mechanism of dye-protein interaction has only been clearly defined for relatively few dye-protein pairs. Cibacron F3G-A, for example, appears to act as a nucleotide analogue with certain

Fig 5.1 Known structures of some dyes investigated.

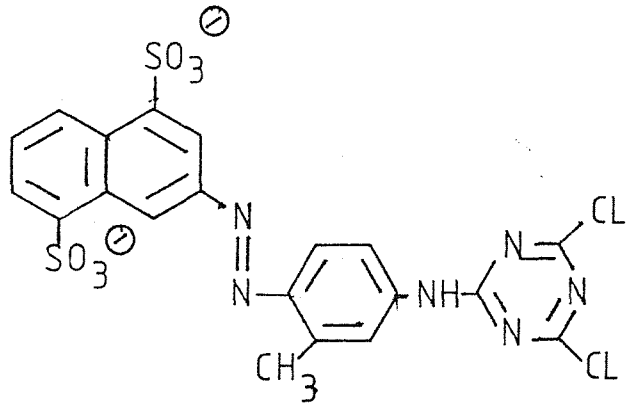
(A)



(B)



(C)



A - Cibacron Blue F3GA,ortho isomer — ref: Lowe & Pearson 1984
Procion Blue HB,meta & para isomers

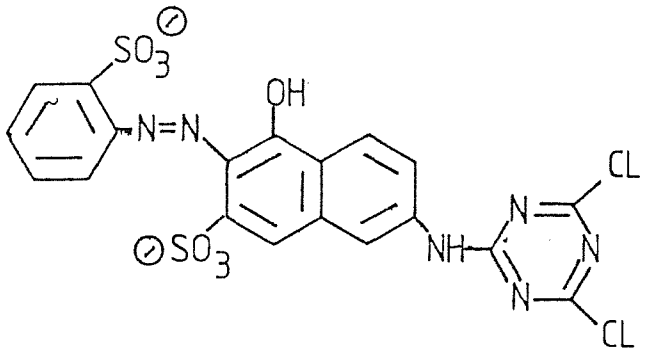
B - Procion Blue MX-R — ref: Lowe & Pearson 1984

C - Procion Yellow MX-R — ref: Qadri & Dean 1980

D - Procion Orange MX-G — ref: Lowe & Pearson 1984

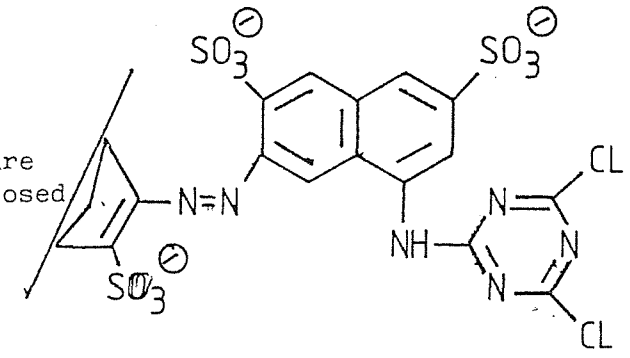
E - Procion Red MX-8B,partial structure — ref: Hughes et al 1984

(D)

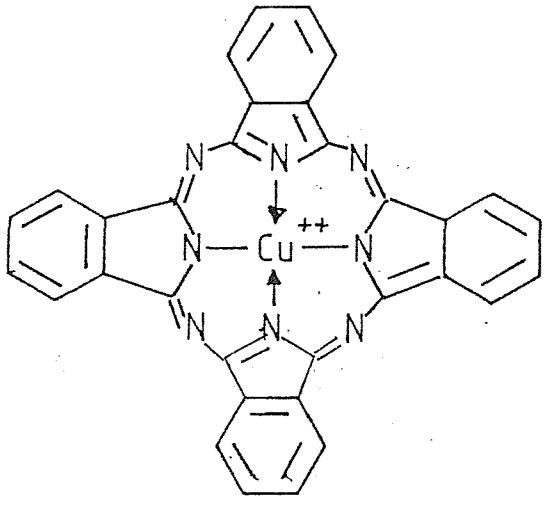


(E)

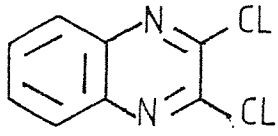
Ring structure undisclosed



Copper phthalocyanine chromophore.



2,3,dichloroquinoxalone



Structures undisclosed:

Procion Red HE-7B

*Procion Green H-4G

*Procion Turquoise MX-G

*Procion Turquoise HA

°Levafix Brilliant Green E-5BNA

* - copper phthalocyanine containing

° - nickel phthalocyanine containing

dehydrogenases. X-ray crystallographic studies of the binding of F3G-A to purified horse liver alcohol dehydrogenase have shown a direct interaction with the dinucleotide fold, the site of NADH binding. The majority of proteins which interact with reactive dyes lack this structural element in their conformation. For these proteins the dye need not necessarily mimic nucleotides alone and some hydrophobic interaction has been implicated (Qadri and Dean 1980). The possession of both aromatic and polar sulphonate groups affers considerable potential for multiple hydrophobic and electrostatic binding. Dye-enzyme interactions may involve binding to regions which are actively involved in the catalytic process, but may equally include regions away from the active site but nevertheless capable of modifying enzyme activity. Some free dyes behave as active site directed inhibitors where they bind at or close to the active site. Inhibition is the result of placing the highly reactive chlorotriazinyl ring in close proximity to a susceptible nucleophile on the enzyme, for example free thiol groups. A time, and dye concentration, dependant inhibition is often seen, which may be inhibited by the addition of a cofactor or substrate or both (Clonis and Lowe 1980).

Immobilized triazine dyes have a number of properties, including the high substitution possible, relative cheapness, high protein binding capacity and reuseability, which make them an attractive choice in protein purification schemes where an appropriate dye-protein interaction is shown (Lowe and Pearson 1984). Approximately 80 triazine dyes are commercially available. Cytidylyltransferase is a nucleotide dependant enzyme which has some hydrophobic properties (Weinhold et al 1986) and any dye interactions might involve the CTP binding site or some hydrophobic interaction. Within this chapter representative dye of both mono- and dichlorotriazine series were screened for suitability as pseudoaffinity ligands.

5.2 Results

5.2.1 Cytidylyltransferase interactions with free triazine dyes

The binding and subsequent elution of a small proportion of soluble human lung cytidylyltransferase to immobilised Procion Blue MX-R, (Section 4.2.6), demonstrated the potential of dye-affinity chromatography in its purification. Reactive triazine dyes of many different configurations are available and it was reasoned that a greater binding affinity might be demonstrated with other dyes. The inhibitory action of free dyes was evaluated as an index of specific binding. Any dye showing active-site directed inhibition might prove invaluable in mechanistic studies with the purified enzyme. Dyes providing substantial inhibition were immobilised to Sepharose CL 4B and investigated as potential affinity matrices.

5.2.1.1 Dye inhibition of soluble rat lung and human fibroblast enzyme

An essential prerequisite for the investigation of dye-enzyme interactions was the removal of serum proteins, especially albumin. Albumin has a high affinity for some triazine dyes, with interactions occuring at bilirubin or fatty acid binding sites (Leatherbarrow and Dean 1980). Rat lung H form enzyme, from gel filtration, and human lung fibroblast 2nd S_{100}, which were essentially free of albumin, were chosen for dye investigations. Ten dyes, fig 5.1, were investigated for their ability to inhibit rat or human lung enzyme.

The patterns of free dye inhibition are shown in Table 5.1 for rat and human enzyme. It is evident that rat and human enzyme are inhibited by dyes from both the monochlorotriazine and dichlorotriazine series. Similarities and differences were apparent with each enzyme. Procion Orange MX-G and procion Red HE7B failed to inhibit either enzyme. Procion Blue HB, by contrast, did not inhibit rat enzyme while producing 40% inhibition of fibroblast enzyme at 100μM. The greatest inhibition, 67% for rat and 85% for human enzyme, was achieved when incubated with Procion Green H-4G. This was the only dye tested which gave greater than 50% inhibition and it therefore showed the greatest potential as an affinity ligand.

Table 5.1

Dye-mediated inhibition of soluble rat and human cytidylyltransferase.

	Dichlorotriazine dyes.	% Inhibition of rat H form enzyme.	% Inhibition of human fibroblast 2nd S_{100} enzyme
	Procion Blue MX-R	17	21.5
	Procion Yellow MX-R	39	-
	Procion Red MX-8B	17.5	-
	Procion Orange MX-G	0	0
*	Procion Turquoise MX-G	30	48
	Monochlorotriazine dyes.		
	Procion Red HE-7B	0	0
	Procion Blue HB	0	40
*	Procion Turquoise HA	22	40
*	Procion Green H-4G	67	85
*	Levafix Brilliant Green E-5BNA	21	40

* - phthalocyanine containing dyes.

Soluble enzyme(500ul)was incubated with free dye(100uM) for 30 minutes at 37°C then assayed for cytidylyltransferase activity as a fraction of dye-free control.

Procion Green H-4G is a copper phthalocyanine containing dye, the precise structure of which ICI will not disclose. It has previously been shown to have a unique, Mg++ dependant, interaction with yeast hexokinase (Clonis et al 1981). This property was exploited in a pseudoaffinity purification of yeast hexokinase. Two other copper phthalocyanines, Procion Turquoise MX-G and Procion Turquoise HA, and the nickel phthalocyanine containing Levafix Brilliant Green E5BNA were among the most inhibitory of the dyes tested with rat and human enzyme. A possible interpretation of this observation is that the possession of the phthalocyanine chromophore, fig 5.1, might facilitate inhibition. The phthalocyanine group is a bulky aromatic structure and potentially involved in either active site interaction or hydrophobic interaction.

5.2.1.2 Time and Concentration dependance of Procion Green H-4G inhibition

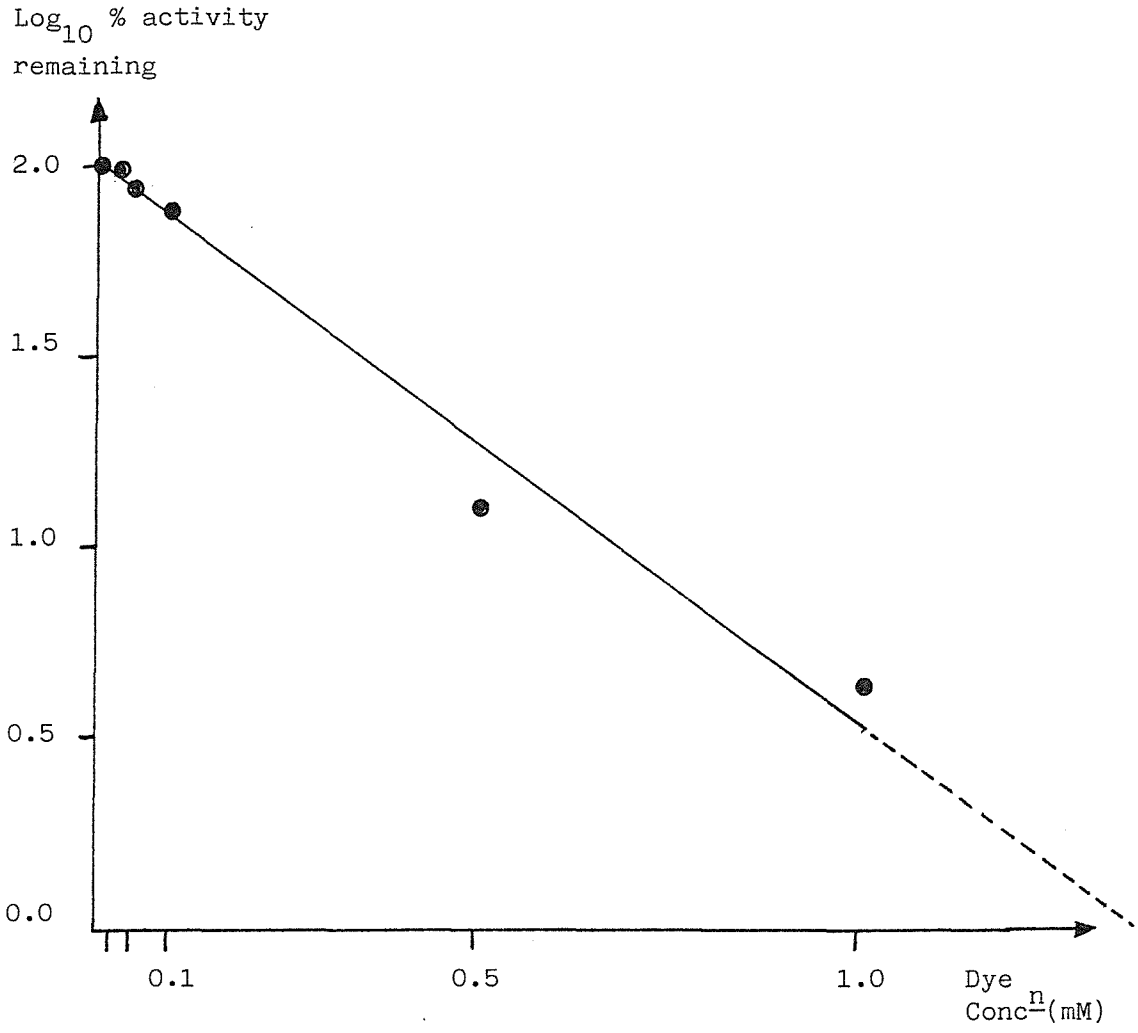
The difference in the extent of Green H-4G inhibition at 100μM between rat and human enzyme, 67% vs 85%, was investigated by increasing dye concentration for rat enzyme. The time course for inhibition of fibroblast enzyme was also evaluated to assess whether progressive inhibition followed the pattern of active site directed kinetics described by Lowe and colleagues (Clonis and Lowe 1980, Clonis et al 1981).

Aliquots of Rat H form enzyme, (550μl) were incubated with Procion Green H-4G between 0 and 1mM. The pattern of inhibition, fig 5.2, was concentration dependant non-linear and a first order relationship was evident. Extrapolation of experimentally determined values indicated that 500μM Procion Green H-4G was necessary to achieve the same degree of inhibition of rat enzyme seen with 100μM dye on human enzyme. In addition dye concentrations above 1.6mM might be necessary to effect complete inhibition.

Accurate kinetic analysis of dye inhibition proved to be too complicated as a result of continued exposure to the dye during the 20 minute assay period, albeit at a lower concentration (56μM). Within these limitations the inhibition, fig 5.3, was rapid and fibroblast enzyme inhibition at 100μM Green H-4G reached its greatest value within five minutes of dye exposure. This inhibition was, however, untypical of the first order, active site directed, processes seen with other enzymes

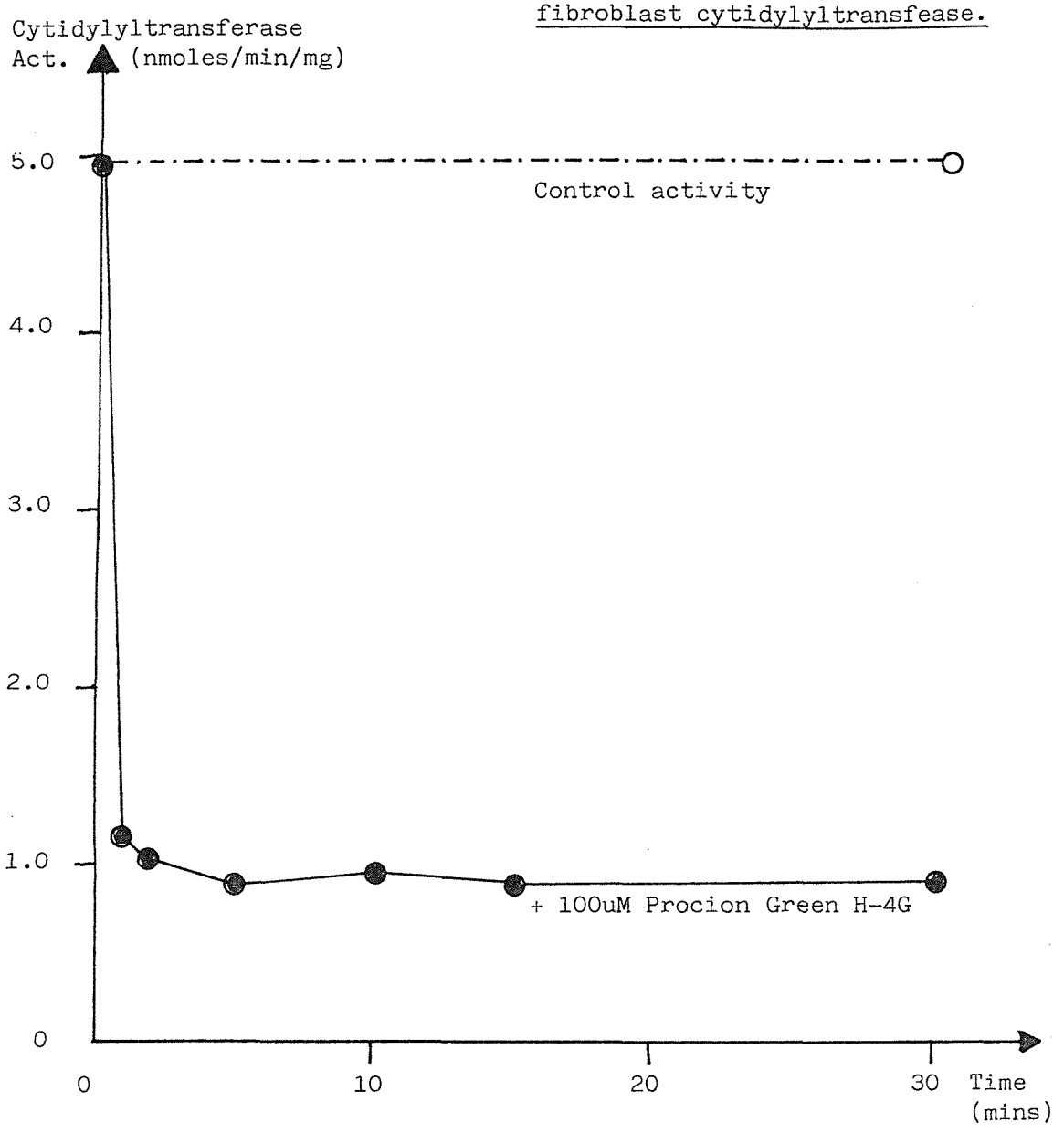
Fig 5.2

Concentration-dependance for Procion Green H-4G inhibition of rat H form cytidylyltransferase.



Rat H form enzyme(500ul) was incubated with Procion Green H-4G (0 - 1mM) for 30 minutes at 37°C,then assayed for remaining activity.

Fig 5.3. Timecourse of Green H-4g inhibition of human lung fibroblast cytidylyltransfease.



Fibroblast 2nd S_{100} enzyme (2mls) was incubated with 100uM Procion Green H-4G at 37°C and duplicate analyses of cytidylyltransferase activity performed after 1,2,5,10,15,and 30 minute intervals.

which are generally slower (Clonis and Lowe 1980, Clonis et al 1981). It appeared, from these results, that the Procion Green H-4G interaction with cytidylyltransferase was not active site directed.

5.2.1.3 Procion Green H-4G inhibition of fibroblast membrane-associated cytidylyltransferase

The interaction of Procion Green H-4G with human, membrane-associated enzyme was of interest, in particular whether or not it behaved in the same way as the soluble enzyme.

Duplicate washed fibroblast membrane-rich fractions, from centrifugation between 10,000 x g x 10 minutes and 100,000 x g x 1 hour (500µl, 2.5mU) were incubated with Procion Green H-4G (100µM) for 30 minutes at 37°C. Inhibition from 1.14mU/mg to 0.2mU/mg was observed. The figure of 82.5% inhibition is comparable to the 85% inhibition seen with fibroblast 2nd S_{100} enzyme. This result appeared to indicate that, irrespective of whether or not dye inhibition is active site directed, the dye had free access to, and was able to inhibit membrane-associated enzyme. The dye binding/interaction site was exposed and available for binding.

5.2.1.4 Protection against Procion Green H-4G inhibition of fibroblast enzyme

The unique, Mg^{++} dependant, inactivation of yeast hexokinase by Procion Green H-4G (Clonis et al 1981) was found to be competitively inhibited by the adenine nucleotides ATP and ADP and the sugar substrates D-glucose, D-mannose and D-fructose but not by non-substrate sugars. This protective effect was subsequently exploited in the use of ATP as a biospecific eluant for hexokinase bound to immobilised Procion Green H-4G (Clonis et al 1981). An assessment of the potential of the dye as a nucleotide or substrate analogue was made by investigation of the protective ability of cytidylyltransferase substrates.

An initial experiment was performed by incubating fibroblast 2nd S_{100} with 100µM Green H-4G in the presence or absence of 20mM MgCTP. The inhibition by the dye was reduced in the presence of MgCTP but at 20mM MgCTP also produce a large substrate inhibition in controls, making

Table 5.2

Protection against Procion Green H-4G inactivation of fibroblast cytidylyltransferase.

Composition of incubation mixture.	Cytidylyltransferase activity(nmoles/min/ml)	Percentage control activity
Control	3.30	100
Enzyme + 100uM Green H-4G	0.65	19.7
Enzyme + 20mM MgCTP	1.50	45.5
Enzyme + 20mM MgCTP + 100uM Green H-4G	1.55	47.0
Enzyme + 20mM Choline phosphate	1.80	54.5
Enzyme + 20mM Choline phosphate + 100uM Green H-4G	0.45	13.6
Enzyme + 20mM CDPcholine	3.00	91.0
Enzyme + 20mM CDPcholine + 100uM Green H-4G	1.35	40.9

Duplicate incubations of fibroblast 2nd S_{100} enzyme(200ul) were performed at 37°C for 30 minutes with the appropriate substrate $\pm$ 100uM Green H-4G in a volume of 300ul. Substrates were then removed by passage through 1cm each of the ion exchange resin Dowex 2X8-200(Cl form) and Sephadex G-25. Dye removal,by binding to Sephadex G-25,was monitored at 675nm(the absorption maximum,molar absorption coefficient 57,400/M/cm.). Duplicate cytidylyltransferase activities were made on column eluates and corrected to initial volume.

interpretation difficult. The problem was overcome by removal of both free dye and substrates from incubated samples before assay. This also enabled CDPcholine and choline phosphate to be assessed without problems of radiolabel dilution during assay.

MgCTP was most effective in prevention of dye inhibition, table 5.2. but the 30 minute exposure to nucleotide at 20mM reduced active anzyme recovery. CDP choline appeared to offer some protection, while choline phosphate failed to prevent inhibition. Two possibilities were suggested by these observations:

1) Procion Green H-4G may act as a CTP analogue which binds to the site of the enzyme. The covalent linkage of the reactive chlorotriazinyl ring to a suitable enzyme nucleophile, perhaps an essential thiol of the type demonstrated for rat liver enzyme (Weinhold et al 1986), might then result in inhibition. This option provides a logical explanation for the observed MgCTP protection and the partial protection offered by CDPcholine. It is an attractive proposition when viewed alongside a similar proposed mechanism for Green H-4G interaction with the ATP binding site of yeast hexokinase (Clonis et al 1981). The rapid rate of inhibition described in section 5.2.1.2 does not support the view of an active site directed inhibition, however, arguing against this interpretation.

2) An alternative view suggested that Green H-4G may interact with a site close to the CTP binding site, or removed from it but able to exert influence on it. In this model MgCTP would either prevent dye access to the proximity of the active site or act to stabilise it against dye-induced conformational change. The dye-enzyme interaction might be hydrophobic in nature, involving the large, aromatic, phthalocyanine chromophore thereby explaining the efficacy of other phthalocyanine dyes.

In both postulated mechanisms the site for choline phosphate binding is envisaged as removed from the dye binding site rendering it ineffective as a protecting ligand.

Table 5.3.

The binding of fibroblast cytidylyltransferase to Sepharose immobilised phthalocyanine containing triazine dyes.

Contents of incubation mixture	% Cytidylyltransferase in supernatant
Enzyme + Sepharose CL4B	100
Enzyme + Sepharose CL4B/Procion Turquoise HA conjugate	100
Enzyme + Sepharose CL4B/Procion Turquoise MX-G conjugate	83
Enzyme + Sepharose CL4B/Levafix Brilliant Green E-5BNA conjugate	71
Enzyme + Sepharose CL4B/Procion Green H-4G conjugate	15
Enzyme + Sepharose CL4B + 10mM MgCTP	97
Enzyme + Sepharose CL4B/Procion Green H-4G + 10mM MgCTP	24

Dye/Sepharose conjugates(100mg moist weight) were incubated,at 37°C for 30 minutes,with fibroblast 2nd S_{100}(900ul,3.6mU/ml)and the percentage unbound cytidylyltransferase determined in the 12,000 x g x 5 minute supernatant. The control incubations contained Sepharose Cl4B alone.

5.2.2 Characterisation of cytidylyltransferase interactions with immobilised phthalocyanine dyes

The potential of Procion Green H-4G as an affinity ligand was further investigated when coupled to Sepharase CL4B. The other phthalocyanines had also shown some inhibitory action and a consequent potential as affinity ligands and they too were conjugated with Sepharose CL4B and their binding abilities assessed. Where binding to dye-Sepharose conjugates has been shown to occur, then conditions for desorption or prevention of binding must be found if it is to prove a suitable dye-affinity matrix. Typical approaches have included use of a variety of potential eluants (Lowe and Pearson 1984) including biospecific conditions, the inclusion of substrates, cofactors etc., and non-specific cluants, including altering ionic strength, pH, EDTA concentration, detergent or temperature changes. No *a priori* assumptions can be made and alteration of one or more parameters will not necessarily lead to a predictable change (Lowe and Pearson 1984). Consequently an empirical approach was undertaken.

5.2.2.1 Cytidylyltransferase interactions with phthalocyanine dye-Sepharose conjugates

Phthalocyanine dye sepharose conjugates were synthesised as described (Lowe and Pearson 1984) and examined for their abilities to bind fibroblast enzyme.

Of the four phthalocyanine conjugates investigated Procion Green H-4G/Sepharose CL4B bound most enzyme (85%),table 5.3. The other Sepharose CL4B conjugated dyes, Procion Turquoise HA, Procion Turquoise MX-G and Levafix Brilliant Green E-5BNA bound 0%, 17% and 29% respectively. Under these conditions, then, Procion Green H-4G/Sepharose CL4B demonstrated the greatest potential as a pseudo-affinity matrix and further investigation was confined to its properties.

5.2.2.2 Binding to Green H-4G/sepharose in the presence of MgCTP

The prevention of free dye inhibition of cytidylyltransferase in the presence of 20mM MgCTP suggested that it might prove a suitable eluant for the dye/Sepharose bound enzyme. In the light of the overall

Table 5.4

Ionic strength effects on the binding of fibroblast cytidylyltransferase to Sepharose CL4B-immobilised Procion Green H-4G

Composition of the incubation mixture	Cytidylyltransferase (mU/ml)in supernatant	% Control activity
Enzyme + Sepharose CL4B (control)	6.45	100
Enzyme + Sepharose CL4B/Green H-4G	1.50	23.3
Enzyme + Sepharose CL4B + 0.5M KCl	5.40	83.7
Enzyme + Sepharose CL4B/Green H-4G + 0.5M KCl	2.3	35.7
Enzyme + Sepharose CL4B + 1M KCl	3.40	52.7
Enzyme + Sepharose CL4B/Green H-4G + 1M KCl	2.70	41.9

Sepharose CL4B/Green H-4G and Sepharose CL4B controls (100mg moist weight) were incubated with fibroblast 2nd S_{100}(900ul) with and without 0.5M KCl and 1M KCl. Cytidylyltransferase was then determined in the 12,000 x g x 5 minute supernatant.

reduction in recovered enzyme from 20mM MgCTP treatment the protective effect was monitored with 10mM MgCTP.

The enzyme activity recovered in the supernatant of MgCTP treated dye/Sepharose incubations, table 5.3, was only 10% greater than the incubation without MgCTP (25% vs 15%). The percentage of unbound enzyme may be even higher since slight inhibition of enzyme in 10mM MgCTP/Sepharose CL4B incubation was noted. The difference in binding on a crude batch analysis warranted further investigation with column chromatography (Section 5.2.3.2).

5.2.2.3 Ionic strength effects on binding Procion Green H-4G/Sepharose CL4B

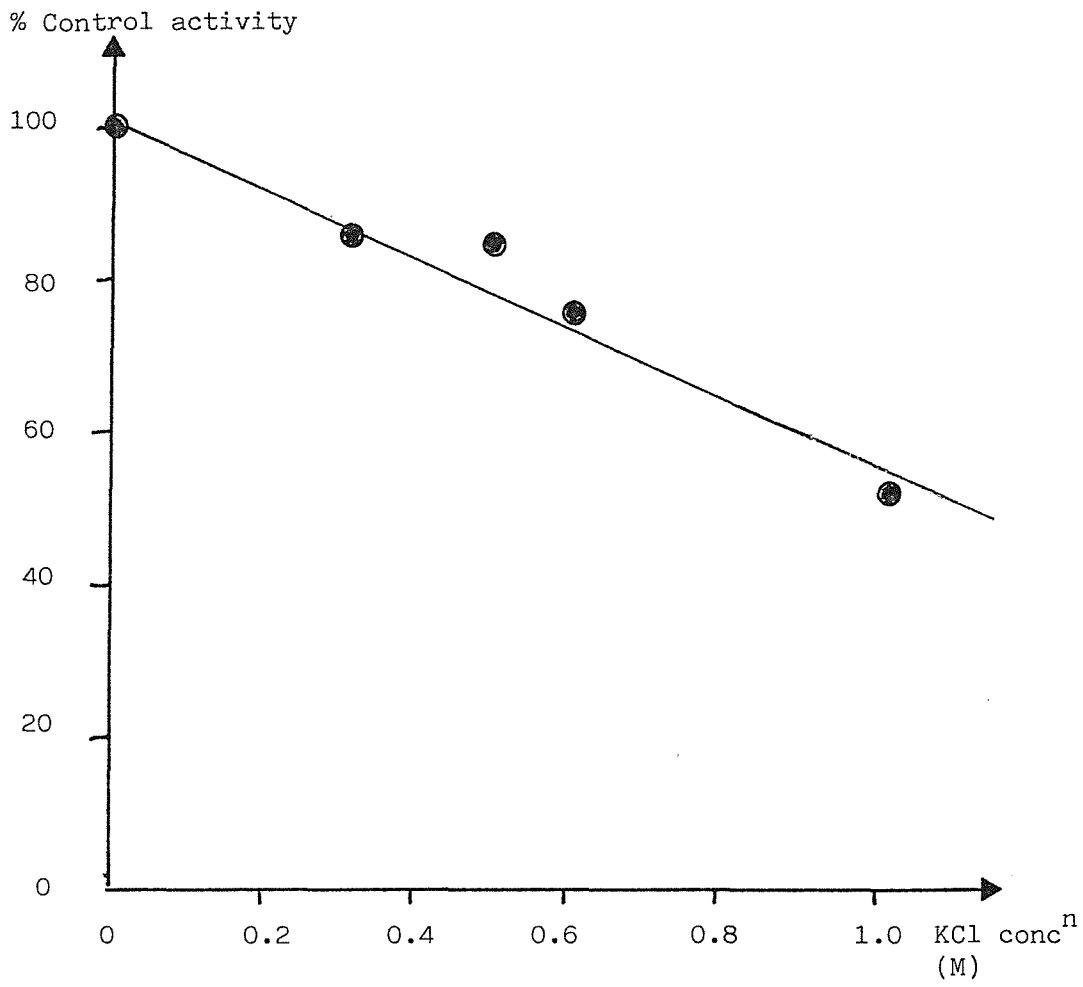
The binding of cytidylyltransferase to the dye/Sepharose conjugate was monitored at high ionic strengths, while the effect of low ionic strength was assessed by attempts to elute bound enzyme.

Increasing KCl, table 5.4, prevented enzyme binding, suggesting some ionic effect, but with a concomitant reduction in total measured activity. Since KCl seemed to effectively prevent binding the extent of KCl inhibition was measured with fibroblast enzyme. The enzyme activity, fig 5.4 was inhibited by KCl in a concentration dependant fashion. Attempts were made to remove KCl by passage of 200µl of 1M incubation through a small (200mg) quantity of Sephadex G-25 equilibrated in buffer A. No increase in activity was recovered indicating that KCl irreveribly inhibited the enzyme.

Elution with low ionic strength buffer B with ethylene glycol was attempted. In addition to stabilising enzymes in low ionic strength buffers ethylene glycol helps reduce hydrophobic interactions with dye affinity matrices (Lowe and Pearson 1984). Dye/Sepharose conjugate (100mg) was incubated with fibroblast 2nd S_{100} (1ml) for 30 minutes at room temperature. After centrifugation at 12,000 x g x 5 minutes the pelleted conjugate was washed with 1ml buffer A and centrifuged again. The supernatants were assayed and the conjugate resuspended in buffer B with ethylene glycol (1ml) and the 12,000 x g x 5 minute supernatant assayed for eluted enzyme. The first supernatant in buffer A contained 18% activity (i.e. 82% bound) the buffer A wash contained 5% while the low ionic strength wash contained no activity. It appeared that lowering

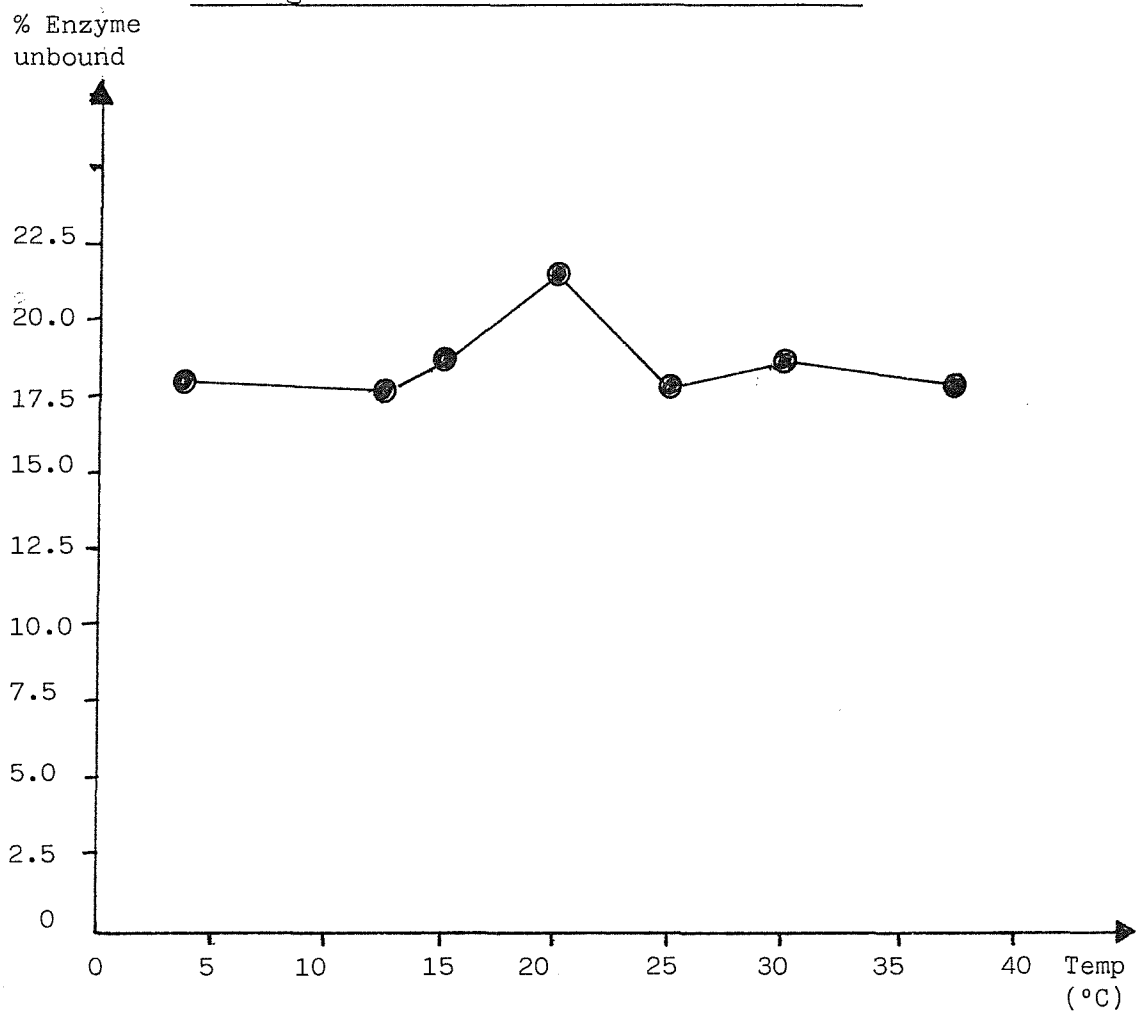
Fig 5.4

KCl inhibition of human lung fibroblast cytidylyltransferase



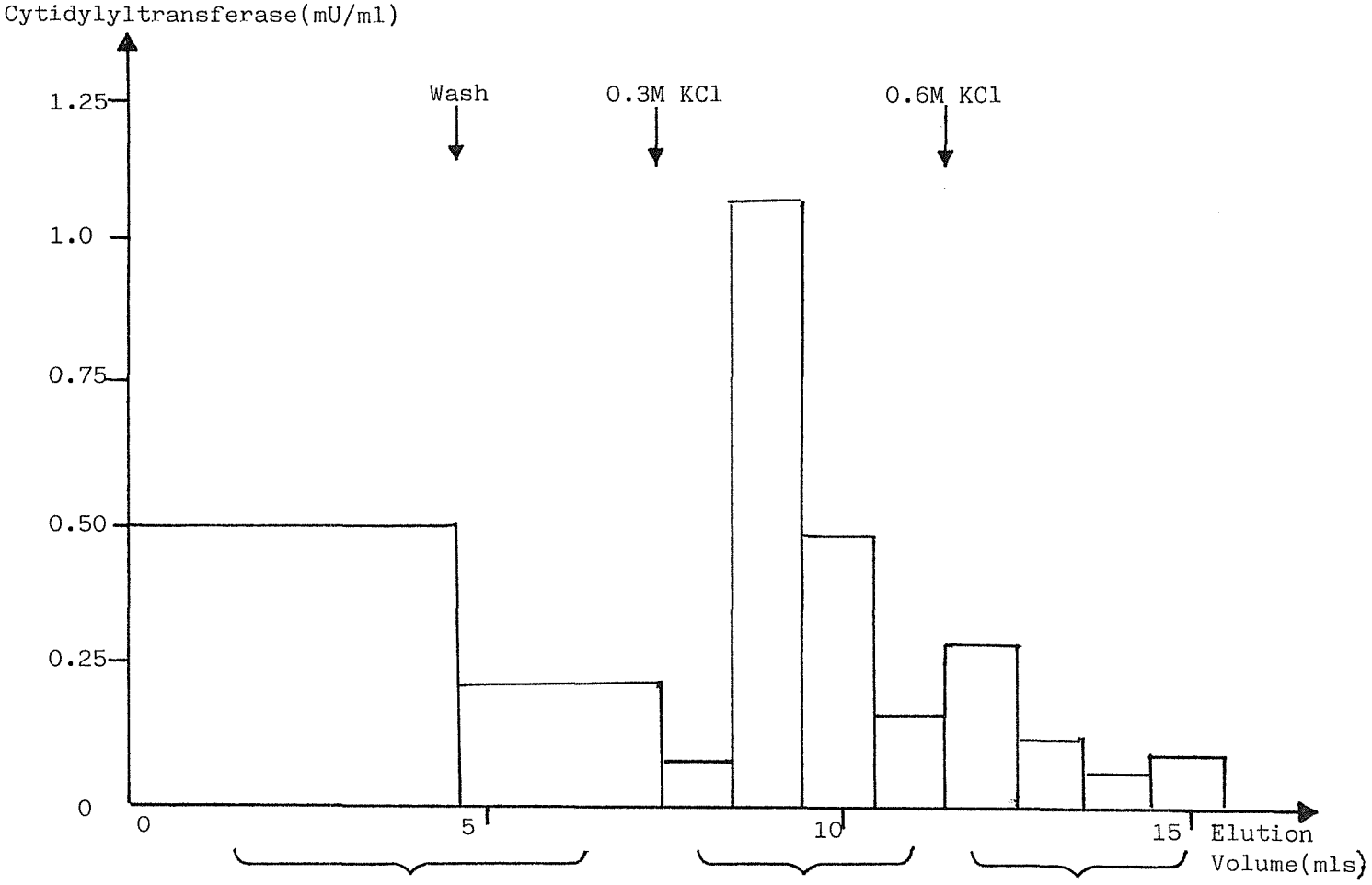
Fibroblast 2nd S_{100} cytidylyltransferase(500ul) was mixed with 0.3M,0.5M,0.6M and 1M KCl and activities determined after 30 minutes incubation at 37°C. Remaining activity was expressed as a percentage of KCl free control.

Fig 5.5 Temperature dependance of fibroblast cytidylyltransferase binding to immobilised Procion Green H-4G.



The temperature dependance of fibroblast 2nd S_{100} enzyme binding to Sepharose CL4B immobilised Green H-4G was investigated at several temperatures between 4°C and 37°C. Aliquots of dye-Sepharose (50mg moist weight) were incubated with fibroblast enzyme(500ul),after 30 minutes pre-equilibration at 4°C,12°C,15°C,20°C,25°C,30°C and 37°C, for 30 minutes. The 12,000 x g x 5 minute supernatants were assayed for cytidylyltransferase,and unbound enzyme activity expressed as a proportion of Sepharose CL4B control incubation.

Fig 5.6 Elution of Sepharose CL4B/Green H-4G bound cytidylyltransferase with KCl



Fibroblast 2nd S_{100} (4mls,4.95mU/mg,19.8mU)applied and eluted with 3ml buffer A,4 x 1ml 0.3M KCl and 4 x 1ml 0.6M KCl

ionic strength did not result in elution of enzyme.

5.2.2.4 Temperature dependance of Green H-4G/Sepharose CL4B binding

The temperature dependance of dye/sepharose binding to enzyme was investigated at several temperatures between 4°C and 37°C. The unbound enzyme showed little variation between 4°C and 37°C, fig 5.5, with a mean, unbound activity of 18.9%. The results did not seem to suggest that temperature change between 4°C and 37°C might be an effective elution step. The greatest proportion of unbound enzyme was observed at 20°C, but a maximum variation in binding of 3.75% in 82%, between 20°C and 25°C, is unlikely to provide a significant yield of pure enzyme. Batch binding of the type described above may not reflect absolute binding characteristics of enzymes allowed to percolate through the affinity matrix. Further analysis was consequently confined to testing elution conditions, singly and in combination, on column chromatography.

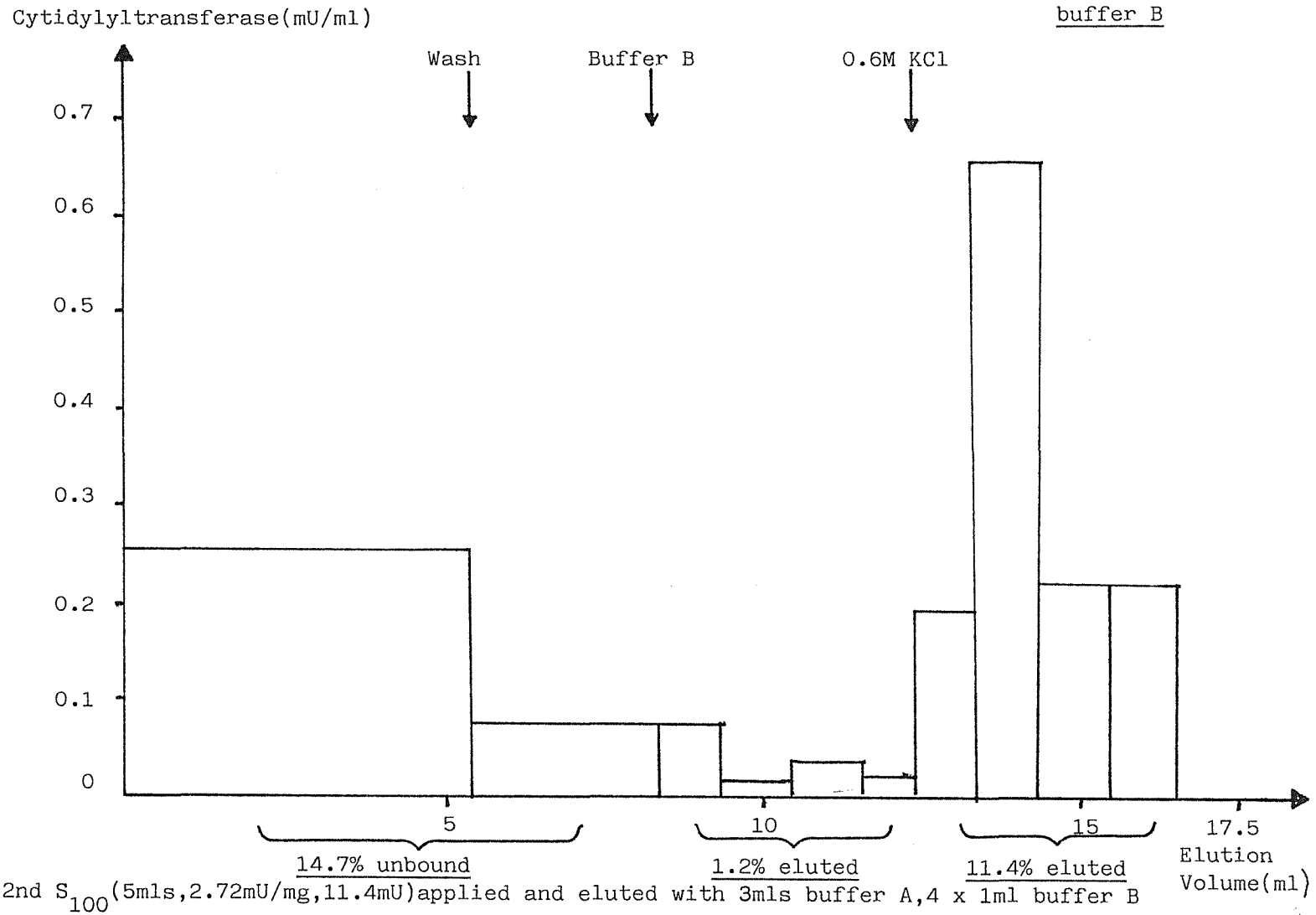
5.2.3 Chromatographic properties of soluble cytidylyltransferase on Sepharose CL4B immobilised Procion Green H-4G

Small columns (2cm x 0.5cm, Econo columns - BIORAD) were made from the Procion Green H-4G/Sepharose CL4B matrix and employed in all subsequent experiments. The columns were equilibrated by irrigation with 30-50ml buffer A. This ensured that all free dye, which might otherwise inhibit enzyme was completely removed. After equilibration no absorbance at 675nm was evident in column washings. Unless otherwise indicated binding and elution attempts were performed at ambient temperature between 20°C and 25°C, typically 22°C. The effects of ionic strength, MgCTP, temperature dependance and CHAPS were all evaluated as potential elution strategies.

5.2.3.1 Ionic strength effects

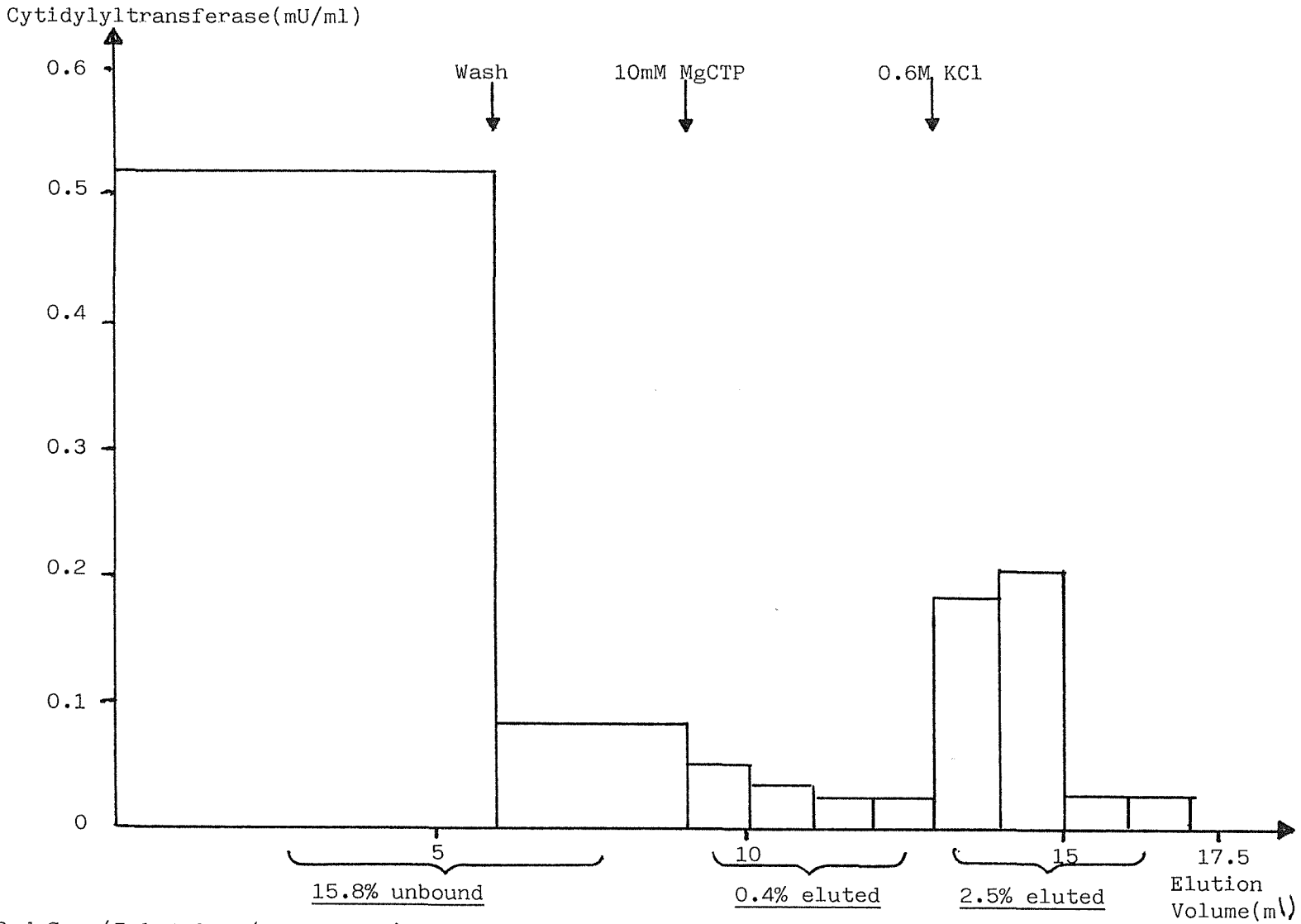
Fibroblast 2nd S_{100} (4ml, 4.95mU/mg, 19.8mU) was applied to a green H-4G column, mixed and flow stopped for 10 minutes to allow maximum exposure to dye/Sepharose. The elution profile is shown in fig 5.6. The first 4.5ml eluted were pooled as were the first 3ml washings and enzyme activities determined to ascertain percentage bound. The column

Fig 5.7 Elution of Sepharose CL4B/Green H-4G bound cytidylyltransferase with low ionic strength buffer B



Fibroblast 2nd S_{100}(5mls,2.72mU/mg,11.4mU)applied and eluted with 3mls buffer A,4 x 1ml buffer B and 4 x 1ml 0.6M KCl.

Fig 5.8 Elution of Sepharose CL4B/Green H-4G bound cytidylyltransferase with MgCTP



Fibroblast 2nd S_{100}(5ml,4.05mU/mg,21.45mU) was applied and eluted with 3ml buffer A,4 x 1ml 10mM MgCTP, then 4 x 1ml 0.6M KCl.

was then eluted with 4 x 1ml 0.3M KCl and 4 x 1ml 0.6M KCl and enzyme determined in each fraction. The specific activity of the void volume fraction was 0.75mU/mg and after washing with buffer A 87.5% of applied activity was not recovered. This indicated a measure of selective binding to the column matrix. The two stepwise KCl pulses yielded a further 11.5% enzyme (14% corrected for KCl inhibition) with at least 73.5% of applied activity unaccounted for. The possibility that the void volume and KCl peaks represented two separated lower activity components of the origi nal applied activity was tested by combining fractions of each. No increase in activity was observed in any recombination experiments.

It was clear from this experiment that a large portion of fibroblast 2nd S_{100} enzyme was separated from the bulk of applied protein and was strongly and selectively bound to the dye-affinity matrix. Irrigation of column with KCl to 0.6M was able to elute a portion of the bound enzyme but did not comprise the optimum elution protocol since the majority of enzyme remained unaccounted for and KCl also inhibits enzyme irreversibly.

Low ionic strength buffer B with 20% ethylene glycol was investigated as a potential eluant despite its poor performance in batch elution. Fibroblast 2nd S_{100} (4ml, 2.72 mU/mg, 11.4mU) was applied to a Green H-4G column and eluted as shown in fig 5.7. In this experiment 85.3% of applied enzyme was bound with only 1.2% eluted with buffer B. A further 11.4% (14.6% corrected for KCl inhibition) was recovered in subsequent KCl pulse, which was comparable to the KCl eluted recovery from the previous experiment. At least 69% of applied activity was retained on the column. It appeared from these results that elution with low ionic strength was ineffective, although the matrix bound enzyme might be purified by buffer B mediated desorbtion of non specifically bound protein. Once again increased ionic strength successfully desorbed a small amount of enzyme but this was partially inactivated by KCl.

5.2.3.2 MgCTP effects on elution

As described in section 5.2.2.2 the presence of 10mM MgCTP offered some protection against enzyme binding to batches of dye/Sepharose. It was possible that MgCTP might release dye/Sepharose bound enzyme and consequently this elution scheme was investigated.

Fibroblast 2nd S_{100} (5ml, 4.05mU/mg, 21.45mU) was applied to a Green H-4G column and elution performed as shown in fig 5.8. 84.2% was retained on the column with 0.4% eluted by MgCTP. 0.6M KCl released 2% activity (2.5% without KCl). Recombination experiments with void volume, MgCTP or KCl fractions showed no increase in activity.

It was evident that MgCTP was not a suitable eluting species for dye/Sepharose bound cytidylyltransferase. This provided more evidence that the dye enzyme interaction was probably not one involving the CTP binding site.

5.2.3.3 *The effect of temperature on binding and CHAPS elution*

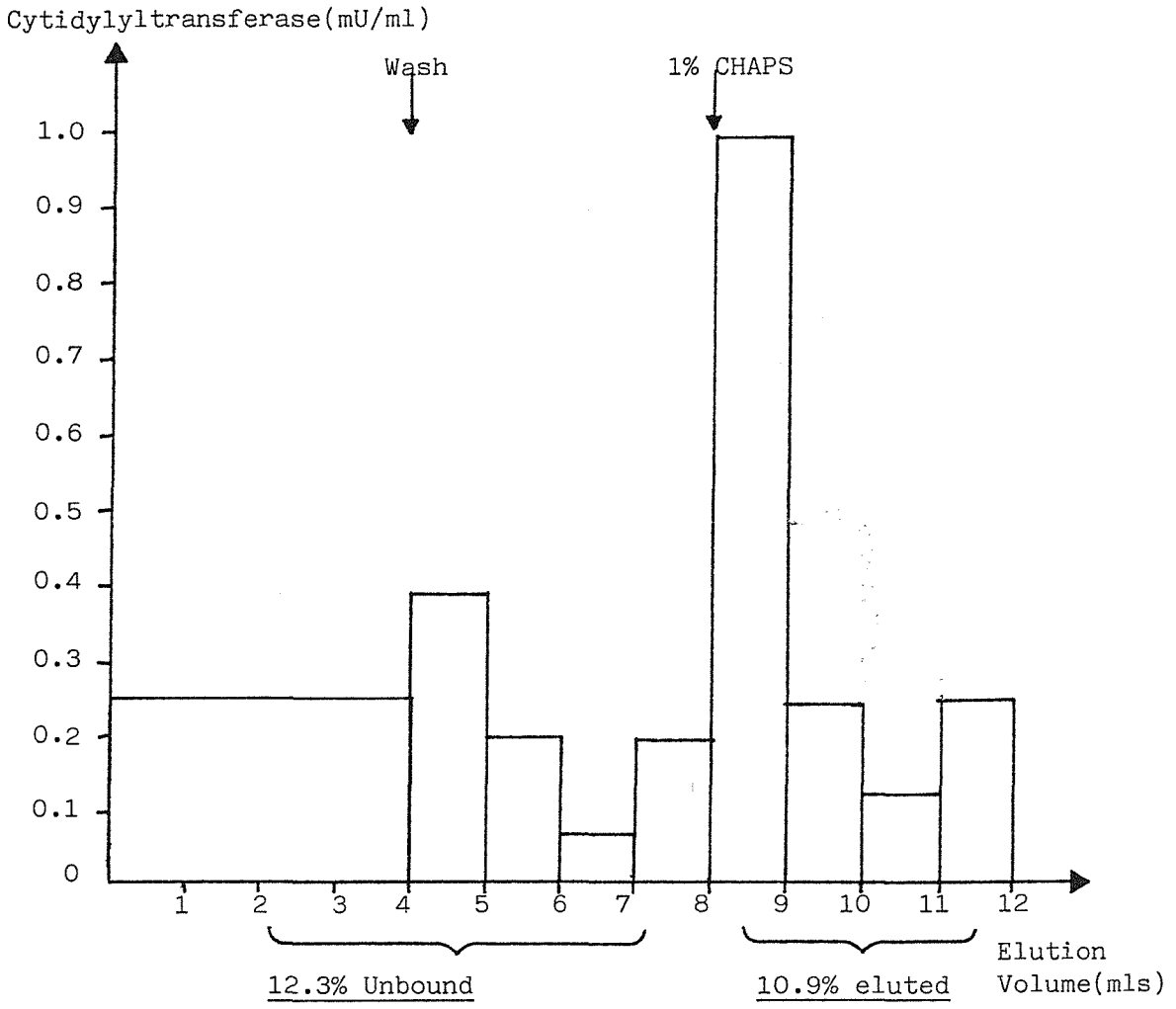
The effect of manipulating binding and elution temperatures between 4°C and 37°C was investigated. The effect on 1% CHAPS on elution at 4°C and 30°C was also studied.

Fibroblast 2nd S_{100} (4ml, 4.2mU/mg, 16.8mU) at 4°C. After washing with 4 x 1ml buffer A, elution was attempted with 1% CHAPS at 4°C. The peak of enzyme activity from CHAPS treatment was precipitated by treatment with Deoxycholate/TCA (Peterson 1983) prior to protein determination and also analysis on 10% PAGE.

A similar protocol was adopted for enzyme binding at 30°C. Fibroblast 2nd S_{100} (3ml, 2mU/mg, 5.92mU) was applied to the column at 30°C and eluted at 30°C with buffer A followed by 1% CHAPS at 30°C.

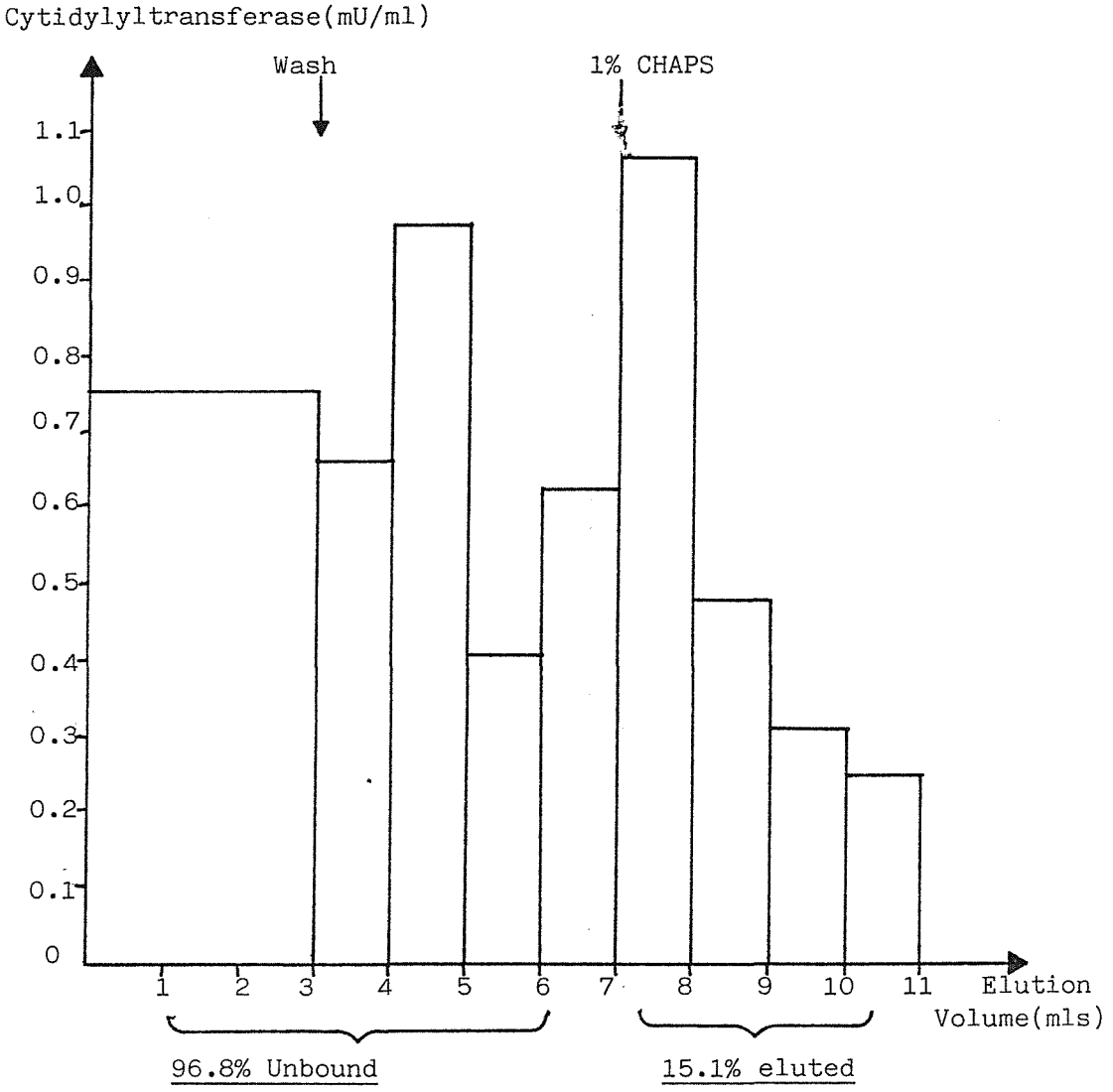
The elution profiles, fig 5.9 and 5.10, demonstrated a striking difference in behaviour between binding and elution at 4°C and that at 30°C. At 4°C 87.7% of applied enzyme was retained while only 3.2% was retained at 30°C. CHAPS eluted 10.9% of activity from the 4°C column but 15.1% at 30°C. It appeared that these results showed a difference in binding between 4°C and 30°C which might potentially be a useful purification step with a good overall yield. The specific activity of the CHAPS fraction from the 4°C elution was 20mU/mg (from 4.2mU/mg applied) representing a 4.8 fold increase in specific activity. Densiometric analysis of this fraction on 10% PAGE, fig 5.11, demonstrated 10 major protein bands and a number of minor bands. This in turn showed a relative lack in specificity in binding.

Fig 5.9 Binding and elution of fibroblast cytidylyltransferase to Sepharose CL4B/Green H-4G at 4°C



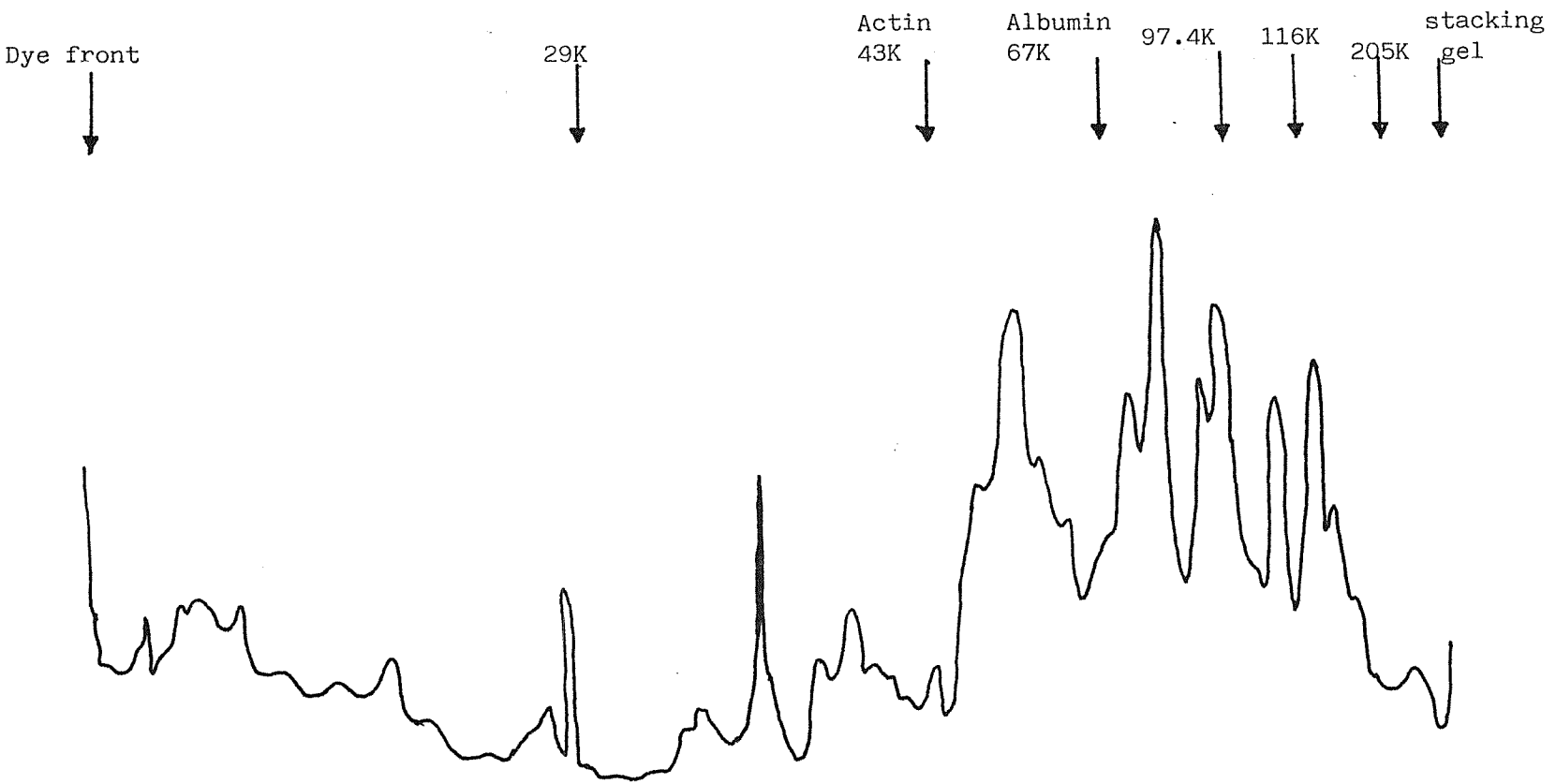
Fibroblast 2nd S_{100}(4ml,4.2mU/mg,16.8mU)was applied and eluted from column following pre-equilibration of column and the irrigation buffers at 4°C. Elution was effected with 4 x 1ml buffer A,then 4 x 1ml 1% CHAPS.

Fig 5.10 Binding and elution of fibroblast cytidylyltransferase to Sepharose CL4B/Green H-4G at 30°C



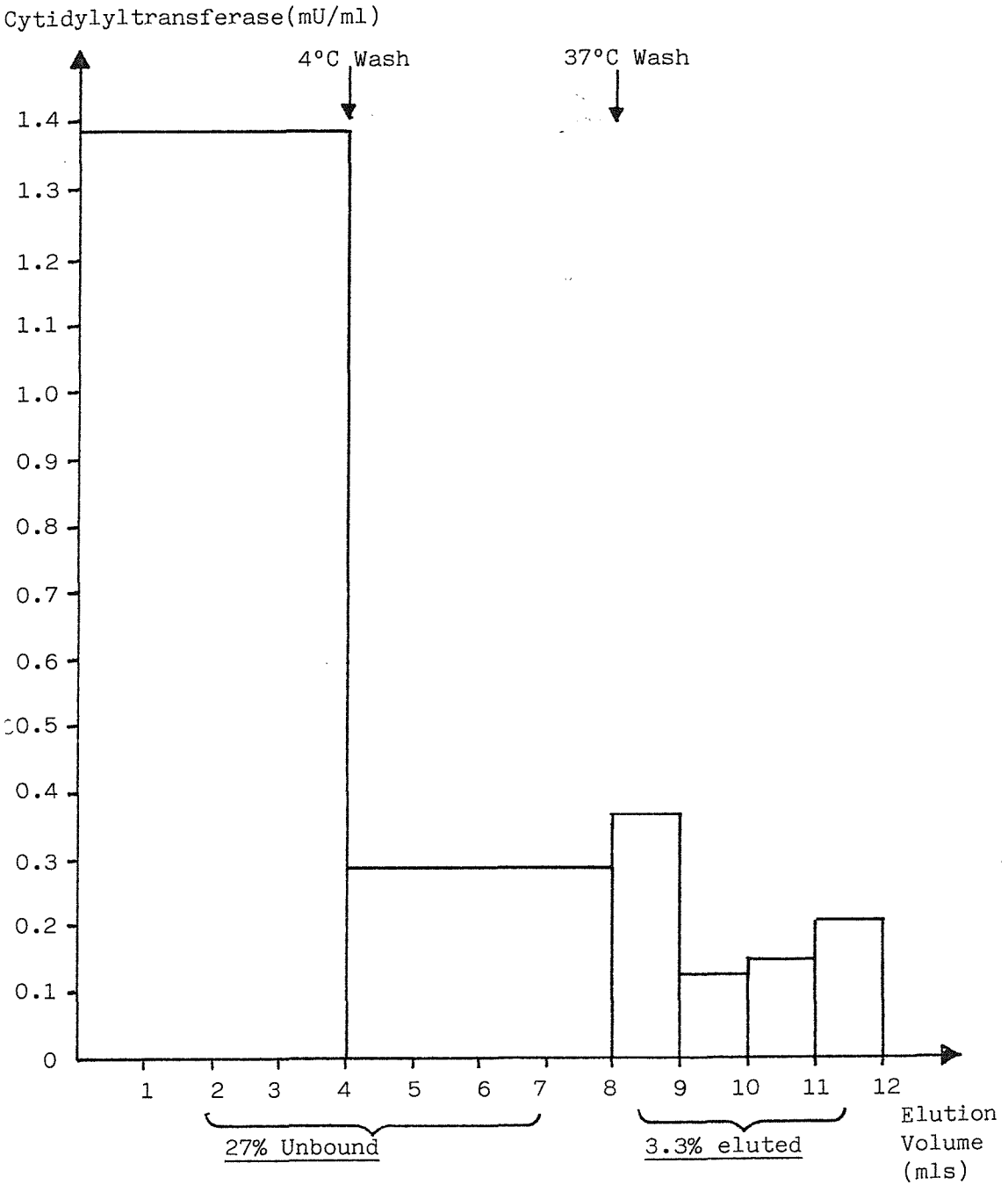
Fibroblast 2nd S_{100}(3ml,2mU/mg,5.92mU)was applied and eluted from column following pre-equilibration of column and the irrigation buffers at 30°C. Elution was effected with 3 x 1ml buffer A,then 4 x 1ml 1% CHAPS.

Fig 5.11 Densiometric scan of SDS PAGE separation of 1% CHAPS fraction from Sepharose CL4B/Green H-4G column



The pooled CHAPS eluate from the previous experiment(2ml,0.83mU,20mU/mg) was precipitated by mixing with deoxycholate and TCA as described for modified Lowry protein analysis. The protein pellet was then separated by SDS PAGE in 10% gel,stained with coomassie brilliant blue and scanned densiometrically at 570nm. Molecular weight standards were separated on a parallel lane.

Fig 5.12 Fibroblast cytidylyltransferase binding to Sepharose CL4B/ Green H-4G at 4°C and its elution at 37°C



Fibroblast 2nd S_{100}(4ml,5.1mU/mg,24.5mU) was applied at 4°C and washed with buffer A at 4°C. The column was then warmed to 37°C and subsequently eluted with 4 x 1ml buffer A at 37°C.

The difference in binding at 4°C and 30°C suggested that binding at 4°C followed by elution at 37°C might avoid drastic changes in ionic strength and obviate the need for detergents while providing a high recovery of enzyme. A more selective desorbtion might result with a greater consequent specific activity.

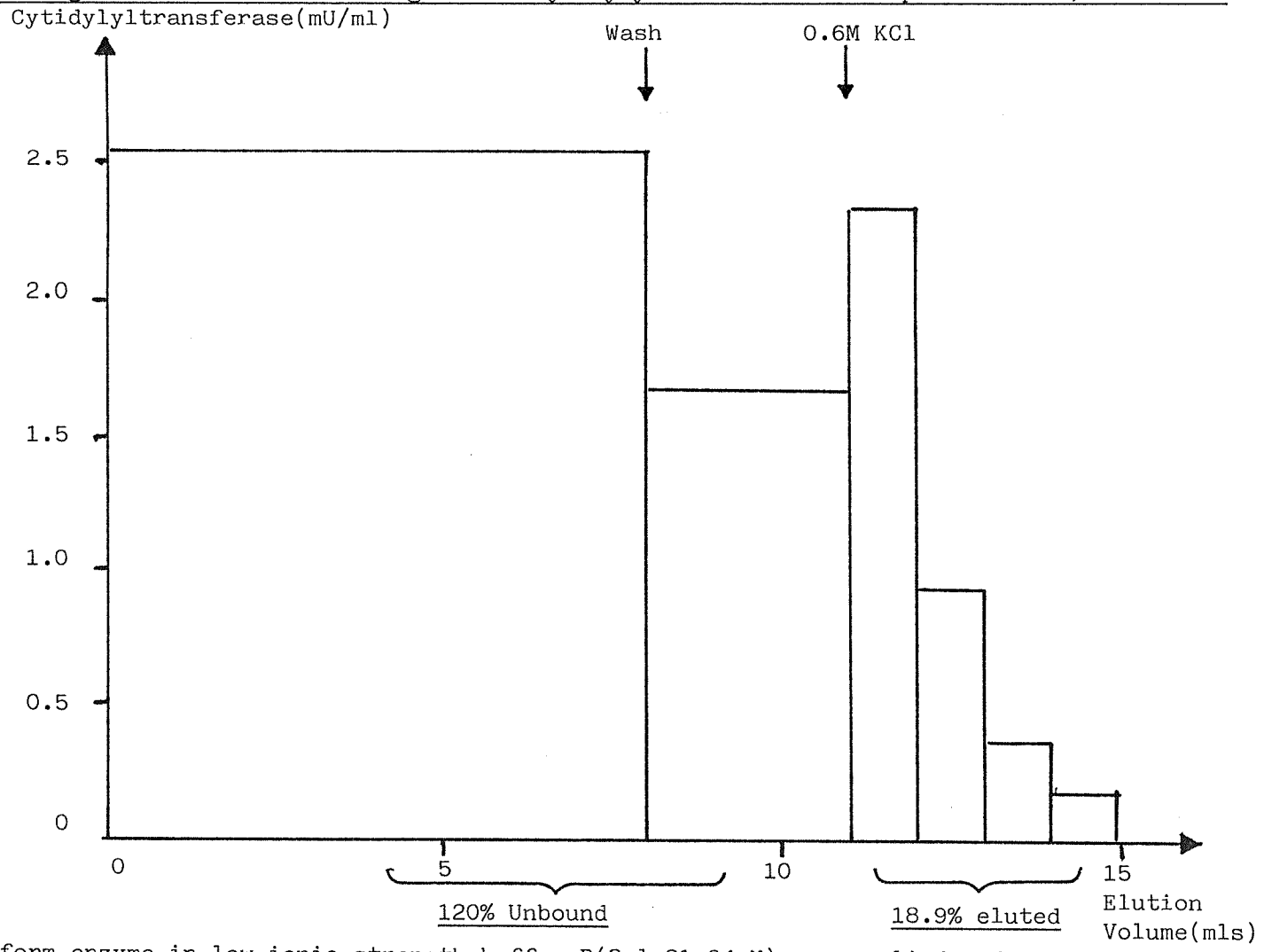
Fibroblast 2nd S_{100} (4ml, 5.1mU/mg, 24.5mU) was applied to a Green H-4G column at 4°C, washed with 4ml buffer A at 4°C. The column was then warmed to 37°C for 1 hour and eluted with 4 x 1ml buffer A at 37°C. The elution pattern, fig 5.12, was not that predicted by the previous two experiments: 6.64mU (27%) of applied activity was unretarded at 4°C while elution at 37°C yielded only 0.82mU (3.3%) extra with 70% of applied activity unaccounted for. One possible explanation of the failure in recovery at 37°C was that the binding of enzyme to the dye matrix at 4°C leads to some physical change in its state. When the temperature was raised, in this scheme the enzyme was desorbed but was much reduced in activity as a result of its dye binding. The enzyme would appear to remain bound to the column matrix while simply inactivated.

5.2.3.4 Rat lung enzyme binding to Procion Green H-4G/Sepharose CL4B

The interaction of rat lung enzyme with free Green H-4G was less than that of fibroblast enzyme (Sections 5.2.1.1, 5.2.1.2) as judged by dye mediated inhibition. The smaller inhibition might not reflect binding and consequently the efficacy of immobilised Procion Green H-4G as an affinity matrix for rat lung enzyme was investigated.

Rat lung H form enzyme in low ionic strength buffer B (8ml, 21.04mU) was applied to a Green H-4G column and washed with buffer B, then 0.6M KCl as shown, fig 5.13. 25.25mU (120%) enzyme activity was unretarded by the column, while 4mU (19%) was recovered with 0.6M KCl pulse. It was evident that the bulk of rat lung enzyme was not bound to Green H-4G/Sepharose CL4B. The dye matrix was not investigated further as an affinity medium for rat lung enzyme.

Fig 5.13 Binding and elution of rat lung H form cytidylyltransferase to Sepharose CL4B/Green H-4G



Rat lung H form enzyme in low ionic strength buffer B(8ml,21.04mU) was applied and eluted with 3 x 1ml buffer B,then 4 x 1ml 0.6M KCl.

5.3 Discussion

Many of the approaches to purification of human and rat lung cytidylyltransferase, detailed in chapter 4, demonstrated the common characteristics of low yield and highly labile enzyme. Even with the best recoveries achieved, successive application of several of these steps would rapidly reduce the overall yield below acceptable levels while providing insufficient purification. The potential of affinity chromatography as a step providing high yield of pure protein was not realised with the immobilised choline phosphate analogues and CTP employed (section 4.2.5). A demonstration of cytidylyltransferase binding to immobilisedProcion Blue MXR was consequently encouraging, despite the low yield (section 4.2.6), because of the vast range of dye structures available (Lowe and Pearson 1984) any one of which might prove more suitable as a dye-affinity ligand. A high yield and readily reversible binding, even if not totally specific, offered the prospect of substantial purification, especially if employed in the later stges of schemes using one or more of the steps investigated in chapter 4.

The interaction and inhibition by free Procion Green H-4G was unique and quickly established this dye as the most promising of ten representative dyes investigated as potential affinity ligands. Mono-chlorotriazine dyes are generally less reactive than dichlorotriazine analogues and this showed a degree of specificity in the Procion Green H-4G inhibition. The characteristics of the inhibition were unusual for while a concentration dependant, hyperbolic inhibition was observed the rate of inactivation was too rapid to be explained in terms of an active site directed mechanism. Indeed, protection offered by MgCTP and CDPcholine while offering some support for an active site involvement was equally explicable by a conformational change. Procion Green H-4G is over twice the size of ATP (Clonis et al 1981) and consequently of CTP. It might, when bound, partially overlap the MgCTP binding site, without a direct interaction. The occupation of the MgCTP binding site might just be sufficient to prevent dye binding and/or inhibition.

The enzyme inhibition by phthalocyanine dyes from the mono-chlorotriazine and dichlorotriazine series as well as a dichloroquinoxalone derivative implicated a phthalocyanine specific hydrophobic interaction. A rapid partitioning of dye into a hydrophobic region of the protein might be prevented by MgCTP or CDPcholine. A

direct demonstration of hydrophobic interaction with triazine dyes is often made by monitoring the spectral shifts which occur upon dye binding to a less polar environment. Pure enzyme is required for this kind of investigation, since the binding of other proteins, evident with fibroblast 2nd S_{100}, fig 5.11, would complicate interpretation.

Immobilised Procion Green H-4G was shown to bind human but not rat enzyme. A consistant finding of up to 30% enzyme unretarded on Procion Green H-4G/Sepharose CL4B columns may reflect enzyme protected by the presence of nucleotides or by its existance in a different conformational state or as inaccessible large aggregates. The binding, typically 70-80% of applied enzyme, was high although not specific, with many protein bands evident when eluted, fig 5.11. A large separation from the bulk of applied protein was noted, however. The observations filled the first criterion, of high binding, for use as a purification step. The sensitivity of the fibroblast enzyme to increased ionic strength, the failure of biospecific elution and relatively low recoveries from detergent treatment or temperature variation were a great disappointment. Rat liver enzyme has been reported to consist of two types of subunit (Weinhold et al 1986). The possibility that the enzyme matrix interaction was separating the enzyme into two inactive components was ruled out by recombination experiments with mixtures of individual fractions which showed no increase in recovery. Clearly none of the attempted eluants were successful in removing sufficient enzyme to make Procion Green H-4G affinity chromatography a useful purification step. It is possible the matrix binding alters enzyme structure irreversibly, rendering it inactive upon elution, equally it may remove other effectors, perhaps actin as suggested in chapters 3 and 4. The column may indeed be a useful affinity matrix but unable to yield an active enzyme.

Cytidylyltransferase interaction with Procion Green H-4G required much more thorough investigation, which was ruled out by time limitations. Potential further investigation might concentrate on one of several approaches. 1) Adding back purified cytoskeletal components to individual fractions. 2) Using affinity partioning, where an aqueous two-phase system using triazine dye-substituted PEG and dextran is used to selectively partition enzymes into the dye bound phase. (Kopperschlager et al 1983). 3) Investigating other triazine dyes in search of a more effective affinity ligand.

CHAPTER 6

SUMMARY AND PERSPECTIVES

Summary and Perspectives

In this thesis some *in vitro* properties of CTP:choline phosphate cytidylyltransferase from fetal and adult human lung have been explored. To date most investigations of mammalian cytidylyltransferase have concentrated on an examination of changes in its distribution between soluble and particulate subcellular fractions under conditions of flux manipulation, a characteristic named 'ambiquity'(Wilson 1978, 1980). Analyses of some of these data have sought to explain the observed variations in terms of a postulated regulatory mechanism,'translocation', operating *in vivo*. A concommitant activation by and binding to the endoplasmic reticulum is believed to be promoted by fatty acids or protein dephosphorylation (Vance and Pelech 1984). Increases in rat lung cytidylyltransferase are reported to accompany the maturation of the surfactant synthesis system (Stern et al 1976, Maniscalco et al 1978, Weinhold et al 1981). In consequence the measurement of human fetal lung cytidylyltransferase at 15 - 16 weeks, which is immature with respect to surfactant synthesis, was compared to values obtained for the adult human lung enzyme.

Soluble and particulate forms of cytidylyltransferase were found in homogenates of fetal human lung, adult human lung and fetal lung-derived fibroblasts. The subcellular distribution between fetal and adult human lung enzyme revealed no significant variation in this parameter. Furthermore a previous study of neonatal lung cytidylyltransferase, limited by a failure to fractionate homogenates (Thom and Zachman 1975) was unable to confirm or reject the possibility of a transient shift in distribution in the immediate pre- and perinatal period. In the absence of available human tissue a parallel investigation of rat lung enzyme established an increase in particulate enzyme from 31% to 40% between d18 gestation and term (d22). While this observation was capable of use in support of a translocation the increase in total soluble enzyme and in particular its greater response to the phospholipid activator PG, fits equally well with the proposed regulatory role for 'cytosolic' enzyme (Chu and Rooney 1985, Rooney 1985, Rooney et al 1986).

A further explanation for the reported translocations was offered by observations of the *in vitro* behaviour and properties of both human and rat lung cytidylyltransferase. The L to H forms of cytidylyltransferase which were found in soluble, S_{100} fractions were consistant with reports of other mammalian cytidylyltransferases (Pelech and Vance 1984a). An L

to H form transition was accompanied by the aggregation of a protein-rich insoluble fraction, identified as predominantly F-actin, reflecting a G-actin/F-actin polymerisation. A strong association of rat lung H form enzyme with the F-actin enriched pellet from the 100,000 x g x 60 minute centrifugation after ageing and a lesser association recorded with human lung H form was strongly suggestive of an *in vitro* and possibly *in vivo* binding to cytoskeletal elements. Examples of similar interactions for other enzymes and complete enzyme systems are increasingly common in the literature (section 1.4.7.) including many enzymes displaying ambiquitous characteristics. Enzymes such as pyruvate kinase, experimentally designated 'soluble', show cytoskeletal interaction at protein concentrations which exist within unfractionated, undiluted cell matrix (Clarke and Morton 1982). The greater association of human lung cytidylyltransferase with F-actin in the presence of the space filling polymer PEG_{6000} suggested that extensive cytoskeletal binding of the enzyme might pertain *in vivo*.

The behaviour of cytidylyltransferase in these and other investigations was entirely consistant with a possible linkage to the endoplasmic reticulum mediated by elements of its integral cytoskeleton, perhaps analogous to the membrane association of the regulatory protein calmodulin (Gazzotti et al 1985). Such a model would compliment the emerging concept of a highly ordered aqueous cytoplasm (Clegg 1984(a)) in which no truely 'soluble' proteins exist *in vivo*. The vast majority of proteins are envisaged as bound, at least transiently, with the cell architecture (Gershon et al 1985, Jacobson and Wojcieszyn 1984). It is tempting to speculate that choline kinase a 'soluble' enzyme might also be cytoskeletally bound *in vivo* either directly or in 'piggy back'fashion akin to glycolytic enzyme binding to PFK (Gerlach and Hofer 1986). The The translocation model would simply require the incorporation of the membrane cytoskeleton holding cytidylyltransferase and the membranous cholinephosphotransferase in close proximity and correct orientation. This arrangement would allow for the direct enzyme/enzyme interactions and substrate channeling characterising many enzyme pathways (Srivastava and Bernhard 1985(a)(b)). In turn flux manipulation at the point of cytidylyltransferase action would only require a change in binding affinity for the cytoskeletal component, coupled with a movement of active sites with respect to one another.

The testing of these possibilities in a defined system required the

purification of human lung cytidylyltransferase as a first step. While several reports of the purification of rat liver cytidylyltransferase have been made, with varying degrees of enrichment (Choy and Vance 1976, Choy et al 1977, Vance et al 1981, Weinhold et al 1986) no description exists of the resolution of lung enzyme beyond the separation of H and L forms. This failure has undoubtably reflected in part the lower overall tissue activity of the pulmonary enzyme, coupled with the low yields inherent in published schemes. Consequently any approach required few steps of high yield and purification factor. A common element of purification schemes has been the interconversion of soluble and particulate forms of cytidylyltransferase (Borkenhagen and Kennedy 1957, Vance et al 1981, Weinhold et al 1986) represented by L and H forms respectively upon gel filtration. Separation of H and L forms of fresh or aged human lung S_{100} by gel filtration revealed latent enzyme activity as high as 3 fold above freshly determined activities. H form human enzyme, which was associated with F-actin, was unstable unlike rat H form which was more stable than the L form. It was apparent that the in vitro behaviour of cytidylyltransferase was dependant on the state of aggregation of the cytoskeletal elements present. The human enzyme appeared most stable in the presence of unpolymerised components, while rat enzyme, from which F-actin was removed lost activity rapidly.

When fractionation of S_{100}s was attempted with ammonium sulphate a loss of rat lung enzyme activity was seen, while the enrichment of human lung enzyme was less than that seen with gel filtration. These observations reflected a sensitivity of the enzyme to high ionic strength typified by the concentration-dependant irreversible inhibition of fibroblast cytidylyltransferase in the presence of KCl seen in the attempted deployment of hydrophobic interaction chromatography. The stability of rat lung cytidylyltransferase was compromised by exposure to high ionic strength during ammonium sulphate treatment or low ionic strength following dialysis. Inhibition recorded after exchanging the Tris for phosphate was also reminiscent of the reported phosphate inhibition of rat liver enzyme (Weinhold et al 1986).

The coprecipitation of cytoskeletal components and cytidylyltransferase in the presence of the space-filling polymer PEG_{6000} provided a convenient route to enrichment and concentration of the enzyme but required the ability to separate the components in a subsequent step. One approach to the separation of H form enzyme from the cytoskeletal aggregates was suggested by the use of biocompatible detergents. One such

detergent, CHAPS, employed above its CMC, proved a useful method for the separation without the inhibitory characteristics of detergents such as SDS (Choy et al 1977, Vance et al 1981) and Triton X_{100} (Stern et al 1976). CHAPS produced a concentration-dependant release of latent cytidylyltransferase from the rat lung H form enzyme, accompanied by the dissociation to lower molecular weight enzyme. A similar effect was also noted with rat lung, membrane-rich P_{100} fractions, providing evidence that freshly determined rat lung cytidylyltransferase assays did not accurately reflect V_{max} activities. CHAPS did not unmask any latent soluble human enzyme activity, while the P_{100} fraction yielded some solubilised enzyme but a reduction in preincubation activity. This latter observation, however, followed a doubling of human fetal lung P_{100} cytidylyltransferase after washing with a high EDTA buffer designed to release extrinsic membrane protein. In addition to reinforcing the inadequacy of fresh human lung cytidylyltransferase activity measurements this appeared to indicate that human fetal P_{100} enzyme was in part more loosely asociated with the membranous fraction than that of the rat lung. The use of CHAPS in solubilising membrane- or cytoskeletally-associated cytidylyltransferase resulted in a less stable enzyme, possibly a consequence of the separation.

The efficacy of affinity chromatography in the purification of pulmonary cytidylyltransferase was investigated with the hope of a rapid and specific purification in order to overcome some of the instability associated with other approaches. A report of rat liver enzyme purification with immobilised glycerophosphocholine has been made (Choy and Vance 1976, Choy et al 1978) although the lack of subsequent recorded use suggested that its use was not without problems. A similar approach with fibroblast 2nd S_{100} enzyme showed only a small binding of applied activity which may have been non-specific. Immobilised p-diazonium phenylphosphorylcholine, a choline phosphate analogue, separated from the gel matrix by a longer 'spacer' arm was also found to be an unsuitable affinity matrix. Agarose-bound CTP was similarly shown to be a non-viable affinity medium, while a partial binding to Sepharose-immobilised Procion Blue MX-R demonstrated the potential of dye-affinity chromatography, successful in the purification of other nucleotide-dependant enzymes (Lowe and Pearson 1984).

An initial screening of triazine dyes representative of the 80 dyes

commercially available, quickly established Procion Green H-4G as a candidate pseudoaffinity ligand. While free dye-mediated inhibition was partially mitigated by MgCTP and CDPcholine, the Green H-4G did not seem to be acting as an active site-directed inhibitor. Rapid, rather than progressive, inhibition and a tendancy for mono- and dichlorotriazine dyes containing the phthalocyanine chromophore to inhibit cytidylyltransferase suggested a hydrophobic component to the binding. Cytidylyltransferase interaction with the bulky aromatic structure, followed by a nucleophillic attack at a crucial region of the enzyme appeared an attractive possibility. Immobilised Sepharose CL4B/Green H-4G typically bound 70 - 80% of fibroblast 2nd S_{100} enzyme although MgCTP, KCl, CHAPS or variation of temperature proved to be unsuitable eluants. Over 80% of rat lung cytidylyltransferase, in contrast, was not retained by the dye matrix. Procion Green H-4G Sepharose binding of human lung cytidylyltransferase was not very specific, as evidenced by the large number of protein bands in 1% CHAPS eluates of bound enzyme. Despite the observation that the best eluant, CHAPS, was able to release only 18% of bound activity the dye matrix offered the potential of use as a purification step following initial high yield enrichment. Realisation of this objective, however, would require the discovery of suitable elution conditions to both liberate enzyme and retain its activity.

The observations recorded within this thesis have revealed some properties of human and rat lung cytidylyltransferase which offer an alternative explanation for the reported behaviour of other mammalian cytidylyltransferases, while posing many new questions. The *in vitro* and potential *in vivo* associations with F-actin containing components have not previously been recognised for cytidylyltransferase, possibly a reflection of the latter's ubiquity. Any actin recognised may have been dismissed as a universal contaminant. The many recent reports of enzyme-cytoskeleton interactions noted in section 1.4.7 offer some general guidance for directions of future investigations with human lung cytidylyltransferase. While the further purification of the enzyme must be pursued, information may be obtained from *in vivo* studies and partially purified enzyme. The effect of both antimicrofilamental agents and of antimicrotubules on PC synthesis in lung-derived fibroblasts would allow an evaluation of exactly which cytoskeletal elements may be involved. Cytochalasins, the conventionally employed antimicrofilaments, can alter other metabolic activities including glucose trnsport and the use of botulinum toxin, shown to prevent actin polymerisation by the specific

ADP ribosylation of non-muscle G-actin in cells such as neutrophils (Al-Mohamma et al 1987), provide an alternative method for testing the importance of an intact cytoskeleton. The behaviour of 'soluble' choline kinase under the experimental conditions elaborated in chapter 3, and in S_{100} fractions concentrated to the theoretical intracellular protein concentration as described for brain glycolytic enzymes (Clarke and Morton 1982) should provide useful information. In particular it might serve to indicate the possible existance of multienzyme complexes of the CDPcholine pathway enzymes *in vivo*. Investigation of any interactions of the membrane enzyme cholinephosphotransferase with the cytoskeleton, perhaps in an analogous fashion to that of the integral membrane enzyme 5'-nucleotidase with F-actin (Dieckhoff and Mannherz 1985), might offer support for such an organised arrangement. The 'ambiquitous' enzyme PAP (Brindley 1985) is another candidate for as yet unrecognised interactions with cytoskeletal elements, and may in part be found with F-actin-rich fractions *in vitro*. Cytoskeletal binding *in vivo* might serve to allow the direct enzyme/enzyme transfer of diacylglycerol from PAP to the membranous cholinephosphotransferase. The reported existance of C-kinase bound to membranes in both cytoskeleton-dependant and independant fashion (Wolf and Sahyoun 1986) suggest that investigation of the nature of cytidylyltransferase binding to the endoplasmic reticulum should include an assessment of a similar possibility.

The stability of human and rat lung cytidylyltransferase during purification appeared, empirically, to be affected by the presence or absence of cytoskeletal components. When activity was lost it was not recovered by recombination with separated fractions however. This failure may have reflected the form in which these components were presented and recombinations of partially purified enzyme with pure actin, tubulin or other cytoskeletal elements in monomeric or polymeric forms should be evaluated. The use of dye affinity chromatography was promising and assessment of other untested dyes may reveal one more effective in reversible cytidylyltransferase interaction. In addition to dye affinity chromatography, the employment of affinity partitioning with dextran- or PEG-immobilised dye in a two phase system(Koppeschlager et al 1983), could provide an alternative strategy for purification. The apparent intimate association of lung cytidylyltransferases with the lung cytoskeleton, at least *in vitro*, shows that any approach to the understanding of regulation or the purification of the enzyme demands a thorough knowledge of the nature and effects of the interaction.

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