STUDIES ON ERYTHROCYTE PHOSPHOLIPID ABNORMALITIES IN PATIENTS WITH NON-INSULIN DEPENDENT MATURITY-ONSET DIABETES MELLITUS AND THE POSSIBLE INFLUENCE OF NON-ENZYMATIC PHOSPHOLIPID GLYCOSYLATION.

by

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STATEMENT

I, EMMANUELLE EMENIKE ORAGUI Jnr., WAS ADMITTED AS A RESEARCH STUDENT OF THE UNIVERSITY OF ST. ANDREWS IN AUGUST 1984 IN ACCORDANCE WITH ORDINANCE GENERAL NO. 12 AND THE RESOLUTION OF THE UNIVERSITY COURT, 1967, NO. 1. THE THESIS WAS COMPLETED IN JANUARY 1986.

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DEDICATION

I HAVE DEDICATED THIS THESIS TO GRAND-MA, AMY EMMA ORAGUI, AND TO THOSE PATIENTS WHO GAVE BLOOD FOR THIS RESEARCH AND I WISH THEM A QUICK RECOVERY.

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ABBREVIATIONS

ADH	Antidiuretic hormone
a.u.f.s.	Absorbance unit full scale
BC	Before Christ
CAD	Coronary artery disease
$CHCl_3$	Chloroform
COOH	Carboxyl group
$CuSO_4$	Copper sulphate
CV	Coefficient of variation
CVD	Cerebrovascular disease
d.f.	Degrees of freedom
DI	Diabetes insipidus
DM	Diabetes mellitus
DNA	Deoxyribonucleic acid
FFA	Free fatty acid
FPG	Fasting plasma glucose
GDM	Gestational diabetes mellitus
GPS	Glycosylated phosphatidyl serine
GTT	Glucose tolerance test
HbA_{1c}	Glycosylated haemoglobin
HDL	High-density lipoprotein
HLA	Human leucocyte antigen
HPLC	High pressure liquid chromatography
IDDM	Insulin-dependent diabetes mellitus
LDL	Low-density lipoprotein
MeOH	Methanol
NAD	Nicotinamide adenine dinucleotide (oxidised form)

ABBREVIATIONS

NADH	Nicotinamide adenine dinucleotide (reduced form)
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
NaOH	Sodium hydroxide
Na_2HPO_4	Disodium hydrogen phosphate
Na_2CO_3	Sodium carbonate
NEG	Non-enzymatic glycosylation
NH_3	Ammonia
NH_2	Amino group
NIDDM	Non-insulin dependent diabetes mellitus
NS	Normal subjects
PC	Phosphatidyl choline
PCD	Poorly-controlled diabetics
PE	Phosphatidyl ethanolamine
PI	Phosphatidyl inositol
PS	Phosphatidyl serine
PVD	Peripheral vascular disease
RBC	Red blood cell
RNA	Ribonucleic acid
SM	Sphingomyelin
S.D.	Standard deviation
TLC	Thin layer chromatography
UV	Ultra-violet
VLDL	Very low-density lipoprotein
WCD	Well-controlled diabetics
WHO	World Health Organisation

SYMBOLS AND NOTATION

l	litre
ml	millilitre (10^{-3} litres)
µl	microlitre (10^{-6} litres)
g	gramme
mg	milligramme
µg	microgramme
mCi	millicurie
µCi	microcurie
M	molar
$<$	less than
^{o}C	degrees Celsius
w/v	weight by volume
rpm	revolutions per minute
%	percent(age)
nm	nanometres
P	probability
$\bar{X}$	mean
Σ	sum of data
^{14}C	radioactive
^{3}H	radioactive
t	student t-test
U	Mann-Whitney test
r	correlation coefficient
µ	micron
Δ	change in
pH	degree of acidity
pKa	degree of dissociation
µm	micrometer

SUMMARY

Although insulin has been used for over fifty years for the treatment of Diabetes Mellitus (DM), these patients continue to suffer serious and often life-threatening complications e.g. cataracts, neuropathy, retinopathy, nephropathy and vascular disease. As a result of these complications, the average life-span of diabetics is reduced by approximately one-third. The biochemical basis for the development of these complications is not clear and much discussion has taken place regarding the relationship between the degree of control of blood sugar and the development of the sequelae.

The recognition that free sugar condenses non-enzymatically with proteins and that this reaction is increased in the diabetic state with attendant hyperglycaemia, has offered improved means for assessing diabetic control and has opened new avenues of investigation concerning the pathogenesis of diabetic complications. Initially, non-enzymatic glycosylation attracted the attention of investigators interested in the management of diabetes mellitus following the demonstration that haemoglobin undergoes post-ribosomal non-enzymatic glycosylation *in vivo* and that the proportion of total glycosylated haemoglobin is increased in diabetic patients with attendant hyperglycaemia. Subsequent reports that other proteins, lipoproteins and nucleic acids are also

subject to glycosylation under physiological conditions, helped support the hypothesis that this might be a model reaction relevant to the pathogenesis of certain complications of chronic diabetes, and may form the biochemical basis of diabetic sequelae. The aim of this work was to study erythrocyte phospholipid abnormalities in patients with diabetes mellitus and to investigate the possible influence of non-enzymatic phospholipid glycosylation.

We have carried out cross-sectional phospholipid studies on poorly-controlled diabetics (PCD), well-controlled diabetics (WCD) and the peripheral vascular disease patients (PVD) by comparing their erythrocyte phospholipid class composition. We have also carried out longitudinal studies on red cell phospholipid composition from poorly-controlled diabetics from the time of first presentation at the diabetic out-patient and during treatment to normalise their blood glucose level. Erythrocyte phospholipids were extracted using chloroform/methanol, separated by two-dimensional thin layer chromatography, and quantitated colorimetrically. Phosphatidyl choline (PC), phosphatidyl ethanolamine (PE), phosphatidyl serine (PS) and sphingomyelin (SM) were resolved completely from each other and from the other minor phospholipid classes. In the cross-sectional studies, it was found that PCD had significantly increased PS when compared with WCD or PVD (PCD: 14.9 ± 1.1/WCD: 12.4 ± 0.6; $p < 0.0005$), (PCD: 14.9 ± 1.1/PVD: 11.7 ± 1.2;

$p < 0.0005$) and significantly decreased PE (PCD: 27.1±1.7/ WCD: 29.7±0.7; $p < 0.0005$), (PCD: 27.1±1.7/PVD: 28.1±1.6; $p < 0.01$). Also significantly decreased SM was found in poorly-controlled diabetics (PCD: 27.2±1.5/ WCD: 28.5±1.0; $p < 0.025$), (PCD: 27.2±1.5/PVD: 29.2±1.0; $p < 0.0005$). PC/SM ratio was found to be significantly increased in PCD when compared with other groups (PCD: 1.15±0.06/WCD: 1.06±0.06; $p < 0.0005$), (PCD: 1.15±0.06/PVD: 1.04±0.04 $p < 0.0005$). No significant differences were found for PC between groups. The results of the longitudinal studies show highly significant differences for PS ($p < 0.0005$) and PE ($p < 0.005$) for individual patients in the "poorly-controlled" and "well-controlled" state. It was also found that for individual patients, the overriding trend is for PS to increase with increasing fasting plasma glucose and vice versa. In the light of these findings, we conclude that erythrocyte phospholipid composition is abnormal in poorly-controlled patients with diabetes mellitus.

We have also synthesised model glycosylated PE and PS in non-physiological conditions with about 30% yield in each case. An _in vitro_ production of glycosylated phospholipids in membranes yielded glycosylated PE (GPE) and glycosylated PS (GPS) in the ratio of 4:1 respectively. Overlap between GPE and PS, and GPS and PE occurred using the initial two-dimensional TLC system.

The resolution of GPE was achieved by a "modified" TLC and "improved" HPLC. An increased GPE was demonstrated in poorly-controlled diabetics by TLC and phosphorus estimation, which correlated with glycosylated haemoglobin ($r = 0.784$; $p < 0.0005$) and fasting plasma glucose ($r = 0.941$; $p < 0.0005$). Our findings show that some of the erythrocyte phospholipid changes observed in poorly-controlled diabetics are consistent with glycosylation of PE and could be important to explain some of the physical abnormalities associated with diabetic red cells.

INTRODUCTION

1. INTRODUCTION

1.1 HISTORICAL REVIEW OF DIABETES

Diabetes has been known to mankind since antiquity /1/. The Egyptian papyrus of Ebers dating from 1550 BC contains dietary remedies for those passing abundant urine. The first clear account of diabetes is given in the writings of Areteaus the Cappadocian in 170 AD who described; "This mysterious affection being a melting down of flesh and limbs into urine, life is short, disgusting and painful, thirst unquenchable and death inevitable" /2/.

In 1799, it was hinted that there was a pancreatic involvement in diabetes and a case was described, which at autopsy, showed marked pancreatic damage and this led to the postulation that pancreatic disease might be causally related to diabetes. The importance of the pancreas was established in 1889 when it was shown how pancreatectomy made a laboratory dog urinate excessively and how the urine attracted flies due to the presence of large amounts of sugar.

The histology of the pancreas had been known twenty years earlier but the significance of the islet tissues was not clear. In 1893 it was suggested that they might produce an endocrine secretion. The development of the concept of hormones in 1904 engineered the extraction of insulin from the pancreas in 1921. This extract lowered the blood glucose in dogs who had been made diabetic by pancreatectomy. This discovery of insulin, one of the

greatest triumphs of twentieth century medicine, was quickly translated into a life-saving remedy, and from the work of the pharmaceutical industry it became available for clinical care throughout the world.

Although a new era had dawned for diabetics, there remained problems to be surmounted since it was apparent that correction of insulin lack and hyperglycaemia per se were not totally successful in treating patients' pathological symptoms. In 1939 nodular lessions were first noticed in the glomeruli of diabetics and by 1944 several more renal vascular lessions were noted and interest had also been awakened to diabetic retinopathy. In 1954 the concept of specific and widespread diabetic small blood vessel disease, affecting especially the kidneys and retina was postulated. It became apparent, therefore, that the price of survival bought with insulin for the diabetic was a life-shortened and crippled by degenerative vascular disease. Although the therapeutic advances with insulin and oral agents have made it possible to control primary diabetic symptoms, they have failed to prevent the serious vascular and other complications of diabetics. Thus the search continues for the fundamental causes of diabetes and its complications.

Much work remains to be done and now-a-days, a child who develops diabetes can lead a full and happy life with insulin treatment. His expectation of life ahead, however, is considerably reduced and his later years may be clouded by ill-health and blindness. Until we can recognise the

cause and prevention of these degenerative changes, continued effort and research will be needed. Finally, the day may dawn when we can recognise those who are susceptible to diabetes and then be able to prevent its occurrence.

1.2 DIABETES - THE DISEASE

Diabetes may be defined as a chronic disorder characterised by raised level of glucose in the blood and urine. Since there are many factors which can influence blood glucose, diabetes can be the outcome of many different causes, some hereditary, some environmental and some hormonal. There are two types of diabetes; diabetes insipidus (D.I.) and diabetes mellitus (D.M.).

1.2.1 DIABETES INSIPIDUS

Diabetes insipidus is due to inability or insufficiency in the secretion of antidiuretic hormone (ADH) often in association with the evidence of pituitary or hypothalamic damage caused by head injury or during hypophysectomy or due to invasion of the region by tumour /3/. It may also be due to a rare inborn error of renal tubular function in which ADH levels are high but the tubules cannot respond to it. In DI the renal threshold for glucose is lowered and this gives rise to glucosuria even in the presence of normal plasma glucose /3/. Further discussion of DI is outwith the scope of this thesis since the patients studied were all suffering from DM.

1.2.2 DIABETES MELLITUS

Diabetes mellitus has been defined by the World Health Organisation (WHO) on the basis of laboratory findings. A patient is said to be diabetic when his fasting plasma glucose is 8 mmol/l or more /4/. Most of the metabolic changes in DM can be explained as a consequence of insulin deficiency and tend to parallel it in severity /4/.

1.2.2.1 CLASSIFICATION AND PATHOGENESIS OF DIABETES MELLITUS

There are three classes of diabetes mellitus: (1) insulin-dependent diabetes mellitus (I.D.D.M.) or type I DM; (2) non-insulin dependent diabetes mellitus (N.I.D.D.M.) or type II DM; (3) gestational diabetes mellitus (G.D.M.). The largest groups of patients with diabetes mellitus are the IDDM and the NIDDM. The proportions of types will vary with the specific population /5/.

1.2.2.1.1 INSULIN-DEPENDENT DIABETES MELLITUS

This class of DM is usually characterised clinically by abrupt onset of symptoms, insulinopenia and dependence on injected insulin to sustain life, and proneness to ketosis. Classically, this type of disease occurs in juveniles, and it was formerly termed juvenile diabetes. IDDM appears to be heterogenous in terms of genetics and environmental factors that precipitate the disease /6/. Abnormal immune responses and autoimmunity are also thought to play an etiologic role, and islet cell antibodies are

are frequently present at diagnosis /7/.

1.2.2.1.2 GESTATIONAL DIABETES MELLITUS

Patients in this class develop diabetes mellitus or abnormal glucose intolerance during pregnancy /8/. It may occur during the second trimester but most usually occurs during the third trimester of pregnancy /9/. GDM occurs in about 2% of all pregnancies. The resistance to insulin action that occurs late in pregnancy due to high levels of circulating steroid hormones and human chorionic somatomammotropin probably decompensates the carbohydrate regulatory system in individuals who have a genetic predisposition to diabetes mellitus.

1.2.2.1.3 NON-INSULIN DEPENDENT MELLITUS

This class of diabetes mellitus usually has its onset after the age of thirty years. It is frequently associated with obesity and not ordinarily associated with ketoacidosis. While insulin treatment may occasionally be required to normalise fasting blood glucose, insulin treatment is not necessary to maintain life /5/. There is no association with specific Human Leukocyte Antigen (HLA) types and circulating anti-islet antibodies are usually not present. A major abnormality is the presence of resistance to insulin action /10/.

The genetic features in non-insulin dependent diabetes mellitus are unclear. All available techniques to measure insulin action show that this is impaired in patients with NIDDM /11/. Initially, it was thought that this was due to a primary decrease in the number of insulin

receptors on the plasma membrane of insulin sensitive tissues, but there is now evidence that individuals with normal fasting plasma glucose but postprandial hyperglycaemia have decreased plasma membrane insulin receptors and that this correlates with insulin resistance /12/. If insulin resistance were the only defect in NIDDM, one might expect that euglycaemia (normal glucose levels) could be maintained by increasing insulin secretion. However, this is not the case because the beta cell in NIDDM has a defect in appropriately recognising glucose as a stimulus to insulin secretion /13/. Thus, NIDDM appears to involve resistance to insulin action and defective glucose-mediated insulin secretion. The studies in this thesis were carried out on patients with NIDDM, so that further discussion will focus largely on this type of diabetes.

1.2.2.2 MANAGEMENT OF DM

1.2.2.2.1 BIOCHEMICAL FEATURES

In the fasting state, insulin production may be normal when the disease is in its mildest form. When insulin deficiency becomes more severe, there is evidence of hyperglycaemia and glycosuria. The glucose content of the proximal tubular fluid exceeds the capacity of the cells to reabsorb it and osmotic retention of water in the tubular lumen occurs. The high osmotic pressure overides the capacity of the anti-diuretic hormone (ADH) mechanisms more distally to absorb water and glucose appears in the urine, together with extra sodium chloride

and water. Dehydration follows the polyuria and thirst is the consequence /3/.

Abnormalities in lipid metabolism as a result of glucose lack occurs and lipolysis is stimulated and plasma free fatty acid (FFA) level rises. In the liver, FFA are converted to acetyl CoA and ketone bodies. If the deficiency of insulin is extremely severe, the patient, if untreated, progresses steadily to ketoacidosis, coma and death /4/.

1.2.2.2.2 DIAGNOSIS OF DM

The diagnosis of DM is simple and unequivocal when the patient has severe clinical symptoms such as polydipsia (excessive water drinking), polyphagia (excessive eating), polyuria (excessive urine) and weight loss accompanied by grossly elevated blood glucose and ketonaemia. However, the diagnosis of DM in the absence of symptoms or in the presence of mild questionable symptoms is much more difficult /13/. A test for glycosuria (e.g. clinitest) and for elevated blood glucose two hours after a normal meal will give an indication of impaired insulin action. Absolute confirmation however, is obtained by the performance of an oral glucose tolerance test before the patient can be clinically diagnosed as diabetic /4/.

In DM the plasma glucose concentration tends to show a higher fasting value, to rise to higher levels, and to fall more slowly than in healthy individuals. The most significant early abnormality is a failure of the concentration to return to normal level after glucose load.

In mild diabetes the peak glucose concentration usually exceeds the renal threshold and glycosuria occurs, but this is not always the case. In severe DM during glucose tolerance test (GTT), all plasma glucose levels are abnormal /3/.

1.2.2.2.3 TREATMENT OF NIDDM

From the therapeutic stand point, three measures are presently available that are capable of ameliorating the insulin resistance in NIDDM. Firstly, a regular program of physical activity is very likely to improve glucose tolerance through an enhancement of insulin sensitivity. Secondly, dietary modification may prove useful in reducing the fasting plasma glucose concentration towards normal levels. Thirdly, the newer generation of sulphonylurea agents appear to provide a promising means of improving the insulin resistance.

1.2.2.2.3.1 SULPHONYLUREA THERAPY OF NIDDM

Although the oral sulphonyl urea agents have been in use for many years, their precise mechanism of action is still unknown. *In vivo* /14/ and *in vitro* /15/ studies have demonstrated that the sulphonylureas, when acutely administered, cause a prompt increase in insulin secretion. The primary effect of the oral sulphonylurea agents is enhancement of the release of preformed insulin.

The sulphonylurea agents appear to have two distinct actions on the beta cell. First, they directly stimulate insulin secretion /15/ and second, they potentiate the stimulatory effect of glucose as well as other secretagogues

/16/ on insulin release. Since the presence of sulphonylureas within cells has not been demonstrated, it has been presumed that they initiate their action on insulin secretion by interacting with the cell surface /17/. The sulphonylurea agents include chlorpropamide, glibenclamide and glipizide. Another class of hypoglycaemia agents include biguanides and fenfluramine.

If the patient remains hyperglycaemic during dietary control, a sulphonylurea is given while continuing the diet, for example chlorpropamide, 100-500 mg per day, or glipizide, 2.5-30 mg per day /18/.

1.3 DIABETIC COMPLICATIONS

Although insulin deficiency can be ameliorated by diet, insulin injection, or oral hypoglycaemic agent therapy, such treatment has not completely prevented the development of chronic complications affecting the eyes, kidneys, nerves and arteries. In the eye, retinal capillary damage leading to edema, new vessel formation, and haemorrhage makes blindness more common among diabetics /19/. Cataracts also occur more frequently in patients with DM and capillary damage in the glomerulus associated with basement membrane thickening makes chronic renal failure with proteinuria more common /19/. In the diabetic peripheral nerves, axonal dwindling and segmental demyelination are associated with a high prevalence of motor, sensory and autonomic dysfunction, including impotence which affects diabetic males /19/.

The complications of DM are recognised as vascular disease, cataracts, retinopathy, nephropathy, neuropathy and "diabetic foot". The tissues involved do not normally require insulin for glucose transport or metabolism and hyperglycaemia therefore produces an elevated intracellular glucose concentration in such tissues. This glucose level is thought to be involved in the pathogenesis of the complications of diabetes. However, the biochemical mechanism still remain obscure. Other factors believed to play a role are, elevated growth hormone, membrane and plasma lipid abnormalities and haematological abnormalities.

1.3.1 VASCULAR COMPLICATIONS

Vascular disease is more common in diabetics than in non-diabetics. Two types of vascular complications occur frequently in diabetic patients and these are micro- and macro-angiopathy.

1.3.1.1 MICROANGIOPATHY

While the discovery of insulin led to the successful therapy of the acute diabetic syndrome and greatly extended the useful life of diabetics, it has so far not resolved the problem of microangiopathy which constitutes the most threatening aspect of the disease. The foundation for an understanding of diabetic microangiopathy was provided in the late 1950's when electron microscopic studies by Bergstrand and Bucht /20/ in Sweden and Farquhar et al. /21/ in the United States revealed that the capillary disease of the renal glomerulus is characterised by thickening of the basement membrane. Subsequent basement

membrane changes were observed in the capillaries of a variety of other tissues of the diabetic including muscle /22/, skin /23/, retina /24/, and ciliary processes of the eye /25/.

1.3.1.1.1 NEPHROPATHY

Renal structure and functional changes have been recognised to occur soon after onset of DM. Renal hypertrophy occurs in early DM /26/ and in these patients, kidney enlargement is noted shortly after the onset of hyperglycaemia /27/. Both glomerular and tubular size are significantly increased with increase in total glomerular volume /27/, an increase in glomerular filtration surface area /28/, and proximal and distal tubular cellular hypertrophy and hyperplasia /29/. Precise glycaemic control by insulin administration or oral agents can reduce the progression of these structural changes in drug-induced diabetic animals /30/ and in diabetic patients /31/.

Renal disease associated with diabetes is characterised by continuous proteinuria and a decreasing glomerular filtration rate. The severity of these factors correlates with the extent of glomerular basement thickening and consequent capillary occlusion resulting in chronic renal failure.

1.3.1.1.2 RETINOPATHY

The mechanisms involved in the pathogenesis and progression of diabetic retinopathy are still speculative. Within days of diabetes, a breakdown of the blood-retinal

barrier occurs and the degree of breakdown correlates with the degree of metabolic control and the duration of diabetes /32/. Early in the course of diabetes, normalisation of blood glucose levels with insulin or oral agents significantly reduces this increased capillary permeability /33/. The morphologic basis for these permeability changes appears to be a diabetes-induced opening in the endothelial cell tight junctions. Decreased active transport of osmotically active substances from the extravascular to the intravascular space may also contribute to the breakdown of the micropinocytotic vesicles in capillary endothelial cells /34/. Increased retinal capillary permeability results in clinically apparent intraretinal edema and hard exudate formation. However before these changes become clinically detectable, fluid accumulation in the retina may significantly reduce oxygen diffusion. As hypoxia progresses, microaneurysm and new vessel formation with haemorrhage can result in permanent retinopathy and blindness.

1.3.1.2 MACROANGIOPATHY

Accelerated large vessel disease in diabetes may be due in part to abnormalities in plasma lipids and perhaps also to changes in the composition and metabolism of the arterial wall. These forms of macrovascular disease have been recognised; coronary artery disease (CAD), peripheral vascular disease (PVD) and cerebrovascular disease (CVD). Insulin deficiency may well influence the progression of atherosclerosis through synergistic pathological mechanisms

involving hyperlipidemia and abnormalities in arterial function /35/.

1.3.1.2.1 PLASMA LIPIDS, LIPOPROTEINS AND ARTERIAL WALL

Abnormalities of plasma lipids and lipoprotein metabolism in diabetes have long attracted attention due to the association between hyperlipidemia and atherosclerosis /36/ and the presence of even modest elevations in plasma lipids over many years is thought to be a major contributory factor to the development of diabetic macroangiopathy.

In non-diabetic patients, endocytosis of cholesterol-rich low-density lipoprotein (LDL) is thought to influence the development of atherosclerosis by affecting both the rate of accumulation and the rate of removal of tissue cholesterol deposits /37/. The existence of high-affinity LDL receptors on fibroblasts allows these and many non-hepatic cells to be supplied with adequate amounts of cholesterol (required for membrane synthesis) while maintaining the lowest possible level of plasma LDL. Low plasma LDL is critical, because the rate of cholesterol deposition in tissues increases linearly with plasma LDL concentration. When normal LDL receptor function is impaired, compensatory increases in plasma LDL concentrations lead to accelerated atherogenesis. Whether or not diabetes affects LDL levels has been less clear but measurement of lipoprotein levels in a random sample of non-insulin dependent diabetic individuals revealed that very low-density lipoprotein (VLDL) cholesterol levels were

elevated /38/. Insulin-dependent diabetics who are poorly controlled have elevated levels of total triglyceride, VLDL cholesterol, and LDL cholesterol as compared with non-diabetics /39/. LDL that has been modified chemically or biologically can be taken up by distinct "scavenger" receptors on macrophages and results in cholesterol ester accumulation. This is thought to contribute to the pathogenesis of atherosclerosis /40/.

1.3.2 NEUROPATHY

Peripheral neuropathy is a frequent and often disabling complication of DM /4/. Abnormalities of neural function in diabetes, such as slowed nerve conduction, may be related to the metabolic derangements in this disease /42/. Diabetic neuropathy is very complex and can arise in a number of clinically distinct forms; symmetrical sensory neuropathy, acute painful neuropathy, diabetic amyotrophy, mononeuropathy, mixed and sensory neuropathy and autonomic neuropathy.

Diabetic neuropathy is characterised by a variety of morphological changes associated with decreased sensory and motor conduction velocities, including axonal degeneration and segmented demyelination /35/. Although a number of biochemical alterations have been described in diabetic nerve, the ultimate sequence of events leading from insulin deficiency and its attendant hyperglycaemia to the functional and structural manifestations characterising clinical neuropathy remains to be elucidated /35/. Ronald et al. /43/ found that levels of glycosylated

haemoglobin (an index of long term glycaemia) correlated with perineal motor conduction velocity in diabetic patients and their findings suggest that the degree of hyperglycaemia of untreated maturity onset diabetes contributes to the motor nerve conduction abnormalities in the disease.

Sorbitol and fructose are present in normal brain and peripheral nerve tissues /44, 45/ and they fluctuate directly in relation to blood glucose concentration /46/. Ward et al. /47/ proposed that hyperglycaemia resulted in increased intracellular accumulation of sorbitol and fructose which in turn produced an increase in osmolality and subsequent schwann cell damage. Direct evidence for a pathophysiological role of sorbitol and fructose accumulation in diabetic neuropathy is lacking. However, the studies of Gabbay /48/ demonstrated that the slowing of nerve conduction velocity in rats fed on galactose is reduced by simultaneous administration of an inhibitor of aldose reductase, an enzyme which converts sugars to sorbitol. These studies suggest that an increased sorbitol pathway activity (see 1.5.1) may play a role in the pathogenesis of diabetic large vessel disease and peripheral neuropathy /49/.

1.3.3 CATARACTS

Cataracts and the opacity of the occular lens occur earlier and more frequently in patients with diabetes. Dische et al. /50/ were the first to note that the proteins of human cataractous lens contained a greater number of

disulphide bonds than did normal lens, which reflects increased sulphydryl oxidation. Since disulphide bonds can function as intermolecular crosslinks, it has been proposed /51/ that these bonds participate in the polymerisation of lens proteins to form high molecular-weight aggregates.

Another mechanism that may participate in the development of diabetic cataracts involves an intracellular accumulation of sorbitol which is produced from elevated amounts of glucose in the diabetic lens /52/. The lens also contains aldose reductase and operates the polyol pathway (see 1.5.1) in which glucose and galactose are reduced to their corresponding sugar alcohols. It is proposed /52/ that intracellular polyol accumulation also causes osmotic swelling of the lens and hence an accelerated opacification.

1.4 HAEMATOLOGICAL ABNORMALITIES IN DM

DM is not normally considered to be a haematological disorder. The interest in the blood of diabetic subjects is based, however, on the fact that many of the symptoms and complications of diabetes appear to be linked to alterations in blood. The multifactorial pathogenesis of vascular complications of diabetes is widely acknowledged, and in addition to the multiple metabolic and hormonal inbalances, a variety of haemorheologic perturbations also have been described and implicated in the pathogenesis of diabetic vascular diseases /35/. Abnormalities in erythrocytes, polymorphonuclear leukocytes, lymphocytes and

platelets, as well as the fluid phase of coagulation (plasma proteins) have been reported by many investigators. These abnormalities include increased erythrocyte aggregation /53/ and decreased deformability /54, 55/, increased level of glycohaemoglobin /56/, decreased effective levels of 2,3 DPG /57/, increased platelet aggregation /58, 59/, increased blood viscosity /60/ accelerated fibrinogen consumption /61/ decreased levels of antithrombin III /62/, increased levels of von Willebrand factor /63/ and decreased fibrinolysis /64/.

1.4.1 ERYTHROCYTES

Erythrocyte factors and plasma protein factors have been proposed to contribute to the increased viscosity seen in the blood of diabetic patients /55, 60/. Increased viscosity and decreased deformability are seen in red cell membranes obtained from diabetic patients. Factors in the erythrocyte that have been implicated in these changes, include changes in sialic acid content /65/, protein abnormalities /66, 67, 68/ and phospholipid abnormalities /69, 70, 71, 71a/.

Abnormalities in red cell deformability could be expected to decrease red cell survival. Erythrocyte survival studies have shown a decrease in red cell survival when patients were hyperglycaemic and when hyperglycaemia was corrected in these patients, red cell survival returned to normal /72/. Since red cells must change shape in order to pass through the capillary bed, considerable interest has been focused on the possibility that the diabetic

state might impair red cell deformability and that stiffened red cells might mechanically traumatise capillaries, thereby contributing to diabetic micro-angiopathy.

1.4.2 LEUKOCYTES

In the era before antibiotics and insulin were available, it was generally accepted that patients with diabetes were more susceptible to staphylococcal, streptococcal and tubercule bacillus infection /73, 74/. It was also generally accepted that in these patients the prevalence of urinary tract infection and bacteriuria was higher. Such observations stimulated investigations of the polymorphonuclear leukocyte in order to determine if a granulocyte abnormality might be present in the hyperglycaemic subject.

The major role of the leukocyte is to protect the host against bacterial infection. To do this effectively, these cells must be present in adequate numbers, must be able to adhere to a surface and move to a site of infection (chemotaxis), must be able to engulf particles (phagocytosis) and must be able to kill ingested organisms /75/. Gilbert *et al*. /76/ reported abnormal responses to the administration of Pseudomonas aeruginosa endotoxin in patients with diabetes. In this study, patients with DM showed a greater decrease in leukocyte cell counts in response to endotoxin than did controls. Further studies have also documented an abnormality in leukocyte adherence in patients with diabetes mellitus. It was

shown that in diabetic patients who were out of control but not acidotic, there was a decrease in leukocyte adherence as compared with that in the controls. The defect, however, was normalised when glucose concentrations were corrected /77/.

Abnormalities in random migration, chemotaxis, phagocytosis and bacterial killing have all been detected in leukocytes obtained from patients with diabetes /78/. Abnormalities of chemotaxis *in vitro* have been reported to subside when insulin has been added to the medium in physiological concentrations /79/, although an *in vivo* effect of insulin administration has not been found. However corrections of abnormalities of leukocyte migration and bacterial killing have also been reported following glucose normalisation in patients with diabetes mellitus /80/. The involvement of membrane fluidity in these processes suggests that membrane defects could account at least in part for the decreased resistance in infection in diabetic patients.

1.4.3 PLATELETS

Platelets play a central role in regard to aspects of normal haemostasis. They initiate haemostasis through their properties of adherence to vascular or foreign surfaces and aggregation with one another. It is thought that the metabolic changes that occur in diabetics could influence the *in vitro* behaviour of platelets. The contribution of platelets to vascular sequelae of diabetes is not quite clear but studies in animals have documented

early increases in platelet adhesiveness and aggregation in rabbits and rats treated with alloxan or streptozotocin /81, 82/. The early appearance of lesions makes it unlikely that vascular disease causes the platelet changes, but rather they could be due to viscosity or metabolic alterations that occur in hyperglycaemic animals. However, it is also possible that platelet abnormalities in diabetes are secondary to the vascular complications concomitant with the disease and do not participate in the genesis of vascular lesions.

It is possible that the metabolic changes that occur in diabetes could influence the _in vitro_ behaviour of platelets. Platelet aggregation increases in the presence of free fatty acids /83/ and cholesterol /84, 85/. Concentrations of cholesterol and triglycerides have been shown to be correlated with the decrease in hyperglycaemia as reflected by glycosylated haemoglobin concentrations in patients with diabetes /86/, and platelets are hyperaggregable in certain states of hyperlipoproteinaemia as well as when imbalance occurs in the ratio between saturated and unsaturated fatty acids in the diet /87, 88/. Fuller _et al_. /89/ have reported reduced platelet counts in maturity-onset diabetic subjects, suggesting increased consumption of these platelets and their involvement in a hypercoagulable state. These changes have been implicated as potential causative factors in the vascular disease that accompany diabetes.

1.4.4 LYMPHOCYTES

The major role of the lymphocytes is to provide a surveillance and destruction mechanism against foreign antigens, whether circulating or cell-associated. Certain lymphocytes may be relatively long-lived, making them potentially vulnerable to biological or chemical changes. Several metabolic abnormalities have been detected in lymphocytes obtained from diabetic patients in poor metabolic control. The lymphocyte of the diabetic subject has been reported to show decreased synthesis of citrate and this defect has also been shown to be corrected by insulin /90/. The relevance of these metabolic alterations in lymphocyte function has not been clarified. Nevertheless, most of the abnormalities are corrected when the serum glucose concentration is normalised /91/.

There is some clinical evidence to suggest that abnormal lymphocyte function in patients with diabetes may be significant. The resistance to staphylococcal sepsis is in part cell-mediated /92/, and the rate of mortality among patients with diabetes who have staphylococcal sepsis is increased compared to that in controls /93/. Also micromycosis occurs in patients with diabetes who are in poor control or in ketoacidosis /94/.

1.4.5 PLASMA PROTEINS

Vascular lessions in diabetes and atherosclerosis contain large deposits of fibrin, the ultimate product of coagulation /95/. Certain plasma proteins have long been known to participate in the coagulation process. These

coagulation proteins which are termed "factors" and designated by Roman numerals, participate in two mechanisms that lead to the formation of a fibrin clot. These mechanisms, called the intrinsic pathway and extrinsic pathway play important roles in haemostasis. Both pathways involve reactions with plasma proteins leading to the formation of thrombin which can cleave fibrinogen to fibrin.

When normal blood is constantly flowing, little or no intravascular coagulation occurs and the concentrations of coagulation factors remain reduced because of dilution. Also numerous natural inhibitors of coagulation enzymes circulate in the blood. These inhibitors, such as antithrombin III and α_2-macroglobin, bind to the proteolytic coagulation factors such as thrombin /96/ and thus terminate their participation in the clotting process. Bannerjee et al. /97/ found significantly lower values for plasma thrombin clotting time, indicating hypercoagulability in patients with maturity-onset diabetes mellitus as compared with the values in normal subjects. However, in contrast, they found no difference in plasma thrombin clotting time values between normal subjects and patients with juvenile-onset diabetes mellitus /98/ suggesting that the presence of macrovascular disease is more prevalent in maturity-onset diabetes and may be a differentiating factor.

1.4.5.1 FIBRINOGEN

Fuller et al. /89/ demonstrated that fibrinogen concentrations in diabetic patients are significantly

increased above those of normal controls and even more significantly elevated in diabetic patients with demonstrable vascular disease /99/. Chakrabarti et al. /100/ studied the course of plasma fibrinogen concentrations during treatment of diabetes and found a significant decrease in fibrinogen concentrations which suggests a reversible phenomenon. The work of Coller et al. /101/ demonstrated a significant correlation between fibrinogen concentrations in plasma and the degree of glycosylated haemoglobin in serum, again suggesting that hyperfibrinogenemia is associated with the level of glycaemia. The increase in fibrinogen has been shown to raise the plasma viscosity, thus increasing the flow resistance of whole blood /102/. The alteration in plasma viscosity is thought to play a role in the pathogenesis of vascular disease.

Jones and Peterson /61/ studied the survival of fibrinogen in diabetics with varying degrees of vascular disease and serum glucose control and found reduced fibrinogen survival when compared with normals. They also demonstrated reversal of the phenomenon when euglycaemia was achieved. These results suggest that the fibrinogen molecule is not altered functionally and that an abnormal plasma or vascular environment is a more likely basis for reduced fibrinogen survival during hyperglycaemia. Correction by Lepasin, however, suggests that reduced fibrinogen survival in this instance is due to the action of thrombin or one of its antagonists, thereby providing indirect evidence for a hypercoagulable state.

1.4.5.2 α_2-MACROGLOBULIN

The concentration of α_2-macroglobulin was found to be increased, and this increase was related to the presence and degree of vascular complications, in diabetic patients /89/. Further evidence was provided by Almer et al. /103/ who also demonstrated increased concentrations of α_2-macroglobulin and suggested a correlation with vascular disease. The increase in α_2-macroglobulin was also found to favour red cell aggregation /103/, thus contributing to diabetic microangiopathy.

1.4.5.3 ANTI-THROMBIN III

This is one of the major regulator proteins of the coagulation systems. It inhibits thrombin, plasmin and plasma factor Xa and it is known to exhibit heparin cofactor activity /104, 105/. Antithrombin absorbs thrombin released during clot formation and blocks the effect of this thrombin on fibrinogen, thus playing an important regulatory role in haemostasis. Deficiency of this protein has been reported in maturity-onset diabetes, and may contribute to a state of increased coagulability /106, 107/. However Corbella et al. /108/ demonstrated increased antithrombin concentrations in diabetic children without vascular disease and suggested that this could be due to an early defence response against the activation of the blood clotting system.

In summary, several haematological abnormalities have been described in diabetic patients, and these changes include abnormal rheology, abnormal leukocyte and lymphocyte

functions, platelet aggregation abnormalities and problems in the fluid phase of coagulation (plasma proteins). Many of these abnormalities are reversible when carbohydrate control is optimised in hyperglycaemic subjects. These findings have definite implications regarding optimum glucose concentrations in diabetic patients who are faced with infection, surgery or wound healing. However, the basis of thse haematological abnormalities and their true clinical relevance remain to be determined.

1.5 THE BIOCHEMICAL BASIS OF DIABETIC COMPLICATIONS

1.5.1 THE ROLE OF THE POLYOL PATHWAY

This biosynthetic pathway, (Fig. 1) also termed the sorbitol pathway, consists of two reactions. Glucose is first reduced to its corresponding sugar alcohol, sorbitol, by the enzyme aldose reductase, with NADPH as the electron-donating coenzyme. The sorbitol molecule is then oxidised to fructose by the enzyme sorbitol dehydrogenase and NAD^+.

$$\text{D-Glucose} + \text{NADPH} + H^+ \xleftrightarrow{\text{Aldose Reductase}} \text{Sorbitol} + \text{NADH}$$

$$\text{Sorbitol} + NAD^+ \xleftrightarrow{\text{Sorbitol Dehydrogenase}} \text{D-Fructose} + \text{NADH} + H^+$$

Significantly elevated concentrations of both sorbitol and fructose have been reported in several tissues of diabetic animals and man, including lens, retina, arterial wall and schwann cell sheath /109, 110, 111, 112/. The activity of this pathway is regulated by the ambient glucose concentration in those tissues not requiring

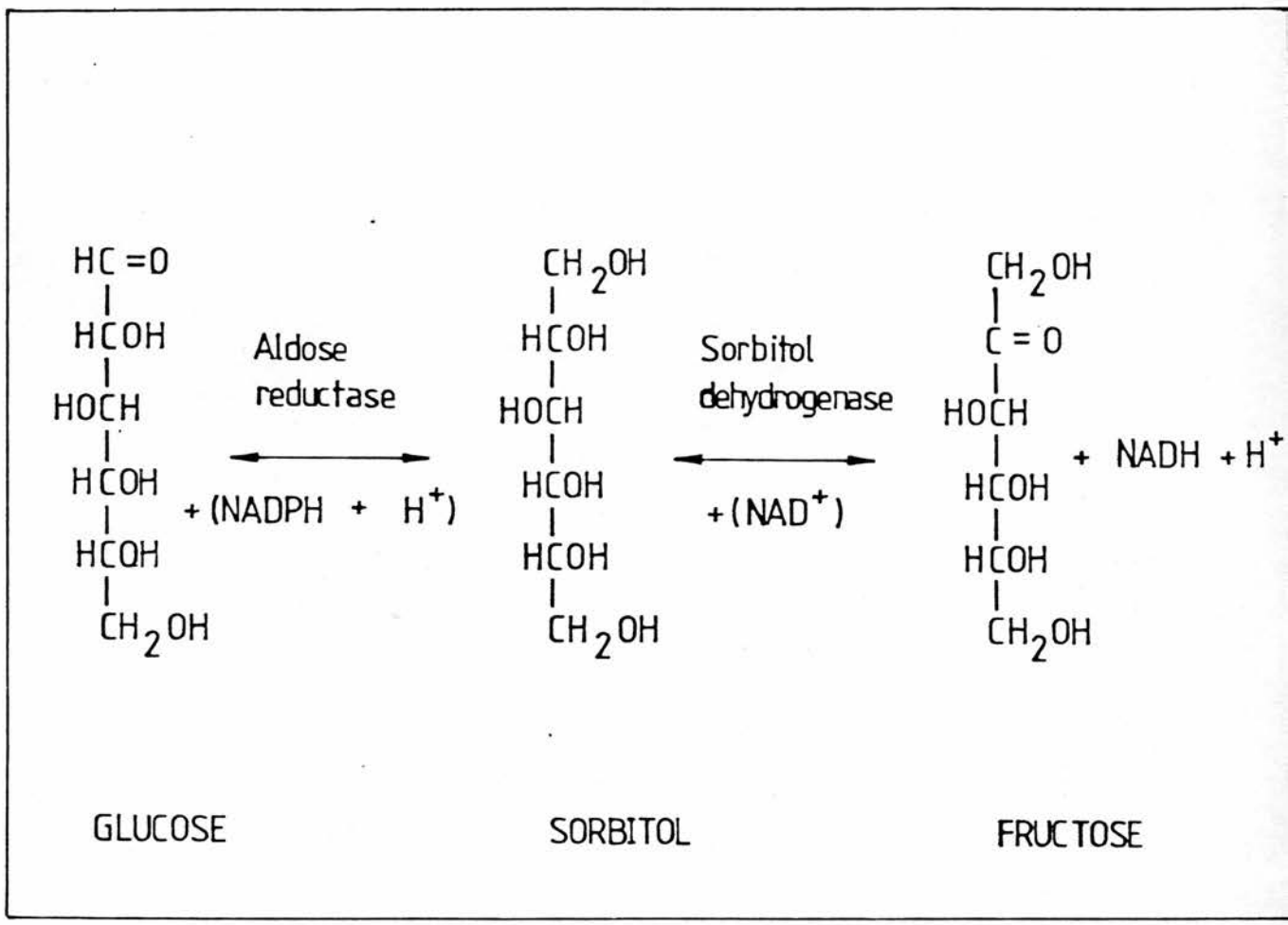


Fig. 1 THE POLYOL PATHWAY.

insulin for glucose transport, such as nerve /113/, since the Km for aldose reductase lies in the range of physiological concentrations of glucose.

Elevated amounts of glucose would result in an intracellular accumulation of sorbitol, since polyols do not readily diffuse across cell membranes. Intracellular accumulation of sorbitol causes osmotic swelling and eventual disruption of cell architecture. Increased sorbitol activity has been implicated in the formation of galactosaemic and diabetic catarracts /52/. It is also thought to play a role in the pathogenesis of peripheral neuropathy /49/. However further discussion of the role of the polyol pathway is outwith the scope of the thesis.

1.5.2 THE ROLE OF NON-ENZYMATIC GLYCOSYLATION (NEG)

1.5.2.1 CHEMISTRY AND KINETICS OF NEG

The non-enzymatic glycosylation reaction, also called the Maillard or Browning reaction /114/ is described as the chemical reactions occuring between reducing sugars and primary amino compounds (Fig. 2). The NEG reaction is very complex, and evolves through different successive or parallel chemical pathways /115/ that can be grouped into two simplified steps. The first step includes the preliminary formation of an unstable aldimine (Schiff base) which can regenerate the precursor molecules. The second step is the formation of a more stable ketoamine (Amadori Compound) by the intramolecular rearrangements of aldimine. The ketoamine is the most important step of this reaction as it is the precursor of all later products.

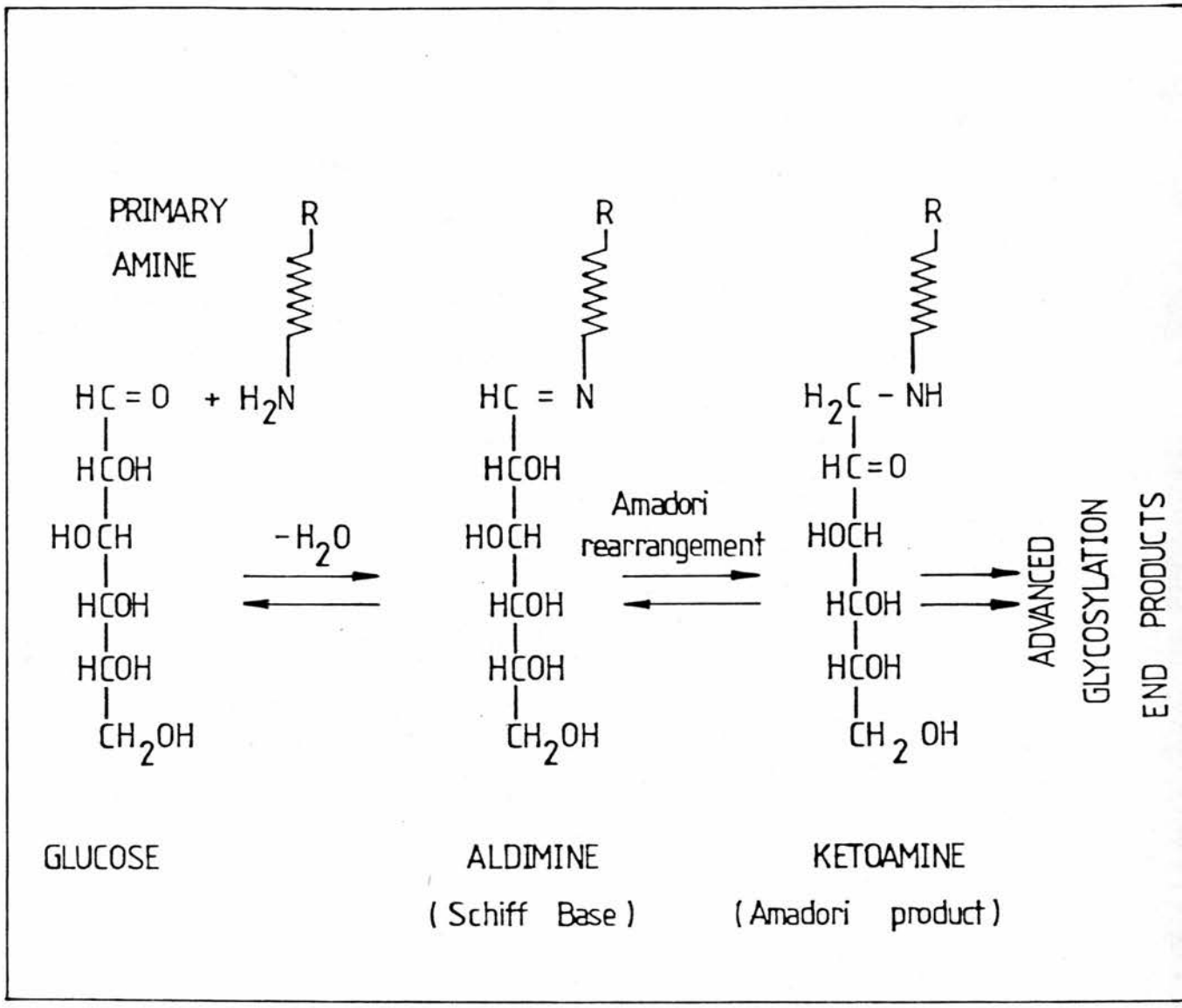
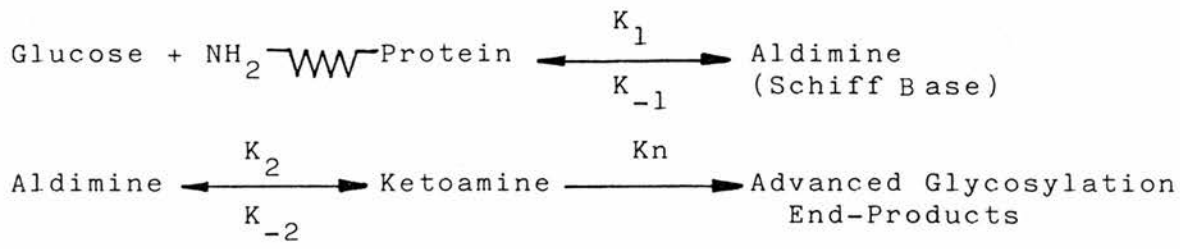


Fig. 2 THE MAILLARD OR BROWNING REACTION.

(MAILLARD L.C. 1912)



Higgins et al. /116/, using human haemoglobin as a model protein have shown that the rate of Schiff base formation (K_1) is approximately equal to the rate of dissociation (K_{-1}). They also demonstrated that over a period of weeks, a slow chemical rearrangement of the Schiff Base occurs, the rate K_2 being approximatley equal to 1.6% of K_1, which results in the accumulation of a stable but chemically reversible sugar-protein adduct, the Amadori product.

Recent chemical studies /117/ demonstrate that the Amadori product undergoes further dehydration, rearrangement and cleavage reactions. The final products in vitro /118, 119/ are highly cross-linked (Fig. 3), insoluble pigmented and fluorescent carbohydrate-protein polymers, termed "advanced glycosylation end products" or melanoidins (hence the Browning reaction). In contrast to the Amadori product, these adducts, once formed, are irreversible, and continue to accumulate indefinitely.

1.5.2.2 THE PATHOPHYSIOLOGICAL CONSEQUENCES OF NON-ENZYMATIC GLYCOSYLATION

NEG has long been recognised and its potential significance in clinical medicine is widely appreciated. Initially, NEG attracted the attention of investigators

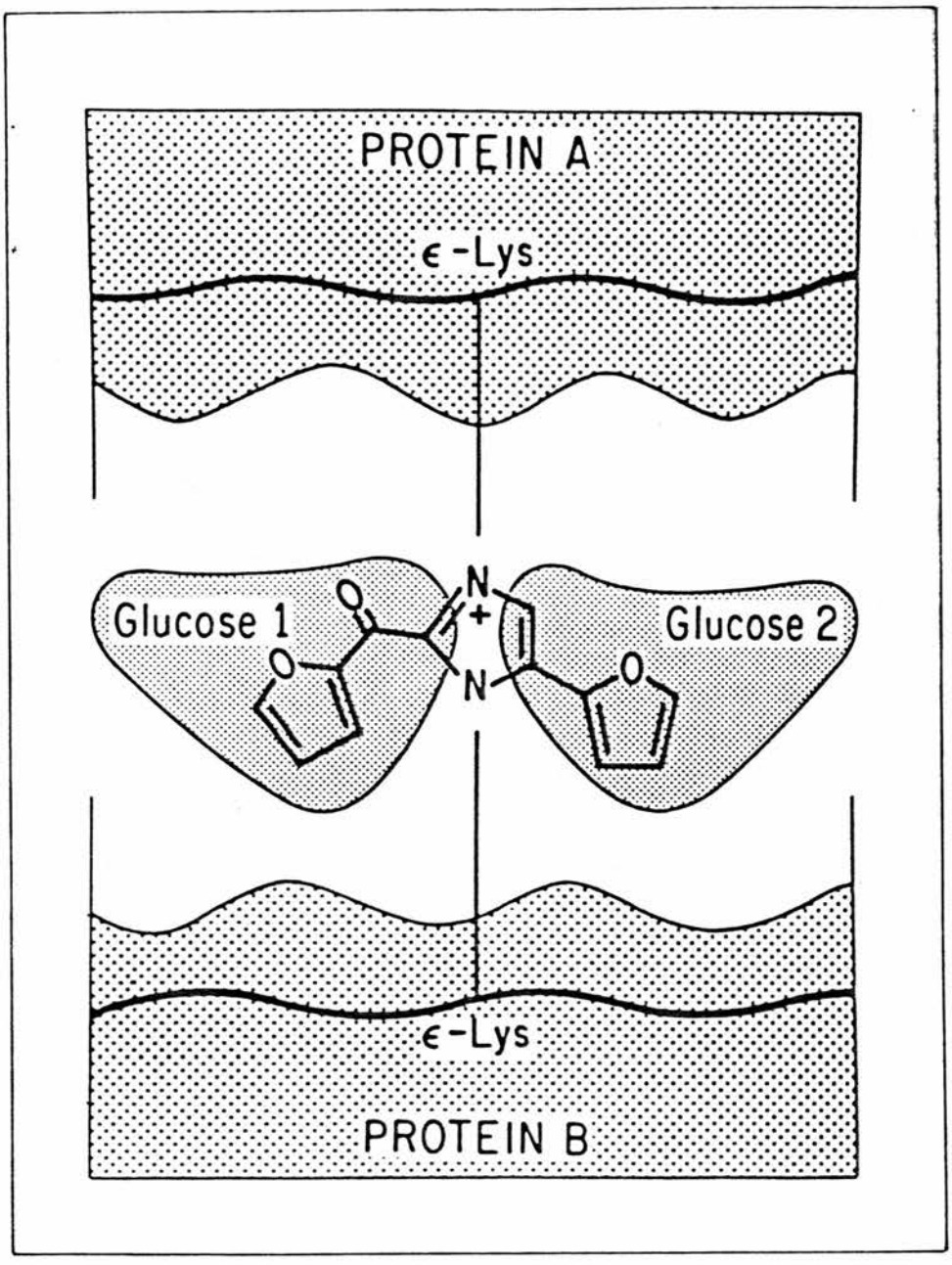


Fig. 3 Glucose-derived protein cross-link (furoyl-furanyl imidazole), a condensation product of two glucose molecules and two lysine-derived amino groups.

(Reynolds T. M. 1965)

interested in the management of DM following the demonstration that haemoglobin undergoes post-ribosomal NEG *in vivo* /120/, that the proportion of total haemoglobin that is glycosylated is increased in diabetic patients with attendant hyperglycaemia /121/, and that measurement of the percentage of glycosylated haemoglobin in an individual patient could reflect ambient glucose concentration over an integrated period of time /122, 123/. Subsequent reports that other proteins such as albumin /124, 125/, erythrocyte membrane proteins /126, 127, 128/, lens crystallins /129, 130/, plasma proteins /131, 132/ basement membranes and collagens /133, 134/ and nerve myelins /135/ are also subject to glycosylation under physiological conditions, helped support the hypothesis that this might be a model reaction relevant to the pathogenesis of certain complications of chronic diabetes.

There is an increasing body of evidence based on both clinical experience with patients /136/ and animal model studies /137/, which indicates that chronic hyperglycaemia may be the major factor contributing to the long-term complications of diabetes. The manner in which excess NEG could contribute to the development of diabetic complications is undefined, although there is considerable speculation and some experimental evidence supporting the hypothesis that this covalent structural modification may alter functional properties of involved proteins, lipoproteins and nucleic acids.

1.5.2.2.1 NON-ENZYMATIC GLYCOSYLATION OF PROTEINS

With definitive evidence that glucose itself can chemically alter proteins, the idea that NEG of haemoglobin was a model reaction relevant to the pathogenesis of various long-term complications of diabetes gained popularity. The list of proteins that undergo NEG in patients or animals with diabetes mellitus is still growing (Table 1).

1.5.2.2.1.1 ERYTHROCYTE PROTEINS

Haemoglobin is the best studied example for which there is conclusive evidence that the level of glycosylated form (HbA_{1c}) is significantly elevated /123/, that the cumulative amount of HbA_{1c} is directly proportional to the ambient glucose concentration during the period before the red cell sample is obtained /138/, and that the percent of total haemoglobin represented by HbA_{1c} increases during periods of poor diabetic control and diminishes in response to optimization of diabetic control /139/.

Haemoglobin is glycosylated at both intra-chain lysine and amino-terminal valine residues /140, 141/, but the primary site of glycosylation of HbA_{1c} is the valine residue at the terminus of the β-chain which is the binding site for 2,3,diphosphoglycerate (2,3,DPG), the physiological regulator of haemoglobin's oxygen affinity. In vitro, glycosylated haemoglobin have been shown to have altered oxygen saturation curve and decreased sensitivty to the allosteric effects of organic phosphate /142/. However, in vivo, the higher oxyen affinity of haemoglobin in diabetic patients appears to be independent of the state of glycosylation of haemoglobin /143/ and whole blood

PROTEIN	PHYSIOLOGIC FUNCTION
Hemoglobin	Oxygen exchange
Red cell membrane	Deformability in microvasculature
Antithrombin III	Inhibition of excessive coagulation
Fibrinogen	Plasma viscosity and clot formation
Fibrin	Clot maintenance
Endothelial cell membrane	Maintenance of vascular integrity
Lens crystallins	Transmission of light to retina
Lens capsule	Focusing of light on retina
Myelin	Nerve impulse conduction
Tubulin	Axonal transport
Glomerular basement membrane	Renal filtration barrier
Collagen	Tissue structural properties; scar and plaque formation
Coronary artery proteins	Vessel integrity for myocardial perfusion
Low density lipoprotein	Lipid transport and metabolism
High density lipoprotein	Lipid transport and metabolism
Albumin	Osmotic regulation; transport of metabolites
Cathepsin B	Intracellular protein degradation
Beta-NAc-D-glucosaminidase	Glycoprotein sugar removal
Pancreatic RNase	Hydrolysis of RNA
Ferritin	Iron storage

TABLE 1. Nonenzymatic Glycosylation of Proteins

oxygen saturation curves for normal and diabetic populations are essentially identical /144, 145/. It is therefore unlikely that glycosylation of haemoglobin has any pathological effects.

An erythrocyte (Figure 4) can pass through vessels smaller than its own diameter because of its ability to alter its discoid shape. Normally 8μm in diameter, it is capable of passing through glass tubes whose diameter is only 3μm /146/. Following passage, the red cell returns quickly to its discoid shape. This property is normally referred to as deformability. Reduced deformability of red cells has previously been demonstrated to occur in thalassaemia, sickle cell disease, spherocytosis /157/ and diabetes /55/. Observations of reduced deformability of diabetic erythrocytes /55/ and in low shear-rate viscometric flow /148/ have suggested that the increased viscous resistance of these erythrocytes to cell deformation might be responsible. Because the viscous properties of diabetic haemoglobin have been found to be normal /149/, the diabetic erythrocyte membrane is thought to be responsible for reduced deformability.

Evans et al. /150/ suggested that the elastic properties of the erythrocyte membrane are produced by the spectrin-actin complex (Figure 5), a protein network located at the inner surface of the plasma membrane, and evidence from Lux et al. /151/ suggested that this network may be responsible for restoring the discoid shape of the red cells.

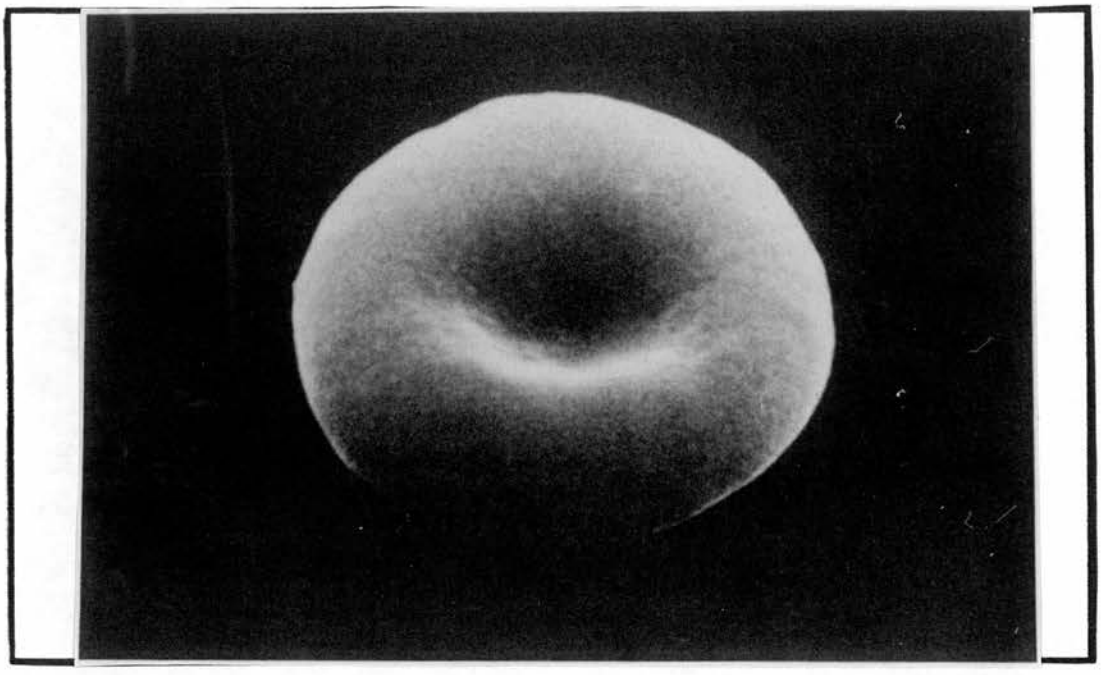


Fig. 4 The normal human erythrocyte (diameter: 8 µm)

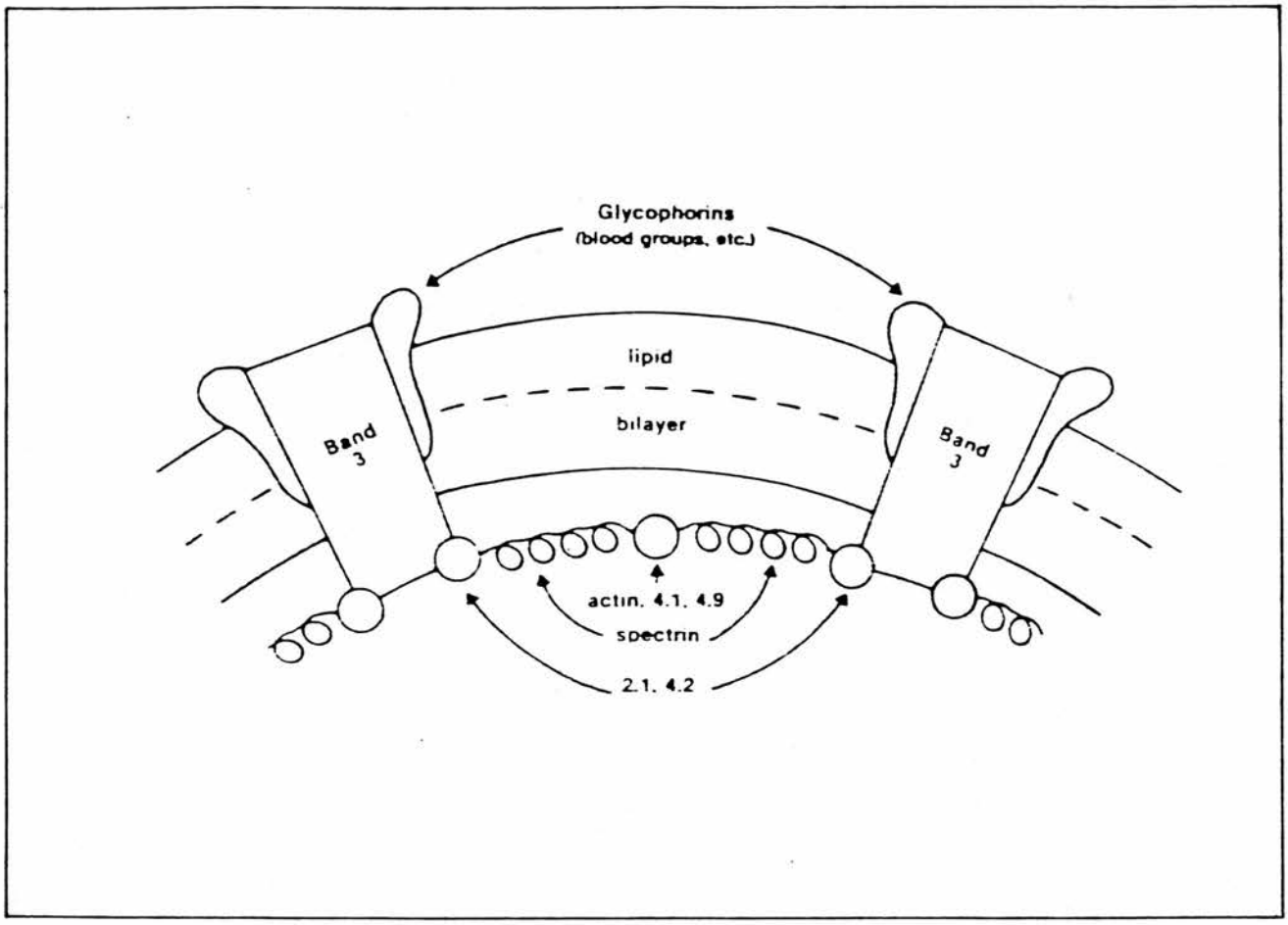


Fig. 5 Organization of the protein structures responsible for the elastic behaviour of the erythrocyte membrane. (Evans and Hochmuth 1976).

Red cell membrane proteins are also subject to increased NEG in diabetes /126, 127, 152/. McMillan et al. /153/ recently demonstrated increased glycosylated spectrin in diabetic red cells and suggested that this may be responsible for the increased number of poorly deformable erythrocytes found among aged diabetic erythrocytes. It is thought, however, that other factors such as pH, changes in the lipid composition of the membrane /69, 70, 71, 71a/, lipid-protein interactions or a direct insulin effect /154, 155/, may also be involved.

1.5.2.2.1.2 PLASMA PROTEINS AND LIPOPROTEINS

Although deposition of albumin and immunoglobins along basement membranes in the kidney /156/, and skin /157/ is a common finding in diabetes, there is no evidence to suggest that this could be due to NEG of either protein. However, alterations in drug /158/ and bilirubin /159/ binding capacity in vitro have been reported for glycosylated albumin.

Heparan sulphate (heparin) in the blood normally enhances the rate of inhibition of thrombin activity by anthrombin III. Glycosylation of antithrombin III produces a significant decrease in thrombin-inhibition activity in vitro /160/. Inhibition of heparin binding to human antithrombin III in hyperglycaemic patients may result in a transient functional deficiency of the protein. This could explain the significant in vivo inhibition of biological function of human antithrombin III that occurs in both insulin-dependent /161/ and non-insulin dependent

/162/ patients. The inhibition of heparin-catalysed antithrombin III activity by NEG, in conjunction with glycosylation-induced changes in susceptibility of fibrin to degradation by plasmin /163/, could play a role in the abnormal accumulation of fibrin reported to occur in several diabetic tissues affected by long-term vascular complications /164/.

Glycosylation of low-density lipoprotein (LDL) has been shown to alter its rate of uptake and degradation by cultured human fibroblasts /165, 166/. These effects resulted from inhibition of LDL binding to the high-affinity receptor involved in regulation of cholesterol metabolism, suggesting a possible defect in receptor-dependent catabolism of LDL and an increased requirement for LDL catabolism by endothelial cells and macrophages in diabetes.

In contrast to LDL, catabolism of high density lipoprotein (HDL) appears to be accelerated by glycosylation /167/, but the effect was demonstrable only after extensive modification of the protein. It is thought that the slightest increase in glycosylation of these proteins in diabetes could have a marginal effect on their catabolism, sufficient to induce the gradual development of vascular disease.

1.5.2.2.1.3 NERVE PROTEINS (MYELINS)

Increased glycosylation of myelin and myelin associated proteins has been reported in both the peripheral and central nervous systems in diabetics /135, 168/. One

hypothesis is that glycosylation of myelin may yield products which act as signals for recognition and degradation by macrophages, thus inducing excessive myelin turnover and demyelination in diabetics. Increased glycosylation of intracellular tubulins has also been reported in diabetic rat brain /169/, thus glycosylation could also affect microtubule-dependent processes in neural tissues.

1.5.2.2.1.4 LENS PROTEINS (CRYSTALLINS)

According to Chiou et al. /170/ NEG does not appear to be a primary factor in the formation of experimental cataracts, since drug-induced diabetic rats fed with aldose reductase inhibitor (sorbitin) did not develop cataracts. However, Liang et al. /171/ observed that incubation of bovine α-crystallins with sugars led to a change in the tertiary configuration of α-crystallins noted by an increase in exposed sulphydryl groups. This was supported by observations made by Ansari et al /172/ who demonstrated increased disulphide-linked proteins in diabetic lens as compared with lens from patients with cataracts. These observations suggest a possible link between glycosylation and sulphydryl oxidation. The increased oxidation of sulphydryl groups has been attributed to a loss of glutathione which occurs as a consequence of osmotic shock and membrane rupture /173/.

Thus it appears that although NEG of crystallins is accelerated in the diabetic lens, its precise role in exposure of sulphydryl groups remains to be elucidated.

Evidence, however, seems to indicate that alterations in the sorbitol pathway (1.5.1) are more significant than increased NEG of the protein.

1.5.2.2.1.5 EXTRACELLULAR MATRIX PROTEINS

Increased glycosylation of diaphragmatic tendon and skin collagens have been demonstrated in diabetic and aged humans /174/, and in drug-induced diabetic rats, similar effects were noted with aortic and glomerulular basement membrane collagens /175/. The increased glycosylation of collagen *in vivo* has been correlated with decreased solubility, elasticity and sensitivity to protease digestion /176/ and increased thermal stability /177/, all of which suggests increased cross-linking of collagen in diabetes. Enhanced glycosylation and cross-linking of collagen by glucose provides an explanation for many of the alterations in the extracellular matrix in diabetes, including basement membrane thickening and stiffening of connective tissue.

Binding of plasma protein constituents such as albumin and immunoglobulins (Fig. 6) to glycosylated perivascular structural proteins may be enhanced *in vivo* by the increase in vascular permeability associated with diabetes in both animals and humans. Accumulation of collagen-related proteins in the glomerular extravascular matrix is the central pathological alteration that characterises diabetic nephropathy. Continual accumulation over many years ultimately results in progressive renal failure due to progressive glomerular capillary occlusion.

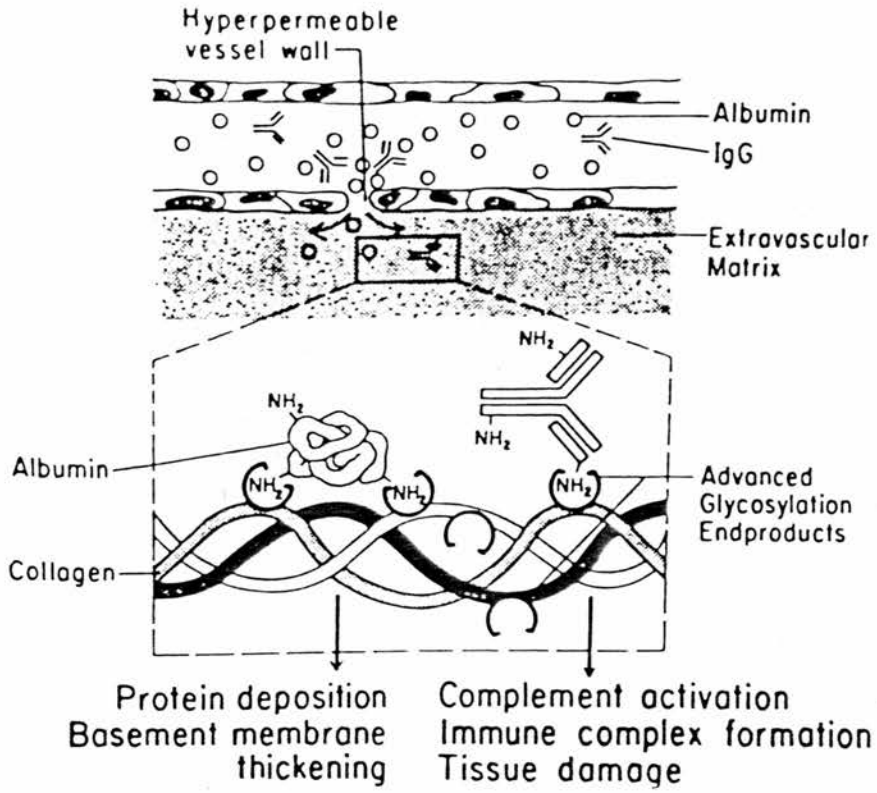


Fig. 6 Covalent trapping of plasma proteins by advanced glycosylation end-products on collagen. (Brownlee, Cerami Vlassara 1984).

1.5.2.2.2 NON-ENZYMATIC GLYCOSYLATION OF NUCLEIC ACIDS

It has recently /178/ been shown that deoxyribonucleic acid (DNA), like proteins and lipoproteins can react with glucose via free amino groups and at a much faster rate with glucose-6-phosphate, with resultant abnormalities of DNA template function.

Because nucleic acids are long-lived molecules in the resting cell, *in vivo* "advanced glycosylation end-products" would progressively accumulate on DNA over time. Such accumulation may be responsible for age-dependent changes in the genetic material that include chromosomal aberrations, DNA strand breaks, and a decline in DNA repair, replication, and transcription /179, 180/. Acceleration of this process by diabetic hyperglycaemia would result in an earlier onset of cellular senescence. The decrease in diabetic fibroblast replicative capacity resembling that associated with normal aging /181/ may be an example.

It is thought that the exposure of the early embryo to high glucose concentrations could lead to an increased reaction of glucose or a glucose metabolite with DNA at critical developmental stages, causing chromosomal breaks and mutagenesis. It is therefore possible that the increased frequency of congenital abnormalities in children of diabetic mothers may be partly due to non-enzymatic glycosylation of nucleic acids.

1.6 AIMS OF RESEARCH PROJECT

From the preceding section, it is apparent that there is no incontrovertible evidence to show that NEG of proteins, lipoproteins and nucleic acids, is a primary factor in the pathogenesis of certain diabetic complications despite considerable circumstantial evidence to this effect. Evidence for glycosylation of erythrocyte membrane proteins /126, 127, 152/, particularly spectrin /153/ and spectroscopic studies on erythrocyte membranes /67, 68/ suggested that this may influence membrane organisation and function, and therefore may play a role in the abnormal deformability, increased aggregation, reduced microviscosity, and reduced cell survival associated with diabetic red cells.

The currently accepted model of the erythrocyte membrane is a mosaic-like structure (Figure 7) in which phospholipids and cholesterol are constrained by repeating protein subunits which are probably linked together both within the membrane and at its cytoplasmic surface to spectrin, thus providing the membrane-associated protein skeleton upon which the material properties of the red cell largely depend. The combined phospholipid-protein interaction confers the discoid shape of the unstressed cell. The contribution of the predominantly fluid lipid to the stability of the protein net-work is not fully understood, yet major alterations in either component may

influence the morphology of the cell as in cholesterol overload. Early reports suggested that specific diseases such as abeta-lipoprotaemia, anaemia and haemoglobinuria produced alterations in erythrocyte lipids.

Since NEG specifically occurs with free primary amino compounds including integral proteins of erythrocyte membranes, one might expect that those amino phospholipids (phosphatidyl ethanolamine and phosphatidyl serine) present on the erythrocyte and other membranes (Figures 8a and 8b) could also become glycosylated in the diabetic state and this may also play an important role in the abnormal physical properties of the diabetic erythrocyte thereby contributing to diabetic microangiopathy. So far, the possibility that these phospholipids could become glycosylated has not been investigated.

A recent cross-sectional pilot study by Hunter et al. /71a/ in our laboratory has demonstrated an abnormal composition in diabetic erythrocyte phospholipids. It was found that phosphatidyl ethanolamine (PE) was significantly reduced and phosphatidyl serine (PS) was significantly increased in poorly controlled diabetics. Sphingomyelin was found to be more significantly reduced in female diabetic subjects. These findings, however, raised the possibility that the phospholipid changes may be a feature of diabetes mellitus in many tissues and may have considerable functional consequences.

It is rather interesting to speculate on, and investigate the origin of these phospholipid abnormalities.

Since erythrocytes are known not to possess the enzymatic machinery for either de novo phospholipid biosynthesis or phospholipid class interconversion, and therefore altered phospholipid composition can only result during erythropoiesis, or from extracellular exchange, or by non-enzymatic mechanisms.

The work of Hunter et al. /71a/ also suggested that PE can become glycosylated and that glycosylated PE co-chromatographed with PS on thin layer chromatography (TLC). This raises the possibility that the reduced PE and/or increased "PS" may be indicative of glycosylation of PE in diabetes. The main aims of this work were therefore:

I To extend the data for the cross-sectional study of erythrocyte phospholipid composition in diabetes.

II To carry out a longitudinal study of phospholipid composition in patients exhibiting changing degrees of glycaemic control before and after therapeutic intervention.

III To synthesise and characterise model glycosylated amino phospholipids.

IV To demonstrate non-enzymatic glycosylation of PE and PS of erythrocyte membranes in vitro.

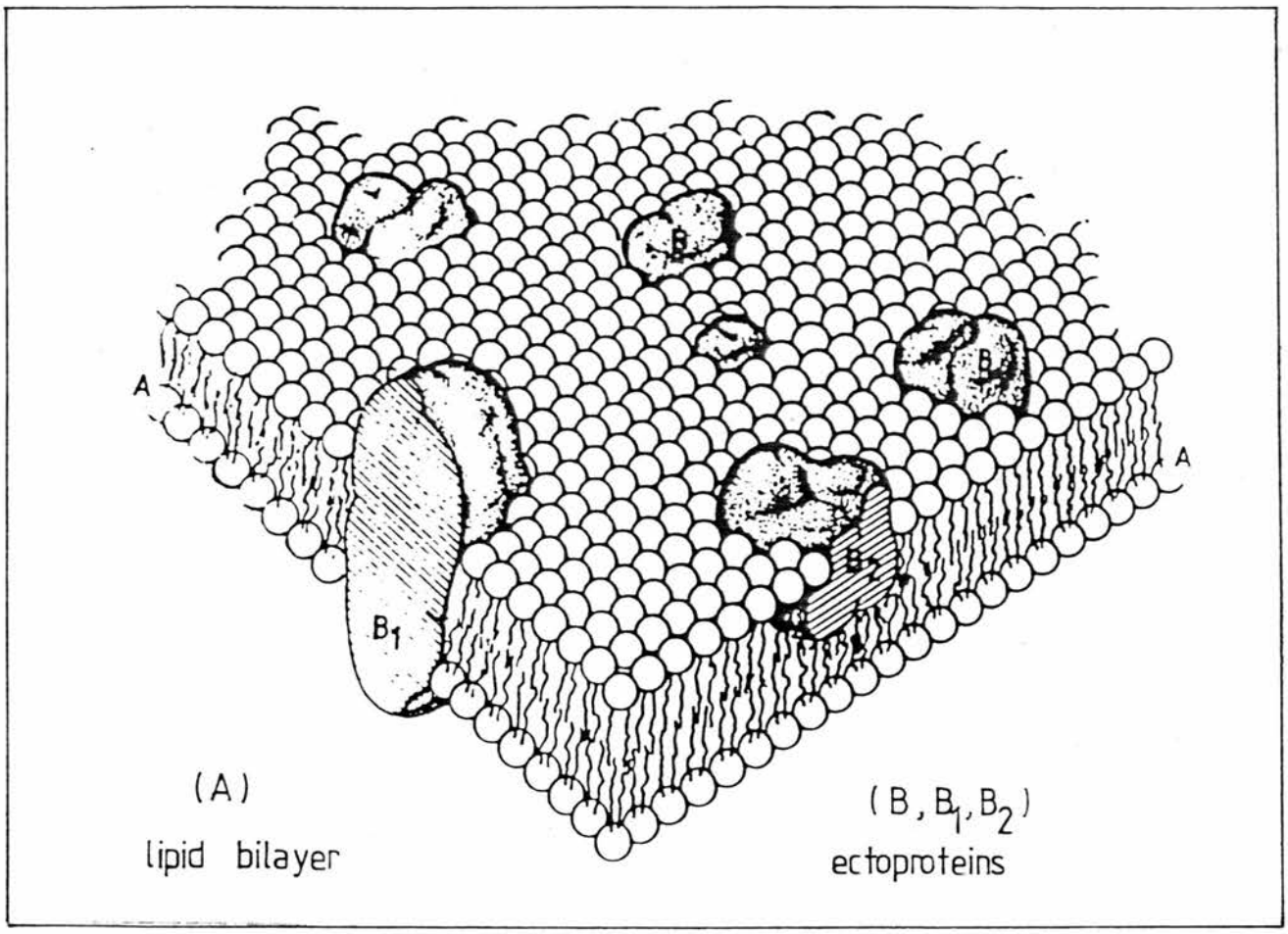
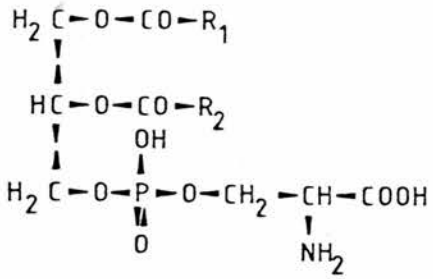


Fig. 7 The fluid mosaic model of membrane structure.

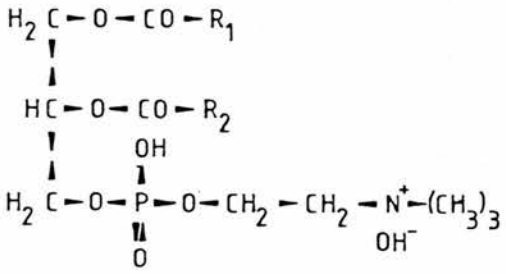
(Singer and Nicolson 1972).

PHOSPHATIDYL SERINE



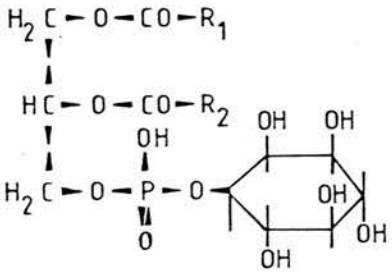
(molecular wt. 313·2 $+R_1$ $+R_2$)

PHOSPHATIDYL CHOLINE



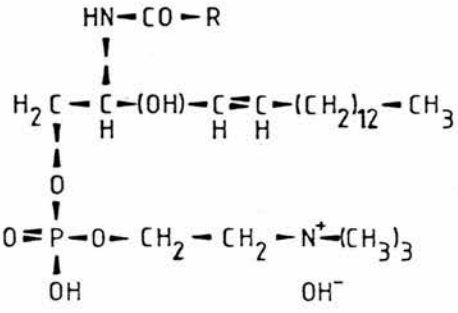
(molecular wt. 329·3 $+R_1$ $+R_2$)

PHOSPHATIDYL INOSITOL



(molecular wt. 388·2 $+R_1$ $+R_2$)

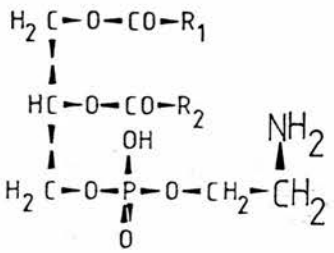
SPHINGOMYELIN



(molecular wt. 509·6 $+R$)

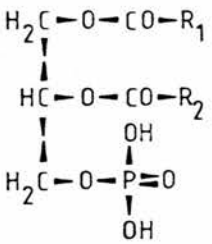
Fig. 8a LIPIDS IN BIOLOGICAL MEMBRANES.

PHOSPHATIDYL ETHANOLAMINE



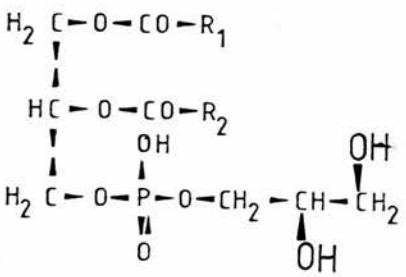
(molecular wt. 269·2 + R_1 + R_2)

PHOSPHATIDIC ACID



(molecular wt. 226·1 + R_1 + R_2)

PHOSPHATIDYL GLYCEROL



(molecular wt. 300·1 + R_1 + R_2)

NON-ENZYMATIC GLYCOSYLATION

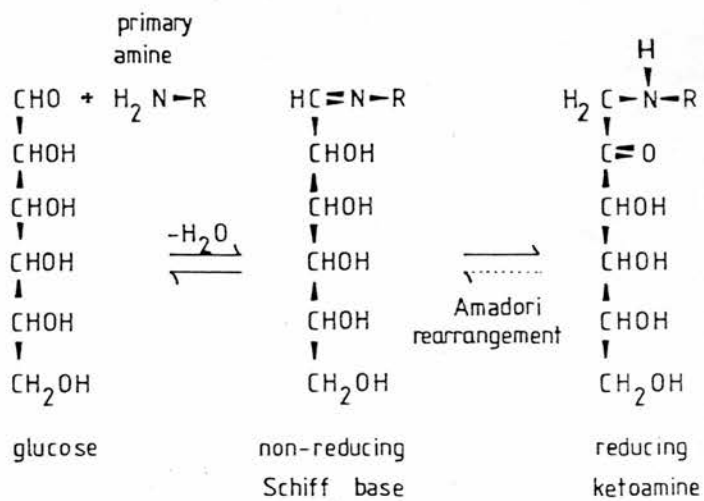


Fig. 8b LIPIDS IN BIOLOGICAL MEMBRANES.

MATERIALS AND METHODS

2. MATERIALS AND METHODS

2.1 THE SUBJECTS

Two groups of patients attending the diabetic out-patient clinic at Ninewells Hospital, Dundee were studied. They were designated poorly-controlled diabetics (PCD) and well-controlled diabetics (WCD). The PCDs were arbitrarily defined as a class of diabetics with fasting plasma glucose (FPG) of 8.0 mmol/l or more and the WCDs were those who had been under treatment (drugs etc.), whose FPG were less than 8.0 mmol/l. All were also participants in the United Kingdom Prospective Diabetic Study /182/. These patients also visited the diabetic out-patient clinic monthly after the first presentation of the disease in a poorly-controlled state until they became well-controlled. The patients in this study were selected as non-insulin dependent maturity onset diabetics and in addition a group of peripheral vascular disease (PVD) patients who had no family history of diabetes were studied.

2.2 BLOOD SAMPLING

Blood samples for erythrocyte phospholipid studies were drawn into heparin tubes from patients who had fasted overnight. Plasma glucose and glycosylated haemoglobin (HbA_{1c}) were determined by the Clinical Biochemistry Laboratory in Ninewells Hospital, Dundee. The blood samples were kept at 4°C and transferred to our laboratory, and phospholipid extraction was performed within three hours of sampling.

2.3 ERYTHROCYTE PHOSPHOLIPID ANALYSIS

2.3.1 LIPID EXTRACTION

Reagents:

Sodium chloride (0.9%)

Methanol (redistilled)

Chloroform (redistilled)

Absolute ethanol

Procedure:

Erythrocyte membrane lipids were extracted using a modification of Broekhuyse's method /183/. Approximately 10 mls of whole blood was spun at 1000 g for 10 minutes. The plasma was removed, as was the buffy coat of white cells and platelet aggregates lying between the supernatant and the packed erythrocytes. The cells were washed three times by resuspension in 0.9% sodium chloride (w/v) and centrifugation. 1.80 mls of packed cells were transferred to each of two 30 ml centrifuge tubes (Quickfit), and 15 mls of methanol was added. The tubes were vortex-mixed intermittently for 10 minutes and then 15 mls of chloroform was added. The tubes were again vortex-mixed, this time over a 15 minute period. The supernatant containing the lipid was removed and stored. The residue was re-extracted using 10 mls of methanol followed by 20 mls of chloroform and centrifuged as before. The combined supernatants were transferred to a round bottomed flask and the solvents removed using a rotatory evaporator under vacuum at 37°C. Any water present was removed by repeated addition and evaporation of absolute alcohol. The dried residue was

redissolved in chloroform-methanol (1:1, v/v), transferred to a small vial and reduced to dryness under a stream of nitrogen at 40°C. The lipid was finally redissolved in chloroform-methanol mixture (2:1, v/v) using 0.1 ml solvent per ml of original packed cells.

N.B. The potassium chloride wash used in Broekhuyses' method was omitted. However, the non-lipid contaminants which would therefore be present in no way interfered with the TLC and subsequent phosphorus determination.

2.3.2 TWO-DIMENSIONAL THIN LAYER CHROMATOGRAPHY

Reagents:

Chloroform (redistilled)

Methanol (redistilled)

Ammonia (Aq. 25%)

Acetic acid (glacial)

Distilled water

Procedure:

Thin layer chromatography (TLC) was carried out using the method of Hunter et al. /184a/. TLC plates were spread with 0.5 mm Thick layers of silica gel (Keiselgel 60 M). 50 g of silica gel were mixed with 110 ml of distilled water (as a slurry) and spread onto glass plates (10 x 10 cm) that had been cleaned with acetone. The plates were then activated for 1 hour at 105°C to remove any water present before use. 25 μl of each sample (approx. 250 μg total lipid) were applied at the lower right corner (2 cm from either side of the plate) using a microsyringe.

The solvent tanks were pre-equilibrated for at least one hour and to aid in this, a paper lining was used inside the tanks which were then sealed with tape to prevent loss of solvent. The solvent systems used were as follows:-

first dimension:

Chloroform	80 ml
Methanol	44 ml
Ammonia (25%)	5.5 ml

second dimension:

Chloroform	100 ml
Methanol	54 ml
Acetic acid	24 ml

The plates were run in two-dimensions at 4°C and while the first dimension took approximately 1½ hours the second took 2½ hours. The plates were dried for 15 minutes after the first dimension and for 30 minutes after the second dimension using a hair-dryer.

2.3.3 DETECTION OF PHOSPHOLIPIDS

After drying the plates, they were placed briefly in a tank saturated with iodine vapour. On exposure of the plates to the vapour, several brown spots appear rapidly and the phospholipids were thus easily identified. Iodine vapour stains non-specifically for lipids.

2.3.4 PHOSPHORUS ASSAY

Reagents:

Perchloric acid (72%)

Disodium hydrogen phosphate

Ammonium molybdate

Ascorbic acid

Distilled water

Procedure:

The method of Rouser et al. /184b/ was employed in this assay. The individual phospholipids separated by TLC were aspirated and the areas of the spots noted, along with a suitable blank area, into Kjeldahl tubes. The scrapings were gently refluxed on an electrically heated digestion rack with 0.9 ml of 72% perchloric acid for 30 mintues. This step converts organic phosphorus to inorganic phosphate. The tubes were cooled and 2 mls of distilled water were then added. At this stage, tubes containing standards (in the range 0-10μg of phosphorus) were also set up using aqueous disodium hydrogen phosphate (Stock Na_2HPO_4, 5μg phosphorus per ml) to give a final volume of 2 mls in each tube.

To the standards, 0.9 mls of 72% perchloric acid were added followed by a further 5 mls of distilled water to both samples and standards. 1.0 ml of 2.5% (w/v) ammonium molybdate and 1.0 ml of freshly prepared 10% (w/v) ascorbic acid were then added to the tubes and the contents of each transferred to 10 ml thick-walled test tubes. The tubes were then stoppered with glass marbles and heated for 5 minutes in a boiling water bath. After cooling, the tubes containing silica gel were spun at 1000 g for 10 minutes to obtain a clear supernatant. The optical densities were then measured at 820 nm.

A blank region of silica of measured area for each plate was routinely analysed to give the background phosphorus content per unit area of absorbent, and the results for the phospholipid spots suitably corrected. The methods were evaluated by repeated extraction and analysis of a large blood sample. Phosphorus recovery was also determined by analysis of six separate 200 μg aliquots of this total lipid mixture not chromatographed, but treated in the same manner as the phosphate standards and TLC spots.

2.4 THE SYNTHESIS OF GLYCOSYLATED PHOSPHOLIPIDS

Reagents:

Phosphatidyl ethanolamine (dipalmitoyl)

Phosphatidyl serine (from bovine brain)

Unlabelled glucose

Labelled glucose (specific activity 275 mCi/mmol)

Chloroform

Methanol

Anthrone

Sulphuric acid (Conc.)

Scintillation fluid

Procedure:

The following method was carried out in an attmept to make glycosylated products of PE and PS *in vitro*. 5 mg of PE (dipalmitoyl) or PS (from bovine brain) were each added to a separate flask containing 20 ml of methanol, 50 mg of unlabelled glucose and tracer amounts (10 μCi) of labelled glucose (^{14}C) (specific activity 275 mCi/mmol

glucose). The flasks were then gently refluxed at 65°C for 24 hours. Control flasks were also set up containing each phospholipid without glucose and glucose alone. After cooling, the methanol was then evaporated using the rotatory evaporator and the dried residue dissolved in 200 μl of chloroform-methanol mixture (2:1, v/v). This gave a total lipid concentration of 25 μg/μl.

250 μg (10 μl aliquot) of this sample were chromatographed by two-dimensional TLC. The spots in the TLC plates were detected with iodine, anthrone reagent (as a spray) and autoradiography. Phosphorus determination was also carried out. 0.2 gm of anthrone were dissolved in 100 ml of concentrated sulphuric acid and used within four hours. A bluish-green colour which develops when the plates are heated at 70°C confirms the presence of carbohydrate. The chromatograms were attached to x-ray films using rubber bands and exposed for 24 hours. The films were then developed using 400 ml of Ilford developer (diluted ¼) and 400 ml of Ilford fixer (diluted ¼). The autoradiograms demonstrated the presence of spots containing $\left[^{14}C\right]$-glucose. The spots on the chromatograms which corresponded to spots on the autoradiograms were scraped into scintillation fluid and counted.

2.5 ERYTHROCYTE MEMBRANE STUDIES

2.5.1 MEMBRANE PREPARATION

Reagents:

Isotonic Tris buffer (0.172 M) pH 7.6

Hypotonic Tris buffer (0.011 M) pH 7.6

Procedure:

The method of Hanahan and Ekholm /185/ was used for the preparation of red cell ghosts. Blood was collected directly into heparinised 10 ml tubes and centrifuged immediately in these tubes at 1000 g for 30 minutes at 4°C. Plasma and buffy coat were removed by careful suction, and the cells were resuspended in 0.172 M (isotonic) Tris buffer, pH 7.6. After mixing well by inversion, the sample was centrifuged again at 1000 g for 30 minutes at 4°C. The supernatant was removed by suction. The washing step was repeated twice more and washed cells were kept on ice, ready for membrane preparation.

5 ml aliquots of the above cell suspension (50%) were transferred to 50 ml polyethylene tubes and 30 ml of 0.011 M (hypotonic) Tris buffer at 4°C were added. The tubes were allowed to stand for approximately 5 minutes before centrifuging at 20,000 g for 40 minutes at 4°C. After centrifugation, and removal of the supernatant, an additional 30 ml of the hypotonic buffer was added, and the suspension was centrifuged again for 40 minutes, at 20,000 g at 4°C. A total of four washes was necessary to obtain colourless membranes.

N.B. An index of the progress of haemolysis is the development of a deep red colour in the soluble portion. The membranes are very light weight and tend to be lost more easily in the initial supernatant: thus the first decantation of supernatant is a little more difficult than with

subsequent washes. If the sample is held so that light shines up through the tube as it is slanted, the membranes can be more easily seen. When as much supernatant is decanted as is possible without losing membranes, the tube is placed on a vibrating mixer to loosen and resuspend the membranes in the remaining buffer. A small fibrin-like skin often attaches to the bottom of the tube; an attempt is made to dislodge it, since it may trap membranes.

2.5.2 PROTEIN ASSAY

Reagents:

Copper sulphate

Trisodium citrate

Sodium carbonate

Sodium hydroxide

Phosphomolybdic phosphotungstate

Albumin standard (4 mg/ml)

Procedure:

The method of Lowry /186/ was used in estimating the protein in the above membrane preparation. Alkaline copper sulphate ($CuSO_4$) solution was freshly prepared (1 in 5 dilution) from a stock solution of 0.5% $CuSo_4$ in 1% trisodium citrate with an alkaline buffer of 2% sodium carbonate (Na_2CO_3) in 0.1 M sodium hydroxide (NaOH). Also Folin-Ciocalteau reagent (phosphomolybdic phospho-tungstate) was prepared fresh by diluting the commercial concentrate 1:1 with distilled water.

An albumin standard curve ranging from 0 - 200 μg

was prepared from the stock solution (4 mg/ml) and the membranes were diluted (1 in 5). 0.5 ml of sample was added to 3 ml of alkaline $CuSO_4$ solution in 5 ml test tubes. The contents of the tubes were mixed and allowed to stand for 10 minutes. 0.3 ml of the diluted Folin-Ciocalteau reagent was added, and the contents of the tubes were mixed and allowed to stand for another 1 hour. The absorbance of the tubes were then read at 540 nm.

2.5.3 MEMBRANE GLYCOSYLATION

Reagents:

Krebs Ringer Phosphate Buffer, pH 7.4

Unlabelled glucose

Labelled glucose (275 mCi/mmol)

Chloroform

Methanol

Scintillation fluid

Procedure:

Krebs Ringer Phosphate Buffer (KRPB) Solutions, pH 7.4 were prepared by the method of Eliott et al. /187/, each containing unlabelled glucose concentrations in the order; 0, 10, 20, 40, 60, 80, 100 mM. 3 ml membranes, (containing 800 μg/ml protein) were incubated in 2 ml of each buffer preparation and inhibiting doses of nystatin (1 μg) and streptomycin (1 μg) were also added in to prevent bacterial and fungal overgrowth. Another tube containing tracer amounts (60 μCi) of labelled glucose (specific activity 275 mCi/mmol glucose) 20 mM glucose, 3 ml membrane, 2 ml buffer was also set up. At the end of the

incubation period (5 days), the contents of the tubes were then centrifuged at 20,000 g for 40 minutes.

The supernatants were decanted, and the membranes were then extracted using methanol and chloroform as in (2.3.1). This was followed by TLC of the lipids and phosphorus estimation as before. Autoradiography was carried out on the TLC plates from the labelled glucose (^{14}C) incubation and radioactive spots on the plates were scraped into scintillation fluid and counted.

2.5.4 MOLECULAR MODELS OF GLYCOSYLATED PHOSPHOLIPIDS

Using a space filling model kit, molecular models were made of glycosylated PE and PS. This was carried out to demonstrate any possible steric hindrance when glucose attaches to the free amino groups and also to compare the size difference between the individual phospholipids and their glycosylated forms to give an idea of possible perturbation of membrane bilayers by such compounds.

2.6 CHROMATOGRAPHIC SEPARATION OF GLYCOSYLATED PHOSPHOLIPIDS

2.6.1 THIN-LAYER CHROMATOGRAPHY

Because earlier work in our laboratory suggested that glycosylated PE (GPE) overlapped with PS in two-dimensional TLC /see 1.6/ an attempt was made to modify the solvent system to achieve resolution of glycosylated phospholipids from their non-glycosylated derivatives. The concentration of the ammonia in the first dimension solvent as used by Hunter et al. /184a/ was increased in order to achieve greater separation of PE and PS.

Hunters System		Modified System	
Chloroform	80 ml	Chloroform	80 ml
Methanol	44 ml	Methanol	44 ml
Ammonia (25%)	5.5 ml	Ammonia (35%)	5.5 ml
Water	5.5 ml		

The modified system was first tested on synthetic phospholipid mixtures and then employed on lipid extracts from *in vitro* membrane glycosylation experiments. Whole blood lipid extracts from PCD, WCD and NS (normal subjects) were also run in this system. Because the levels of glycosylated products are likely to be low *in vivo* relative to the other phospholipids, the loading of lipids onto the TLC plate was quadrupled to attempt quantitation of the glycosylated phospholipids by phosphorus assay.

2.6.2 HIGH PRESSURE LIQUID CHROMATOGRAPHY (HPLC)

Reagents:

N-hexane

Isopropanol

Distilled water

Procedure:

The isocratic system used by Andrews /188/ for the separation of phospholipids was modified into a gradient system. While the isocratic system consisted of hexane-isopropanol-water in the ratio of (6:8:1.15), the two solvents (A and B) used in the gradient system had ratios of (6:8:0.92) and (6:8:1.33) respectively. All solvents were of HPLC grade and prior to use were filtered through

a 0.2 μm millipore filter and degassed by sonication for 30 minutes. Both solvents and columns were maintained at room temperature and the gradient profile (Figure 9) was maintained at a flow rate of 1.5 ml/min.

In our laboratory, the HPLC system consisted of a Gilson Model 303 pump with a Rheodyne injection system and an automatic gradient controller for gradient elution. The column used was a Zorbax Sil column (silica) supplied by Du Pont Instruments Limited. The guard column was also packed with 15-25 μ silica. The detector used in this system was also a Gilson 303 ultraviolet (UV) detector at a wavelength of 206 nm. The direct detection of phospholipids at such low UV wavelength according to Geurts Van Kessel et al. /189/ is dependent not only on the degree of unsaturation of the fatty acid side-chains of the phospholipids, but also on the functional groups such as carbonyl, carboxyl, phosphate, amino and quartenary ammonium of each molecule. The detector was set at 1.0 absorbance unit full scale (a.u.f.s.) deflection, and quantitation was by integration of peak areas using the Shimadzu Integrator Chromatopac C-R1B, which also gave automatic readout of retention times.

The standards which were supplied by Sigma Limited (England) were made from natural sources and the following concentrations were prepared for HPLC analysis; PE (egg yolk): 1 μg/μl, PS (bovine brain): 5 μg/μl, PC (egg yolk): 10 μg/μl, SM (bovine brain): 10 μg/μl, PI (Soya bean): 5 μg/μl. 1.0 ml of each individual

SOLVENT A		SOLVENT B	
Hexane	360 ml	Hexane	360 ml
Isopropanol	480 ml	Isopropanol	480 ml
Water	55 ml	Water	80 ml

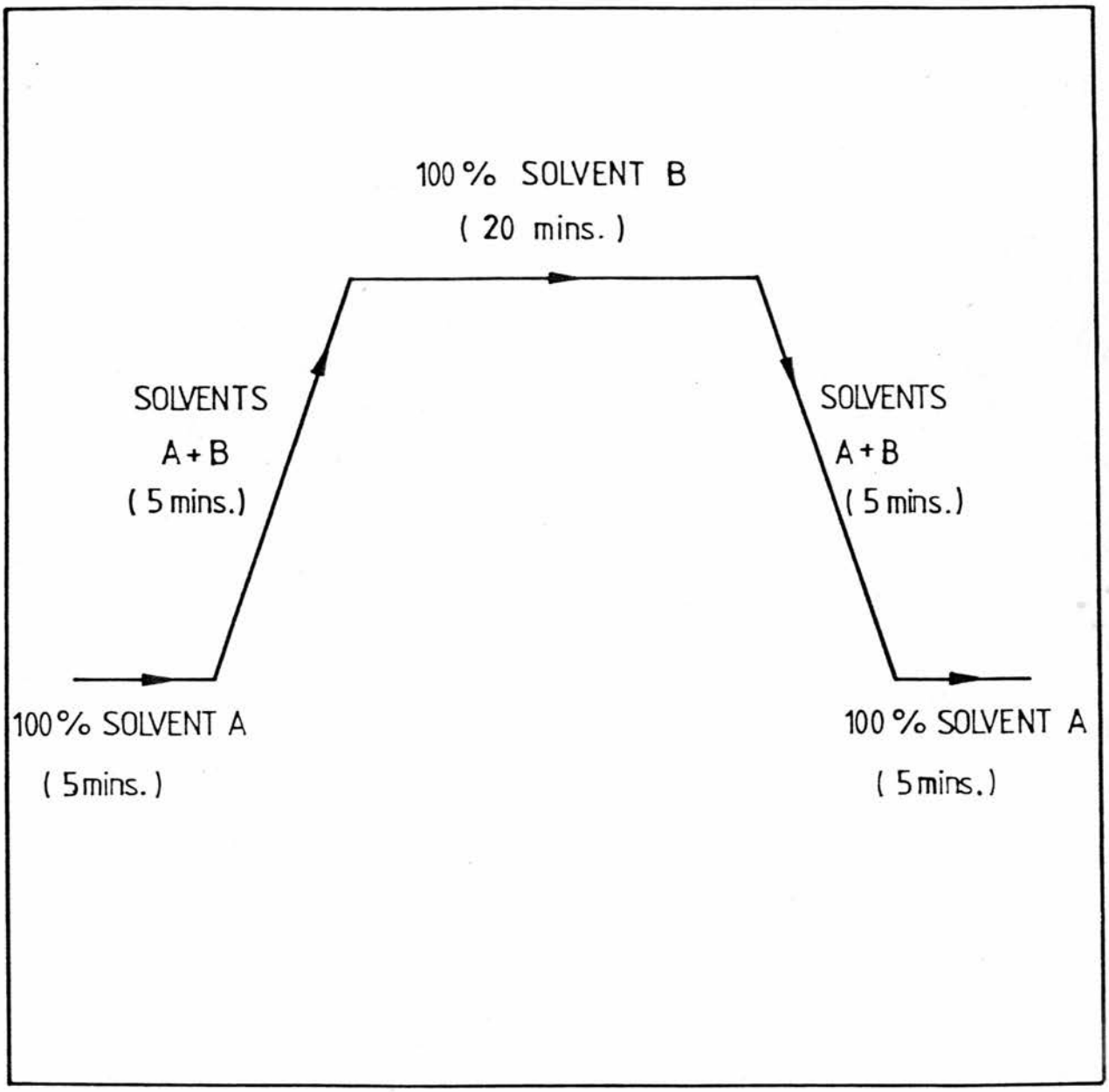


Fig. 9 H.P.L.C. GRADIENT ELUTION PROFILE FOR PHOSPHOLIPID SEPARATION.

standard were mixed together and dried under nitrogen at 40°C and then reconstituted in 1.0 ml of HPLC solvent to be used as a composite working standard. 5 μg/μl of synthetic glycosylated PE and 8 μg/μl of diabetic erythrocyte lipids were freshly redissolved in HPLC solvents. Each sample was then injected into the HPLC fitted with a 20 μl injection loop. The loop was flushed with the solvent after each injection in order to eliminate any possible contamination between samples.

2.7 STATISTICAL ANALYSIS

All measurements were carried out in duplicate from which all graphs and scattergrams were plotted.

n = number of results

x = an individual result

Σ = sum of

$\bar{x}$ = mean of a set of results

S.D. = Standard deviation

(c.v.) = Coefficient of variation

$$\text{Mean } (\bar{x}) = \frac{\Sigma x}{n}$$

$$\text{Standard deviation (S.D.)} = \sqrt{\frac{\Sigma(x)^2 - \frac{(\Sigma x)^2}{n}}{n-1}}$$

$$\text{Coefficient of variation (c.v.)} = \frac{\text{S.D.}}{\bar{x}} \times 100.$$

2.7.1 STUDENT t-TEST (t)

This is a parametric test of the difference between the means of two independent samples. In essence the student t-test measures the size of the difference between

the means of the two samples and converts this into a standard measure of deviation /190/.

$\bar{x}$ = the mean of one set of results

X = the last value of above set of results

$\bar{y}$ = the mean of other set of results

Y = the last value of above set of results

m = the number of duplicates taken to give one mean

n = the number of duplicates taken to give other mean

(m + n) - 2 = Degrees of freedom (d.f.)

S.Dp = the standard deviation of the method, often the pooled standard deviation of the two sets of data.

The standard deviation was derived as follows:

$$S.Dp = \frac{(X-\bar{x})^2 + (Y-\bar{y})^2}{(m-1) + (n-1)}$$

Thus the student t-statistic was calculated as follows using a program on the VAX computer (G. Kemp, personal communication).

$$t = \frac{(\bar{x} - \bar{y})}{S.Dp} \cdot \sqrt{\frac{n.m}{(n + m)}}$$

The values of t were looked up against the degrees of freedom in the statistical tables /190/ to find the probabability (p) that one set of results may be significantly different from the other set.

A large value of t signifies a marked difference between the sample means and, correspondingly, a low

probability that the samples vary purely by chance. If this probability falls below 0.05 the chance hypothesis may be rejected in favour of the alternate hypothesis that there is a genuine and reliable difference between the levels of performance in the two experimental conditions /190/.

2.7.2 THE MANN-WHITNEY TEST (U)

This non-parametric test has been used to test the more general hypothesis that one set of results tends to be higher or lower than the other set. It is used in specific cases where the distribution of the data in the groups being compared is known to be or may possibly be non-gaussian. By finding the sum of the ranks of one of the samples, the Mann-Whitney test allows us to determine the probability that a given separation between the ranks of the two samples could have arised by chance /190/.

n_1 = the size of the smaller group of results

n_2 = the size of the larger group of results

R = the sum of the results for the smaller group of results

When n_2 is less than 20 then;

$$U = n_1 n_2 + \frac{n_1(n_1 + 1)}{2} - R$$

When n_2 is more than 20 then;

$$Z = \frac{U - \frac{n_1 n_2}{2}}{\sqrt{\frac{n_1 n_2 (n_1 + n_2 + 1)}{12}}}$$

The value of U and Z were looked up in the statistical tables /191/ to find the probability that the observed difference between the two sets of data could have occured by chance (two-tailed test).

2.7.2 CORRELATION COEFFICIENT (r)

Although inspection of a scattergram gives a clear impression of the degree of relationship between two variables, quantitation was carried out more precisely using a single numerical index that will allow the comparison of the strength of different correlations. This was done by calculating the coefficient of correlation, which is represented by r.

x = a causative or independent variable

y = a dependent variable

n = the total number of subjects

The coefficient of correlation is calcualted as follows;

$$r = \frac{\Sigma xy - \frac{(\Sigma x)(\Sigma y)}{n}}{\sqrt{\left[\Sigma x^2 - \frac{(\Sigma x)^2}{n}\right]\left[\Sigma y^2 - \frac{(\Sigma y)^2}{n}\right]}}$$

r is an index of the strength of the linear relationship between x and y and the statistical tables /189/ were used to check whether the observed relationship was a reliable one (i.e. significantly above zero) /190/.

RESULTS

3. RESULTS

3.1 EVALUATION OF METHODS FOR PHOSPHOLIPID DETERMINATION

A photograph of a typical TLC plate of a normal sample is shown in Figure 10. Five classes of phosphorus-containing membrane lipids were detected in iodine staining:

Phosphatidyl choline	(PC)
Sphingomyelin	(SM)
Phosphatidyl ethanolamine	(PE)
Phosphatidyl serine	(PS)
Phosphatidyl inositol	(PI)

PS and PI run close together especially in old samples but were normally satisfactorily resolved.

The absorbance readings for standards in the phosphorus assay (Table 2) were used to plot a standard curve (Figure 11). Thereafter it was ensured that all readings taken in the phospholipid determinations fell into this linear plot and thus the optical density (at 820 nm) within this range can be taken as being directly proportional to the phospholipid content; each molecule of most species of phospholipid contains only one phosphate group (Figures 8a and 8b).

The coefficient of variation for the determination of each phospholipid class was calculated for both the colorimetric phosphorus assay alone (Table 3a) and for the combined TLC and phosphorus assay (Table 3b). The percentage recovery for the method is shown (Table 4).

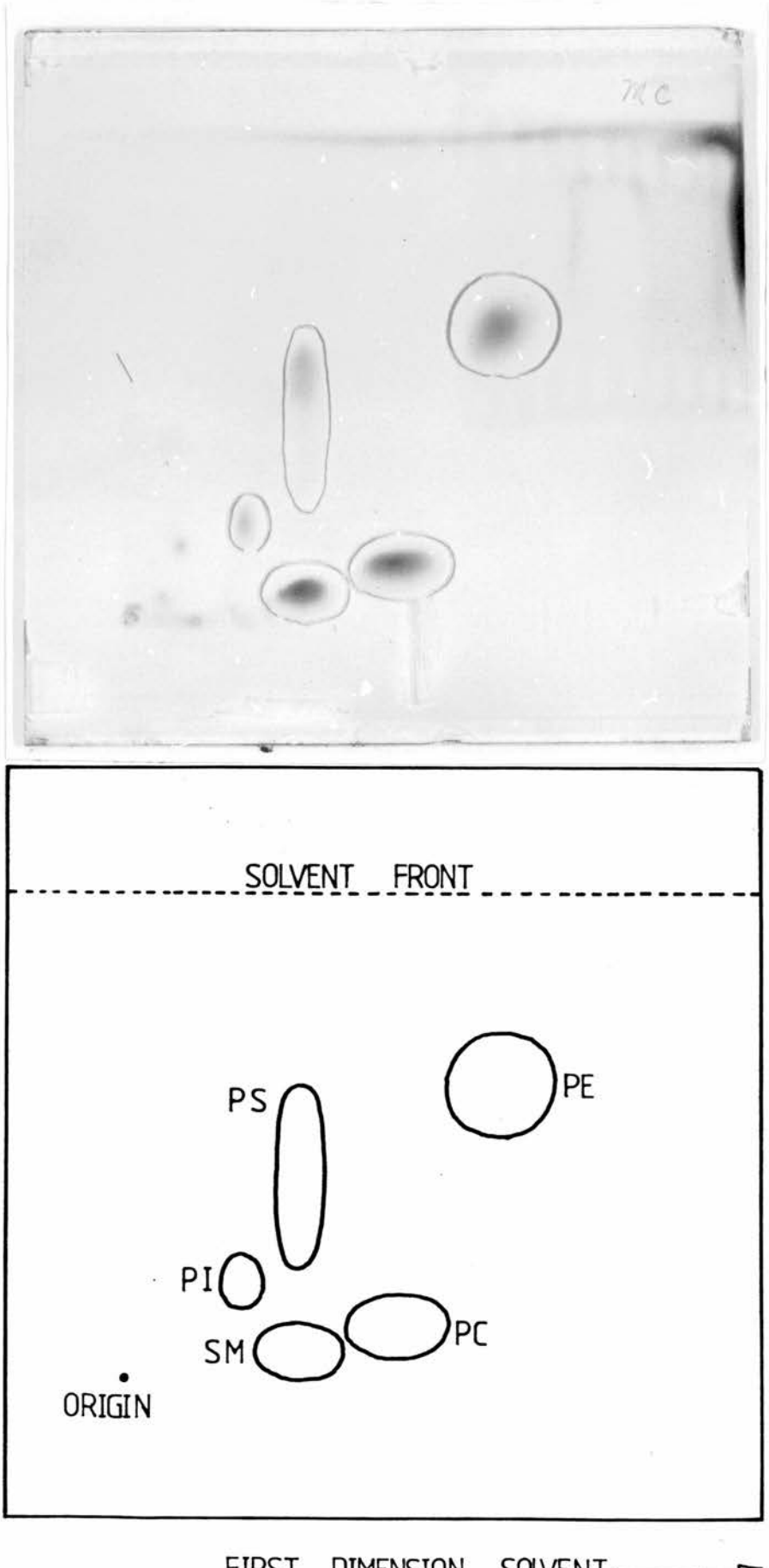


Fig. 10

A TYPICALLY STAINED PLATE.

250µg total phospholipid from erythrocytes

Table 2 Phosphorus standard assay

μg Phosphorus	0	2	4	6	8	10
Absorbance units	0.00	0.06	0.12	0.18	0.24	0.30

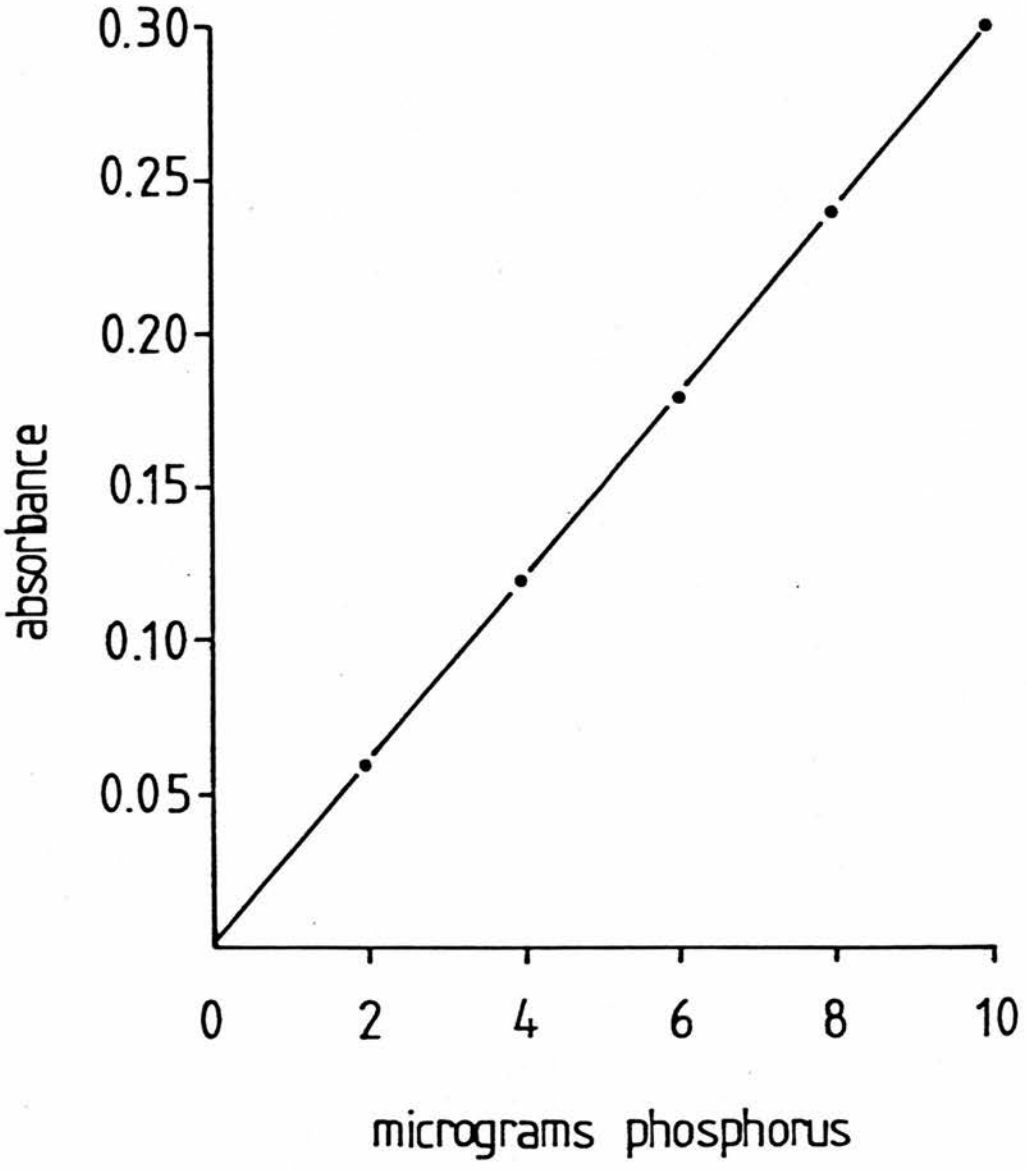


Fig. 11 Phosphorus standard curve

Table 3a Phosphorus assay

(Determination of coefficient of variation)

n = 12	SM	PC	PS	PE
mean ($\bar{x}$)	24.4%	32.4%	13.8%	29.4%
Standard deviation (SD)	0.26	0.33	0.60	0.38
Coefficient of variation (CV)	1.08%	1.0%	4.3%	1.3%

Table 3b **Evaluation of methods for phospholipid estimation**

(Determination of coefficient of variation)

Lipid extraction

(and phosphorus assay)

n = 12	SM	PC	PS	PE
mean ($\bar{x}$)	24.3%	32.8%	13.8%	29.3%
Standard deviation (SD)	0.46	0.42	0.65	0.81
Coefficient of variation (CV)	1.9%	1.3%	4.7%	2.8%

Table 4 Phosphorus recovery

Sample	% Recovery
1	98.2
2	98.8
3	97.6
4	99.0
5	99.2
6	98.4
Mean	98.5%

Percentage recovery = 98.5% ± 0.8

3.2 ERYTHROCYTE PHOSPHOLIPID COMPOSITION

3.2.1 CROSS-SECTIONAL STUDY

The composition of erythrocyte membrane phospholipids is shown for the three classes of subjects studies; well-controlled diabetics (Table 5), poorly-controlled diabetics (Table 6) and peripheral vascular disease patients (Table 7). The proportion of each class of phospholipid for each group of patients is also shown is Figures 12, 13 and 14. Table 8a shows the mean values for each group in this study and the means for pooled data from this and previous study /71a/ are shown in Table 8b. The statistical evaluation of the data by the student t-test and the Mann-Whitney test on each group are also shown (Tables 9a, 9b, 10a and 10b).

Considering the data from this cross-sectional study alone evaluated using the student t-test; PE was significantly decreased in PCD compared with WCD; PS was significantly elevated in PCD compared with both WCD and PVD; SM was significantly decreased in PCD compared with other groups, as was the PC/SM ratio. The differences were almost the same when the pooled data were evaluated. This comparison also showed that data for PVD (this study) and controls (previous study) did not differ significantly. Statistical testing using Mann-Whitney confirmed exactly parallel trends for both this and the pooled studies.

Table 5 **Cross-sectional phospholipid studies on Well-Controlled diabetics**

Name	Age (years)	Sex M = male F = female	Phospholipid class as percentage of total				PC/SM	FPG mmol/l	HbA_{1c} %
			PE	PS	PC	SM			
Egan	51	**M**	30.3	12.1	29.5	28.0	1.05	7.0	9.0
Angus	62	**F**	30.3	13.5	30.8	26.5	1.16	6.6	7.7
Rattray	49	**F**	29.8	12.3	31.0	29.4	1.05	7.3	8.4
Bowman	54	**M**	28.5	12.5	29.5	29.6	1.00	7.3	7.2
Widdis	58	**M**	29.3	12.7	29.3	28.8	1.02	6.5	8.6
Gorrie	63	**F**	30.5	11.3	29.0	29.5	0.98	6.6	6.8
Smith	59	**M**	30.0	12.5	30.0	27.6	1.09	6.5	6.8
Mustard	53	**M**	29.1	12.3	30.0	28.8	1.04	5.4	6.6
Fairlie	58	**M**	29.3	12.6	31.2	28.0	1.11	5.3	5.3
Brash	56	**F**	29.5	13.0	29.0	28.5	1.02	6.6	6.5
n = 10									
Mean ($\bar{x}$)			29.7	12.4	30.0	28.5	1.06	6.5	7.4
Standard deviation (SD)			±0.7	±0.6	±0.8	±1.0	±0.06	±0.7	±1.2

Table 6 Cross-sectional phospholipid studies on Poorly-Controlled diabetics

Name	Age (years)	Sex M = male F = female	Phospholipid class as percentage of total				PC/SM	FPG mmol/l	HbA_{1c} %
			PE	PS	PC	SM			
Glen	48	F	28.0	14.0	31.0	27.0	1.15	28.2	13.7
McCormiskie	31	F	27.0	14.8	32.0	26.1	1.22	13.0	10.1
Roxburgh	39	F	26.5	15.1	31.3	28.0	1.15	12.4	11.7
Cook	65	F	26.8	14.6	30.0	28.8	1.12	20.0	8.9
Burry	47	M	24.6	15.9	31.3	28.3	1.11	10.2	8.7
Stead	37	F	26.4	15.0	31.0	27.7	1.12	21.0	13.0
Mitchell	42	F	26.6	17.0	30.9	25.4	1.22	17.5	17.9
Crichton	50	M	28.0	16.5	30.9	27.6	1.12	9.5	11.6
Gray	64	F	26.3	14.0	31.2	28.5	1.20	9.5	7.8
Chick	62	F	27.3	15.0	30.0	27.5	1.09	14.6	11.2
Tavendale	39	M	28.5	13.4	30.4	27.7	1.18	9.9	9.6
Whitelaw	59	M	23.9	15.3	30.6	30.1	1.02	8.6	7.6
Donaldson	44	M	24.8	16.8	31.5	26.9	1.17	9.4	13.0
Watson	62	F	25.9	14.8	32.2	27.2	1.18	11.8	7.1
Beharrie	53	M	25.3	14.6	30.6	29.5	1.04	10.1	7.3
Joss	63	M	27.1	15.0	29.8	28.2	1.06	12.5	10.9
Sherriff	62	F	30.6	15.5	30.0	24.7	1.21	8.6	7.0
Grace	42	M	27.3	14.8	29.0	24.1	1.20	11.8	11.6

Table 6 Cross-sectional phospholipid studies on poorly-controlled diabetics (contd.)

Name	Age (years)	Sex M = male F = female	Phospholipid class as percentage of total				PC/SM	FPG mmol/l	HbA_{1c} %
			PE	PS	PC	SM			
Anderson	59	**F**	28.5	15.0	29.6	27.0	1.10	8.1	-
Van de Bon	44	**F**	29.0	15.8	30.0	24.9	1.20	10.3	11.1
Taylor	52	**F**	29.1	13.9	30.6	26.4	1.16	18.0	-
Wilkie	58	**M**	29.6	13.0	30.8	26.3	1.17	9.0	7.4
n = 22									
Mean ($\bar{x}$)			27.1	14.9	30.7	27.2	1.15	13.0	10.4
Standard deviation (SD)			±1.7	±1.1	±0.8	±1.5	± 0.06	± 5.1	±2.8

Table 7 Cross-sectional phospholipid studies on peripheral vascular disease patients

Name	Age (years)	Sex M = male F = female	Phospholipid class as percentage of total				PC/SM	FPG mmol/l	HbA_{1c} %
			PE	PS	PC	SM			
Clark	72	F	31.0	10.1	31.0	28.1	1.10	4.8	-
Greensmith	76	M	30.0	11.0	30.1	28.9	1.04	9.3*	-
Turner	52	M	28.0	11.9	30.0	30.3	1.00	4.5	-
Airlie	-	-	26.6	12.5	31.0	31.0	1.00	-	-
Corrigan	66	M	27.8	12.8	31.2	28.3	1.10	4.9	-
Merchant	64	M	27.6	10.1	30.8	29.5	1.04	4.7	-
McCord	-	-	27.1	13.2	30.5	28.5	1.04	-	-
Russell	74	F	28.7	12.3	30.0	29.0	1.03	6.3	-
Souther	-	-	27.1	11.8	30.2	30.9	0.98	-	-
n = 9									
Mean ($\bar{x}$)			28.1	11.7	30.6	29.2	1.04		
Standard deviation (SD)			±1.6	±1.2	±0.5	±1.0	±0.04		

Table 8a Mean values for each group of subjects

Phospholipid	WCD n = 10	PCD n = 22	PVD n = 9
PE	29.7±0.7	27.1±1.7	28.1±1.6
PS	12.4±0.6	14.9±1.1	11.7±1.2
PC	30.0±0.8	30.7±0.8	30.6±0.5
SM	28.5±1.0	27.2±1.5	29.2±1.0
SM/PC	1.06±0.06	1.15±0.06	1.04±0.04

Table 8b Mean values for each group of subjects

(* pooled data from this and previous studies)

Phospholipid	WCD* n = 22	PCD* n = 35	NS n = 21
PE	29.1±1.6	27.2±1.9	28.4±1.9
PS	12.6±1.4	14.9±1.7	12.2±2.1
PC	30.3±1.10	30.8±1.3	31.0±1.8
SM	28.2±1.4	27.4±1.6	28.6±2.8
SM/PC	1.07±0.06	1.10±0.18	1.10±0.19

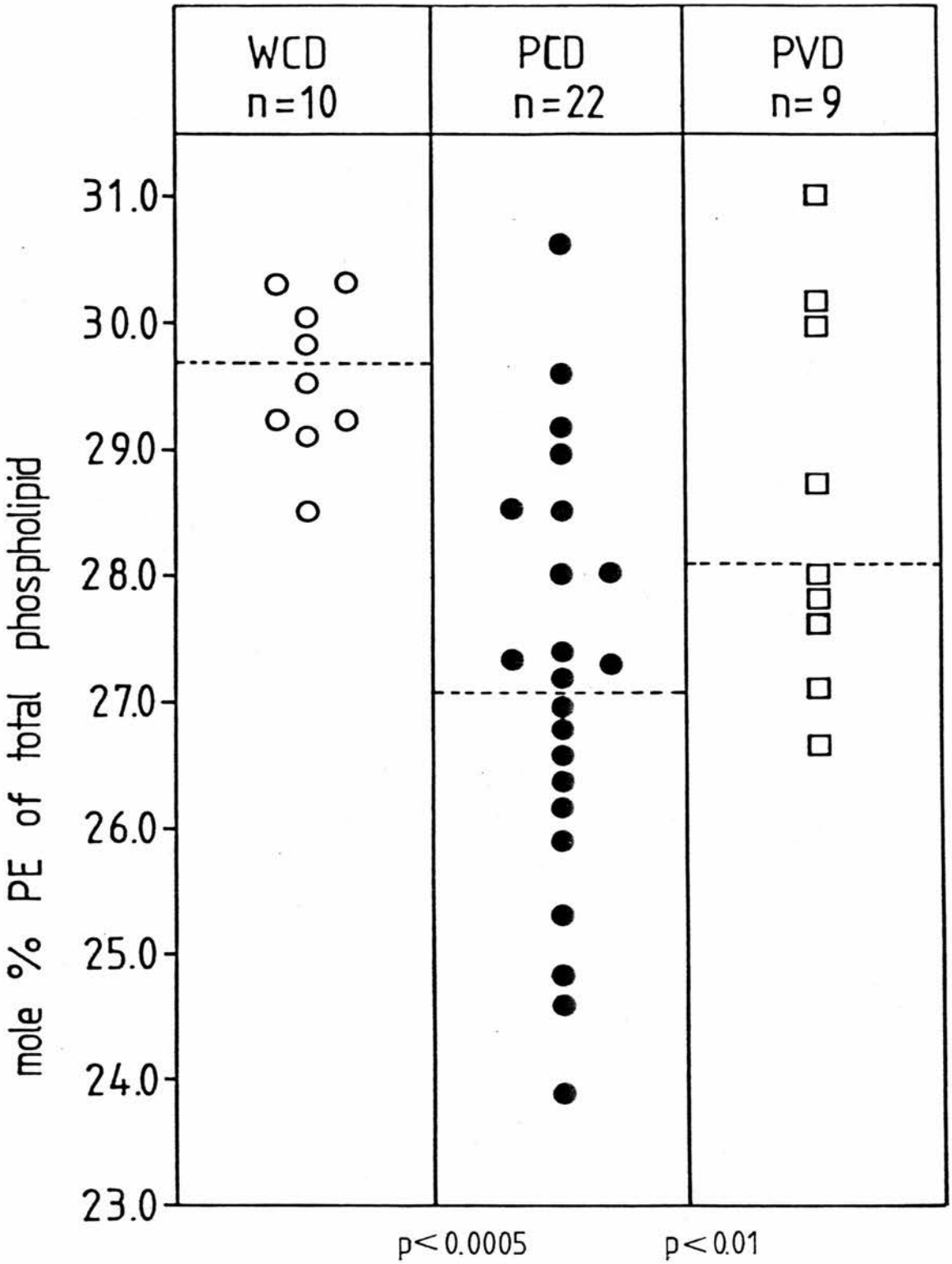


Fig. 12 The proportion of PE in erythrocytes from WCD, PCD and PVD patients.

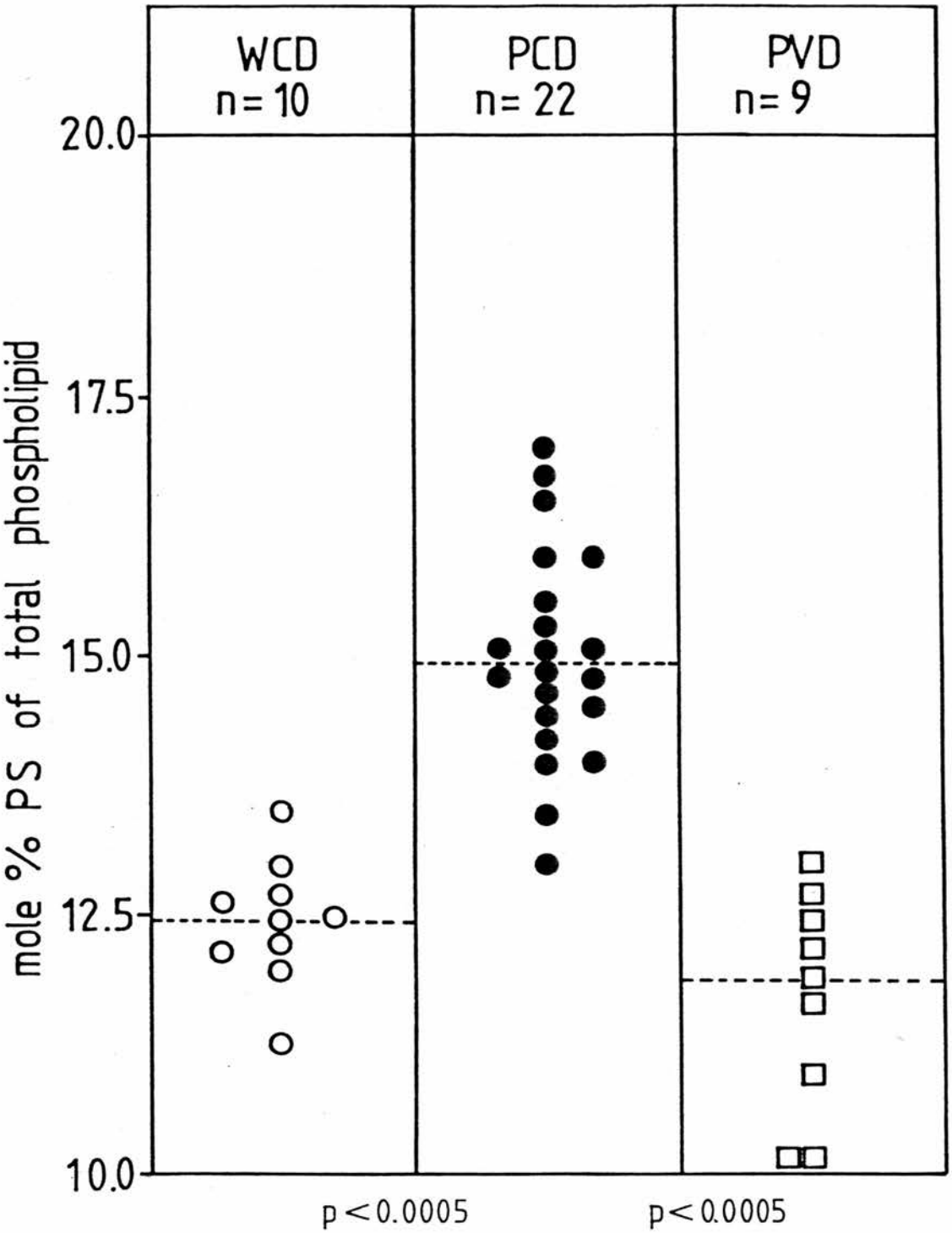


Fig. 13 The proportion of PS in erythrocytes from WCD, PCD and PVD patients.

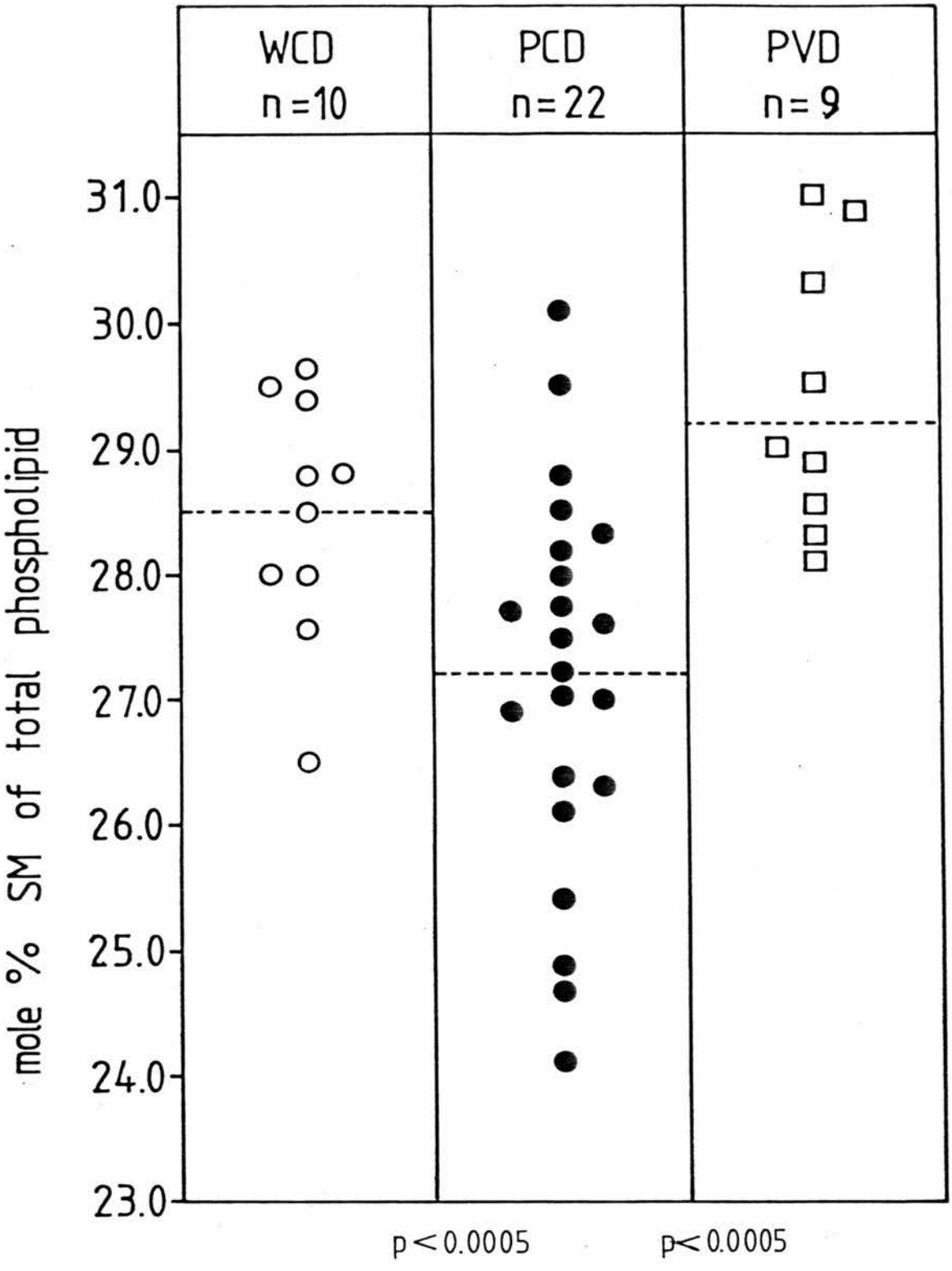


Fig. 14 The proportion of SM in erythrocytes from WCD, PCD and PVD patients.

Table 9a Student t-test

Phospholipid class	Groups Compared			Probability (P)
PE	WCD	vs	PCD	0.0005
	WCD	vs	PVD	0.1
	PCD	vs	PVD	0.01
PS	WCD	vs	PCD	0.0005
	WCD	vs	PVD	0.05
	PCD	vs	PVD	0.0005
SM	WCD	vs	PCD	0.025
	WCD	vs	PVD	0.25
	PCD	vs	PVD	0.0005
PC/SM	WCD	vs	PCD	0.0005
	WCD	vs	PVD	0.25
	PCD	vs	PVD	0.0005

Table 9b Student t-test (pooled data)

Phospholipid class	Groups Compared			Probability (P)
PE	WCD	vs	PCD	0.0005
	WCD	vs	PVD	0.1
	PCD	vs	PVD	0.01
PS	WCD	vs	PCD	0.0005
	WCD	vs	PVD	0.15
	PCD	vs	PVD	0.0005
SM	WCD	vs	PCD	0.025
	WCD	vs	PVD	0.05
	PCD	vs	PVD	0.0005
PC/SM	WCD	vs	PCD	0.0005
	WCD	vs	PVD	0.1
	PCD	vs	PVD	0.0005

Table 10a Mann-Whitney test

Phospholipid class	Groups Compared			Propability (P)
PE	WCD	vs	PCD	0.0002
	WCD	vs	PVD	0.10
	PCD	vs	PVD	0.02
PS	WCD	vs	PCD	0.0006
	WCD	vs	PVD	0.20
	PCD	vs	PVD	0.0006
SM	WCD	vs	PCD	0.02
	WCD	vs	PVD	0.20
	PCD	vs	PVD	0.0006
PC/SM	WCD	vs	PCD	0.0008
	WCD	vs	PVD	0.10
	PCD	vs	PVD	0.0002

Table 10b Mann-Whitney test (pooled data)

Phospholipid class	Groups Compared			Probability (P)
PE	**WCD**	vs	**PCD**	0.0002
	WCD	vs	**PVD**	0.10
	PCD	vs	**PVD**	0.02
PS	**WCD**	vs	**PCD**	0.0006
	WCD	vs	**PVD**	0.18
	PCD	vs	**PVD**	0.0006
SM	**WCD**	vs	**PCD**	0.02
	WCD	vs	**PVD**	0.04
	PCD	vs	**PVD**	0.0006
PC/SM	**WCD**	vs	**PCD**	0.0008
	WCD	vs	**PVD**	0.14
	PCD	vs	**PVD**	0.0006

3.2.2 LONGITUDINAL STUDY

The phospholipid composition in the longitudinal phospholipid studies on PCD patients is shown in Table 11. For a given patient the highest and lowest fasting plasma glucose concentration recorded during the study were taken arbitrarily to represent a "poorly-controlled" and "well-controlled state" respectively. The results of a paired student t-test for this data (Table 11a) show highly significant increased PS ($p < 0.0005$) and decreased PE ($p < 0.005$) for individual patients in the poorly controlled as compared to the well-controlled states. The changes in mole percent PS with glucose control in sequential samples from these patients are also shown (Table 12 and Figure 15). For these purposes the proportion of PS at entry to the study was taken as the basal value and changes are related to this. Figure 15 shows that for individual patients the overriding trend is for PS to increase with increasing fasting plasma glucose and vice versa.

Table 11 Longitudinal phospholipid studies on diabetic patients

Name	Age	Sex	Date of blood sample	PE	PS	PC	SM	FPG mmol/l	HbA_{1c} %
Glen	48	**F**	31.10.84	28.0	14.0	31.0	27.0	28.2**	13.7
			27.11.84	28.2	13.6	29.8	28.4	10.3*	-
			05.12.84	28.8	13.0	30.1	28.1	-	-
McCormiskie	31	**F**	06.11.84	28.0	14.0	31.0	27.0	13.0**	10.1
			04.12.84	29.8	12.7	30.0	27.6	10.1	-
			29.01.85	30.0	12.3	30.0	27.8	9.0	10.0
			20.02.85	29.5	12.8	29.9	27.8	4.8*	-
			23.04.85	29.2	12.8	31.0	27.0	6.1	11.1
Stead	37	**F**	12.12.84	27.0	14.8	32.0	26.1	12.3	9.0
			29.01.85	26.5	15.10	31.3	28.0	21.0**	13.0
			12.03.85	26.8	16.6	30.0	26.8	14.0	13.5
			14.05.85	29.3	12.6	31.2	28.6	10.7*	13.7
Gray	64	**F**	31.10.84	25.8	15.3	29.8	29.1	15.0**	13.9
			31.11.84	26.3	14.0	31.2	28.5	9.5	7.8
			29.01.85	26.5	13.9	30.5	29.1	9.0*	8.0
Chick	62	**F**	05.09.84	32.6	12.1	33.4	22.1	13.8	12.9
			14.11.84	27.3	15.0	30.0	27.5	14.6**	11.2
			08.05.85	29.2	12.8	28.8	28.2	10.2*	7.9
Tevendale	39	**M**	18.07.84	27.7	13.9	29.9	23.7	10.0	12.1
			04.12.84	28.5	13.4	30.4	27.7	9.9*	9.6
			14.05.85	26.7	15.0	29.8	28.5	14.2**	11.4
Beharrie	53	**M**	23.11.84	24.6	15.9	31.3	28.3	18.5**	-
			06.02.85	27.6	13.5	30.0	29.0	8.2*	6.2
			14.05.85	29.5	13.0	29.0	28.5	10.1	7.3

* "well-controlled" state = lowest recorded FPG during study

** "poorly-controlled" state = highest recorded FPG during study

PE: $p < 0.005$

PS: $p < 0.0005$

Table 12 Longitudinal studies on poorly-controlled diabetics

(variation of PS with glucose control)

Name of Patient	Date of blood sampling	Fasting plasma glucose (mmol/l)	Δ PS (%)
Glen	31.10.84	28.2	0
	27.11.84	10.3	-3.0
	05.12.84	-	-7.1
McCormiskie	06.11.84	13.0	0
	04.12.84	10.1	-9.3
	29.01.85	9.0	-12.1
	20.02.85	4.8	-8.6
	23.04.85	6.1	-8.6
Stead	12.12.84	9.0	0
	29.01.85	21.0	+2.0
	12.03.85	14.0	+12.2
	14.05.85	10.7	+14.9
Gray	31.10.84	15.0	0
	13.11.84	9.5	-8.5
	29.01.85	9 0	-9.2
Chick	05.09.84	13.8	0
	14.11.84	14.6	+24.0
	08.05.85	10.2	+14.0
Tavendale	18.07.84	10.0	0
	04.12.84	9.9	-3.6
	14.05.85	14.2	+7.9
Beharrie	23.11.84	18.5	0
	06.02.85	8.2	-15.1
	14.05.85	10.1	-18.2

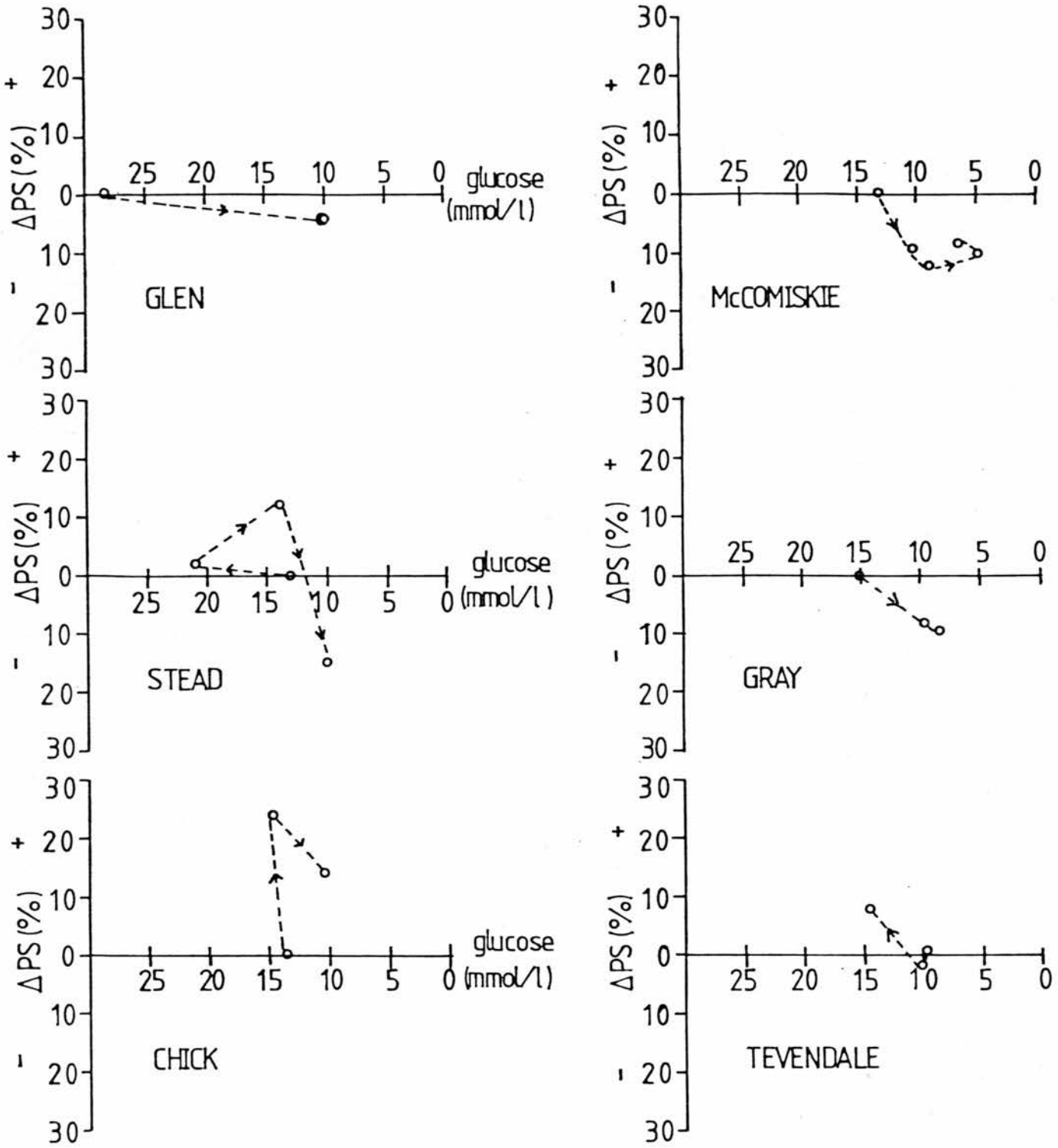


Fig.15 LONGITUDINAL PHOSPHOLIPID STUDIES ON POORLY-CONTROLLED DIABETICS.

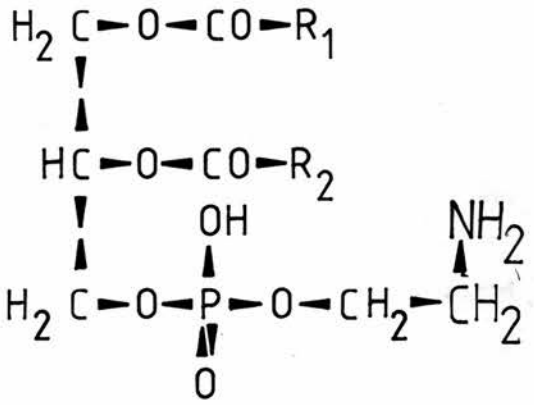
(Variation of PS with glucose control).

3.3 IN VITRO GLYCOSYLATION OF PHOSPHOLIPIDS

3.3.1 MOLECULAR MODELLING

Figures 16a, 16b, 17a and 17b demonstrate the glycosylation of PE and PS. The construction of these models served to illustrate; (i) the substantial increase in head group size caused by glucose addition; (ii) that the carboxyl (-COOH) group of serine might offer some steric hindrance in the approach of glucose to the amino ($-NH_2$) group.

PHOSPHATIDYL ETHANOLAMINE



(molecular wt. 269·2 + R_1 + R_2)

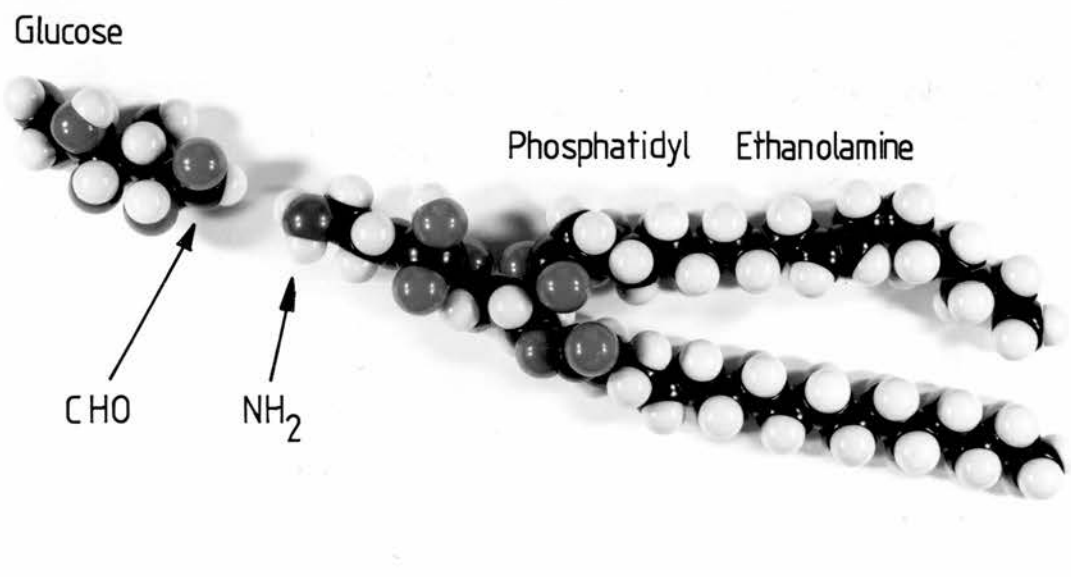
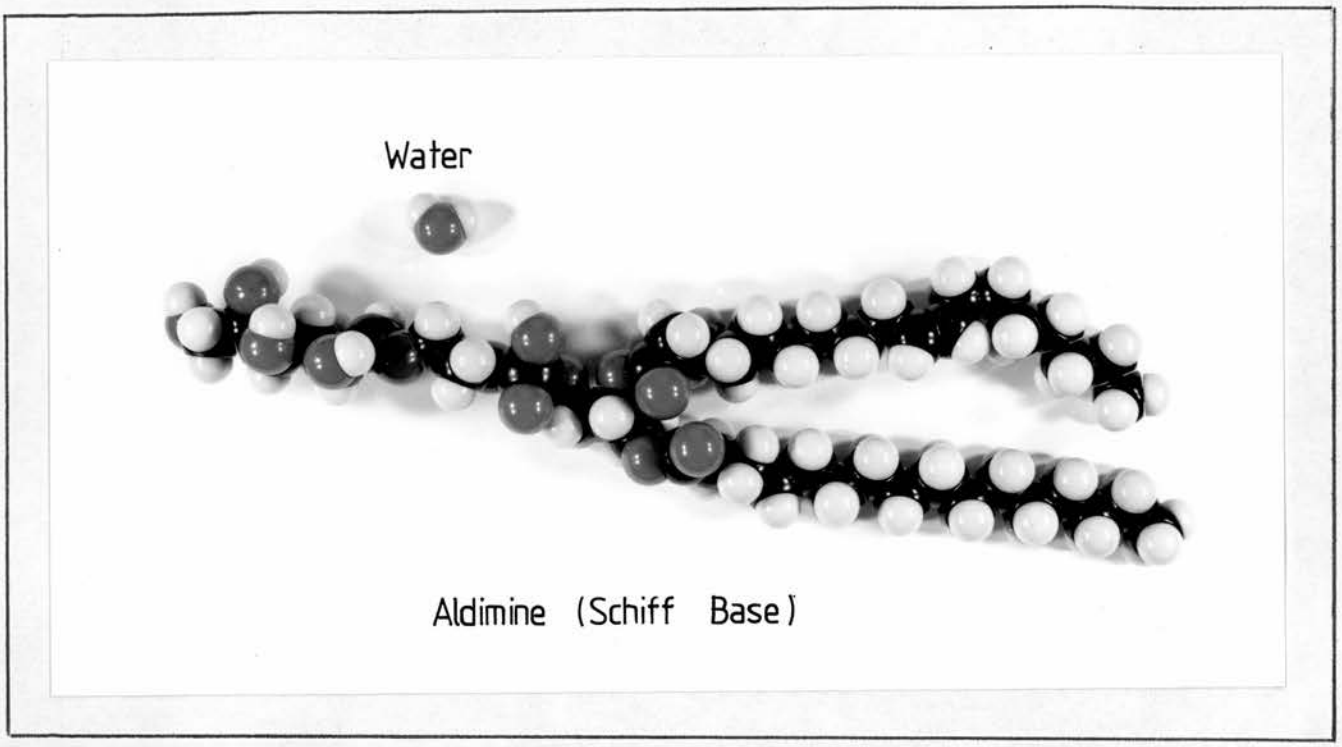


Fig.16a THE NON-ENZYMATIC GLYCOSYLATION OF PHOSPHATIDYL ETHANOLAMINE.



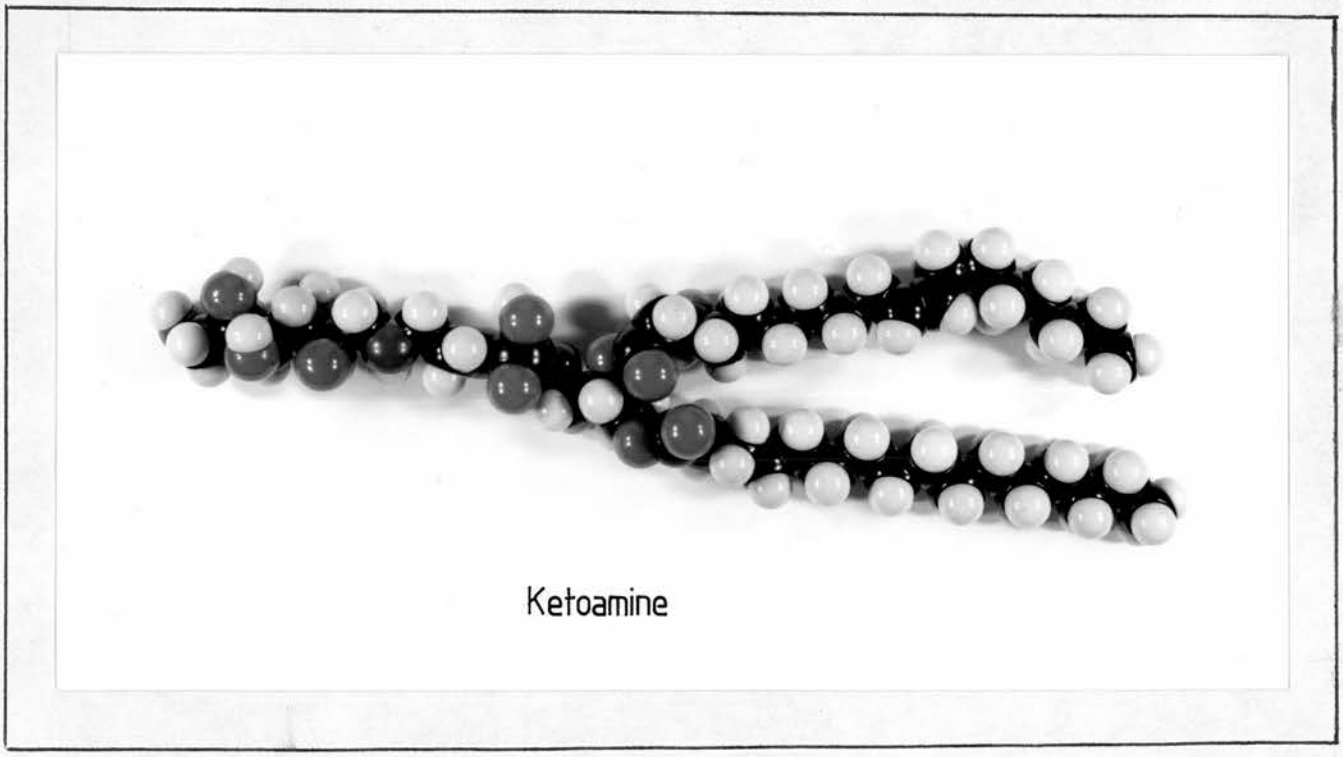
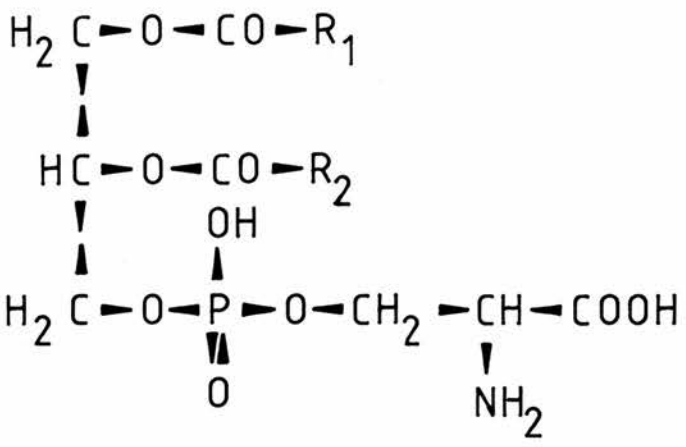


Fig.16b GLYCOSYLATION PRODUCTS OF PHOSPHATIDYL ETHANOLAMINE.

PHOSPHATIDYL SERINE



(molecular wt. 313·2 $+R_1$ $+R_2$)

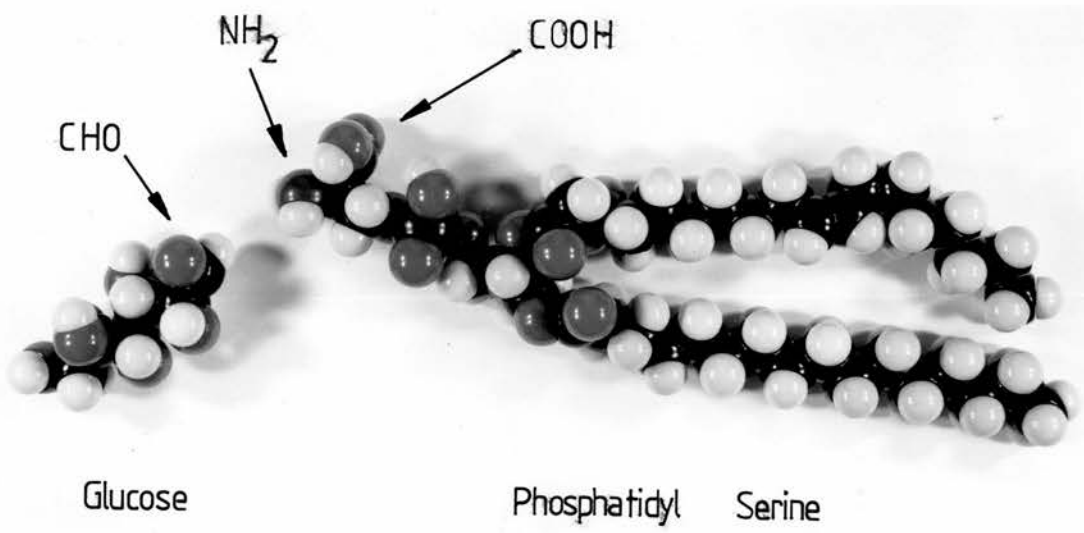
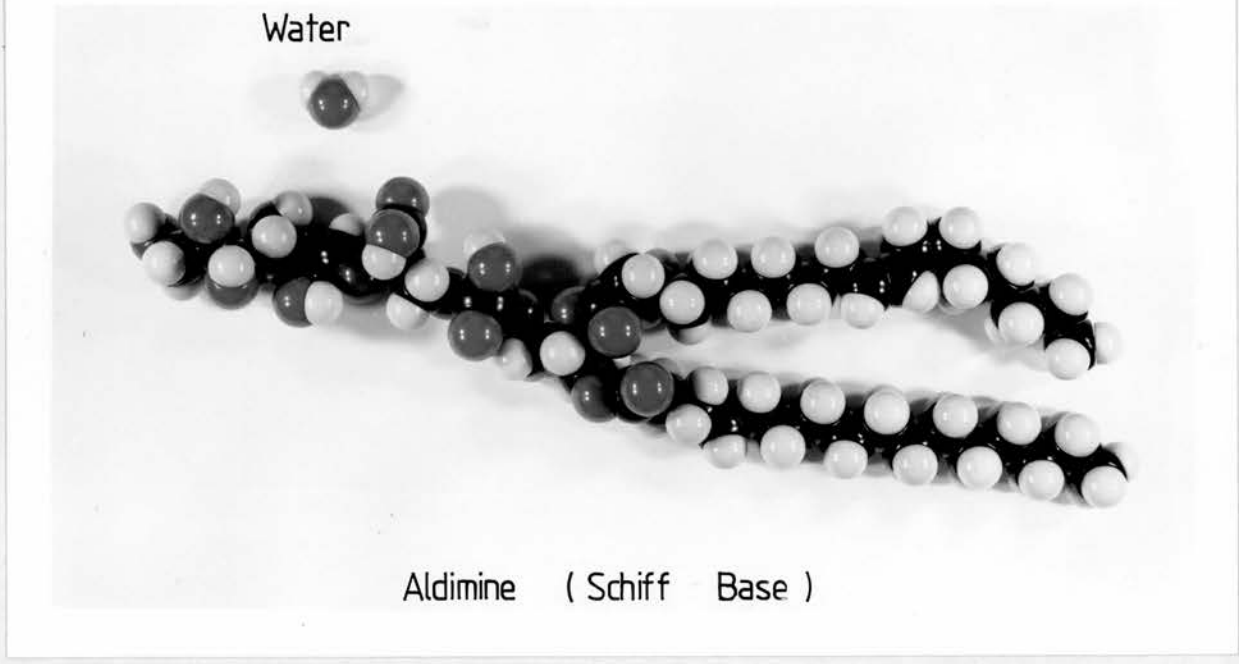


Fig.17a THE NON-ENZYMATIC GLYCOSYLATION OF PHOSPHATIDYL SERINE.



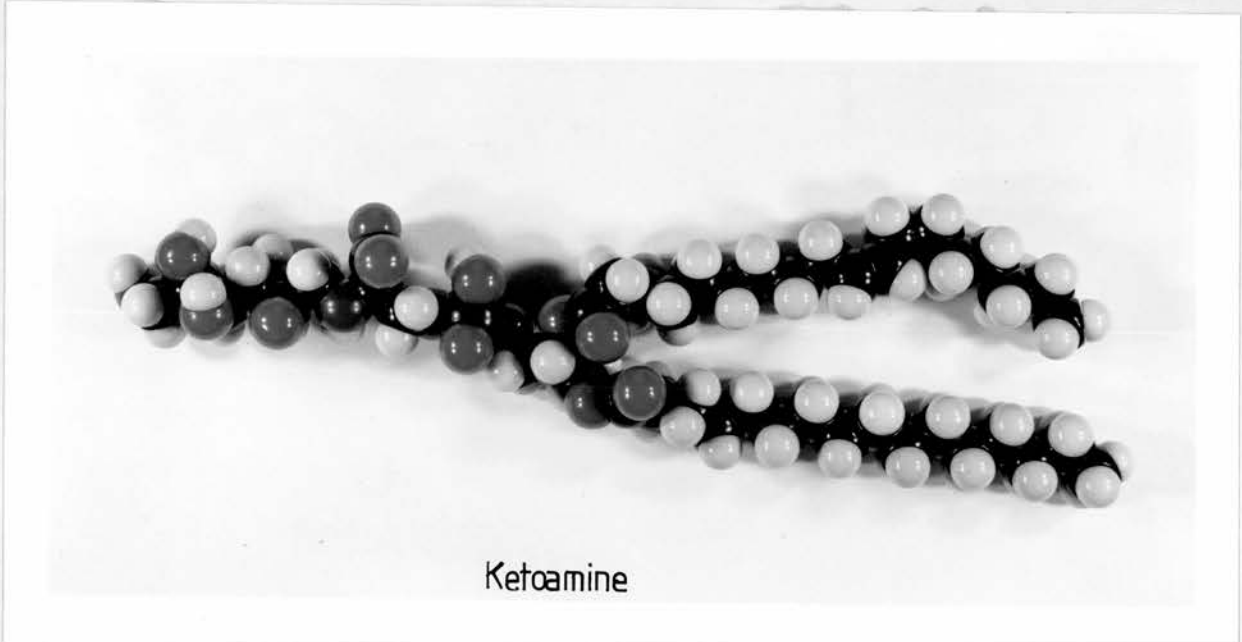


Fig.17b GLYCOSYLATION PRODUCTS OF PHOSPHATIDYL SERINE.

3.3.2 GLYCOSYLATION OF ISOLATED PHOSPHOLIPIDS IN NON-PHYSIOLOGICAL MEDIA

3.3.2.1 IDENTIFICATION OF GLYCOSYLATED PHOSPHOLIPIDS

Figures 18 and 19 show chromatograms of the reaction mixtures after reacting 5 mg of PE (dipalmitoyl) or PS (from bovine brain) with 50 mg of unlabelled and labelled glucose (10 μCi) in methanol at 65°C for 24 hours. The spots labelled glycosylated PE (GPE) and glycosylated PS (GPS) are tentatively identified thus on the basis of the following evidence:-

(i) Rapid and positive iodine staining - confirms the lipid nature.

(ii) Positive anthrone reaction (bluish-green colour) - confirms the presence of carbohydrates.

(iii) Positive phosphorus estimation - confirms the presence of phospholipid and allows quantitation.

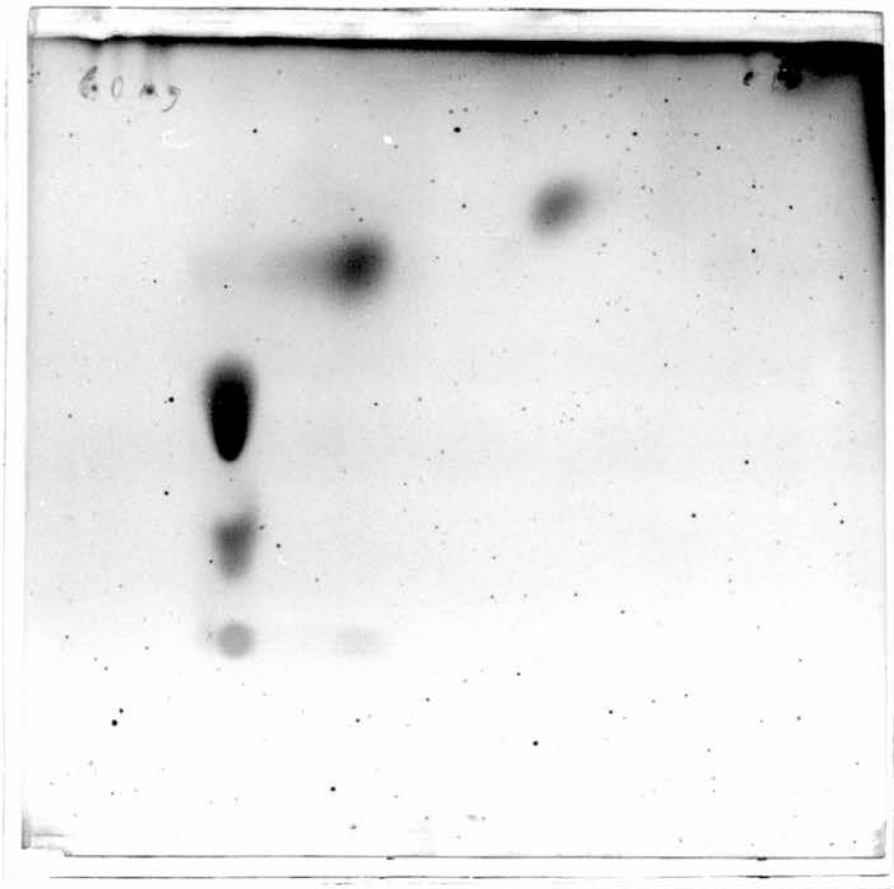
(iv) Autoradiography - demonstrates the incorporation of radioactive glucose.

The behaviour of these glycosylated products is rather interesting because in the solvent system used, the GPE co-chromatographs with PS and GPS co-chromatographs with PE. Figure 20 demonstrates spots for glucose control which were treated under the same experimental conditions. Similarly, single spots were observed for PE and PS controls.

Fig. 18

GLYCOSYLATION OF PE IN NON-PHYSIOLOGICAL MEDIA.

250µg total phospholipid (PE



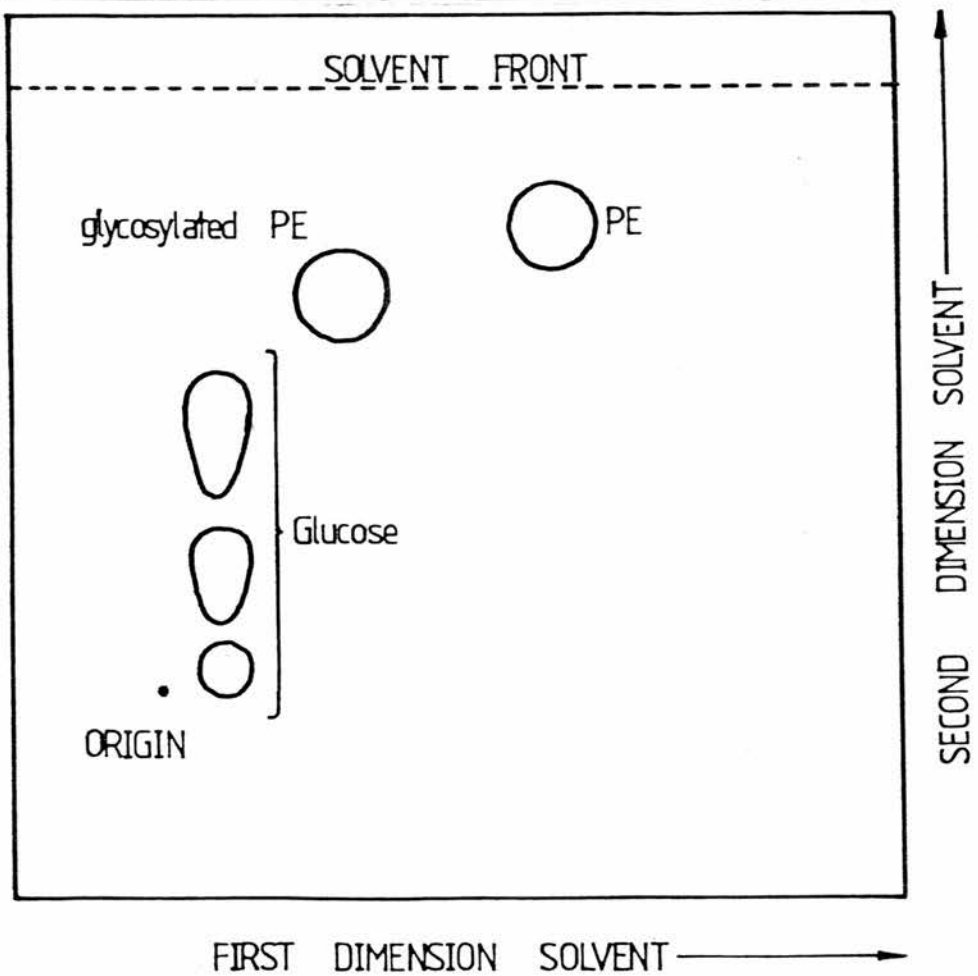
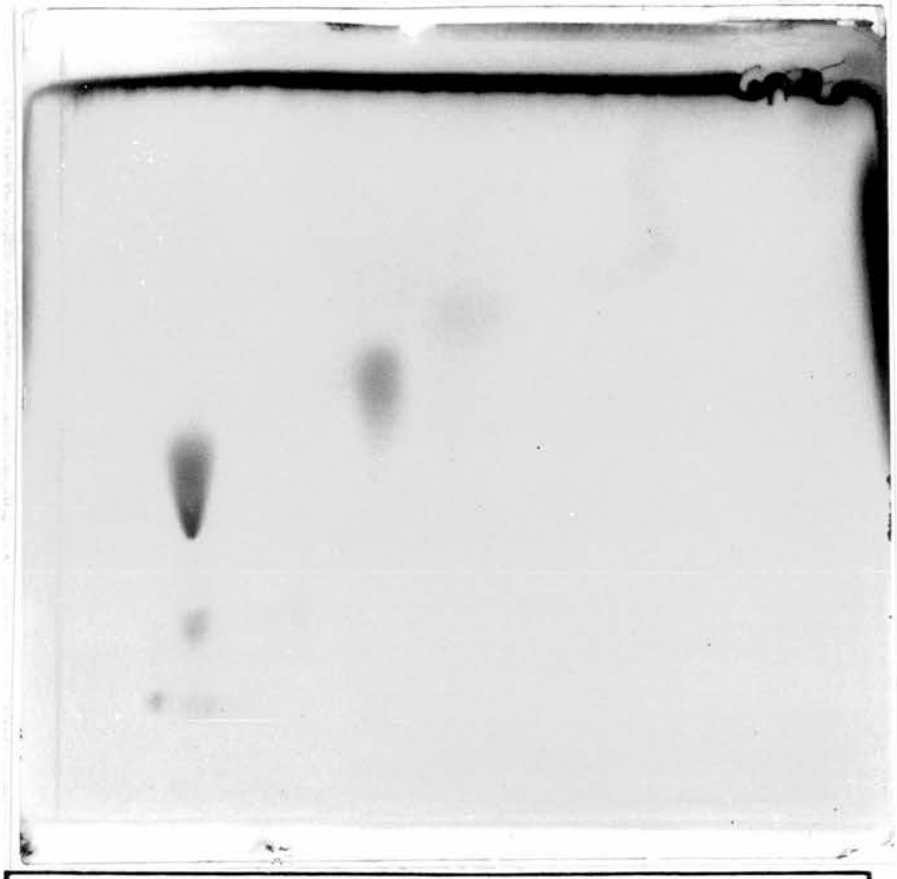
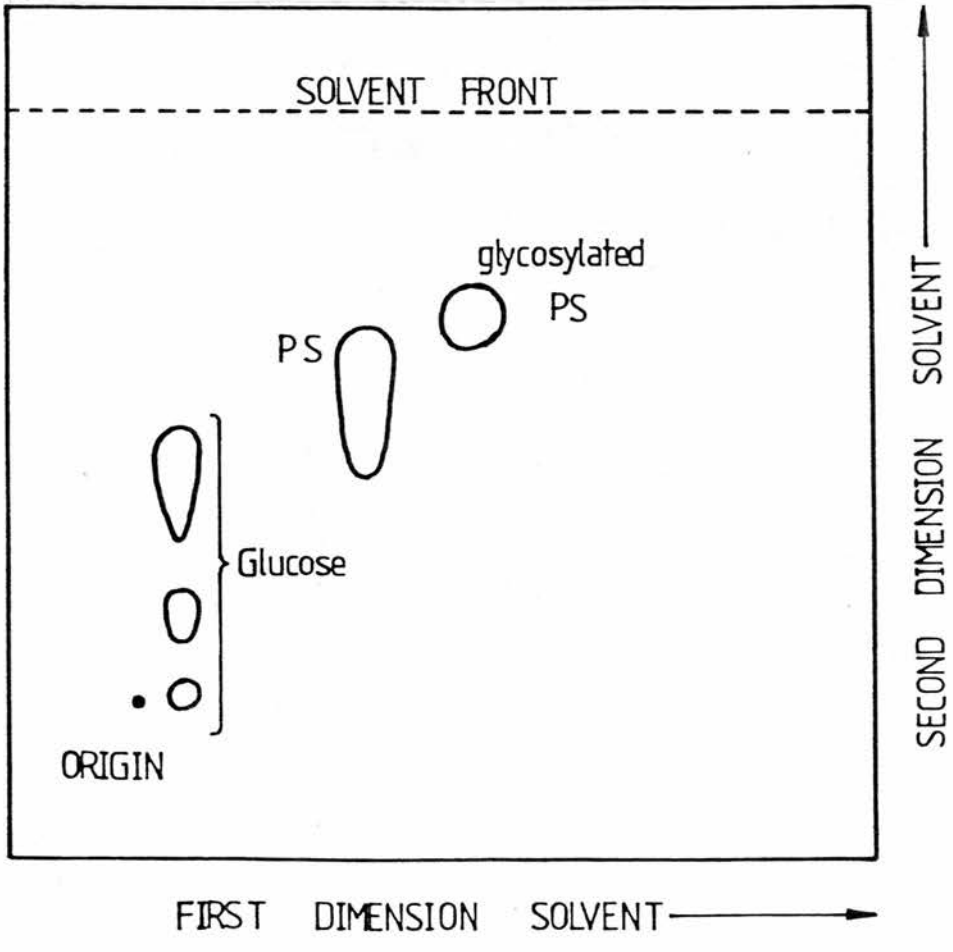


Fig. 19

GLYCOSYLATION OF PS IN NON-PHYSIOLOGICAL MEDIA.

250µg total phospholipid (PS)





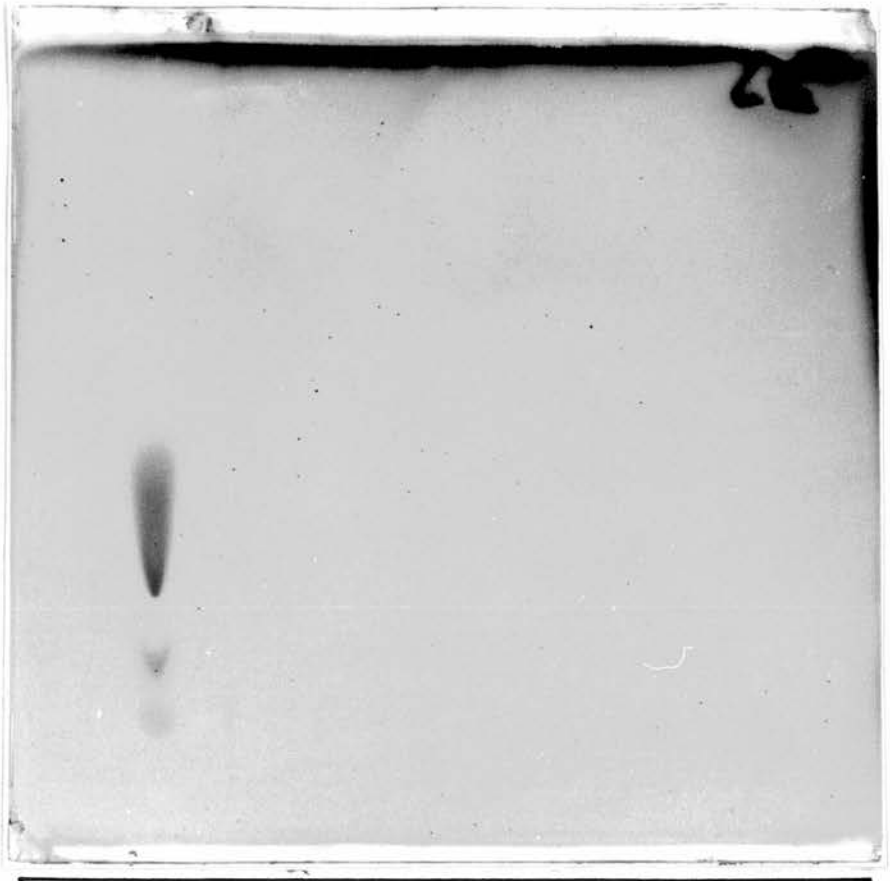
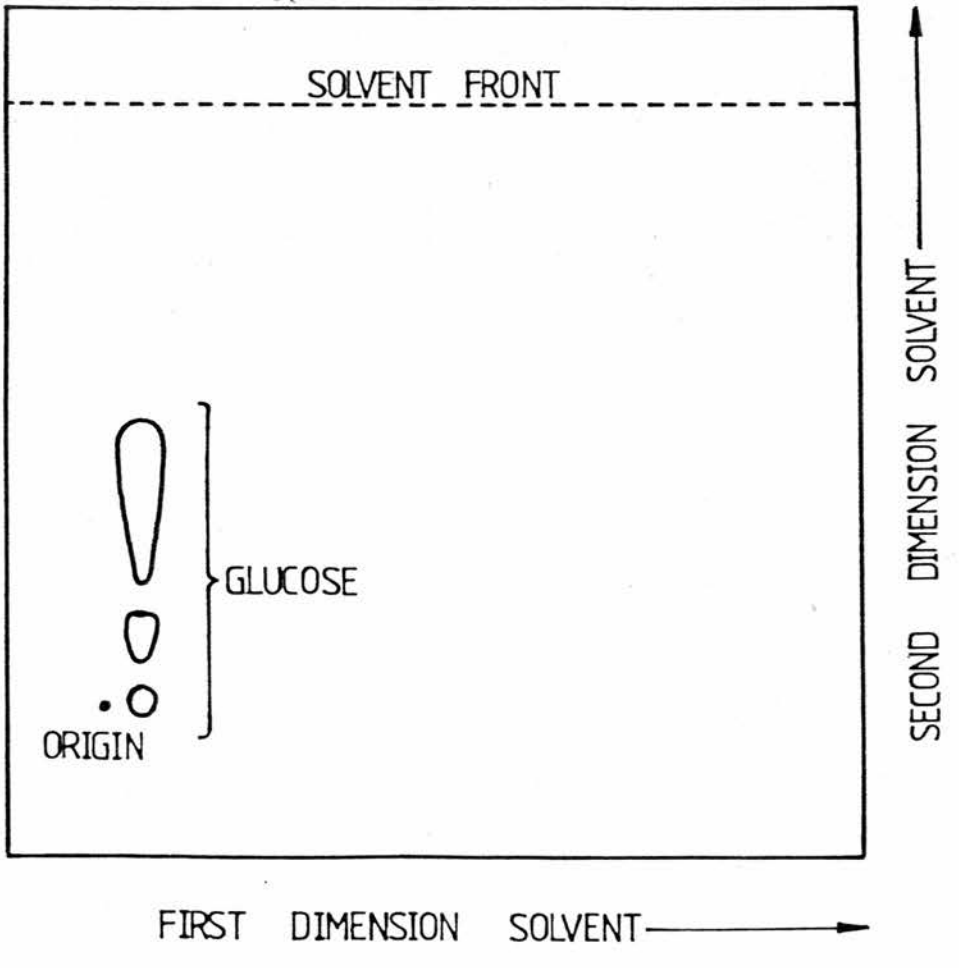


Fig. 20

GLUCOSE CONTRO

ON TLC PLATE



3.3.2.2 YIELD OF GLYCOSYLATED PE AND PS

The percentage incorporation of glucose and the percentage yield of glycosylated phospholipid are shown in Table 13. The yields of putative glycosylated derivatives of PE and PS were calculated in two ways; (i) by phosphorus analysis and (ii) from incorporation of radioactive glucose. Table 13 shows good agreement between the figures calculatedby either method and shows that approximately 30% conversion is achieved for both phospholipids under the conditions employed here.

Table 14 and Figures 21 and 22 show that for increasing concentration of glucose up to 5 mg/ml there is a linear increase in the proportion of glycosylated derivatives of PE and PS.

Table 13 **Yield of glycosylated phospholipids after glycosylation of pure synthetic phospholipid in non-physiological media**

Glycosylated Phospholipid	% conversion of Phospholipid	
	from phosphorus analysis	from ^{14}C glucose incorporation
GPS	25.0	31.3
GPE	30.0	32.5

Table 14 Glycosylation of isolated phospholipid in non-physiological media with varying concentration of glucose

(a) **PE**

Glucose (mg/ml)	0	1	2	3	4	5
Mole % **GPE**	0	7.0	12.0	18.5	25.5	32.7

(b) **PS**

Glucose (mg/ml)	0	1	2	3	4	5
Mole % **GPS**	0	7.5	13.6	21.0	27.5	34.2

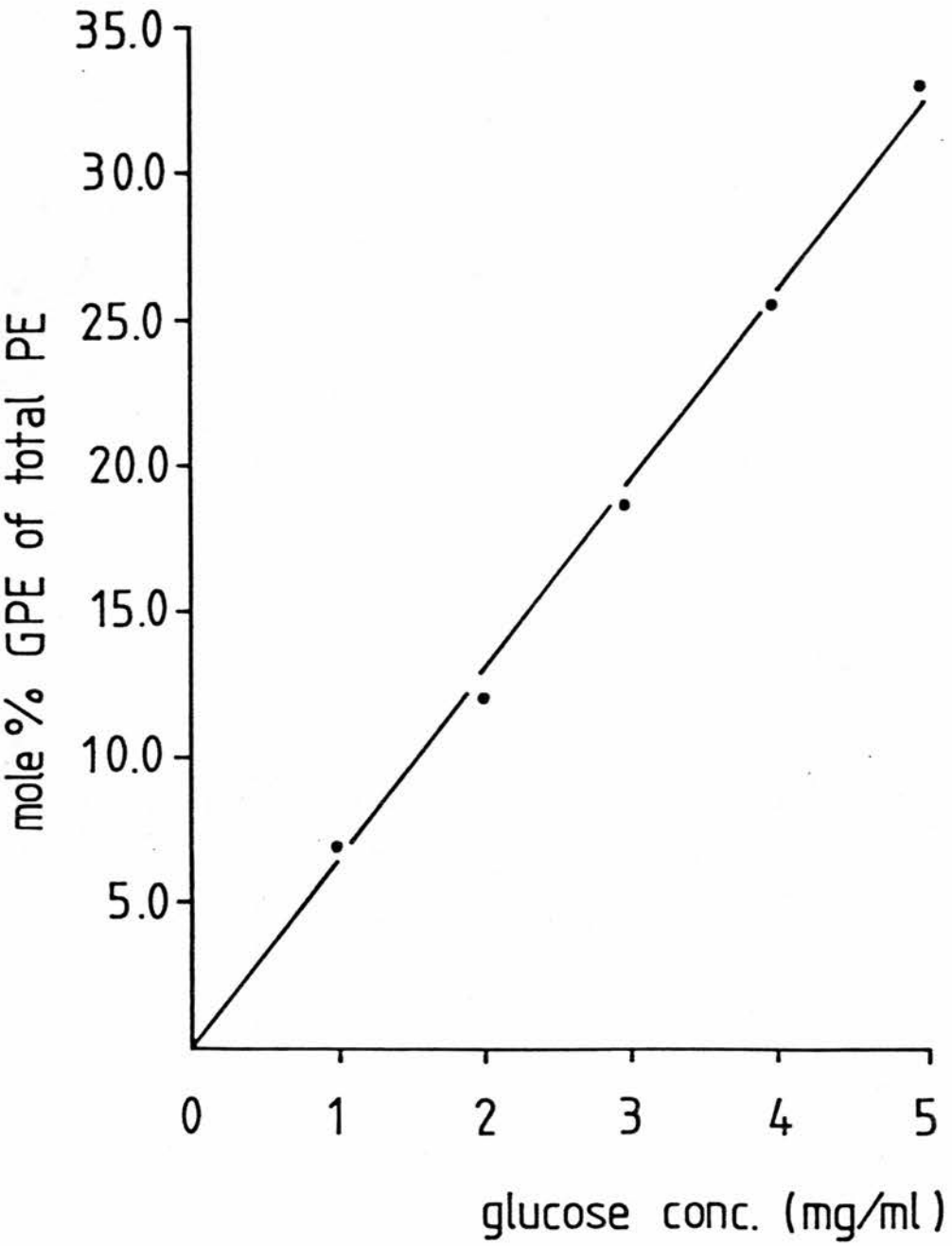


Fig. 21 Variation in the percentage glycosylated PE (GPE) with glucose concentration.

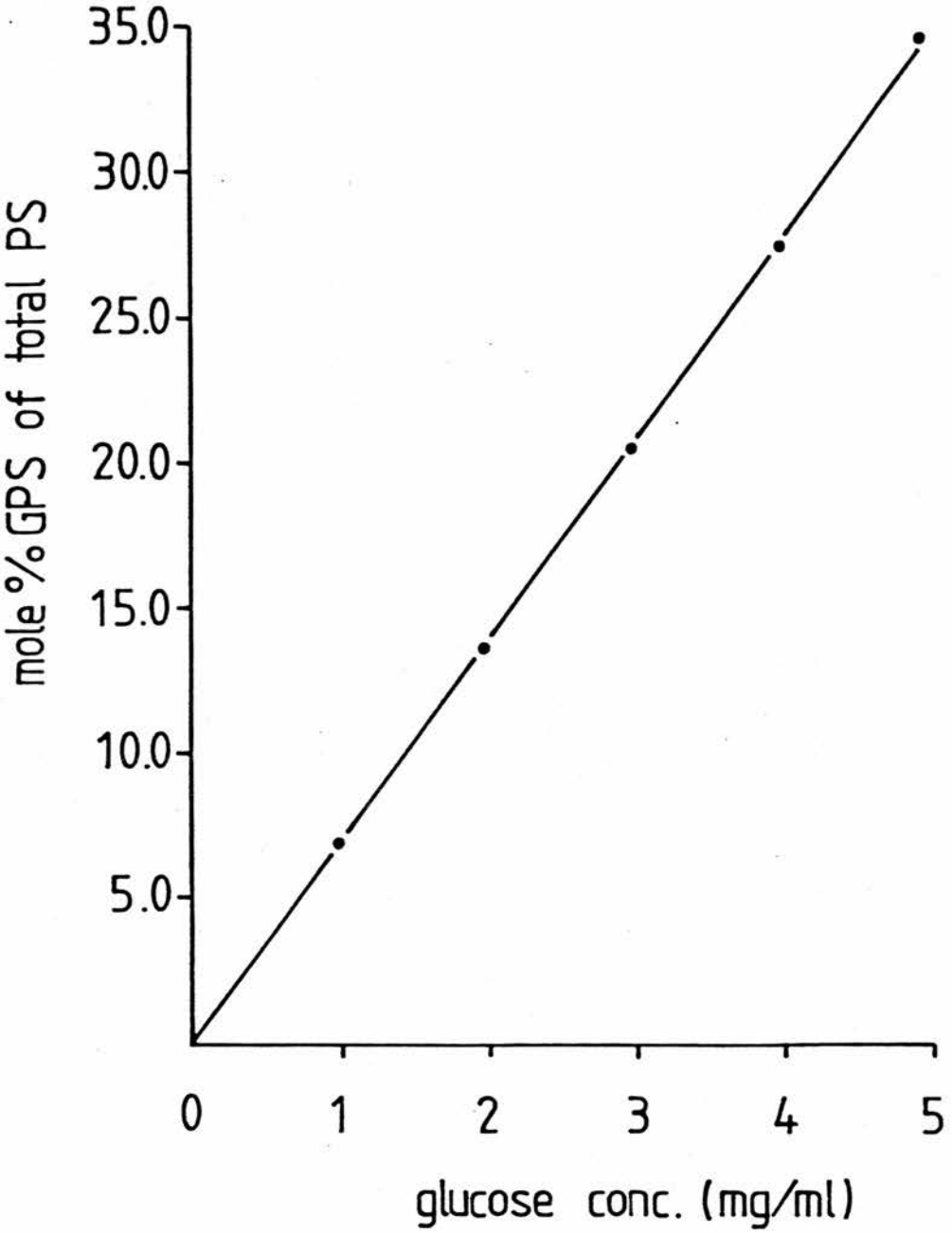


Fig. 22 Variation in the percentage glycosylated PS (GPS) with glucose concentration.

3.3.3 GLYCOSYLATION OF MEMBRANE PHOSPHOLIPIDS IN PHYSIOLOGICAL MEDIA

The protein content of the membrane preparations was assayed using bovine serum albumin as standard. Data for the calibration graph are given in Table 15 and Figure 23. The increase in mole per cent of membranes PS and decrease in mole per cent PE with glucose concentration is shown in Table 16 and Figures 24a and 24b. The percentage conversion of PE to its glycosylated derivative with increasing glucose concentration is shown in Table 17 and Figure 25. Two methods of calculation were used, based on the decrease in PE and the apparent increase in PS, and it can be seen that there is close agreement between the results whichever basis is used. The glycosylation reaction appears completely linear with increasing glucose concentrations well in excess of the physiological.

Incubation of membranes (800 μg protein/ml) with tracer amounts of glucose (^{14}C specific activity 275 mCi/mmol) for 5 days followed by lipid extraction, separation and counting of radioactivity was carried out as shown in Figure 27. Autoradiography of the separated lipids gave a strong radioactive spot for GPE and a much weaker one for GPS (Figure 26). Recovered counts showed that the ratio of label for glucose in PE, PS and protein was 4:1:5, indicating an increased affinity of PE for glycosylation compared with PS, even taking into account their relative abundance in the membrane (Figure 27).

Table 15 Albumin standard assay

Albumin conc. (μg/ml)	0	40	80	120	160	200
Absorbance units	0	0.06	0.12	0.18	0.24	0.30

Erythrocyte membrane proteins were read from the above graph as 800 μg/ml.

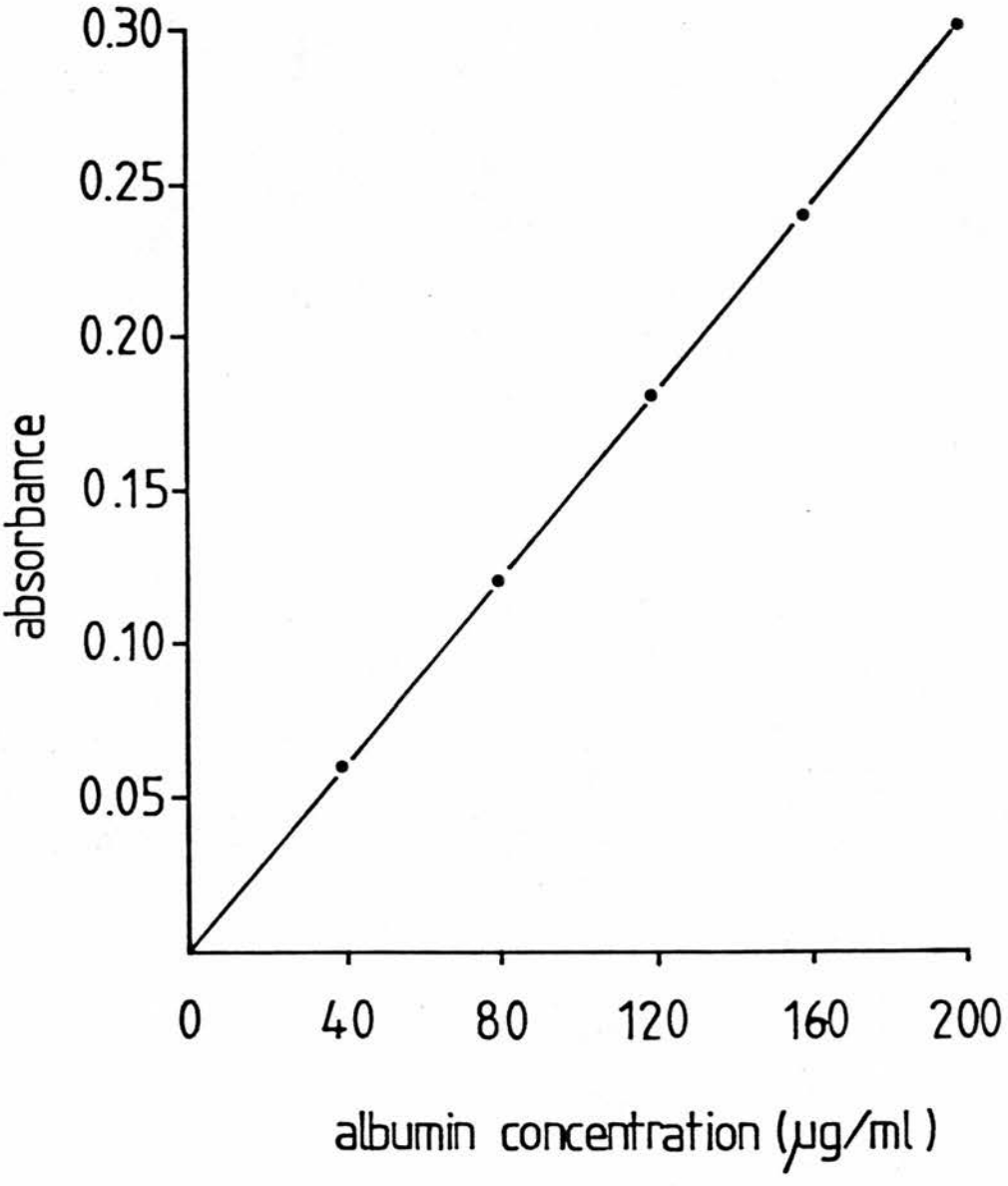


Fig. 23 Albumin standard curve

Table 16 **Glycosylation of erythrocyte membrane phospholipids in physiological media**

(Variation of membrane PS and PE with glucose conc.)

Glucose (mmol/l)	0	10	20	40	60	80	100
+ Δ PS (%)	0	0.5	1.2	1.8	3.2	3.7	4.7
- Δ PE (%)	0	0.3	1.0	1.5	2.5	3.1	4.0

% Δ PS = new PS - basal PS.

% Δ PE = basal PE - new PE.

Table 17 **Glycosylation of erythrocyte membrane phospholipids in physiological media**

(variation of mole % glycosylated membrane PE with glucose concentration)

Glucose (mmol/l)	0	10	20	40	60	80	100
$+\Delta$ PS %	0	1.8	4.2	8.5	12.1	16.4	19.0
$-\Delta$ PE %	0	1.4	3.4	6.8	11.0	13.5	17.4

$+\Delta$ **PS** = Percentage conversion of PE to GPE calculated from the apparent increase in PS.

FORMULA: $+\Delta PS = \frac{\Delta PS}{\text{new } PE + \Delta PS} \times 100$

$-\Delta$ **PE** = Percentage conversion of PE to GPE calculated from the apparent decrease in PE.

FORMULA: $-\Delta PE = \frac{\Delta PE}{\text{new } PE + \Delta PS} \times 100$

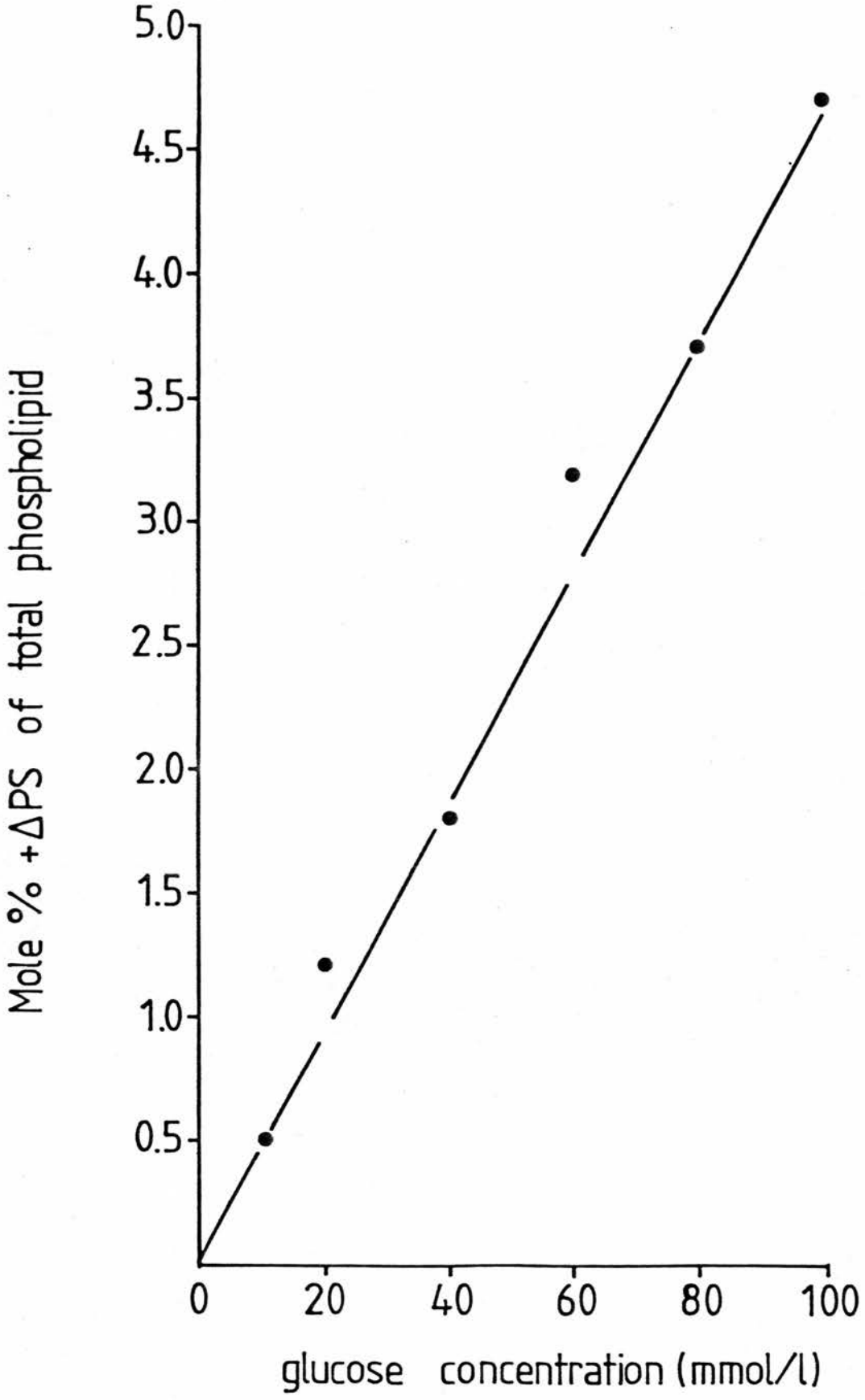


Fig. 24a Variation in membrane PS with glucose concentration.

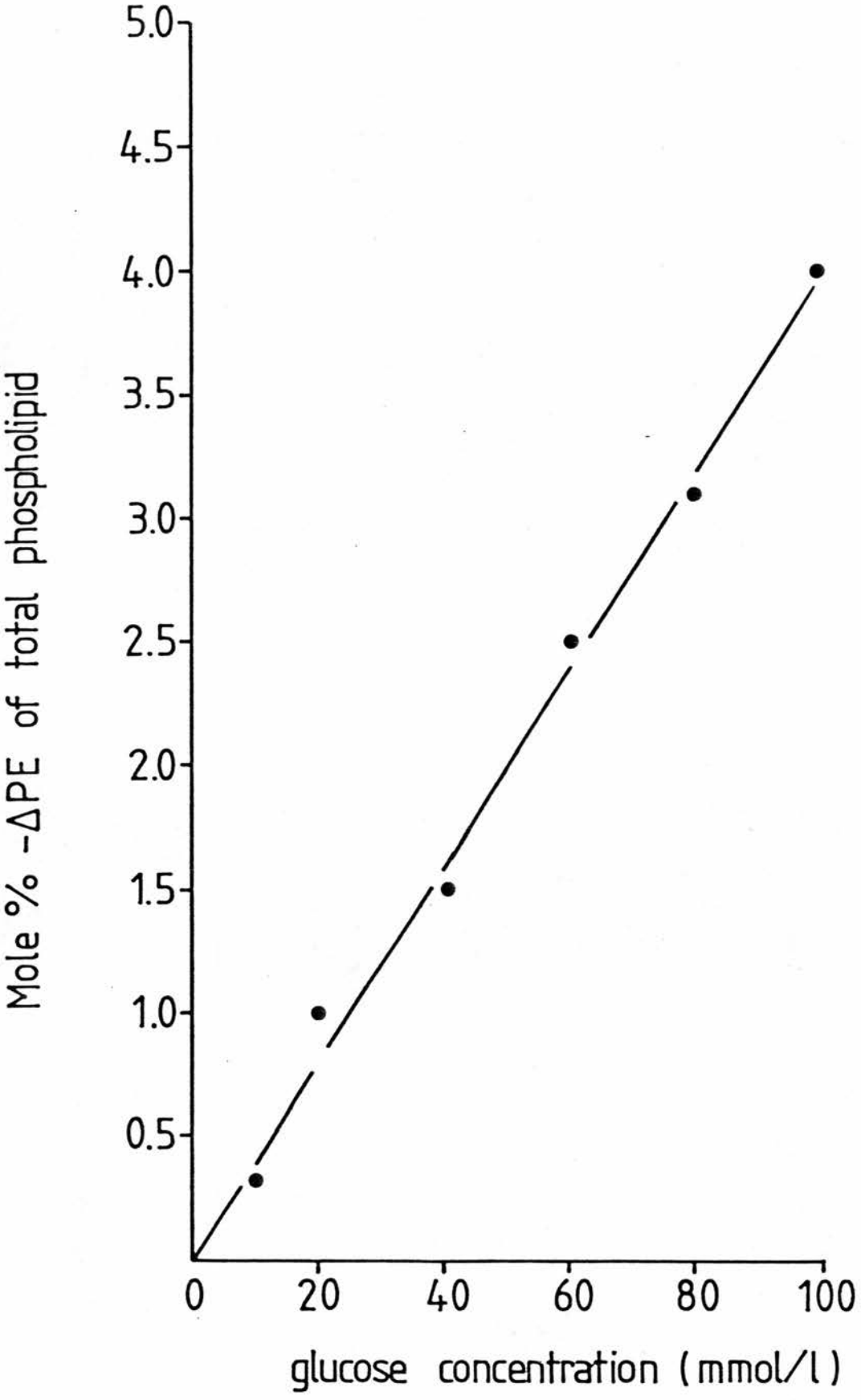


Fig.24b Variation in membrane PE with glucose concentration.

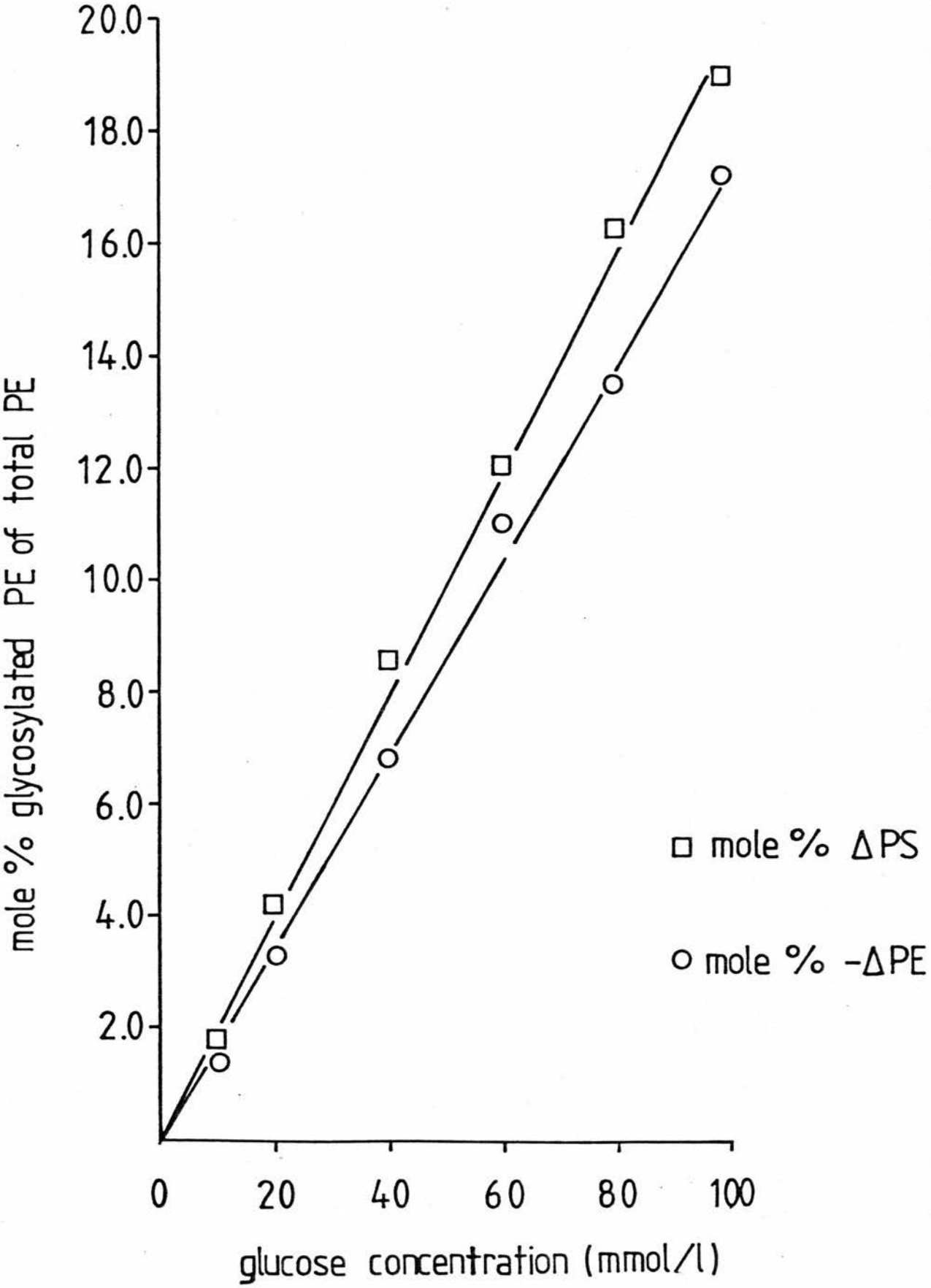


Fig. 25 Variation in mole % glycosylated membrane PE (GPE) with glucose concentration.

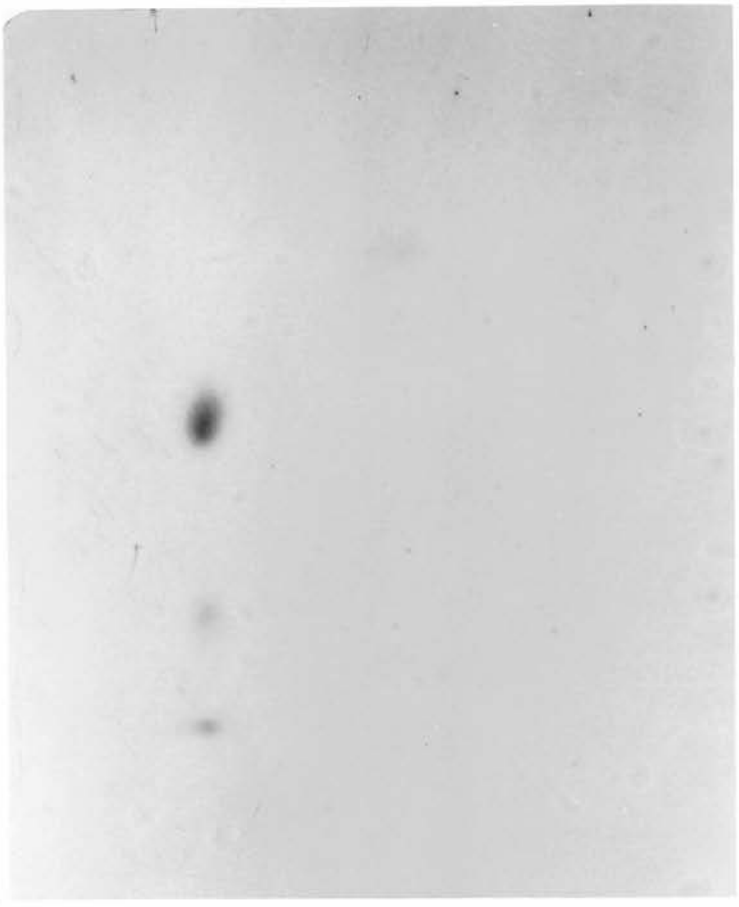
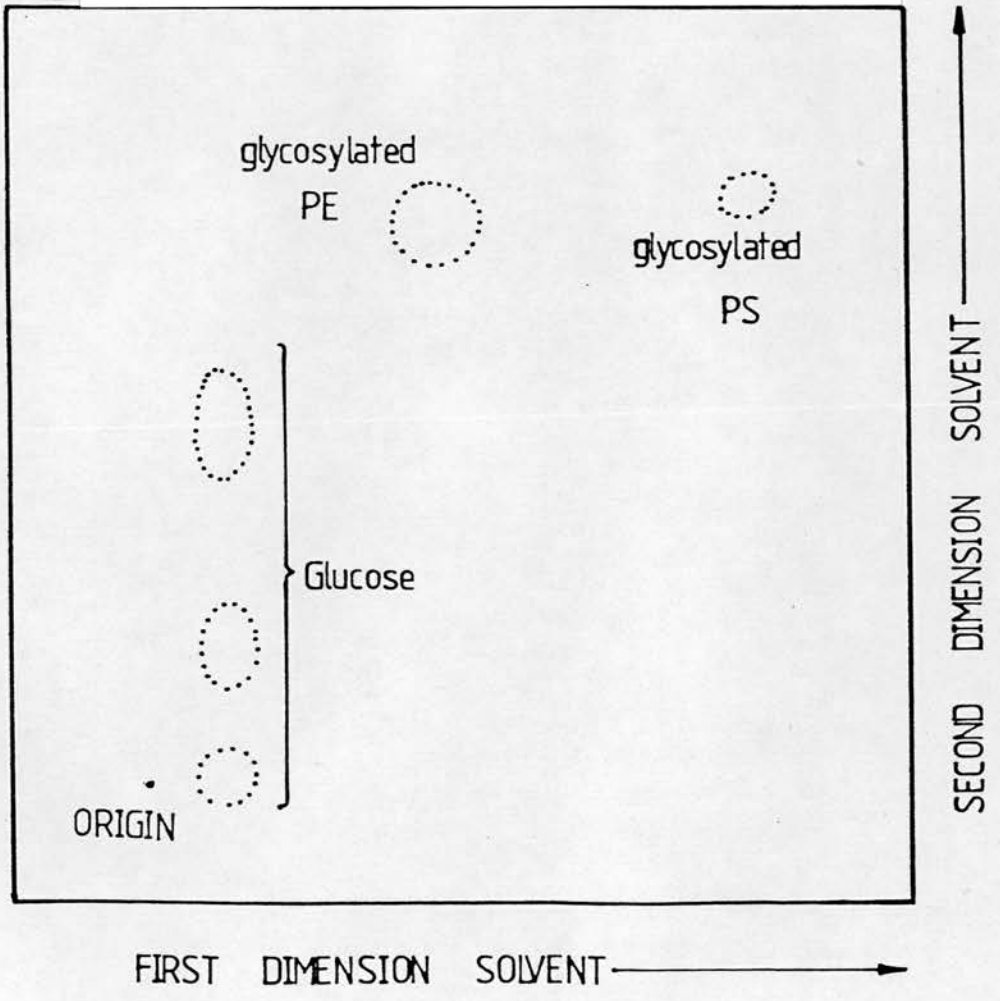


Fig. 26

AUTORADIOGRAM OF GLYCOSYLATED MEMBRANE PHOSPHOLIPIDS.



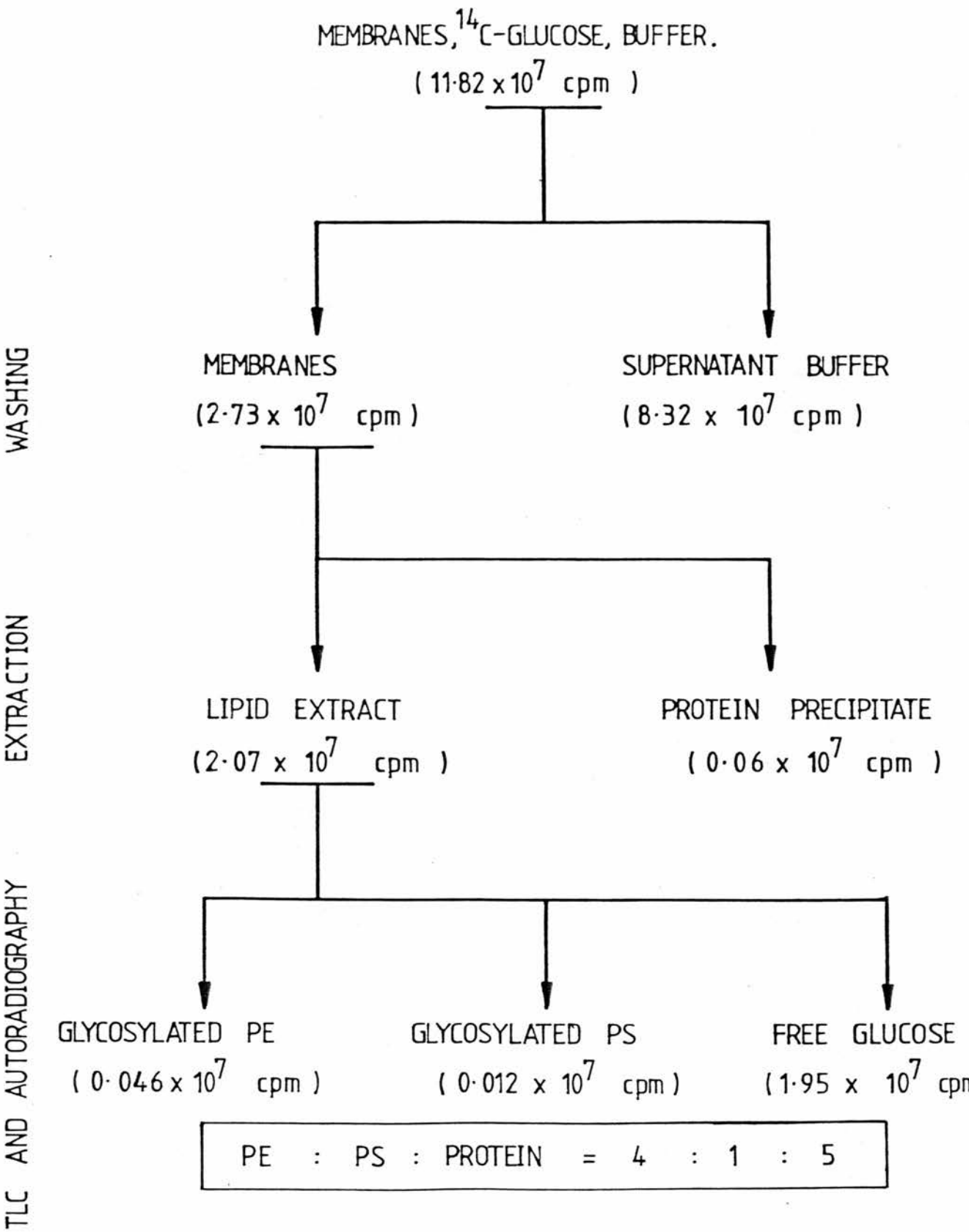


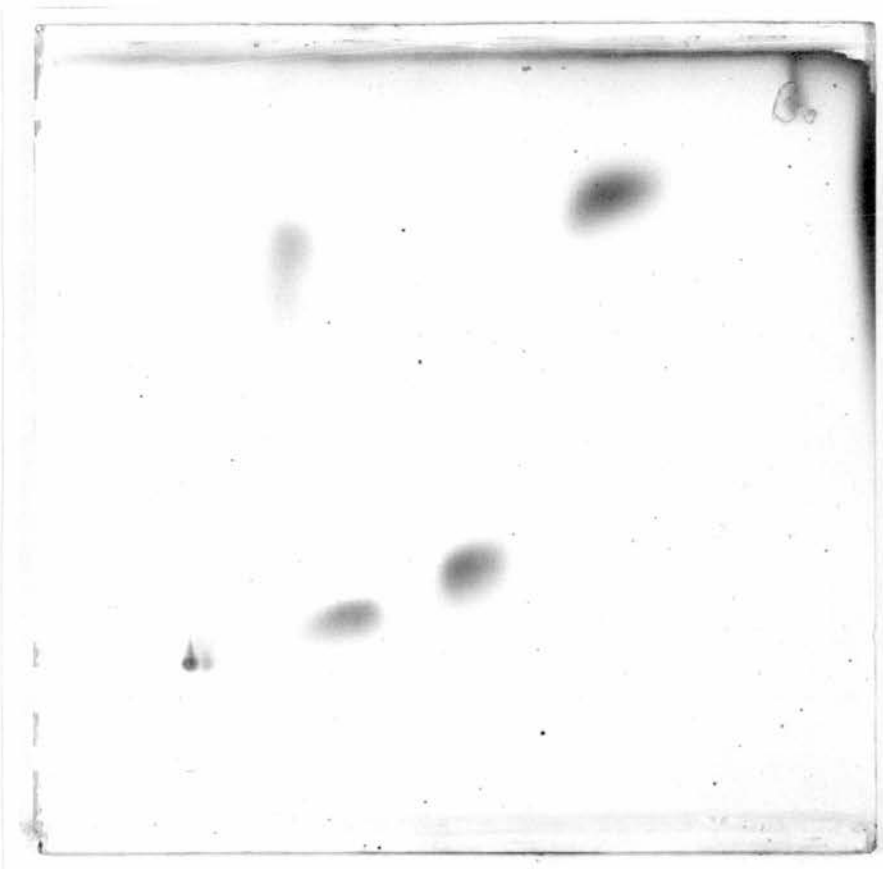
Fig. 27 FLOW DIAGRAM FOR ERYTHROCYTE MEMBRANE GLYCOSYLATION.

3.4 THE MEASUREMENT OF GLYCOSYLATED PHOSPHOLIPIDS

3.4.1 "MODIFIED" TLC SYSTEM

Modification of the existing two-dimensional TLC system in our laboratory was essential for mixtures containing PS plus GPE since these components overlapped. Modification was found only to be necessary in the first dimension where the proportions of components were changed from $CHCl_3$/MeOH/25% NH_3/H_2O (14.5:8:1:1) to $CHCl_3$/MeOH/35% NH_3 (14.5:8:1). Resolution between PS plus GPE was achieved by more extensive separation of PS from PE as shown for red cell lipids in Figure 28. The separation of normal erythrocyte membrane phospholipids and *in vitro* glycosylated erythrocyte membrane phospholipids (20 mM glucose for 5 days) at double the loading in Figure 28 are shown in Figures 29 and 30 respectively. Figure 30 demonstrates an additional glycosylated phospholipid (GPE) spot that co-chromatographs with our putative synthetic GPE.

Fig. 28



"MODIFIED" TLC for PHOSPHOLIPIDS.

iodine-stained

250µg total phospholipid (red cell lipids)

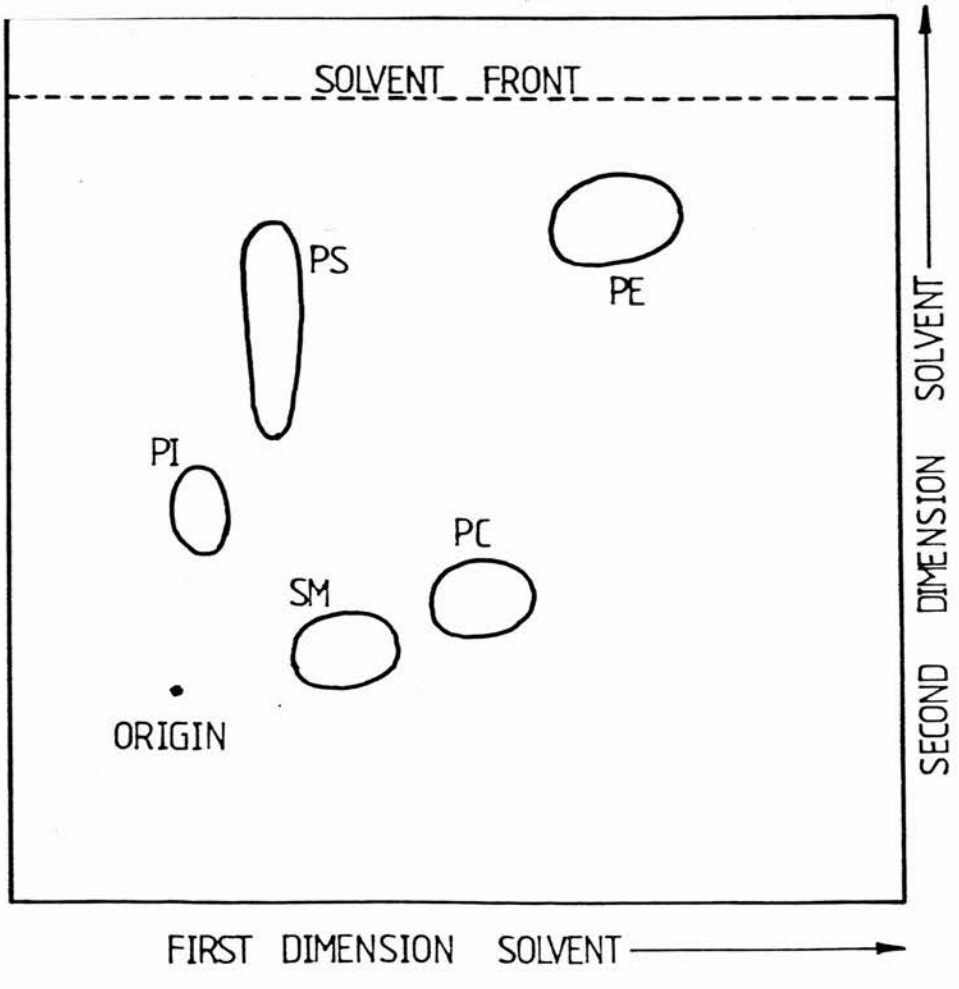
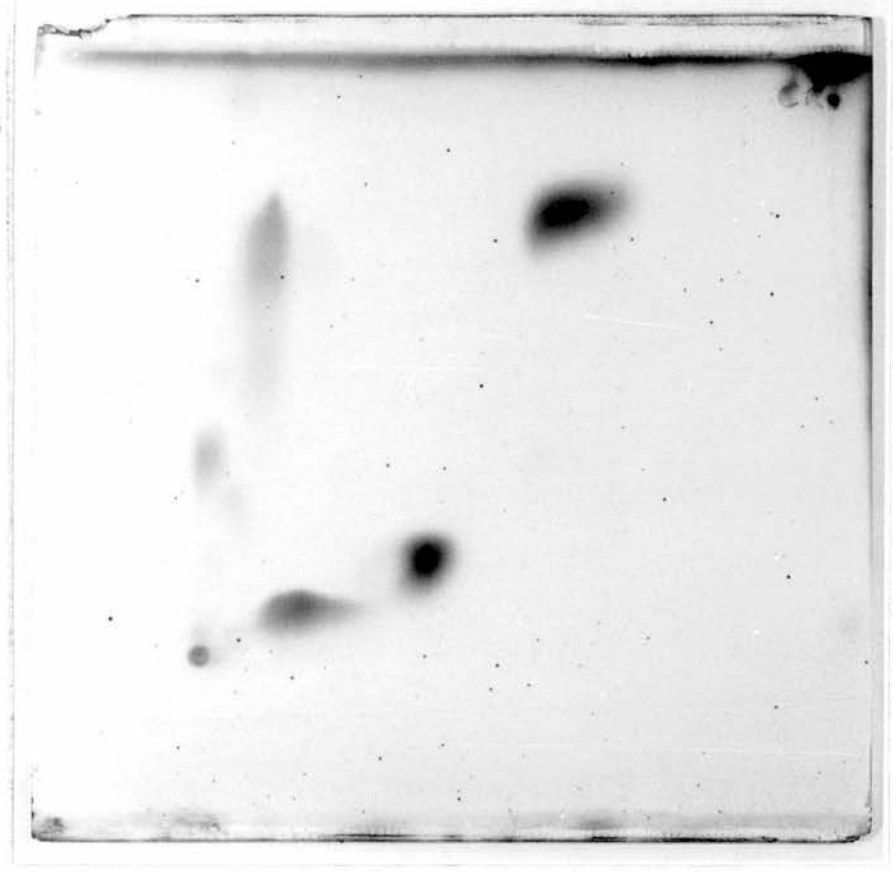


Fig. 29

ERYTHROCYTE MEMBRANE PHOSPHOLIPIDS ON 'MODIFIED' TLC.

iodine-stained

500 µg total phospholipids



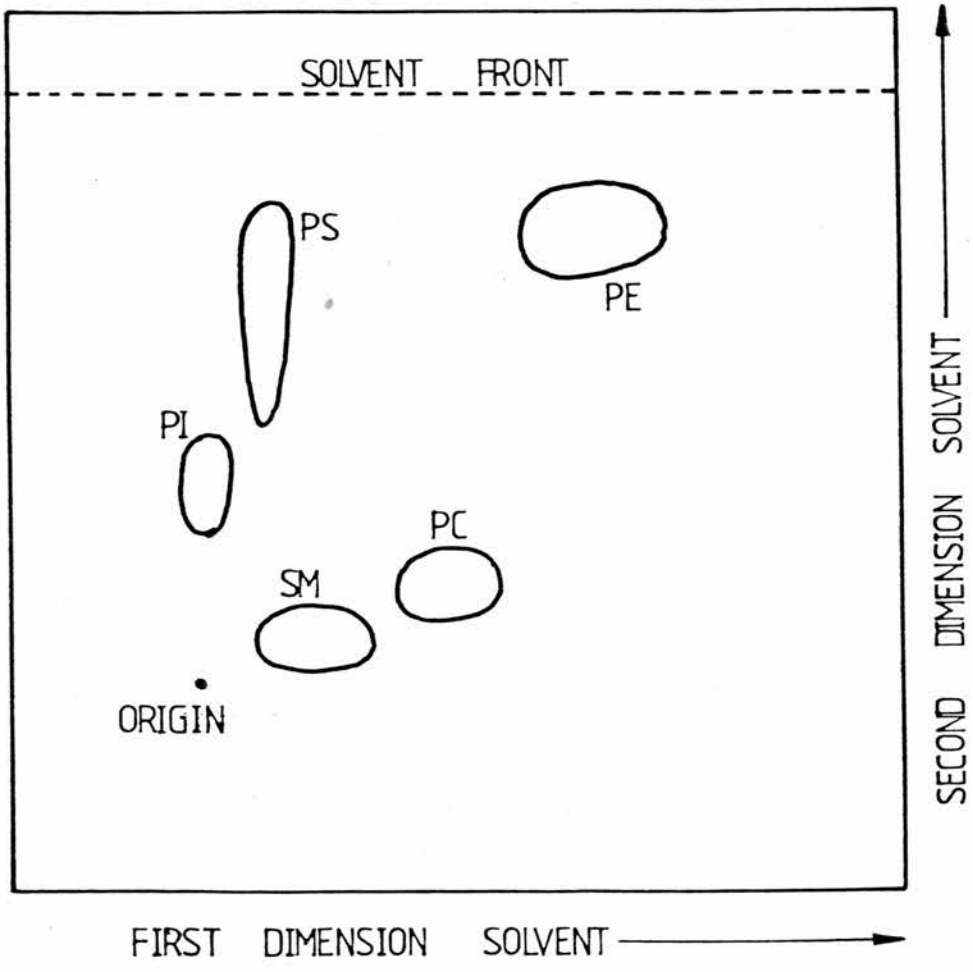


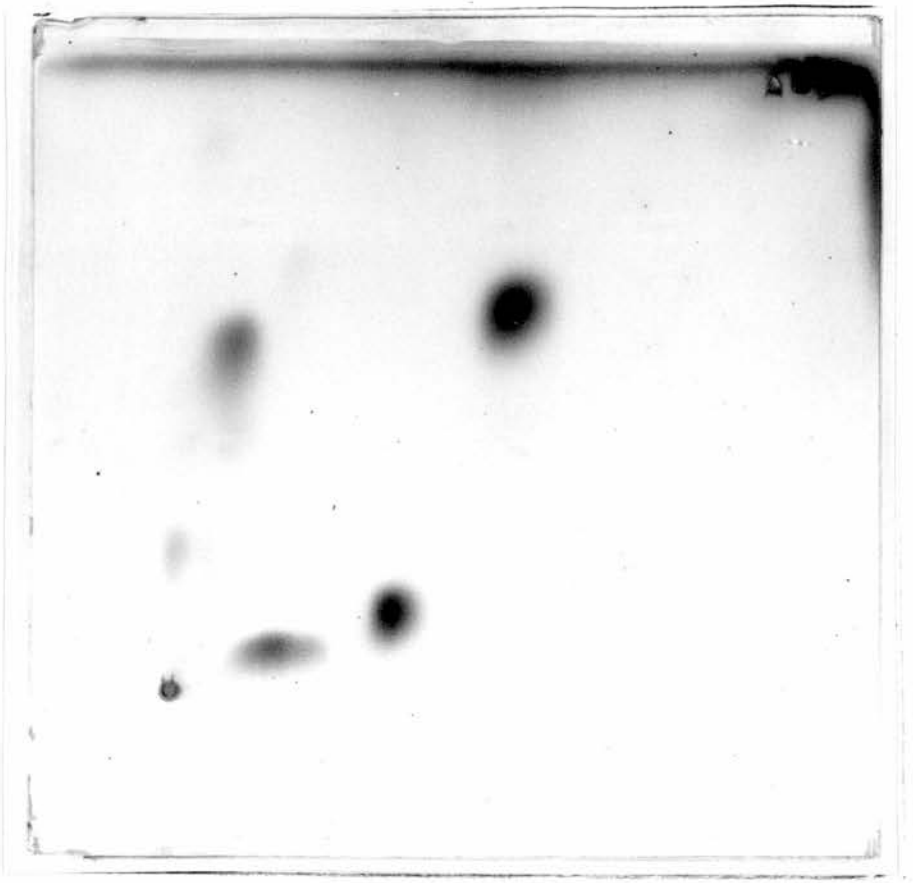
Fig. 30

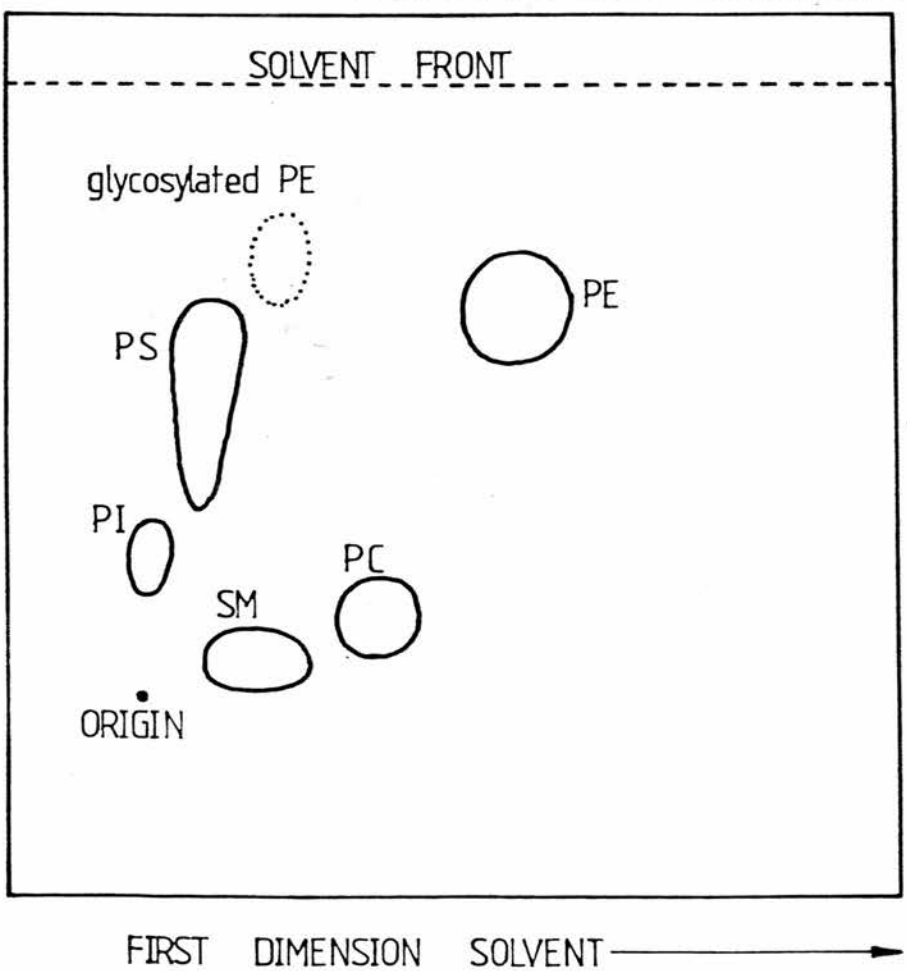
GLYCOSYLATED MEMBRANE PHOSPHOLIPIDS ON "MODIFIED" TLC.

(glucose 20 mmol/l

iodine-stained

500 µg total phospholipids





3.4.1.1 QUANTITATION OF GPE USING THE "MODIFIED" TLC SYSTEM

3.4.1.1.1 IN VITRO INCUBATION OF ERYTHROCYTE MEMBRANES WITH GLUCOSE

Table 18 and Figure 31 show that there was a linear increase in GPE with glucose concentration as measured by TLC in the new system and colorimetric phosphorus estimation.

3.4.1.1.2 COMPARISON OF WELL-CONTROLLED AND POORLY-CONTROLLED DIABETICS

The proportion of total red cell PE constituted by GPE for the two groups of diabetic patients is shown in Tables 19 and 20. The increased GPE seen in PCD as compared to WCD is highly significant ($p < 0.0005$). In addition it is important to note that there is no overlap between the ranges for the two groups of patients. Furthermore, Figures 32 and 33 show a correlation between both FPG ($r = 0.941$, $p < 0.0005$) and HbA_{1c} ($r = 0.784$, $p < 0.0005$) and GPE for both sets of patients.

Table 18 "Modified" TLC for glycosylated membrane phospholipid

(Variation of GPE with glucose conc.)

Glucose (mmol/l)	0	20	40	60	80	100
Mole % GPE	0	0.60	0.98	1.80	2.5	2.8

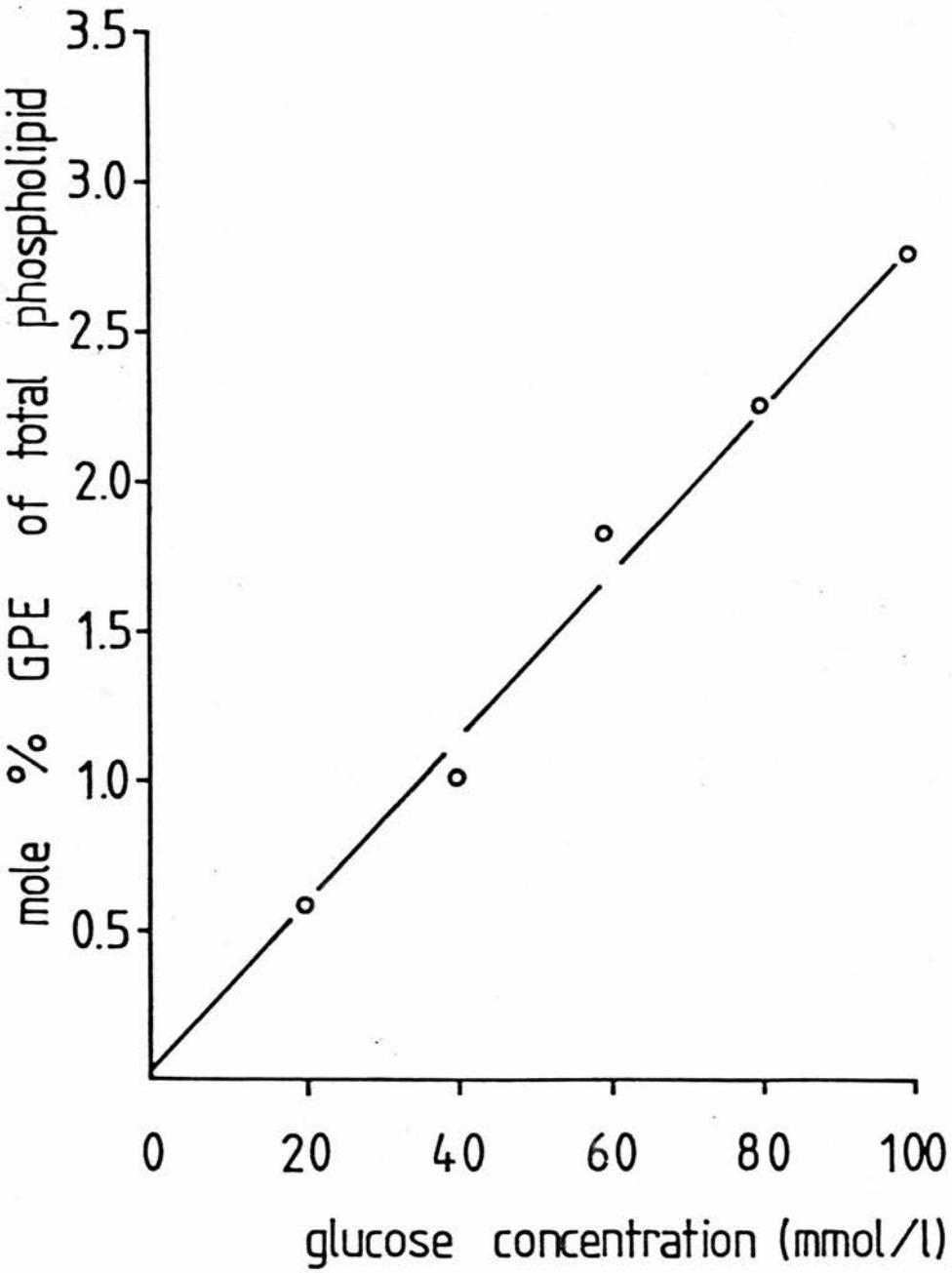


Fig.31 Measurement of glycosylated membrane PE by "modified" TLC system.

Table 19 "Modified" TLC for glycosylated phospholipids (GPE) in well-controlled diabetics

Name	Age (years)	Sex	FPG (mmol/l)	HbA_{1c} %	GPE (mole %)
Brash	56	F	6.6	6.5	1.2
Fairlie	58	M	5.3	5.3	1.0
Downie	56	M	5.6	6.9	1.3
Mustard	53	M	6.6	5.4	0.8
Smith	59	M	6.5	6.8	0.9
Gorrie	63	F	6.8	6.6	1.2
Widdis	58	M	6.5	8.6	1.1
n = 7					
Mean ($\bar{x}$)			6.1	6.8	1.1
Standard deviation (SD)			±0.6	± 1.0	±0.2

Table 20 "Modified" TLC for glycosylated phospholipids (GPE) in poorly-controlled diabetics

Name	Age (years)	Sex	FPG (mmol/l)	HbA_{1c} %	GPE (mole %)
Taylor	52	**F**	18.6	11.6	2.6
Glen	48	**F**	28.2	13.7	3.1
Grace	42	**M**	11.8	11.6	2.2
Joss	63	**M**	12.5	10.9	2.2
Watson	62	**M**	11.8	7.1	2.0
Chick	62	**F**	14.6	11.2	2.4
Mitchell	42	**F**	17.5	17.9	2.4
Stead	37	**F**	21.0	13.0	2.9
Cook	65	**F**	20.0	8.9	3 0
Roxburgh	39°	**F**	12.4	11.7	2.3
McCormiskie	31	**F**	13.0	10.1	2.0
n = 11					
Mean ($\bar{x}$)			16.4	11.6	2.5
Standard deviation (SD)			±5.1	±2.9	±0.4

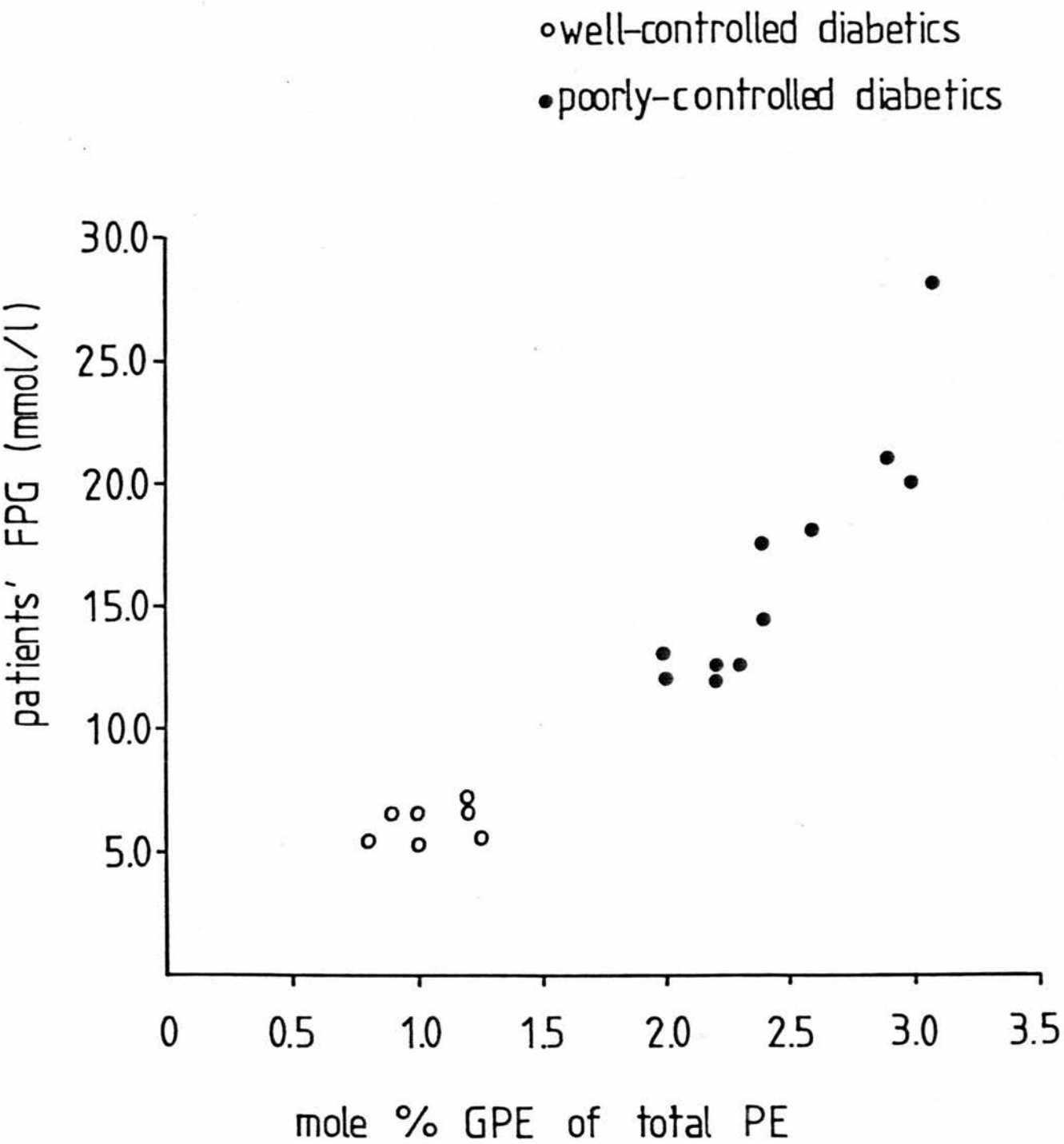


Fig. 32 Correlation between patients' fasting plasma glucose (FPG) and glycosylated PE (GPE).

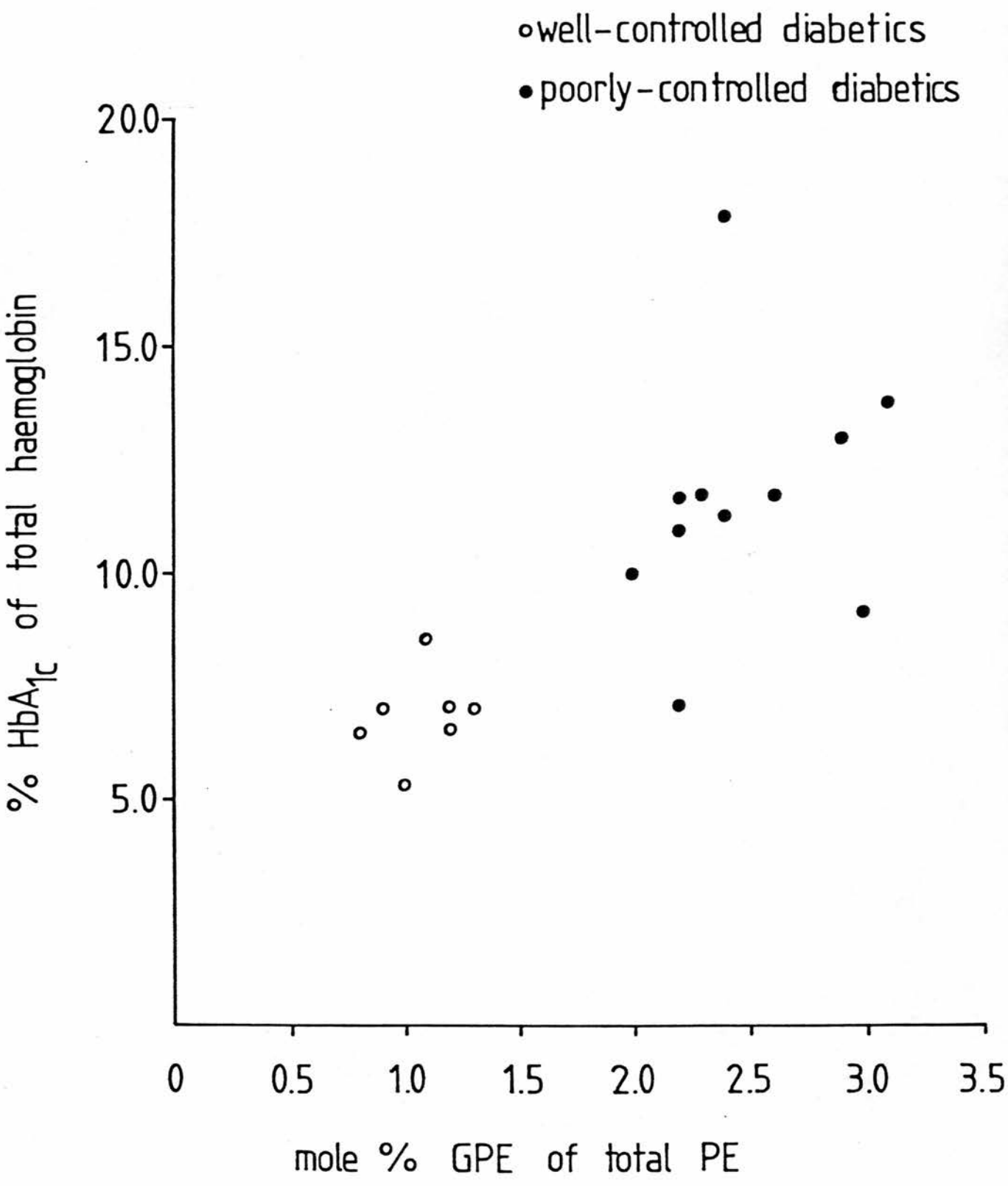


Fig. 33 Correlation between patients' glycosylated haemoglobin (HbA_{1c}) and glycosylated PE (GPE).

3.4.2 HIGH PRESSURE LIQUID CHROMATOGRAPHIC SYSTEM

In parallel with the "modified" TLC work an attempt was also made to develop a more specific, sensitive and rapid method, using HPLC, for the accurate and precise measurements of GPE in erythrocyte extracts. The method utilised hexane/isopropanol/water mixtures as a gradient (varying between 6:8:0.92 and 6:8:1.33) as shown in Figure 39. Detection was by absorbance at 206 nm using the Gilson HPLC (model 303). Figure 34 shows that, with the exception of some overlap between PC and SM, satisfactory resolution of the four major erythrocyte phospholipid classes (as synthetic standards) was obtained in less than 30 minutes. The shape of the PS peak was rather asymmetric, however. Similar results were obtained with total erythrocyte lipids (Figure 35) where the symmetry of the PS peak was improved.

Figure 35 also shows a chromatogram of a synthetic reaction mixture containing putative GPE, PE plus glucose. In addition to PE, a rather broad peak with a retention time of 13.58 minutes was seen. This was absent from controls for glucose which gave no peaks at 206 nm and PE alone, and is therefore tentatively presumed to be GPE. Interestingly, the area ratios of PE/GPE (3:2) are in very close agreement to the ratio of phosphorus (3:2) in the two components as separated by TLC.

The erythrocyte lipid chromatogram (Figure 35) is of an extract from a poorly-controlled diabetic and this also shows a component of the same retention time as our putative synthetic GPE. In the few diabetic patients' samples analysed (Table 21), there seems to be some correlation between the percentage area (of the total PE) constituted by the GPE peak and fasting plasma glucose. However, further work needs to be done to generate further data and confirm this preliminary observation.

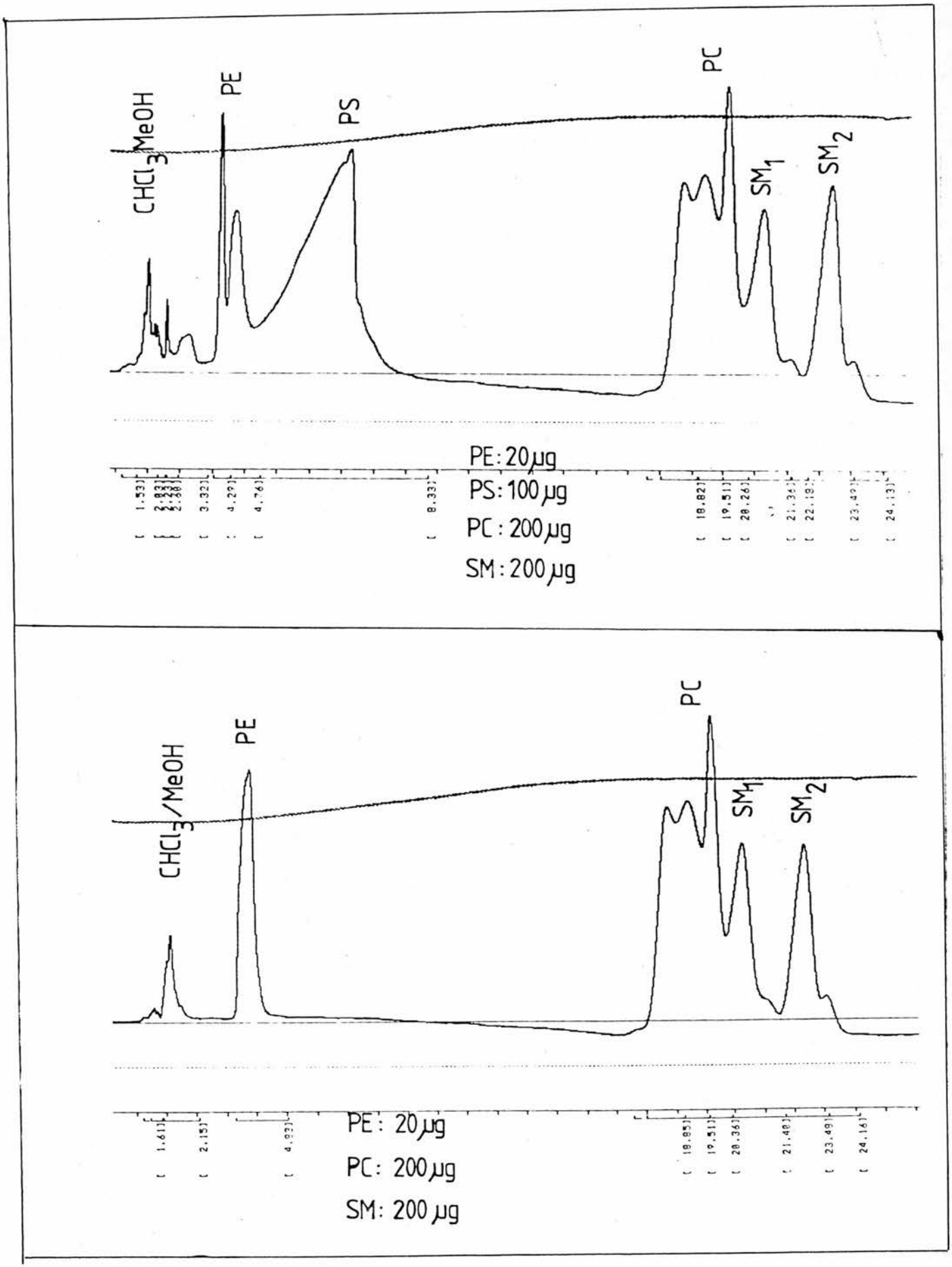


Fig. 34 GRADIENT ELUTION CHROMATOGRAM OF PHOSPHOLIPID STANDARDS.

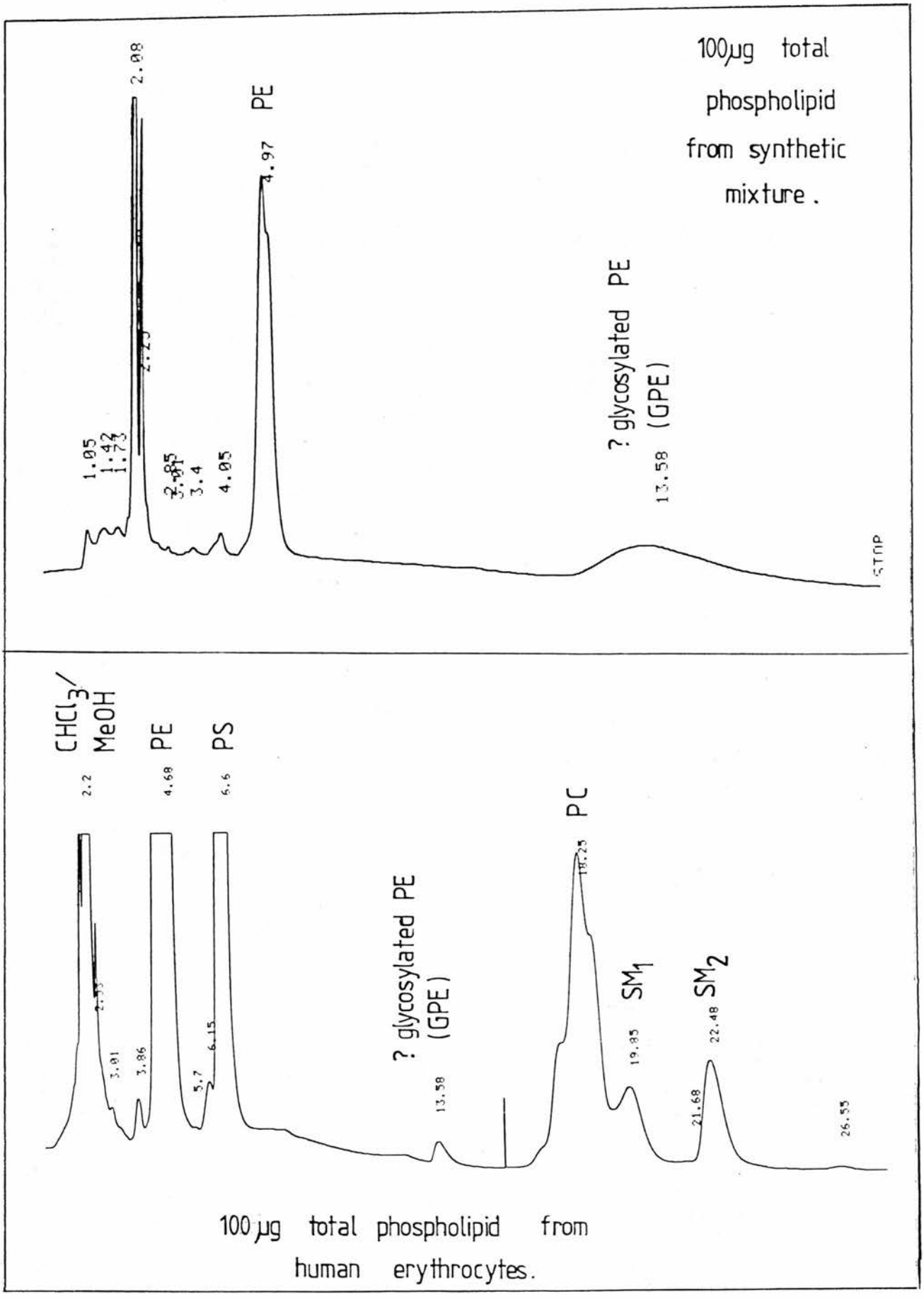


Fig. 35 H.P.L.C. SEPARATION OF GLYCOSYLATED PE FROM SYNTHETIC AND HUMAN SOURCES.

Table 21 HPLC analysis of glycosylated PE (as peak areas) in diabetic patients

Name	FPG (mmol/l)	% GPE
Green	13.7	3.0
McShaffer	8.8	1.7
Sunter	7.0	1.9
Stead	6.1	1.3

DISCUSSION

4. DISCUSSION

4.1 ERYTHROCYTE MEMBRANE PHOSPHOLIPID COMPOSITION IN DM

The erythrocyte membrane is known to vary, among mammalian species, in its phospholipid and fatty acid composition, and to differ in permeability in correlation with these two chemical characteristics. Phospholipids are the major form of lipid in all cell membranes, and their composition and disposition within membranes, of particular species, are fixed. For example, only four classes make up more than 95% of the phospholipids in human red-cell membranes.

These membrane phospholipids are well known to be organised in an asymmetric fashion such that the majority of the free amino group containing classes, PE and PS are located in the inner lamella (Cytoplasmic leaflet), whilst the choline-containing classes, PC and SM are predominantly associated with the outer lamella (extra-cytoplasmic leaflet) of the bilayer /192, 193/.

The results of our cross-sectional study demonstrate significantly decreased SM and PE and significantly increased "PS" in PCDs when compared with WCDs or PVDs. These results are consistent with those obtained by Hunter *et al*. /71a/ in a pilot study using the same two-dimensional TLC and colorimetric phosphorus estimation. The degree of significance in the results remained the same when data from both studies are pooled together. The PVDs were particularly chosen as a group in this study

in order to eliminate the possibility that the phospholipid abnormalities observed in this study are primarily due to diabetes or hyperglycaemia rather than some form of vascular disease.

Early studies by Chandramouli et al. /194/ on chronically diabetic rats did not show any significant changes in erythrocyte phospholipids when compared with controls. An initial study on humans by Kamada and Otsuji /69/ using poorly-controlled diabetics, well-controlled diabetics and control subjects demonstrated decreased SM and increased PE in poorly-controlled diabetic erythrocytes when compared with well-controlled diabetic or normal erythrocytes. In the same study, they also reported increased PE and decreased PS in diabetic patients with nephropathy when compared with normal individuals or patients with retinopathy. However, SM was found to be decreased in both patients with nephropathy and retinopathy when compared with controls. This report contradicts one subsequent report presented by the same authors /70/, in which they studied diabetic patients with and without retinopathy. In this study, Otsuji et al found increased SM in these diabetics as a whole when compared with normal subjects, and an even more significant increase was observed when diabetic patients with retinopathy were compared with normal subjects or those patients without retinopathy. They also found a significant decrease in PE in the diabetic patients as a whole and a more significant decrease in those diabetic patients with retinopathy when compared to controls or patients

without retinopathy. In a later study, Otsuji et al. /195/ found a significant increase in SM and a significant decrease in PE in diabetic patients with and without retinopathy when compared with normal controls.

These two subsequent studies by Otsuji et al. /70, 195/ employed a one-dimensional TLC with densitometry, while their initial study /69/ employed two-dimensional TLC followed by phosphorus estimation. The inconsistency of the results of the present study and others may therefore be due at least in part to the different methods used.

Taking the conflicting previously published data overall, the decrease in SM as observed in PCD in this study and that of Hunter et al. /71a/ is consistent with one report by Otsuji et al. /195/ while the decrease in PE is consistent with other reports by Otsuji et al. /70, 195/, although in some cases these parameters were found not to be statistically significant by them, and also they did not detect any changes in PS. The apparent discrepancies in the published data may be due to the grouping or matching of subjects according to age, sex or even duration of diabetic state. It may also be due to different methodological approaches. Two-dimensional TLC combined with spectrophotometric estimation of lipid phosphorus, as used in this study, eliminates the possibility of overlapping lipid components associated with, and is undoubtedly more accurate than, one-dimensional TLC coupled with densitometry. The co-

efficients of variation obtained with this methodology (Table 3) are all considerably lower than those reported by Otsuji et al. /69/. Whatever the possible reasons for these discrepancies, the results of the present study convincingly demonstrate significant phospholipid abnormalities in the diabetic erythrocyte.

In order to evaluate the data from the longitudinal study, the highest fasting plasma was taken as the "poorly-controlled state" and the lowest fasting plasma glucose was taken as the "well-controlled state" and the phospholipid composition at these times compared by a paired student t-test. There was a highly significant decrease in "PS" as blood glucose decreased with compensatory increase in PE. The converse was even true of one patient whose blood glucose rose between successive visits; i.e. his "PS" increased concomitantly. Figure 15 shows the relationship between the changes in PS (from entry to the study) and the fasting plasma glucose measured on successive visits to the clinic. It can be seen that for individual patients, the overriding trend is for "PS" to increase with increasing fasting plasma glucose and vice versa. Again, these results demonstrate abnormalities in the diabetic erythrocyte phospholipids which are dependent on blood glucose concentration. Phospholipid abnormalities have also been seen in other cell types in diabetes. Brown et al. /196/ found a number of abnormalities in the lipid composition of nerves in diabetic neuropathy. They found that total endoneurial

lipid composition was lowered in these patients. They also demonstrated specifically significant decreases in cholesterol, cerebroside, SM, PE, PS and PI. PC which is not a major myelin constituent was found to be reduced to a lesser degree than were major myelin lipids.

Early studies by Nordoy et al. /197/ on diabetic platelet phospholipids demonstrated increased amounts of phospholipid in both juvenile and maturity-onset diabetics. They found that in patients with juvenile diabetes, the increase was mainly caused by an increase in PS and in the maturity onset group, all main phospholipid fractions (PE, PS, PC and SM) were increased. Only moderate changes were observed in the fatty acid pattern of the various phospholipids. More recently, Kalafoutis et al. /198/ reported increased PE and PS in poorly-controlled diabetic platelets and they also found significant decreases in palmitic and linoleic acid in both poorly-controlled and well-controlled diabetics. Increased arachidonic acid was demonstrated in both groups.

Decreased SM and increased PS have also been demonstrated in the tissues of alloxan diabetic rat /199, 200/ and hence, altered membrane phosphospholipids may be a feature of diabetes or hyperglycaemia. Since erythrocytes do not possess the enzymatic machinery for either de novo phospholipid biosynthesis or phospholipid class interconversion /201/, altered erythrocyte class composition can, therefore, only result during erythropoiesis or from exchange with phospholipids associated with plasma

lipoproteins or by non-enzymatic reactions. The exchange mechanism would only be possible for those phospholipids (PC and SM) which are localised in the extra-cytoplasmic leaflet while non-enzymatic glycosylation would be restricted to those phospholipids (PE and PS) which have free amino groups. This possibility is suggested by the fact that glucose has long been known to attach to free amino groups of protein /120/ lipoproteins /165/ and even nucleic acids /178/ by a non-enzymatic reaction to form initially an unstable aldimine (Schiff base) which undergoes Amadori rearrangement to form a more stable ketoamine (Figure 2).

4.2. IN VITRO GLYCOSYLATION OF PHOSPHOLIPIDS

The formation of Schiff base products of PE with aldehydes other than glucose is known to occur /119/, which suggests that *in vitro* and possibly *in vivo* glycosylation of PE or PS can also occur. In such a reaction, formation of a Schiff base adduct would be predicted along with variable amounts of the more stable ketoamine form depending on the nature of the reaction conditions employed.

In this study, isolated PE and PS were each reacted separately with radioactive and non-radioactive glucose in methanol at 65^{o}C for 24 hours. The contents of the flask were then concentrated, two-dimensional TLC carried out and spots were detected with iodine, anthrone reagent, phosphorus detection reagent and by autoradiography. The behaviour of the putative glycosylated

products in our two-dimensional TLC is rather interesting because glycosylated PE (GPE) co-chromatographs with PS and glycosylated PS (GPS) co-chromatographs with PE (Figures 18 and 19). It is paradoxical that attachment of glucose to PE should generate a more polar product and yet the putative glycosylated PS is less polar than the parent phospholipid in the chromatographic system used here.

Using phosphorus estimation and scintillation counting, it was found that approximately 30% of each phospholipid was glycosylated. The formation of glycosylated products at constant concentration of PE or PS was also found to be directly proportional to the glucose concentration (Figures 21 and 22).

Since synthetic GPE and GPS co-chromatograph with PS and PE respectively, on TLC, a likely explanation for the apparent increase in PS and decrease in PE seen in poorly-controlled diabetics might be preferential glycosylation of PE *in vivo*. To test this, isolated red cell membranes were incubated at 37^{o}C in Krebs Ringer Phosphate Buffer (pH 7.4) containing unlabelled glucose in the range 0 - 100 mM for 5 days and a control was also set up in the absence of glucose from which the "baseline" phospholipid composition can be obtained. Incubation was then followed by phospholipid extraction, thin layer chromatography and phosphorus estimation. It was found that SM and PC remained constant but there was a progressive

increase in "PS" and decrease in PE as the glucose concentration increased (Figures 24a and 24b). From the unlabelled incubations assayed by phosphorus estimation only, it appears that, predominantly, PE had become glycosylated.

Assessing the extent of glycosylation either by the increase in "PS" (+ Δ PS) or the decrease in PE (- Δ PE), the yield of GPE (+ Δ PS or - Δ PE) was calculated using the two expressions on Table 17, and Figure 25 shows that both methods of calculation of the degree of glycosylation agree well and the linearity of the graph over the wide range of glucose concentration extending well into the non-physiological is good evidence that the reaction is non-enzymatic.

The autoradiogram (Figure 26) demonstrates more radioactivity in the PS spot (GPE) and far less in the PE spot (GPS). Although both PS and PE have primary amino groups, they may not necessarily be glycosylated to the same extent since the pKa of the groups may differ. The phospholipids are the most polar of the lipids and they all carry a phosphate acidic grouping with pKa 1-2 which has a negative change at 7.0. PE additionally has an amino group with pKa value of 10 which is positively charges at neutral pH, giving rise to dipolar zwitterions with no net charge. Serine has one negative and one positive charge at pH 7.0, and phospholipids containing serine such as PS consequently carry a single net negative charge /202/.

It has already been shown that, for proteins, the formation of the Schiff base adduct requires an unprotonated amino group. On the basis of the relative abundance of PE and PS in the membrane /202/ one would predict that the amount of GPE formed would be twice that of GPS if both phospholipids are equally reactive. However, the finding from the labelling experiment with membranes of a four-fold excess of GPE as compared with GPS means that PE is more reactive with glucose than PS in physiological conditions. A possible explanation for this is that the pKa of their amino groups is different and that a greater proportion of PE is present as the unprotonated form. The glycosylation of PS by molecular modelling (Figure 17a) also shows that the carboxyl (-COOH) group of serine might offer some steric hindrance in the approach of glucose to the amino ($-NH_2$) group. This might be even more important when one considers the constraints imposed by the orientation of the molecules in a lipid bilayer.

This labelling experiment also has important implications for previous results. It should be emphasised that, if as the glucose labelling shows, PS is also glycosylated, to a much less extent, then any estimate of the yield of GPE in physiological conditions based on either increase in "PS" or decrease in "PE" is likely to be an underestimate, given that GPS co-chromatographs with PE and some genuine PS will be lost as it is glycosylated. This would apply to the membrane incubation and to the patient studies.

The only other report which has even considered the possibility of lipid glycosylation was by Miller et al. /127/ who showed that the treatment of diabetic erythrocyte membranes with tritiated borohydride ($[^3H]BH_4$) resulted in the incorporation of radioactivity into both proteins and lipids. The incorporation of tritium from $[^3H]BH_4$ into membrane proteins is considered a reliable detector of glycosyl-ketoamine linkage suggesting non-enzymatic glycosylation of membrane proteins. The labelling of membrane lipids as observed by Miller et al. may be, however, due to the conversion of lipids to fatty alcohols and lysolipids by sodium borohydride /203/ or due to the presence of glycosyl-ketoamine linkage of the lipids as suggested by our labelling experiment with $[^{14}C]$ glucose.

The quantitation of GPE from in vitro glycosylated membranes and erythrocyte extracts from well-controlled and poorly-controlled diabetics as measured by our "modified" TLC gave a good correlation with glucose concentrations and HbA_{1c} (Figures 31, 32 and 33), and with our "modified" HPLC method, the trend also appears to be significant. This is strong supportive evidence, in the absence of rigorous chemical characterisation, that the material chromatographing between PS and PE is GPE.

The area ratio of the PE and (?) GPE peaks on HPLC in all probability is the molar ratio since the U.V. absorbance depends predominantly on fatty acid composition

which should be identical. The results obtained by the "modified" TLC system for direct estimation of GPE in patients blood (Figure 31) closely agrees with that obtained using Hunter's TLC system (Figure 25) which indirectly quantifies GPE. The slight discrepancies may be due to experimental errors, arising from the pooling of GPE spots in our "modified" TLC system in order to achieve a valid absorbance in phosphorus estimation. However, both TLC methods demonstrate elevated levels of GPE in poorly-controlled diabetic erythrocyte membrane phospholipids.

Spectroscopic studies on diabetic erythrocyte membranes /67, 68/ suggested that non-enzymatic glycosylation of membrane protein /126, 127, 152, 153/ may influence membrane organisation and may have considerable functional consequences. With increased knowledge of the structural complexity of cell membranes, it has become apparent that the composition and micro-viscosity (fluidity) of membrane lipids must be critically controlled for all cell functions. Blood hyperviscosity, excessive aggregation of the erythrocytes and reduced erythrocyte deformability in diabetes have been suggested to be important in the pathogenesis of diabetic micro-angiopathy. An elevation of either intraerythrocyte or membrane viscosities is thought to be the possible cause of reduced deformability in diabetes.

Studies by Otsuji et al. /70/ on erythrocyte membrane microviscosity in diabetes, suggest that the decrease

in membrane fluidity (which is equivalent to an increase in microviscosity) appears to be in conformity with the heterogeneous change of distribution of phospholipid fractions. Their studies also suggested that the contributing factors to the decreased erythrocyte membrane fluidity in diabetic patients were suggested to be not only an increase of membrane cholesterol/phospholipid mole ratio but also multifactorial changes involving membrane phospholipid fractions and other complex factors. The decreased erythrocyte membrane fluidity appears to be related to poor metabolic control and is associated to diabetic microangiopathy.

The data in this study suggest that erythrocytes (like erythrocyte membranes) exposed to an abnormally high glucose concentration, as in non-regulated diabetics, may undergo alterations of phospholipids by enlargement of their head groups due to glycosylation as shown by our molecular modelling (Figures 16b and 17b). This may result in changes in membrane physical state and modification of functional properties and could be important in explaining some of the abnormalities associated with diabetic red cells.

4.3 METHODOLOGICAL PROBLEMS AND FUTURE WORK

In spite of the fact that we have synthesised, characterised and measured what are very probably glycosylated phospholipids, there remain problems to be surmounted. We do not know whether the chromatographically separable "GPE" in this work represents the Schiff base or keto-amino or a mixture of both. Further work is therefore

necessary to study the kinetics of Schiff base and ketoamine formation and to attempt resolution of the two. A pilot study in our laboratory for the assay of ketoamines employed the use of Nitroblue tetrazolium reagents, and produced promising results which require further development. Such a method would be useful in a kinetic study.

Finally, the more sensitive HPLC method also requires further development. The peaks obtained for PS (Figure 34) and GPE (Figure 35) in the synthetic mixtures were not well resolved. The gradient system and other analytical conditions need further readjustments in order to obtain better resolution of the individual peaks. Also a large number of patients' samples need to be studied in order to obtain satisfactory, statistically valid data.

4.4 CONCLUSIONS

The results of our cross-sectional and longitudinal studies demonstrate a significant increase in PS and a significant decrease in PS and SM, in poorly controlled diabetics. We conclude that erythrocyte phospholipid composition is abnormal in poorly controlled maturity-onset diabetics. We have also shown that model GPE and GPS can be synthesised in non-physiological and physiological conditions. The *in vitro* physiological production of GPE and GPS in membranes as demonstrated by our labelling experiment is consistent with some of the phospholipid changes observed when membranes were incubated with increasing concentrations of glucose. We

therefore conclude that some of the phospholipid changes observed in the cross-sectional and longitudinal studies in poorly-controlled diabetics are consistent with and may be due to the glycosylation of PE. We have also resolved GPE by TLC and HPLC (?) and the raised "GPE" obtained in poorly-controlled diabetics by our TLC correlated with HbA_{1c} and FPG.

The functional significance, however, is unclear but it is rather interesting to speculate that glycosylation which may be related to PE (but cannot be related to SM) changes could account for the erythrocyte membrane abnormalities observed in diabetics and may also be found in other membranes. Our findings therefore raise the possibility that phospholipid changes as seen in the erythrocyte, may be a feature of diabetes mellitus in other tissues and *in vivo* glycosylation may have considerable functional consequences, particularly in the red cells and the neurons.

Finally, although the present study has provided clear evidence for phospholipid abnormalities in erythrocytes from diabetic patients and strongly suggests that this is related to phospholipid glycosylation, it is vital to prove whether or not such changes have functional significance. Since it is difficult if not impossible to separate the effects of lipid and protein glycosylation in native membranes, *in vivo* or *in vitro*, a better alternative approach would be to make model liposomes from isolated synthetic or natural glycosylated

phospholipids and to examine the permeability and enzyme activities of proteins which have been reconstituted into such liposomes.

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