CELL SURFACE PROTEINS OF THE NEUTROPHIL IN RELATION TO CHRONIC MYELOID LEUKAEMIA

A thesis submitted to the University of Glasgow for the degree of Doctor of Philosophy in the Faculty of Medicine.

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DECLARATION

I declare that the work presented in this thesis is my own and has not been submitted in support of any other degree or qualification at this or any other University or Institute of learning.

Signed:

J.J. WALERS

Summary

A large number of studies now suggest that non-random chromosomal changes are associated with human cancers. Certain chromosomes are involved more frequently in rearrangements, duplications and deletions than would be expected by chance. The most consistently observed chromosome abnormality is the Philadelphia chromosome, (Ph') which is found in a number of heamopoetic cell lineages in some 90% of patients sufferring from chronic myeloid leukaemia, (CML). Despite the diagnostic significance of the Ph' chromosome virtually nothing is known about its role in the origin and development of the disease.

Well established phenotypic alterations in the mature neutrophil in CML are limited to the finding of a reduced level of alkaline phophatase in these cells. However, membrane-related phenomena, including phagocytosis and lectin agglutination have been shown to be altered.

This study was undertaken to determine whether simple consistent alterations in cell membrane proteins were detectable at the molecular level which might underlie the membrane-related phenomena noted above and could be related to the chromosomal changes which occur in this leukaemia.

Labelling of the membrane proteins of the cell chosen for study,the neutrophil, revealed at least thirteen relatively high molecular weight polypeptides bands which were identified using the ^{125}I-lactoperoxidase labelling technique, on SDS-PAGE.

To demonstrate that the labelled polypeptides were present on the cell surface, three independent methods were used: plasma membrane isolation, trypsin sensitivity and labelling in the presence and absence of exogenous enzymes.

Extraction with the non-ionic detergent, Triton X-100 considerably reduced problems of handling and proteolytic digestion associated with

this cell. Evidence is presented that non-ionic detergents, (TX-100 and NP-40), extract polypeptides selectively which may have structural significance. One major coomassie blue staining band , of mol. wt. 85K, a minor band of mol. wt. 25K and one broad radiolabelled band of mol. wt. 55-60K were particularly selectively retained in a residual pellet.

Experiments using Concanavalin A (Con A) affinity chromatography showed that most of the labelled polypeptides were glycosylated. One major band was unique in that it showed no binding to Con A either by affinity chromatography or by the use of ^{125}I-Con A overlay on SDS-PAGE. It is the major cell surface receptor for Wheat Germ Agglutinin (WGA) which binds to terminal sialic acid residues. When labelled cells were treated with neuraminidase there was an apparent decrease in the mobility of this polypeptide and binding to WGA was abolished. No other labelled band showed significant alteration following neuraminidase treatment.

The above evidence suggests that this glycoprotein of mol. wt. 115K approx., (Gp 115K), is the major sialoglycoprotein at the cell surface of the human neutrophil. This finding may be of general interest since it shares a number of the features, described above, with other 'glycophorin-like' sialoglycoproteins reported in the literature. The function of this unusual class of cell surface glycoproteins is not clear, but they appear to make a significant contribution to the net negative charge at the cell surface and to lack secondary or tertiary structure in the extracellular portion.

Membrane related phenomena of the human neutrophil appear to undergo alterations in CML. Cell surface polypeptides were labelled directly using the ^{125}I-LPO method and radio-labelled lectins (Con A and WGA) were overlayed on whole cell detergent extracts run on SDS-PAGE, in order to try and detect alterations in glycosylation. The results suggest that large scale alterations in the expression of glycosylated, and in particular cell-surface, proteins do not occur. No evidence for novel gene

products or marked alteration in glycosylation of neutrophils in CML was found.

Chapter 1 Introduction

Section 1.1 General Introduction

A large number of studies now suggest that non-random chromosomal changes are associated with human cancers. Certain chromosomes are involved more frequently in rearrangements, duplications and deletions than would be expected by chance, (Rowley, 1977; Mitelman and Levan, 1978 and Rowley, 1982). The most consistently observed abnormality is the Philadelphia chromosome (Ph'), (see fig. 1.3.), which is found in a number of haemopoietic cell lineages in chronic myeloid leukaemia (CML). Some 10% of cases have further chromosomal changes as well at this stage of the disease, (Sandberg, 1980). Despite the diagnostic significance of the Ph' chromosome virtually nothing is known about its role in the origin and development of the disease.

Well defined phenotypic alterations in the mature neutrophil in CML are limited to the finding of a markedly reduced level of alkaline phophatase, (Koeffler and Golde, 1981) and the finding that these cells are quantitatively deficient in their production of lactoferrin, which regulates granulocyte-macrophage proliferation, (Pelus et al., 1982). A number of membrane-related phenomena including phagocytosis, (Dotten et al. 1982) and lectin agglutination (Taub et al. 1980) are apparently altered in these cells.

This study was undertaken with two aims in mind. Firstly, to determine whether simple consistent alterations in cell membrane proteins, detectable at the molecular level, might underlie the membrane-related phenomena noted above. Secondly it was hoped that by using suitable controls, such alterations might be related to chromosomal changes which have occurred in these cells. The neutrophil was chosen for study because

large numbers of relatively pure cells are reasonably easy to obtain and the problems of what constitutes a control cell which are a problem in many studies of this type are avoided.

Relatively little is known about the chromosomal organization and control of genes coding for cell surface proteins. A number of cell-surface antigens, including the histocompatibility loci and the blood group antigens, have been mapped to specific human chromosomes, (see Human Gene Mapping Workshops, e.g. de la Chapelle et al., 1980, for ref.). However it is not always known if cell surface antigens are products of genes for membrane proteins or other components of the cell surface. For example the ABO blood group, assigned to human chromosome 9, (Westerveld et al., 1976) is not due to the protein component but to glycosyl transferases which add the antigenic determinants to the structural protein. Other membrane proteins have been mapped using somatic cell hybridization techniques, e.g. membrane proteins of WI-38 fibroblasts, (Owerbach et al., 1979). Mapping information about genes expressed in human neutrophils is limited. A qualitative gene dosage effect, for a cell surface protein, has been noted in the neutrophils of patients with CML having monosomy 7 in the marrow, (de la Chapelle et al, 1980). There was a marked reduction in the labelling of a major band, (Gp 130 on SDS-PAGE) compared with leukaemic and non-leukaemic controls. Post-translational covalent modification of cell surface glycoproteins obviously adds a further level of complexity to the interpretation of such apparent gene dosage effects.

Why study the expression of cell surface proteins in particular? Knowledge of the biochemical structure and antigenicity of cell surface proteins of haemopoeitic progenitor cells and their mature progeny may help in the understanding of cell differentiation and the functioning of specific cell surface molecules. It can also aid in the identification of

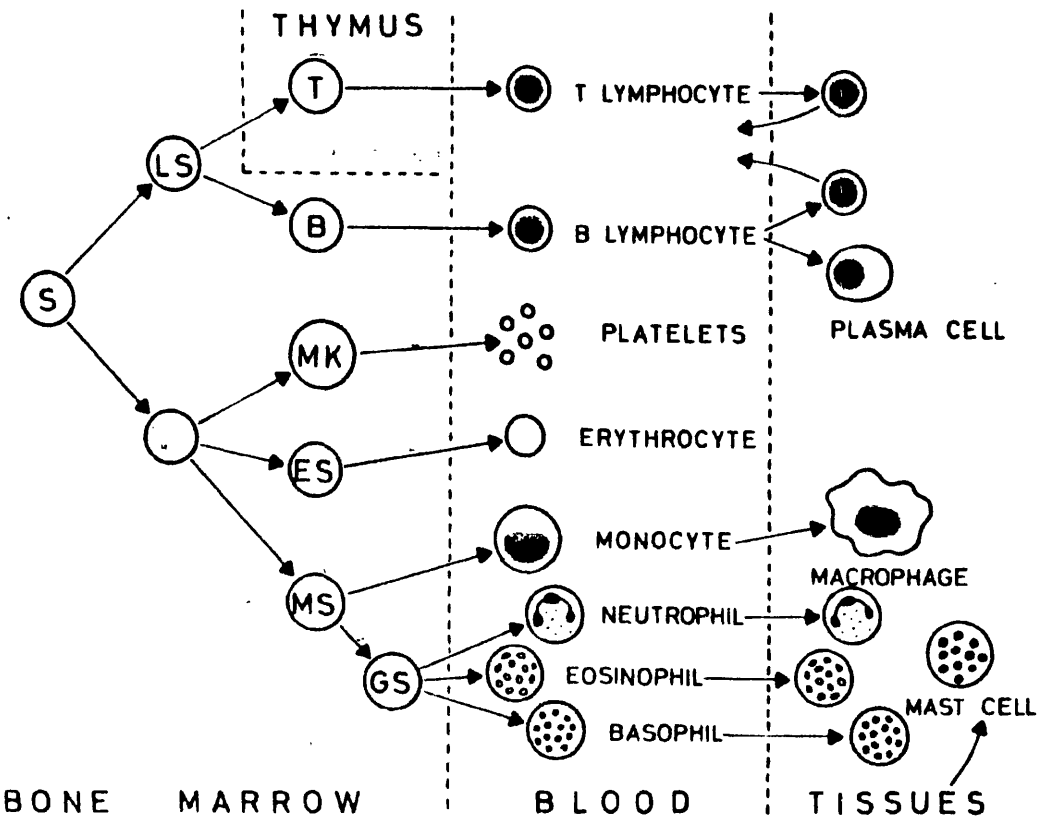
haematological malignancies, and potentially in the diagnosis and treatment of such diseases.

Considerable progress has been made in the understanding of the heterogeneity of lymphatic cells and their malignant counterparts, (for review see Knapp et al., 1981 and and Greaves and Janossy, 1978). It is well established that lymphatic leukaemias and lymphomas can now be further subdivided into clinically relevant subgroups by the use of antigenic markers defined by monoclonal antibodies, (Nadler et al., 1981 and Greaves, 1981). Although myeloid leukaemias represent a considerable proportion of the haematological malignancies of man, relatively few research groups have investigated the antigenic and biochemical characteristics of myeloid cell surfaces, (Knapp et al., 1981). The following sections expand on this outline of the present study.

Section 1.2 Leukaemia: A Heterogenous Cancer of Haemopoietic Cells

Leukaemia is a cancer of haemopoietic cells and like most tumours there is a great deal of heterogeneity with respect to initial clinical and haematological features, the course of the disease and the response to therapy. Part of this difference could be ascribed to genetic differences among the patients themselves, (e.g. drug metabolism affecting drug sensitivity, hormonal factors, immune response differences etc.). In the main however it is thought that differences between patients probably reflect intrinsic or acquired characteristics of the leukaemic cell population itself, (Greaves, 1979).The phenotypic properties of leukaemic cells may be determined by a number of factors: the particular cell lineage of the leukeamia, the degree of maturation arrest within that lineage, the proliferative capacity of the leukaemic cells, and the amount of intraclonal heterogeneity present, (Kerbel, 1979).

Fig. 1.1. Simplified outline of the haemopoietic system



S Stem cell; LS Lymphoid cells; MK Megakaryocyte; ES Erythroid stem cell; MS Myeloid stem cell; GS Gramulocyte stem cell. Adapted from Playfair, (1981)

Table 1.1

Categories of Human Haemopoietic Malignancies*

(from Greaves and Janossy, 1978)

Leukaemias:	Acute disease	Lymphoid-Acute Lymphoblastic Leukaemia(ALL)
		Myeloid -Acute Myeloid Leukaemia(AML)
		Monocytic-Acute Monocytic Leukaemia(AMoL)
		Undifferentiated -Acute undifferentiated Leukaemia (AuL)
	Chronic disease	Lymphoid-Chronic Lymphocytic Leukaemia(CLL)
		Myeloid**-Chronic Myeloid Leukaemia(CML)
Lymphoma-Lymphosarcoma		Lymphoid Hodgkins' Disease
Histiocytic Lymphoma		Monocytic non-Hodgkins' Lymphoma
Reticulum Cell Sarcoma		
(Multiple) Myeloma		Plasma Cells

*Distiguished by morphological and histochemical differences.

**Some 90% of cases have the Philadelphia (Ph') chromosome (22q-). These cases usually develop later into acute leukaemias. This metamorphosis may be sudden (blast crisis). The resulting acute leukaemia may have a myeloid, lymphoid, mixed or undifferentiated morphology.

Section 1.3 The Haemopoetic System and the Categories of Human Haemopoetic Malignancies

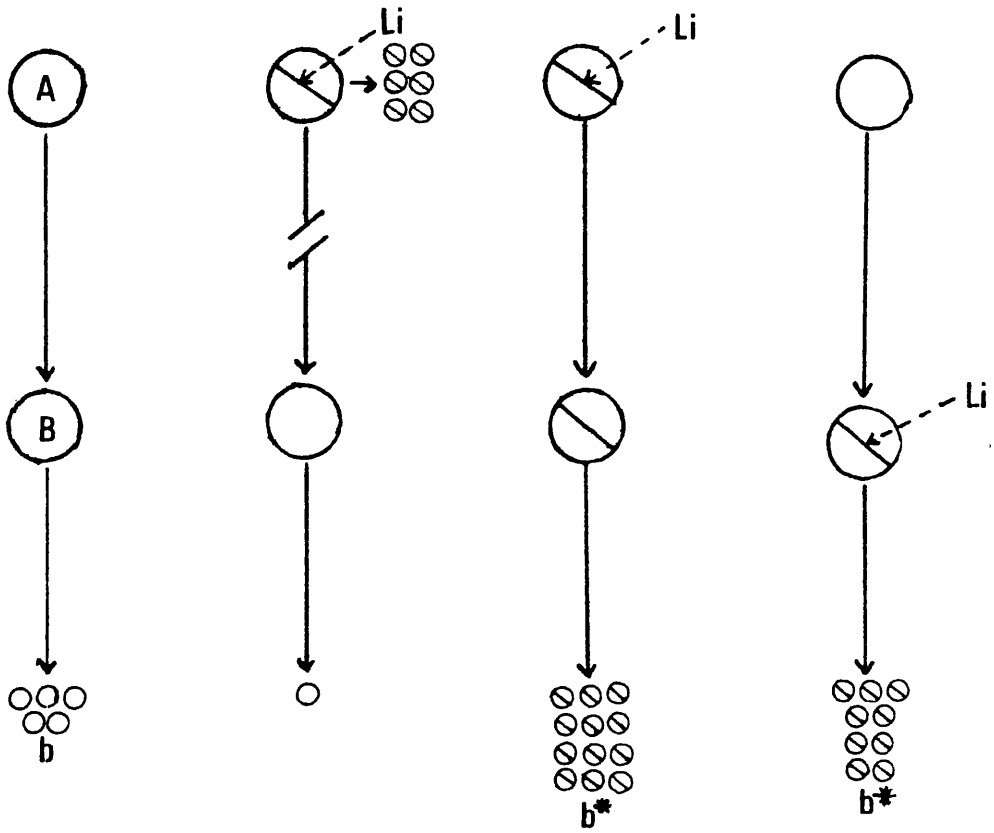
The haemopoietic system consists of at least six distinct cell types (T lymphocytes, B lymphocytes, monocytes, granulocytes, erythrocytes and platelets). Within any given lineage are several subsets of cell types, all of which contain cells at different stages of maturity. These types may all be heterogenous but between them share common precursor and stem cells, (see Greaves and Janossy, 1978 and Greaves, 1979 for review.) and see fig. 1.1.

The potential 'target' range of cells in which a leukaemia may occur is then very large encompassing a number of different cell lineages in any one of which an elaborate, closely defined, maturation process takes place. In practice lymphoid malignancies (i.e. T cells, B cells and their precursors) and myeloid malignancies (granulocytes, monocytes and their precursors) are much more common than those of red cell and platelet precursors. The categories of human haemopoetic malignancies are outlined in Table 1.1.

Leukaemias, when clinically evident, are in common with almost all cancers, monoclonal in origin, (see Koeffler and Golde, 1981). The leukaemic clone, at least to start with, is relatively homogenous involving one dominant cell type whose characteristics are determined by its cell lineage commitment and developmental status. This dominant cell type may not however represent the 'target cell' in which the disease initiated. This cell may retain capacity for further differentiation (along a particular lineage pathway) so that at any one time the predominant cell type or types may represent the developmental progeny of any common target cell. This concept is outlined in Fig. 1.2.

In contrast to a leukaemia which results in the overproduction of

Fig. 1.2. Diagrammatic representation of potential cellular origins, differentiation competence and cellular phenotypes in leukaemia.



A Normal stem cells. B Normal lineage commited precursor cell, b mature lineage progeny. Leukaemia: Li Site of leukaemia initiation resulting in overproduction of stem cells or mature progeny, b^*. From Greaves and Janossy, (1978).

mature or relatively mature cells, in common with many malignant tumours, there is often a complete or partial maturation arrest. The developmental level at which this arrest occurs varies from patient to patient with similar leukaemias and significantly between subclones of one patient at any one time and sequentially during the course of the disease. In some leukaemias a shift occurs from overproduction of a relatively mature cell (e.g. neutrophils in chronic myeloid leukaemia - the subject of this thesis) to an immature but committed precursor cell (e.g. myeloblasts in acute myeloid leukaemia). This acquired change has been seen as evidence of dedifferentiation by some workers whereas others, most notably Greaves (1979) have argued that intraclonal selection may give rise to a dominant subclone of cells which is relatively immature.

Whether the 'maturation arrest' that occurs in leukaemia can be reversed is unclear although some evidence exists for such a process to occur 'in vitro', (Sachs, 1978 and Sachs, 1978). A number of leukaemic cell lines e.g. murine Friend erythro-leukaemic cells, and the human promyelocytic leukaemic cell line HL-60, (see Gahmberg and Andersson, 1979) can be induced to undergo morphological and functional differentiation by various low molecular weight compounds.

To summarize, the phenotype of the dominant cell type in any given leukaemia at any one time is a product of the lineage commitment of the cell type in which the disease was initiated plus the degree of developmental maturity along that cell lineage pathway.

Section 1.4 Chronic Myelogenous Leukaemia

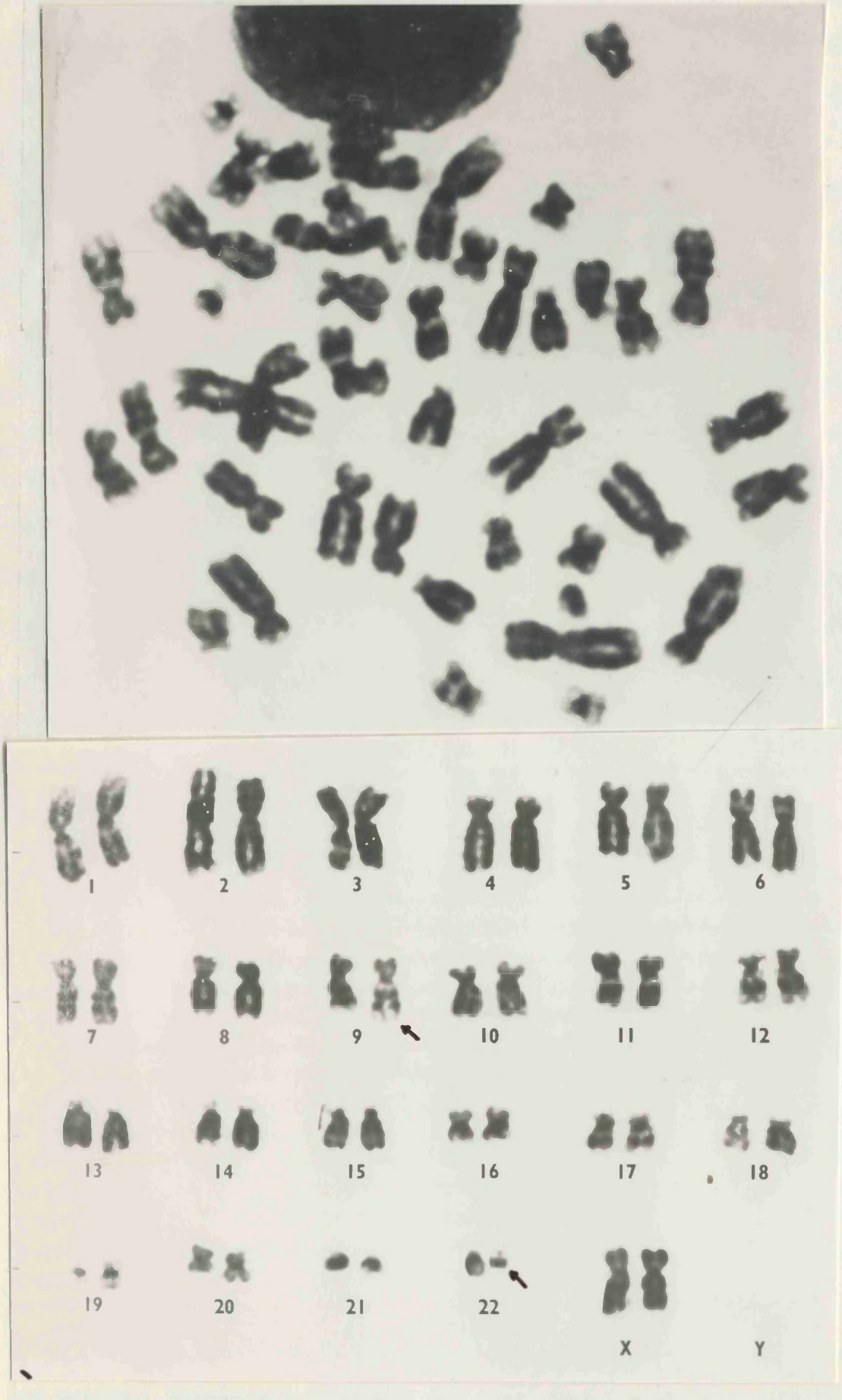
Chronic myelogenous leukaemia (CML), (for review see Koeffler and Golde, 1981) is a clonal myeloproliferative disorder arising as a result of a somatic mutation in a pluripotential stem cell which can give rise to both myeloid and lymphoid progeny, (Koeffler and Golde, 1981 and Greaves, 1982).

CML is characterized clinically by a marked overproduction of granulocytes (normally largely neutrophils). The leukocytosis is often accommpanied by splenomegaly. Patients commonly present with malaise, fever, night sweats, weight loss and abdominal fullness, (Koeffler and Golde, 1981). The disease was probably first identified in 1845 by Cragie, Bennett and Virchow in three separate reports, quoted in Koeffler and Golde (1981).

Within a mean of three years after diagnosis, the relatively benign chronic phase of the disease gives way to either an accelerated period of disease or a phase known as 'blast crisis'. The definition of blast crisis is not precise, but this stage is characterised by increasing numbers of blasts, (immature committed cells - myeloblasts), in the peripheral blood and bone marrow, by progressive anaemia and thrombocytopaenia and by a lack of response to therapy, (Koeffler and Golde, 1981). Factors that may herald the onset of the accelerated phase include fever, lymphadenopathy, basophilia in excess of 20%, elevation of leukocyte alkaline phosphatase, new chromosome abnormalities and myelofibrosis, (Koeffler and Golde, 1981) A practical difficulty here arises in relation to this project in that although blastic transformation may give rise to secondary chromosomal changes, the number of neutrophils in the peripheral blood tends to decrease rapidly removing the possibility of biochemical analysis of these cells.

Leukocyte alkaline phosphatase is decreased or absent in the neutrophils of patients with CML at the time of diagnosis, (Koeffler and Golde, 1981). With infection, inflammation, secondary malignant disease, peripheral-blood remission as a consequence of chemotherapy or the onset of blast crisis, leukocyte alkaline phosphatase can rise to normal or high levels, (Koeffler and Golde, 1981). Fehr and Grossman (1979) suggest that the level of alkaline phosphatase is a marker of cell maturity, and Sato et al. (1982) provide evidence that low alkaline phosphatase activity of

Fig. 1.3. Typical metaphase spread and karyotype of a 46,XX, Ph'+ve cell



Note the extra material translocated on to the end of the long arm of one chromosome 9. Courtesy of Dept. of Medical Genetics, University of Glasgow.

neutrophils of patients with CML is due to the relative immaturity of the cells.

Lactoferrin regulates granulocyte-macrophage proliferation by reducing monocyte-macrophage colony stimulating factor (GM-CSF), (Pelus et al., 1982). Mature granulocytes from patients with CML are quantitatively deficient in their production of lactoferrin, (Pelus et al., 1982). Whether this finding merely reflects the relative immaturity of these cells is not clear.

These findings are clearly of considerable importance as one might expect this immaturity to be reflected in the expression of cell surface proteins.

Perhaps the outstanding feature of this disease is the presence of a consistent chromosome abnormality in the chronic phase of the disease. Nowell and Hungerford (1960) described an abnormal, small chromosome (the Philadelphia, or Ph' chromosome) in patients with this disorder, (see fig. 1.3.). The Ph' chromosome is detectable in about 90% of patients with the clinical picture of CML, (Koeffler and Golde, 1981). There is some evidence that those patients suffering from CML with the Ph' chromosome live longer and are significantly younger than those lacking it, (Schilling and Crowley, 1979). The Ph' chromosome normally arises because of translocation of chromosomal material from the long arm of one of the 22 chromosomes, (Caspersson et al., 1970). In, again, approximately 90% of cases the 22q material is translocated to the long arm of one of the No. 9 chromosomes. The involvement of chromosome No. 9 in the translocation, t(9;22)(q34;q11), was first recognized by Rowley (1973). No evidence for variation in the breakpoint on 22 at 22q11, determined by Watt and Page, (1978), or for the recipricocity of the translocation was found from gene mapping studies using somatic cell hybrids, (Geurts van Kessel et al., 1981). No evidence for deletion of structural genes known to map to chromosome 22 or 9 was found. Other chromosomal rearrangements,

duplications and deletions are known to occur during the chronic phase of the disease in addition to the Ph' chromosome in about 5-10% of cases, (see for example de la Chapelle, 1979 and collected findings in Sandberg, 1980)). Cases in which additional chromosomal anomalies occur before treatment has been started have been found as well as after treatment has started, (Sandberg, 1980). It was hoped that suitable cases would become available during the course of this study. Clearly cases which were monosomic or trisomic for a particular chromosome would be very useful in a search for gene dosage effects.

Evidence for the clonal origin of the leukaemia is provided by cytogenetic and isoenzyme studies. Fluorescent markers and chromosomal polymorphisms have been used to demonstrate that the same No. 9 and No. 22 are involved in the translocation in each Ph'-positive cell in any given patient, (Lawler et al., 1976). Isozyme studies of glucose-6-phosphate dehydrogenase (coded for by the X chromosome) in women with CML who were heterozygous for this enzyme only have Type A or B enzyme in myeloid cells, platelets and erythrocytes. This suggests a clonal origin from a common haemopoeitic stem cell, (Koeffler and Golde, 1981). Recent evidence from a variety of studies suggests that the B lymphocyte lineage is also involved in the chronic phase of the disease, (Koeffler and Golde, 1981). The monoclonal nature of the disorder allows chromosomal findings derived from immature replicating cells to be correlated with the expression of cell surface proteins of the mature end cell, the neutrophil.

Because of the typical clinical picture and the presence of a specific chromosome abnormality, CML is perhaps the best defined of the leukaemias. Nevertheless technical problems remain in cytogenetic analysis in this disorder, as is the case for all leukaemias. Despite the diagnostic significance of the Ph' chromosome virtually nothing is known about its role in the origin and development of the disease.

Section 1.5 Rationale for the Study of the Cell Surface

Knowledge of the biochemical structure and antigenicity of cell surface proteins of haemopoetic progenitor cells and their mature progeny may help in the understanding of cell differentiation and the function of specific surface molecules. It can also aid in the identification of cells and cell lineages, in the classification of haematological malignancies, and potentially in the diagnosis and treatment of such diseases. Analysis of such proteins will also provide, together with cell binding studies a means of comparing the specificity of monoclonal antibodies raised in different laboratories against a given cell type as suggested by Greaves (1981). These reasons for the study of cell surface proteins in leukaemia are gone into in more detail below.

A) Classification of tne Disease State

Until recently diagnosis of a given leukaemia has relied largely on conventional haematological criteria. Increasingly more accurate diagnosis, in terms of defining the leukaemic cell type(s), is now becoming available through the use of panels of biochemically and/or immmunologically defined 'markers'. These allow a more accurate description of the leukaemic cell to be made, both in terms of membership of a particular cell lineage and as to the level of maturation arrest. In this respect use has been made of the 'OKT' series of monoclonal antibodies to dissect the T cell lineage pathway and hence to define the level of maturation arrest in T cell leukaemias, (Reinherz et al., 1980). Heteroantisera and monoclonal antibodies that recognize ALL-associated antigens have been developed, (Fitchen et al., 1981, for review). On the basis of analysis of these antigens and other

surface markers several subsets of ALL, (acute lymphocytic leukaemia) have been identified. In some instances, subclassification according to surface marker characteristics of the leukaemic cells appears to be of prognostic value. Patients with T-ALL have a worse prognosis than those of non-B, non-T-ALL. Patients with c-ALL antigen-positive non-B, non-T-ALL may have a more favourable prognosis than those who lack this determinant. Thus, improved diagnosis may lead to subsequent benefits in terms of therapeutic approaches and patient management.

Although myeloid leukaemias represent a considerable proportion of the haematological malignancies of man relatively few research groups have investigated the antigenic and biochemical characteristics of myeloid cell surfaces, (Knapp et al., 1981).

B) The Potential Use of Leukaemic 'Markers' in Disease Therapy

'Leukaemia-associated' markers as well as allowing more accurate diagnosis, potentially have a role in immunotherapy. A number of reports have demonstrated the antitumour effects of passive antibody administration, (Wright and Bernstein, 1980). The major limitations of this approach have been the difficulty in preparing xenogenic antisera with specificity for tumours and the fear of administering large amounts of foreign protein to patients. The use of monoclonal antibodies of known class, avidity and specificity against defined markers, may surmount these problems. Use has already been made of a conventional antiserum, anti-c-ALL (anti-common-acute lymphocytic leukaemia) in combination with autologous bone marrow trasplantation, (Fitchen et al. 1981). Enrichment, collection and cryopreservation of selected cell populations, for future use either by reintroduction into the same patient or as potential donor material may be possible. Allogeneic bone marrow transplantation after selective removal of T cells 'in vitro,

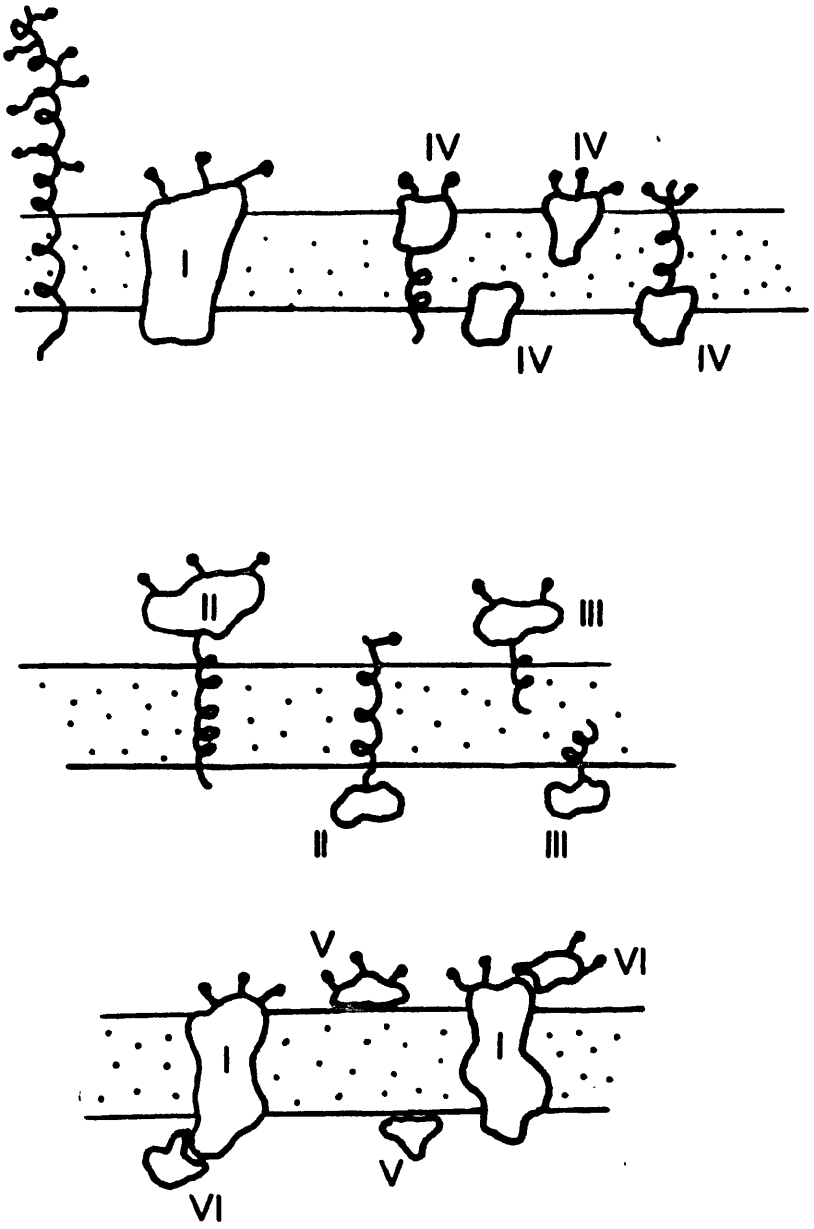
thus abrogating GVHD (Graft-versus-Host-Disease), has been reported, (Fitchen et al. 1981).

C) To Provide a Better Understanding of the Intrinsic Defects in Leukaemia

The search for cell surface correlates of malignancy has been a dominant theme of cancer research during the last decade. The plasma membrane is a selective barrier between the cell interior and the external environment and so any external signal, whatever its form may be,must interact with a receptor on the cell surface or pass through it to some internal receptor. It seems probable therefore that the changes in growth control often associated with malignancy may well be reflected in changes in the structure and composition of the surface membrane. Cairns (1981) and Klein (1981) have suggested that most human cancers may result from genetic rearrangements rather than mutations. The finding of a non-random distribution of chromosomal changes with some cancers is one area of evidence for this view, (Rowley, 1977 and Mitelman and Levan, 1978). The Ph' chromosome is the most consistently observed chromosomal abnormality found in a human cancer and yet very little is known about the role of this chromosomal rearrangement in the causation or development of the disease. Whether such rearrangements result in the expression of novel gene products or result in the increased expression (or functional activity) of a normal cellular product, is not known. It may be noted here that the genes for human light-chain (lambda) immunoglobulin chains have been assigned to chromosome 22, (Erikson et al., 1981) The immunoglobulin gene complexes undergo extensive DNA rearrangements during B-lymphocyte ontogeny and this may be related to a high frequency of chromosome breakage at these sites, (Cox et al. 1982).

It is likely then that the primary lesion causes a variety of alterations in regulatory controls of cell proliferation and

Fig. 1.4. Classes of integral and peripheral proteins



Integral (I-IV) and Peripheral (V-VI). Top left, speculative representation of glycophorin-like integral sialoglycoproteins. Examples, I erythrocyte anion transport protein (Band 3); II aminopeptidase; III cytochrome b_5 reductase; IV adenylate cyclase; V pyruvate oxidase; VI erythrocyte glucose-6-phosphate dehydrogenase.

Parallel lines enclosing stippled area, lipid bilayer. Open spaces represent globular folded portions of the molecule.

Fibrous tails or anchors are shown as twists of α-helix integrated into the phospholipid bilayer. Carbohydrate attached to proteins shown as ? (from Houslay, 1981).

differentiation. Such alterations may be found at the cell surface and may have implications for the 'correct' functioning of the cell with respect to its external enviroment. Thus while alterations at the cell surface may not be themselves sufficient to account for 'transformation', tumourigenicity or malignancy of cells, it is probable that the expression of cell surface proteins in any given leukaemia will reflect the level of maturation arrest of the leukaemia and may provide information about the expression of cell surface proteins in normal cells.

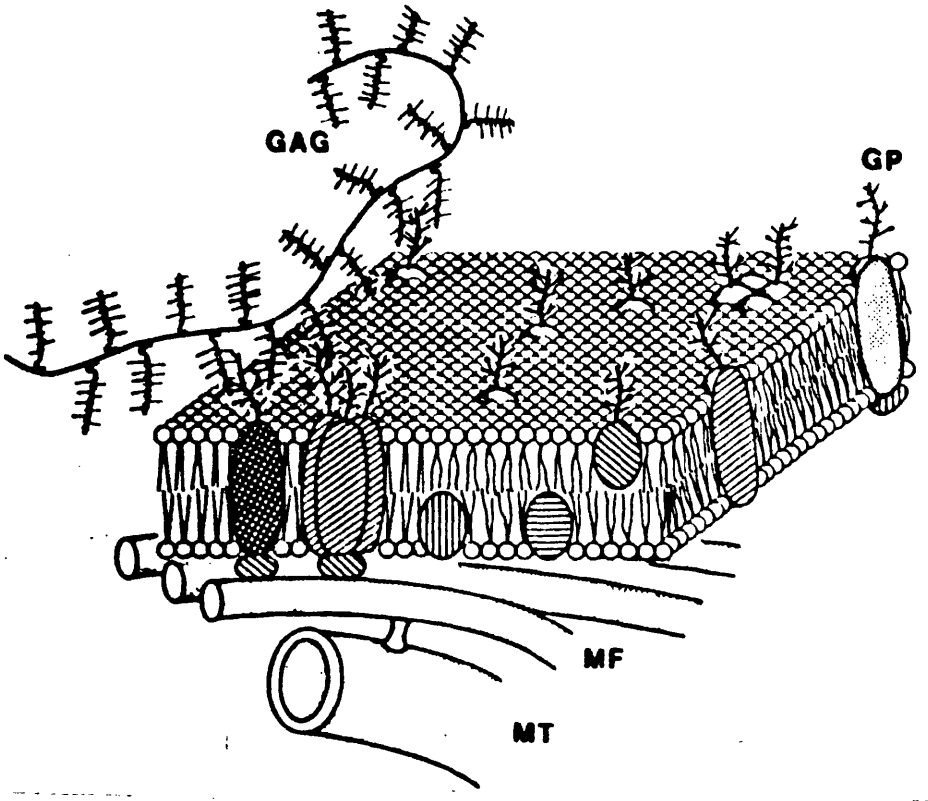
Section 1.6 Structure and Function of Cell Surface Glycoproteins

Section 1.6.1. Organization of the cell surface

The structure and organization of cellular membranes conforms to several basic principles, (Bretscher and Raff, 1975; and Lotan and Nicolson, 1979). These are summarised in Fig.1.5. and outlined below:

a)the major class of membrane lipids, the phospholipids, are arranged in a planar bilayer configuration which under physiological conditions

Fig. 1.5. Hypothetical structure of the plasma membrane



Singer and Nicolson Fluid Mosaic Model from Lotan and Nicolson, (1979).

Hypothetical structure of a plasma membrane including possible interactions between glycoproteins (Gp) and glycosaminoglycans (GAG) at the outer surface as well as between Gp and membrane-associated microtubules (MT) and microfilaments (MF) systems involved in transmembrane control of cell surface receptor mobility and distribution.

is maintained in a fluid state.

b)numerous proteins or glycoproteins are inserted into this bilayer.

c)the distribution of proteins, glycoproteins and glycolipids in the inner and outer halves of this bilayer is asymmetric in most if not all cellular membranes.

d)cell membrane proteins are heterogenous structures and may exist in various states of aggregation.

Membrane proteins may be subdivided into two classes on the basis of whether or not they enter the lipid bilayer:

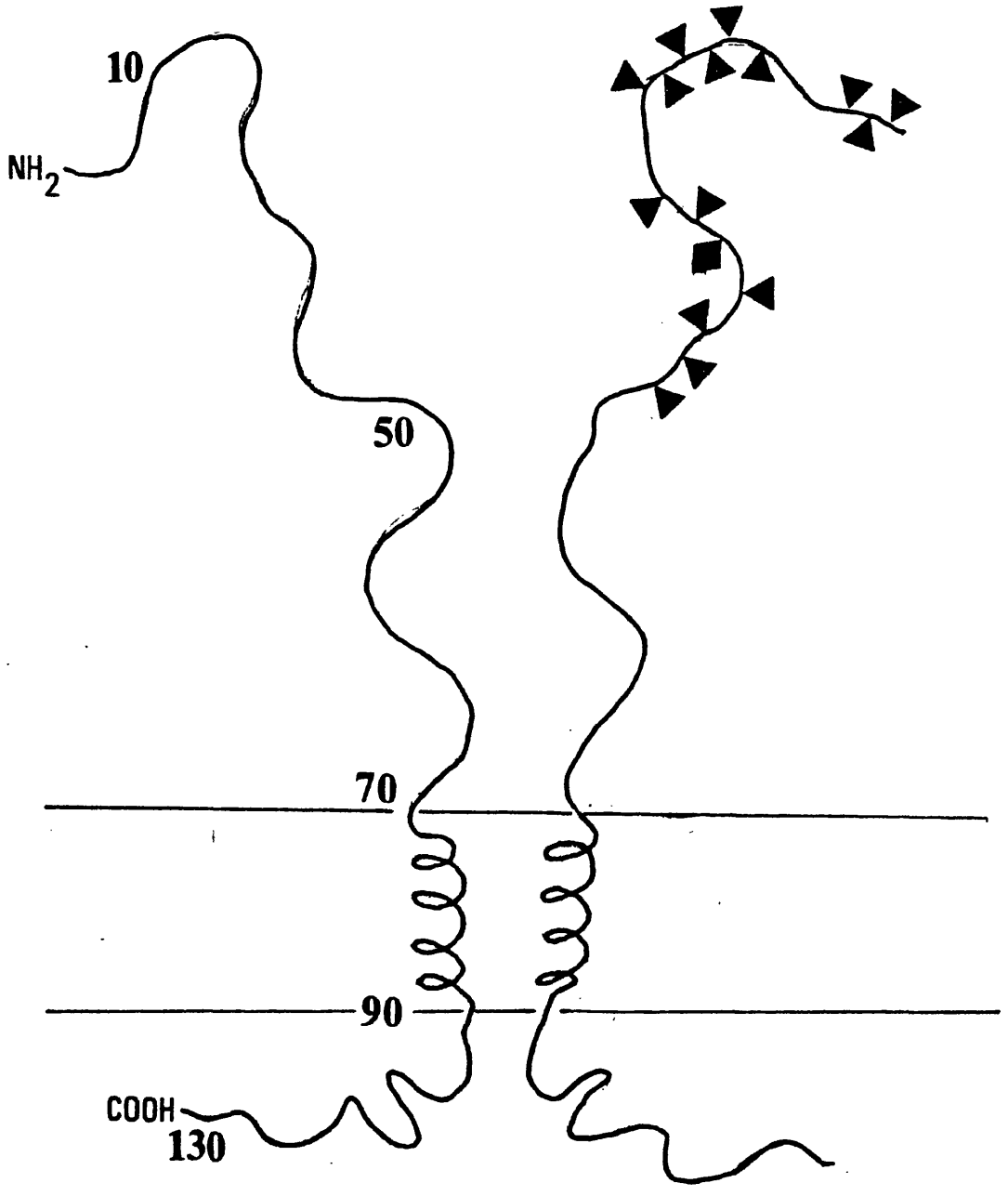
-Integral membrane proteins are firmly embedded in the lipid bilayer

-Peripheral membrane proteins are only loosely attached to the interior or exterior face of the lipid bilayer, (See Fig.1.4.).

Cells have evolved systems to restrict the mobility of membrane proteins in the lipid bilayer to a greater or lesser extent. Specific interactions between intrinsic membrane proteins and cytoskeletal elements are thought to exist, (Flanagan and Koch, 1978; Mescher et al., 1981 and Sheterline and Hopkins, 1981). Controlled attachment and detachment of the cytoplasmic portion of integral membrane proteins to internal peripheral membrane proteins (Fig.1.4. and 1.5.), and cytoskeletal elements may occur, (Houslay, 1981). The maintainance of such specific membrane protein-cytoskeletal interactions may mediate responses to intra- and extracellular stimuli, (Houslay, 1981).

Basic membrane structure is regarded as being an elaboration of the 'fluid mosaic model' first put forward by Nicolson and Singer (1975), (see Lotan and Nicolson, 1979) and summarized in Fig. 1.5. Additional extracellular and intracellular components are shown such as large proteoglycans or glycosaminoglycans (GAG), which are thought to partially cover the surface of some cells, and cytoskeletal elements such as microtubules (mt) and microfilaments (mf). Some oligomeric transmembrane complexes are shown as well as 'bridging' molecules linking cytoskeletal

Fig. 1.6. Schematic representation of the major red cell sialoglycoprotein, Glycophorin A.



The polypeptide is thought to be dimeric in its native state. The N-terminal is extracellular,,the C-terminal segment is intracellular, the do double line representing the lipid bilayer. The numbering refers to amino acid residues. The triangles and square represent O-linked and N-linked oligosaccharide sidechains respectively.

elements together.

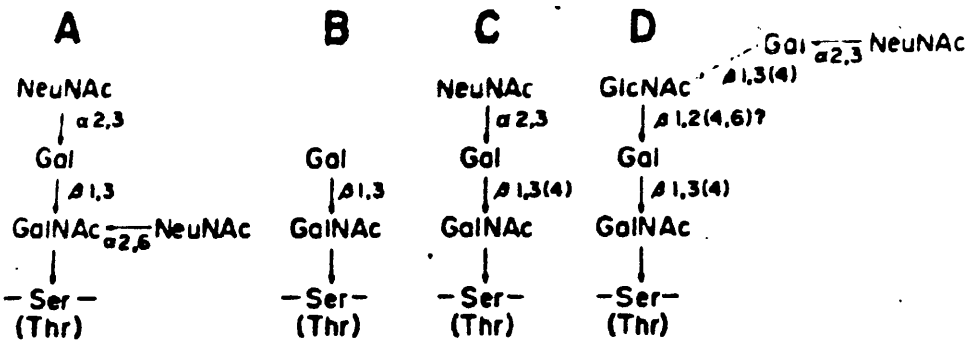
A number of membrane proteins have now been fully or partially sequenced either by direct sequencing of amino acid residues, e.g.: Glycophorin A, (Tomita et al., 1978), erythrocyte anion transport protein, (Band 3), (Braell and Lodish, 1982) and Thy-1, (Campbell et al., 1981) or indirectly through the sequencing of cDNA or genomic DNA known to code for membrane proteins, e.g.: membrane-bound IgM (SmIg), (Rogers et al., 1980), HLA, (Pober et al., 1978), H-2, (Coligan et al., 1981) and influenza haemagglutinin, (Gething and Sambrook, 1981).

Glycophorin A, the major red cell sialoglycoprotein, has tended to serve as a model for membrane proteins that span the lipid bilayer although it may be rather atypical with respect to the extracellular (glycosylated) region, see Figs. 1.4. and 1.6. The glycophorin monomer polypeptide is 131 amino acid residues long, (Harrison and Lunt, 1980). The amino acid composition is not strikingly different from soluble glycoproteins, (Lotan and Nicolson, 1979). However the amino acid sequence of glycophorin, and other membrane proteins so far studied, is quite distinct from that of soluble glycoproteins having an unusually high proportion of charged, hydrophilic amino acid sequences at both ends of the molecule, with a predominantly hydrophobic sequence inbetween. Thus the glycophorin molecule may be considered in three segments:

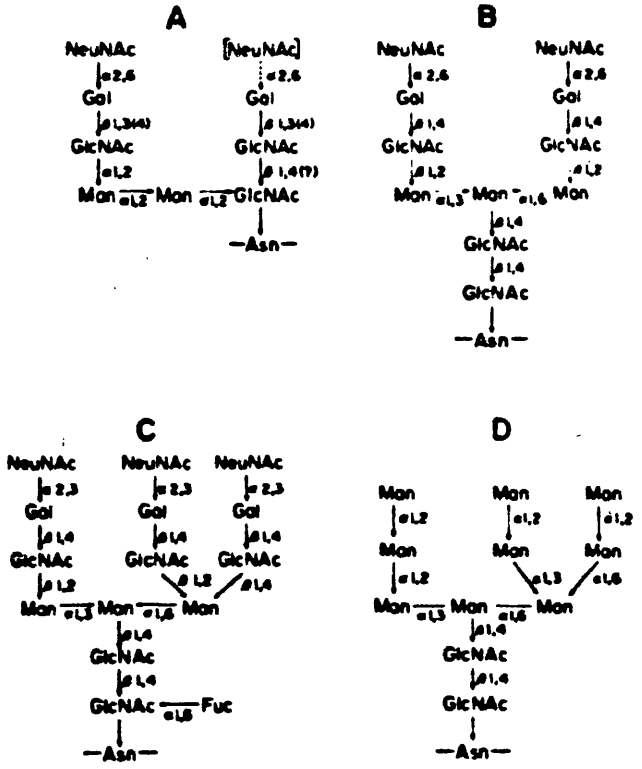
a) a hydrophilic, extracellular, N-terminal portion of 72 amino acid residues that contains 22 residues of serine or threonine, 15 of which serve as oligosaccharide linkage sites. In this portion is also found an N-glycosydically linked oligosaccharide chain, (see fig. 1.8.).

b) an internal sequence between residues 73 and 92 that is composed predominantly of hydrophobic amino acids probably arranged in the form of an helix. This segment is thought to be entirely embedded in the lipid bilayer and to be in strong association with lipid hydrocarbon tails. The presence of a cluster of acidic amino acid residues at the

Fig. 1.7. Examples of N- and O-glycosidically bound sidechians found in glycoproteins



O-linked sidechains: A) glycophorin (major red cell sialoglycoprotein); B) - D) TA-3 mammary carcinoma cell surface epiglycanin.



N-linked sidechains: A) glycophorin (red cell sialoglycoprotein); B) human serum transferrin; C) Vesicular Stomatitis viral envelope glycoprotein D) thyroglobulin unit A and isolated from human diploid fibroblasts.

From Lotan and Nicolson (1979)

Table 1.2.

Some Features of the Glycosylation of Proteins

Carbohydrate-Protein Linkage	Occurrence	Mode of Biosynthesis
A) O-Linked		
Linkage via the hydroxyl group of serine or threonine (alkali-labile)	Fetuin, Membrane sialoglyco-proteins, Ig A, ABO and Lewis Blood Group Substances	Sequential glycosyltransferase activity
Other Remarks: In general, small, often tetrasaccharide sidechains. No evidence for role in folding of nascent polypeptide chain. Membrane sialoglycoproteins may be relatively extended in solution - no evidence for extracellular 'domains'.		
B) N-Linked		
Linkage via the amide group of asparagine (alkali-stable)	Secreted, soluble glycoproteins e.g. α_2M*, αfp** Some enzymes, e.g. lactoperoxidase, ribonuclease. Most membrane glyco-proteins.	Involvement of intermediate lipid carrier in transfer of oligosaccharide to protein.
Other Remarks: There are two subclasses of, N-linked, oligosaccharide sidechains: 'High Mannose and 'Complex'. Possible role in the ability of membrane proteins to fold properly during biosynthesis - correct glycosylation may be essential if proteins are to obtain correct tertiary structure.		
C)Linkage via the hydroxyl group of hydroxylysine	Collagen, Basement membrane. Not found in membrane glycoproteins.	

* α-foetoprotein

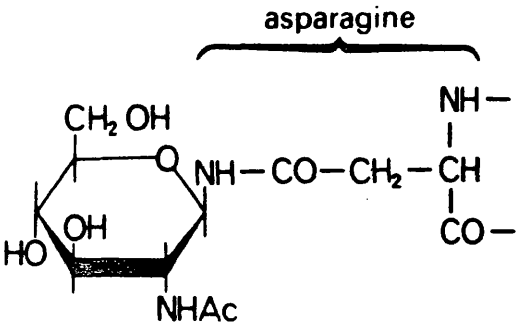
** α_2-macroglobulin

N-terminal (extracellular side) end of the internal hydrophobic segment and of four basic amino acid residues at the C-terminal (intracellular side) end of this segment may be important in the stabilizing of the protein in the phospholipid bilayer, (Coligan, 1980).

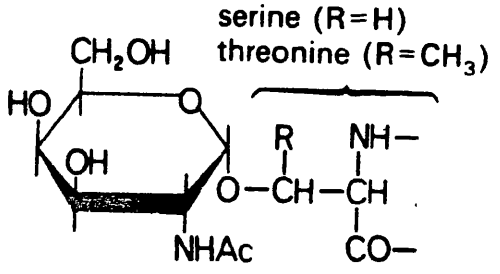
c) a **hydrophilic** C-terminal segment that probably participates in interactions with peripheral membrane components at the cytoplasmic side, and with cytoskeletal elements. This may involve the phosphorylation of serine residues; such phosphorylated residues have been reported in this portion of H-2, (Houslay, 1981) and HLA, (Pober et al., 1978).

These findings for Glycophorin A are outlined in Fig. 1.6. Glycophorin exhibits little or no secondary or tertiary structure (it posseses no intracellular disulphide bonds) and probably takes up an extended conformation in solution, (Brown et al., 1981). Brown et al. (1981) also suggest that such molecules may be of functional significance for mediating the behaviour of circulating cells. In contrast to Glycophorin most transmembrane proteins, on the basis of primary structure and other properties, appear to take up a 'domain' conformation in the extracellular portion. A 'domain' being defined as a 'stable folding unit', (Rossiman et al., 1981). Another atypical feature of Glycophorin is the nature of the majority (15/16) of its protein-carbohydrate linkages. These are short tetrasaccharide O-linkages, (see Fig. 1.7a. and Fig. 1.8.). In contrast most membrane glycoproteins generally contain longer N-linked residues, (see fig. 1.7b and Table 1.2. for a comparison), (Lotan and Nicolson, 1979). The finding of a sialoglycoprotein on rat thymocytes, (Brown et al., 1980) with glycophorin-like biochemical properties has resulted in speculation about the possible function of such atypical proteins on circulating cells.

Fig. 1.8 Glycosidic linkages in membrane proteins



Alkali-stable glycopeptide bond involving an *N*-glycosidic linkage between asparagine and *N*-acetylglucosamine.



Alkali-labile glycopeptide bond involving an *O*-glycosidic linkage between serine or threonine and *N*-acetylgalactosamine.

From Harrison R. and Lunt G.G., (1980), p 137.

Section 1.6.3 Role of glycosylation of membrane proteins

The assymmetric distribution of membrane glycoproteins with respect to the lipid bilayer and in particular the finding that glycosylation of these proteins is limited to the extracellular portion of the molecule has lead to speculation about the function of these carbohydrate moieties.

Glycoproteins in general, function in a wide variety of cellular and physiological processes. Many glycoproteins that are responsible for specific biological processes have been identified such as enzymatic activities, plasma membrane receptors, hormones, clotting factors, determinants of intracellular recognition and immunoglobulins, (see Atkinson and Bramwell, 1981 and Hughes, 1976, for review).

Using inhibitors of protein glycosylation, recent studies using mammalian cells in culture, have shown that the metabolic pathways responsible for introducing the carbohydrate sidechain into glycoproteins are critical for the maintenance of a variety of cellular processes. These are listed in Firestone and Heath (1981) and include: cell morphology and substratum adhesion, expression and function of specific plasma membrane proteins, cell differentiation, protein secretion and conformational and proteolytic stability **and transport to correct cellular location.**

In some instances studies have shown, (see Firestone and Heath, 1981 for review) that the non-glycosylated forms of membrane proteins exhibit increased susceptibility to proteases or are not normally mobilized for assembly into membrane structure, e.g. in fibronectin in chick fibroblasts, and in vesicular stomatitis virus (VSV) glycoproteins, Of potential interest to the work of this thesis is the finding by Firestone and Heath, (1981) that the non-glycosylated form of alkaline phosphatase (a plasma membrane glycoprotein in mouse L-cells) is intrinsically more sensitive to endogenous proteases than the glycosylated form of the molecule.

The role of carbohydrate in membrane antigens is largely undefined. While the antigenicity of the ABO blood group system on red blood cells is dependent on the carbohydrate component of the molecule, (see Harrison and Lunt, 1980 for review), this is not the case for the MN blood group system, (Anstee, 1981). In general when antibodies are raised to membrane glycoproteins they react with the polypeptide component. Although there is no apparent role for the carbohydrate units of the histocompatibility antigens, (Nathenson and Cullen, 1974), those of the immunoglobulin molecules seem to be important in complement induced cytotoxicity, (Kolde, Nose and Muramatu, 1977). Wilson et al. (1981) have suggested that the glycosylation of the **haemagglutinin** membrane glycoprotein of influenza virus modulates recognition of the **haemagglutinin** by the immune system.

The functional significance of alterations in glycosylation is important in many cases. In the case of glycoproteins at the cell surface, such changes may lead to alterations in the insertion, configuration and rate of turnover which may have a profound effect on normal cell behaviour.

Section 1.7. Cell Surface Proteins of the Neutrophil

Section 1.7.1. Cell Surface Proteins: Biochemical Identification

During the course of this study a number of pieces of work were published which identified the cell surface proteins of the neutrophil in man, (Andersson and Gahmberg, 1978); rabbit, (Willinger and Frankel, 1979) and pig, (Sheterline and Hopkins, 1981). These studies can be related to the work **described** in this thesis and so will be described here in some detail.

Andersson and Gahmberg (1978), and Andersson et al. (1979), studied the surface glycoprotein patterns of a number of different types of human

Table 1.3

Labelling of neutrophils with Na^3HB_4

Apparent Mol.Wt. (x 10^3)	Neuraminidase pretreatment + GO	Periodate
GP 155	++	++
GP 130	+++	-
GP 110	-	+
GP 105	++	+++

+/++/+++ Relative labelling intensity

(Taken from Andersson and Gahmberg, 1978 and Andersson et al., 1979)

Table 1.4.

^{125}I-lactoperoxidase labelling of neutrophils

Rabbit peritoneal neutrophils (Willinger and Frankel, 1979)		Pig venous neutrophils (Sheterline and Hopkins, 1981)
Band No.	Approx. mol. wt. x 10^{-3}	Approx. mol. wt. x 10^{3}
1	>200	215-220
2	150-180	169-172
3	120	144-149
4	90	105-115
5	66-68	78-80
6	60-62	45-46
7	45	32-36
8	35	25-32
9	25	
10	>20	
11	>18	

leukocytes using a rather more selective method than the one used in this thesis. Two complementary labelling methods (using ^{3}H substitution reactions) were employed. The galactose oxidase-tritiated borohydride (GO-$NaB^{3}H_{4}$) labelling method labels galactosyl residues of exposed glycoproteins by oxidation with GO followed by reduction with $NaB^{3}H_{4}$, (Gahmberg and Hakomori, 1973). Sialic acid residues of surface glycoproteins were labelled with $NaB^{3}H_{4}$ after oxidation with sodium periodate under conditions where the periodate did not penetrate the intact membrane, (Gahmberg and Andersson, 1977). The results of their experiments with respect to neutrophils are shown in Table 1.3.

The authors suggest that the most strongly labelled band,in both systems (Gp 130/Gp 105 respectively) is a single species apparently rich in sialic acid residues, (Andersson and Gahmberg, 1978). No heavily labelled bands of higher molecular weight were seen.

Some cell surface proteins are thought to be directly involved in phagocytosis, (Willinger, Gonatas and Frankel, 1979 and Shaw and Griffin, 1981). It follows therefore that one might expect this functional conservation to be reflected in a comparison of the cell surface proteins of the neutrophil from different mammals. Thus it was of some interest to compare cell surface proteins from other animals.

Hawkins and Sauve (1978), Willinger and Frankel (1979) and Thrall et al. (1980) have labelled rabbit neutrophils using the ^{125}I-lactoperoxidase method and use various criteria to determine the plasma membrane location of labelled components. Unfortunately only Willinger and Frankel (1979) provide a detailed description of the labelled proteins. Sheterline and Hopkins (1981) have labelled pig neutrophils using the ^{125}I-lactoperoxidase technique. These two sets of data are shown in Table 1.4.

Willinger, Gonatas and Frankel (1979) in a companion paper to their paper on the biochemical identification of cell surface proteins, demonstrated by radiolabelling cells and then allowing phagocytosis to take place, that the membrane fraction of these cells was missing 5 out of 13 cell surface proteins. Isolated phagosomes were deficient in 6 of these proteins which were enriched in a dense surface fraction. They suggest that their results indicate that a nonrandom interiorization of ^{125}I-LPO labelled surface proteins takes place during phagocytosis.

In humans, Gahmberg and co-workers have provided evidence for the functional involvement of Gp 130 (a glycoprotein of mol. wt. 130K) in phagocytic and locomotor activity of neutrophils, (de la Chapelle et al., 1980). They noted that in two patients with haematological disorders in whom most, if not all, bone marrow mitoses were monosomic for chromosome 7, labelling of Gp 130 was markedly reduced. They suggested that this might be a 'dosage effect' and that monosomy for chromosome 7 and possibly the region 7q22-7qter is associated with a decrease in labelling of the glycoprotein, Gp 130 and with a reduction of locomotor activity.

Using the promyelocytic cell line HL-60, (Gahmberg et al., 1979) demonstrated that by induction with DMSO (dimethylsulphoxide) the expression of Gp 130 correlates with the appearance of phagocytic and chemotactic activities of these cells.

A number of groups of workers have now produced hybridoma derived monoclonal antibodies against various myeloid cells, including neutrophilic granulocytes, (see review in Knapp et al. 1981). The biochemical characterization of antigenic determinants recognized by monoclonals produced against neutrophils is so far limited. Dalchau et al. (1980a and 1980b) have described two monoclonal antibodies, F10-89-4 and F10-44-2, which recognize determinants which have been biochemically identified.

The antibody F10-89-4, (Dalchau et al., 1980a) is directed at the human leucocyte common antigen, a major glycoprotein of human leucocytes with a molecular weight of 190K to 215K. The antigen is widely distributed amongst leucocytes, being found on T and B lymphocytes, thymocytes, monocytes and granulocytes, but is not found in other tissues. Homologous antigens with virtually identical tissue distribution have been found in both the rat and mouse, (Dalchau et al., 1980a).

The antibody F10-44-2, (Dalchau et al., 1980b) is directed at an antigen found predominantly on brain, granulocytes and T lymphocytes. It is a major glycoprotein of human lymphocytes, has a molecular weight of 105K and is almost certainly the human homologue of the rat W3/13 antigen, (Dalchau et al., 1980b). The W3/13 antigen is a 'glycophorin-like' sialoglycoprotein, (Brown et al. 1981) and is discussed in relation to my results in Section 5.

Kay (1981) has discovered that an antigenic determinant (mol. wt. 62K approx.) present on senescing red blood cells (RBCs) is also present on stored platelets, lymphocytes and neutrophils. It functions in RBCs as a marker for a recognition system that selectively removes senescent RBCs from the circulation. Whether it has a similar role in leucocytes is not yet clear.

Clearly the use of monoclonal antibodies raised against the antigenic determinants described above would allow the purification and characterisation of such determinants and might provide information in a comparison of single determinants on neutrophils in controls and CML.

Section 1.7.3. Biochemistry of Cell Surface Receptors

Knowledge of the biochemistry of the cell surface receptors of the neutrophil is limited and there is still a considerable gap in our understanding between the functions of the cell surface receptors of the

neutrophil and the biochemistry of those receptors.

Two types of IgG-Fc receptors, (Barrett et al., 1979), C_{3b} and C_{3d} receptors, Ross et al., 1978) are expressed on the cell surface of the human neutrophil. They allow the recognition of suitably opsonized particles which may result in attachment, phagocytosis and metabolic changes, (Willinger, Gonatas and Frankel, 1979).

Ross et al. (1978), conclude from their work that Ia-like determinants CR_1 (immune adherence, C_{4b}-C_{3b} receptors), and CR_2 (C_{3d} receptors) are expressed, during neutrophil maturation,in the order:

$$Ia - CR_2 - CR_1, CR_2 - CR_1$$

Ross et al. (1978) noted that this sequence of expression was not rigidly linked with morphological maturation and suggest that the regulation of membrane differentiation may be independent from the events of nuclear and cytoplasmic maturation as defined by standard morphological criteria. Barrett et al. (1979) again working on human neutrophil receptors reach the different conclusion that their may be distinct subsets of neutrophils resulting in cell surface heterogeneity of neutrophil populations for IgG-Fc, C_{3b} and C_{3d} receptors.

Despite work on receptor identification little is known about the biochemistry of the above receptors.

In work on human alveolar macrophages, Schneider et al. (1981) have isolated a biologically active receptor for C_{3b}, the major cleavage component of complement (C3). The receptor is accessible to labelling with ^{125}I-LPO, is a glycoprotein (determined by incorporation of ^{14}C-glucosamine) and migrates as a broad band in the 58K-68K molcular weight region on SDS-PAGE. They also noted that no alteration in the mobility of the band was caused by running under non-reducing conditions suggesting that interchain disulphide bonds were not present. Since human neutrophils

also express receptors for C3b, (Ross et al., 1978), it is interesting to note that a broad band of similar mol. wt. 55-60K, can be identified in the results presented in Section 3.2 and 3.3.

Section 1.8. The Neutrophil in CML: Studies on the Cell Surface

Malignant cells may have qualitatively or quantitatively unique properties residing in new, altered or missing molecules which can explain their apparent regulatory failure. Greaves (1979) has emphasised the need for adequate controls in such a study in the form of normal cells of the same cell lineage, at the same developmental or maturational compartment and in the same growth or cell cycle phase.

It was with these points in mind that the neutrophil was chosen for study. Relatively homogenous (>93%) populations of 'post-mitotic' neutrophils can be obtained, by simple separation procedures, in large enough quantities for biochemical analysis from normal individuals and most CML patients.

Barrett et al. (1979) have provided evidence that peripheral blood neutrophils are composed of subsets with different membrane marker reactivities (2 types of IgG-Fc receptors and 2 activated C components, C_{3b} and C_{3d}) but these apparent differences may merely reflect differences in cell age or maturation.

The question of whether we are comparing 'like with like' is obviously an important one in a study such as this. There are a number of pieces of work that suggest that this is not strictly the case which may be relevant to any likely findings at the cell surface.

Sato et al. (1982) suggest that neutrophil alkaline phosphatase (NAP) activity may be a marker for cell maturity in neutrophils. They suggest that low NAP activity of neutrophils derived from CML patients is due to the immaturity of these cells.

Other workers have suggested that the lifespan of the mature neutrophil

may be longer than normal in CML, (Koeffler and Golde, 1981 for review). It is also clear that there are an increased number of immature cells in the peripheral circulation, (Greaves, 1979).

Impairment of phagocytosis has been documented in CML, (Dotten et al., 1982). Phagocytosis is a complex process which, in the case of opsonized particles, involves specific receptors on the cell surface. Rosner et al., (1970) and Whittaker et al., (1974) postulated that in CML neutrophils had a biochemical or lysosomal defect, resulting in impaired ingestion.

A number of rather fragmentary pieces of evidence suggest that the expression of cell surface proteins may be altered in neutrophils in CML. Reijden et al. (1979) describe the loss of a granulocyte specific alloantigen in Chronic Granulocytic Leukaemia. They argue that this is not an effect of relative cell immaturity since normal promyelocytes (and even possibly myeloblasts) express the antigen. The loss of the antigen which has not been biochemically characterised may correlate with impending blastic transformation.

Morstyn et al. (1981) have attempted to quantify the binding of a number of monoclonal antibodies using a fluoresence activated cell-sorter. The leucocyte-common antigen recognized by F10-89-4, appears to show a quantitative increase with maturation of the myeloid series. The antigen appeared to be present in equal amounts on the corresponding cells of one patient examined with CML.

However the brain lymphocyte-granulocyte antigen, recognized by F10-44-2 (see section 1.7.), present on both progenitor and mature granulocytes, diminished in concentration as the progenitor cells differentiated to form mature granulocytes. The study of the myeloid cells of the one patient with CML revealed that the leukaemic blast cells and progenitor cells had a lower concentration of the antigen than normal blast cells and progenitor cells. When studying mature neutrophils, of the CML patient, they found that there were two populations of polymorphonuclear cells, one

Table 1.5

Nylon fibre adherence of granulocytes

Diagnosis	Range of non-adherent cells (%)
Normal (n=9)	0.6 - 13.0 (x = 4.4)
Leucocytosis (n=4)	0.6 - 1.8 (x = 1.5)
CML (n=7)	17.9 - 47.3 (x = 26.4)

Table 1.6.

Lectin agglutination of granulocytes

Lectin	CML	Leukocytosis
Peanut (PNA) galactosyl residues	reduced agglutination	increased agglutination
Concanavalin A mannose/glucose residues	reduced agglutination	ND
Limulin sialic acid residues	increased agglutination	no differencee

Data taken from Taub et al., (1980)

fluorescent (i.e. antibody binding) and the other not. Since all granulocytes from normal individuals are positive for the antigen and show a single broad peak of antigen on the cell sorter, Dalchau et al. (1980b) this is of potential interest.

Taub et al. (1980) have compared in a semi-quantitative way the ability of various lectins to agglutinate peripheral blood neutrophils and nylon-wool adherence of these cells, from patients with Ph' +ve CML, benign leucocytosis and normal individuals.The results of these experiments are shown in an abbreviated tabulated form in Table 1.5. Treatment of neutrophils, from patients with CML, with neuraminidase partially reversed their defect in adherence towards normal. Neuraminidase pretreatment had little effect on cells from normal donors or leukocytotic patients.

CML and normal neutrophils were then compared with respect to the ability of three different lectins to agglutinate these cells. CML neutrophils showed the results shown in Table 1.6. with respect to controls. As with nylon column adhesion, after neuraminidase pretreatment the response of CML cells to treatment with PNA is the same as control cells.

On the basis of their data, Taub et al. (op. cit.) suggest the possibilty that in CML, sialic acid or hypersialylated glycoproteins may mask cell surface receptors that may mediate granulocyte adhesion. They suggest that inhibition of such receptors may be involved which may be involved in neutrophil margination and migration from the capillaries, and in retention of immature forms in the bone marrow, might account in part for altered granulocyte kinetics and function in CML. Gahmberg and Andersson (1979) using the labelling technique described in Section 1.7 have compared neutrophils from normal individuals with 'leukaemic' cells from CML patients and suggest that in the latter Gp 130 is more strongly labelled and more 'diffuse'. Gp 130 is the major labelled component after pretreatment with neuraminidase and the above authors suggest that it is a

sialoglycoprotein.

A number of other pieces of work may be relevant to the interpretation of Taub's results. Fishman et al. (1981) have demonstrated that Con A receptors are immobilized during the differentiation of neuroblastoma cells. This finding may well be related to altered agglutination properties. Hoessli et al. (1980) have noted that during T-lymphocyte differentiation their is an increase in sialic acid constant of the Thy-1 antigen.

Section 1.8 Purpose of the Present Study

The study of any one aspect of a disease depends upon an understanding of both the biological and clinical nature of the disease in general. With this in mind current understanding of the biology of leukaemias (Section 1.2 and 1.3), and in particular of Chronic Myeloid Leukaemia (Section 1.4) has been described.

In broad outline , a rationale for the study of cell surface proteins in leukaemia and the potential benefits that such studies may bring has been provided, (Sections 1.1 and 1.5). The structure and function of cell surface proteins has been briefly outlined and in particular the role of glycosylation of these proteins has been reviewed, (Section 1.6). In Section 1.8 work is described which suggests that some defects of the neutrophil in CML that are thought to occur may involve alterations in the cell surface.

This study was undertaken to determine whether simple consistent differences in the expression of cell surface proteins could be detected between neutrophils isolated from patients suffering from chronic myeloid leukaemia and controls.

The aims of this study are threefold. Firstly, biochemical identification and characterisation of cell surface proteins particularly with respect to glycosylation from control cells is intrinsically

interesting, will provide new information about the organization of the cell surface of the neutrophil and will allow for comparison with leukaemic neutrophils.

Secondly having characterised control cells using the techniques described in Section 2 (Materials and Methods) a comparison of control and leukaemic neutrophils will be made. Thirdly it was hoped that this study might provide gene mapping information through a search for a correlation between the non-random chromosomal changes found in CML and any dosage effects in the expression of membrane proteins.

Section 2.1 Alphabetical List of Suppliers

(1) Amersham International Ltd., The Radiochemical Centre, Amersham, Bucks.

(2) Armour Pharmaceuticals, Hampden Park, Eastbourne, Sussex.

(3) Argyll Glass Co.,1125 Argyll St., Glasgow G3., U.K.

(4) BDH Chemicals Ltd., Poole, Dorset., U.K.

(5) BioRad Laboratories Ltd., Caxton Way, Watford, Herts., U.K.

(6) BRL (Bethesda Research Laboratories UK. Ltd.), P.O. Box 145, Science Park, Cambridge, U.K.

(7) Calbiochem, La Jolla, California, USA.

(8) Decon Ltd., Portslade, U.K.

(9) DuPont UK Ltd., Photoproducts Dept., Wedgewood Way, Stevenage, Herts.

(10) Evans Medical, Speke, Liverpool.

(11) Ferba Chemicals, Heidelberg, GDR

(12) Fisons Pharmaceutical, Norwich, U.K.

(13) Gurr, Hopkins and Williams Ltd., Romford, Essex.

(14) Hopkins and Williams Ltd., Romford, Essex

(15) Kodak Ltd., Hemel Hempstead, Herts, U.K.

(16) Miles-Yeda, Israel

(17) NEN (New England Nuclear), 2 New Road, Southampton, Hants.

(18) Oxoid, Ltd., Southwarkbridge Road, London.

(19) Phamacia Fine Chemicals, Hounslow, Middlesex.

(20) Sigma London Chemical Co., Poole, Dorset.

(21) Worthington Biochemical Corporation, New Jersey, USA.

Section 2.2 Materials for Cell Separation and Identification

2.2.1 Collection and Preparation of Blood.

Evans heparinised 20 ml bottles (Evans Medical) were used for whole blood collection.

Buffy coat samples were obtained in sealed leukocyte transfer packs (courtesy of Law B.T.S.).

2.2.2 Separation of Neutrophils from Whole Blood.

Heparin without preservative (Evans Medical), 500 i.u. per 10 ml blood or buffy coat.

Dextran (Dextraven 110, Fisons, dextrans of mol. wt. 110,000 6% w/v in 0.9% NaCl.)

Ficoll-Hypaque ('lymphoprep') Pharmacia Fine Chemicals.

PBS A Prepared in Media Preparation Room, M.R.C. Institute of Virology, Church St., Glasgow.

NaCl	8.0 $g.l^{-1}$
KCl	0.2 $g.l^{-1}$
$NaHPO_4.12H_2O$	2.31 $g.l^{-1}$
KH_2PO_4	0.2 $g.l^{-1}$

Saline Solution: 0.9% w/v NaCl. Autoclaved prior to use.

Tris-NH_4Cl (selective red cell lysis) - 9 vol. 0.83% w/v aqueous NH_4Cl: 1 vol. Tris buffer: (20.594g Tris base/l. adjusted to pH 7.65 with HCl). The pH of the final mixture is adjusted to 7.2. 100 ml. were prepared every three weeks and stored at 4°C.

2.2.3 Cell Identification

Phosphate Buffer Solution (pH 6.8.) was prepared by addition of one buffer tablet (Oxoid) to 500 ml deionised water.

Lieshmann Stain (Gurr) was diluted 1:2 with phosphate buffer prior to use.

2.2.4 Counting of Cells

Cells were counted in an improved Neubauer pattern haemocytometer, (depth 0.1 mm, area 1/400 mm^2). The counter plate and coverslips were stored in 70% alchohol when not in use.

2.2.5 Assessment of Cell Viability

0.4g of trypan blue (Gurr) was dissolved in 100 ml of 0.9% saline

Section 2.3 Materials for Radiolabelling of Whole Cells

2.3.1 Radiochemicals

$Na^{125}I$, carrier-free, (100 mCi/ml) obtained from Regional Radionuclide Dispensary, Western Infirmary, Glasgow (Obtained from NEN)

2.3.2 Stock solutions

Lactoperoxidase (Calbiochem) was made up in PBS A at a concentration of 1 mg/ml. and stored at -70 ^{o}C in 50ul aliquots.

Glucose Oxidase (Worthington) was made up in PBS A at a concentration of 100 U/ml (183 U/mg). This was stored at -70 ^{o}C in 5 ul aliquots. Immediately prior to use it was diluted with 195 ul of PBS A to give a working concentration of 5 U/ml.

PBS A + 25mM Glucose (BDH, AnalaR).

PBI (10 mM NaI)(Hopkins and Williams, AnalaR).

PBS A + 1% (vol/vol) Triton-X 100 non-ionic detergent.

Phenylmethylsulphonylfluoride (PMSF) (Sigma). A 0.2 M stock was prepared in AnalaR ethanol and stored at 4 ^{o}C. To produce a final concentration of 2 mM it was added to solutions in the ratio 1:100.

Section 2.4 Post-Labelling Treatment of Whole Cells

Trichloroacetic acid (TCA) - 5% stock solution stored at 4^{o}C for protein precipitation on Whatman glass fibre (GF-1) 2.5cm discs.

Trypsin (Bovine Pancreas - Sigma) Stored in PBS A 100 ul aliquots (1 mg/ml) at -70 ^{o}C.

Acetone (BDH AnalaR); BSA carrier (Armour Pharmaceuticals or Sigma).

Neuraminidase (from Cl. perfringens Type V -Sigma) Stored in PBS Complete in 100 ul aliquots (10 Units) at -70^{o}C

-D-N-acetylgalactosaminyl-oligosaccharidase (BRL) Stored lyophilized at 4 ^{o}C 0.1 Unit in 50 mM phosphate, pH 6.5

Section 2.5 Plasma Membrane Isolation

Homogenization Buffer

10mM Phosphate (PBS A)

0.34M Sucrose (Hopkins and Williams)

500 ug/ml BSA (Armour Pharmaceuticals, Fraction V or Sigma)

1mM EDTA, 2 mM PMSF and 5 i.u. Heparin/ml Buffer

Sucrose solutions

Sucrose solutions, 15%, 35%, 45%, 50% and 70% (w/v) were buffered in PBS A containing 50 ug/ml BSA, 1 mM EDTA and 5 i.u./ml Heparin.

Overlay Buffer (8.6% (w/v) Sucrose) was prepared in an identical buffer.

In all cases PMSF and Heparin were added immediately prior to use.

Section 2.6 Immunoprecipitation Experiments

F10-44-2 monoclonal antibody was a gift from Dr. J. Fabre, John Radcliffe Infirmary, Oxford. 100 ul of antibody (immune ascites - IgG fraction obtained by ion-exchange chromatography) was obtained in 0.15M NaCl, 0.25M Tris, 0.02% NaAzide, pH 8.5. This was stored at -70°C until use, in 10ul aliquots.

2.6.1 Staph. A Method

Formalin fixed, heat-killed Staph. A (Stapholococcus aureus) were a gift from Dr. A. Cross, Institute of Virology, University of Glasgow.

STN buffer: 0.15M NaCl, 0.01M Tris pH 7.4, 0.25% NP-40. Nonidet P-40 was kindly supplied by Dr. M. Davies.

2.6.2 'Second antibody' (HT-2) Method

Anti-Mouse IgG (Miles-Yeda) was reconstituted in dI H_2O and stored in 100 ul aliquots at -70°C.

Mouse serum was kindly supplied by Dr. M. Davies. This was stored in 25 ul aliquots at -70°C.

Section 2.7 Protein Separation and Identification on SDS-PAGE

2.7.1. Protein Determination

Solution A

Na_2CO_3	20g
NaOH	4g
Na (alkaline tartrate)	2g
dH_2O	to IL

Solution B

$CuSO_4.5H_2O$	5g
dH_2O	to 1L

Solutions A and B stored at 4°C. Solution C prepared fresh immediately prior to use.

Folin's Reagent (80%)(BDH)

Protein Standards BSA (Armour Pharmaceuticals, (Fraction V) or Sigma) 0.5, 1.0, 2.0 4.0, mg/ml.

2.7.2. Preparation of Slab Gels

Chemicals

Acrylamide (BDH) (electrophoresis pure grade)

Bis-Acrylamide (as above)

Tris (Trizma Base) (Sigma)

Ammonium Persulphate (BDH)

TEMED (N,N,N,N, tetraehtylenediamine) (BDH)

Glycerol (BDH)

SDS (sodium dodecyl (lauryl) sulphate) (Farber)

Solutions

Sample buffer, (final concentrations in brackets): 20% SDS, 0.3mls (4%); 1M Tris, pH 6.8, 0.24mls (.16M); 1M DTT, 0.24mls (0.2M); Glycerol, 0.3mls, (20%); 0.2% bromophenol blue, 15ul; PMSF, 0.2M in ethanol, 15ul, (2 mM); deionised water, 0.345mls. Total volume 1.5mls. The sample buffer was prepared fresh just before use.

Acrylamide 30% (w/v) in deionised water

gradient gels: (5% bis-acrylamide)

single concentration gels: (2.5% bis-acrylamide)

Running Gel Buffer

Tris 181.5g / SDS 4.0g bring total volume to 1 litre after

pH'd to 8.9 with Hcl

N.B. Samples in solution were treated with sample buffer without deionised water. See Section 2.15.2 for details of order of addition etc.

Spacer Gel Buffer

Tris 59.0g / SDS 4.0g as Running gel Buffer but pH'd to 6.7 with Hcl

Ammonium Persulphate 10% (w/v) in deI water

Tank Buffer

Tris 6.32g / Glycine 4.0g / SDS 1.0g Total volume to 1 litre with deI water. Not pH'd.

Fixative with or without Stain

Coomassie brilliant blue R250, (BioRad) 0.2% in methanol: distilled water: glacial acetic acid (50:50:7)

Destain

Methanol	100 mls
glacial acetic acid	140 mls

distilled water up to 2 litres

2.7.3 Visualization of Radiolabelled Polypeptides

Kodak XH-1 Film (All films were preflashed prior to exposure)

Intensifying Screens ('Cronex' Lightning Plus- DuPont), (see Laskey and Mills, 1977).

Section 2.8 Chromatographic Techniques

2.8.1 Columns

For desalting of labelled species prior to further processing 'agar' pipettes were used. These have a nozzle diameter about three times that of a standard pipette which provides a useful drop size for rapid desalting. Routinely these were cut down withm a diamond pencil prior to use and plugged with glass wool.

For affinity chromatography glass mini 'econo-columns' (BioRad) were used.

2.8.2 Chromatographic Media

For desalting, preswollen (3 hours minimum) Sephadex G25-Coarse (Pharmacia) was used. It was preswollen in PBS A + .1% BSA. Prepared columns were stored, partially immersed in buffer, PBS A + .1% BSA, (+ .05% NaAzide as preservative) for up to 7 days.

Sepharose 4B conjugated to Con A was obtained commercially, (Pharmacia Fine Chemicals), supplied in acetate buffer, (0.1M, pH 6, 1M NaCl, 1mM $CaCl_2$, 1mM $MgCl_2$, 1mM $MnCl_2$, Merthiolate, 0.02% as preservative).

Washing Buffer: 0.02M Tris-Hcl, pH 7.4, 0.5M NaCl, 1mM $MgCl_2$, 1mM $CaCl_2$, 1mM $MnCl_2$, 1% Triton X-100.

Elution Buffer: As above except 0.5 -D-Mannose, 0.5% TX-100.

Storage Buffer: 0.1M NaAcetate, pH 6.0. 0.5M NaCl, 1mM $CaCl_2$, 1mM $MnCl_2$, 0.05% NaAzide, (no TX-100)

All buffers and conjugates stored at 4^oC.

Section 2.9 ^{125}I-Lectin Overlay on SDS-PAGE

2.9.1 Chloramine-T Iodination of lectins

Lectins (WGA or Con A) were prepared in 25 ul aliquots at a concentration of 3 mg/ml in PBS A and stored at -70^oC until immediately before use.

^{125}I (carrier free) 100mCi/ml, (NEN - obtained via Radionuclide Dispensary, Western Infirmary).

0.25M phosphate buffer

Chloramine-T (5 mg/ml)

Sodium metabisulphite (12 mg/ml)

Both the above were stored preweighed at room temperature and solubilised prior to use in PBS A.

KI (20 mg/ml) - Prepared prior to use in PBS A + .1% BSA

Column washing buffer: PBS A + .1% BSA

2.9.2 SDS-PAGE Overlay with ^{125}I-Lectin Conjugates.

Amberlite Monobed Resin-MB1 (BDH) for pretreatment of acrylamide solutions

Buffers used for gel processing were as follows:

Equilibration Buffer:

Con A: .1M Tris (pH 7.0), .15M NaCl, 1 mM $MnCl_2$, 1 mM $CaCl_2$

WGA: .1M Tris (pH 7.0), .15M NaCl

Incubation Buffer:

50 ml Equilibration Buffer

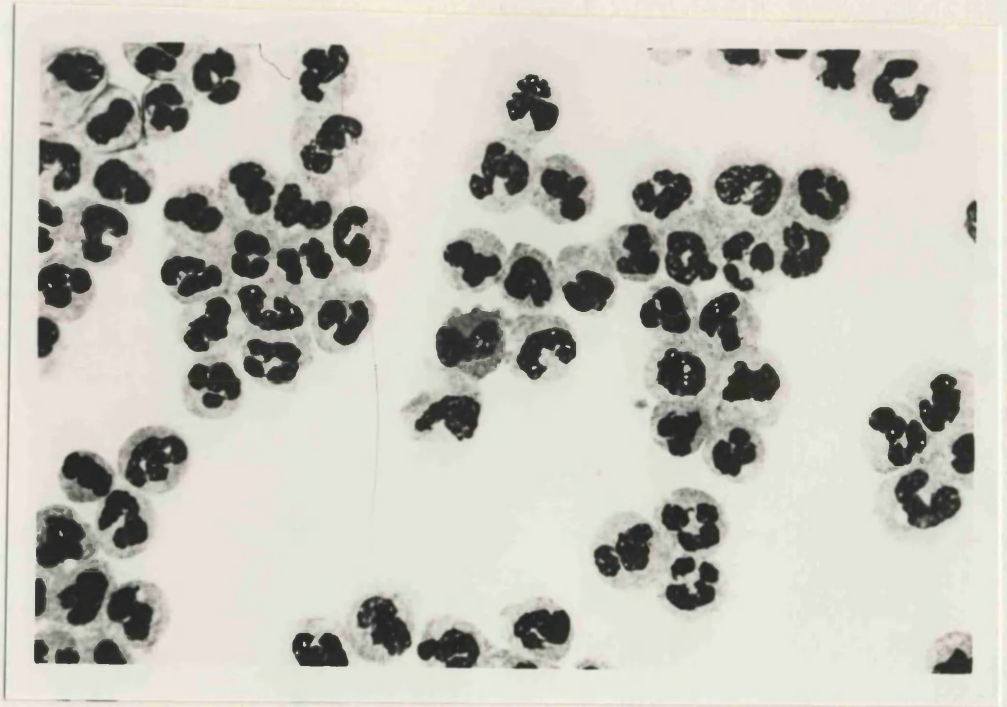
BSA (500 ug/ml)

^{125}I-lectin (approx 15-20 ug at 5-15 uCi/ug specific activity)

Washing Buffer:

Equilibration Buffer +0.5M NaCl

Fig. 2.1 . Purified neutrophils



x 4000.

A contaminating eosinophil, with darker stained cytoplasm, may be seen in the middle of this field.

METHODS

Section 2.10 Blood Preparation and Collection

Venous blood was collected from leukaemic patients or from normal controls. Blood was stored in bottles coated with a thin layer of heparin (Evans heparinised bottles) and processed as soon as possible.

Buffy coat samples from one unit of donated blood were collected from Law Blood Transfusion Service, Lanarkshire, Scotland, via the Blood Donor Centre, St. Vincent St., Glasgow. The buffy coat samples were contained in sealed leukocyte transfer packs and processed as soon as possible.

Cells obtained from venous blood samples were always separated and radiolabelled, if necessary, on the same day that the sample was obtained.

'Buffy Coat' samples were prepared by centrifugation at Law during the latter part of the afternoon, stored at room temperature overnight and then obtained from St. Vincent St. the next morning. No decrease in cell viability was noted in comparison to freshly obtained venous blood.

Section 2.11 Cell Separation and Identification

2.11.1 Separation of Neutrophils

The method of Boyum (1976), modified by D. Smillie (personal communication) was used. This method allows the separation of a 90 - >95% pure population of neutrophils, (see fig. 2.1.). Other granulocytes e.g. eosinophils and basophils make up most of the contaminating cells from normal individuals.

1) Liquid heparin, 500 i.u. (5000 i.u. per ml) per 10 ml of blood and

Dextran, 1 ml per 10 ml of blood were added to the sample and mixed well. The prepared blood was then poured into a 10 or 50 ml syringe that had been presealed at the nozzle. Sedimentaion of erythrocytes was allowed to take place at 1g at room temperature for a minimum of 45 mins. Buffy Coat samples took longer to process at this stage per volume of material.

2) The leukocyte-rich plasma (LRP) was then removed and layered directly onto Ficoll-Hypaque in siliconized glass, round-bottomed, 4 x 1/2" test tubes. 4 ml of LRP was layered onto each 3 ml Ficoll-Hypaque cushion. The samples were then centrifuged at 400g (1400 rpm) for 20 min. using an 'M.S.E. minor' bench centrifuge.

3) The supernatant, interface layer containing monocytes, lymphocytes and platelets and the Ficoll cushion were aspirated off. The aspirator unit contained a large trap to which was added fresh Chloros before experiments were carried out. The granulocyte and red cell pellet was resuspended in a very small volume of PBS A, care being taken not to reintroduce mononuclear cells, some of which remained further up the tube. The suspensions were then pooled in clean tubes and centrifuged at 150g (850 rpm) for 5 min.

4) Remaining red cells were removed by selective hypotonic lysis with Tris-NH_4Cl. Cells were resuspended in 5 ml. Tris-NH_4Cl for 30 min at room temperature. Cells were then pelleted at 200g (1000 rpm) for 2 min. They were then resuspended in Tris-NH_4Cl and pelleted again. Occasionally it was necessary to resuspend the cell in Tris-Nh_4Cl for a further 15 min.

5) The cells were then washed twice in PBS at 200g for 2 min, before examination. Routinely 0.5 - 1.5 x 10^7 neutrophils were obtained per 10 ml. of whole blood.

Buffy Coat samples were processed in the same way, although the relative yield per volume of whole blood was less good.

2.11.2 Staining of Cells for Haematological Identification

Cells were smeared onto glass slides using a Cytocentrifuge (Shandon-Elliot). Cells were stained with Leishmann stain (Gurr) for 2 minutes and then in Stain diluted 1:2 v/v with phosphate buffer (pH 6.8) for a further 3 minutes before rinsing the slides in phosphate buffer.

2.11.3 Total Cell Counts

The total number of cells in a suspension were determined microscopically using an improved Neubauer pattern haemocytometer (depth 0.1 mm, area 1/400 mm^2). A minimum number of 200 cells were counted over 5 large squares on each side of the counter.

2.11.4 Assessment of Cell Viability

Viable cells were assayed with the technique of dye exclusion. A 0.1 ml suspension containing 1-2 x 10^5 cells wasmixed 0.1 ml of 0.4% trypan blue stain and left for 5 min. at room temperature. Cells were carefully resuspended and the solution was observed microscopicaly using a haemocytometer. The fraction of non-viable cells was assessed (minimum 200 cells counted) and if they exceeded 10% of the total cell population the sample was discarded.

Section 2.12 Radiolabelling of Whole Cells

2.12.1 Determination of activity of ^{125}I stock

Prior to each labelling experiment a known dilution (counted sample is $1:10^4$ of stock solution, 1mCi/ml PBS A) of stock ^{125}I was counted in a bench top gammma counter, (Mini-instruments). Routinely a figure of 30,000 counts.s^{-1} was obtained, for this sample. The volume of diluted stock

solution added to the reaction mix was adjusted if necessary, to give an approximate input of 200uCi per reaction mix. Stock solutions were used within 7 days.

12.2 Standard Iodination Procedure

1) After separation, identification and counting, whole cells were spun down at 200g (1000 rpm) for 2 min. in a MSE 'minor' bench top centrifuge.
2) The cells were carefully resuspended in ice-cold PBS A, in plastic leighton tubes and left on ice.
3) The Reaction Mix was then added immediately in the following order:

Glucose Oxidase	50 ul (5 U/ml)
Lactoproxidase	50 ul (1 mg/ml)
PBS A + 25 mM Glucose	200 ul (final reaction mix: 5mM Glucose)
PBS A + 200 uCi $Na^{125}I$	200 ul

4) The tubes were then left on ice for 15 min. Every 5 min. the tubes were gently revolved to resuspend the cells. The reaction was then stopped by the addition of 5 ml of ice-cold PBI + 2 mM PMSF.
5) The leighton tubes were then respun at 200g for 3 min.
6) The supernatant was then decanted down the sink in the fume hood. The washing step was repeated twice, firstly in PBI + 2 mM PMSF and then in PBS + 2 mM PMSF.
7) The cell pellet was then resuspended in 200 ul of PBS A (+ 1% TX 100) + PMSF, briefly whirlimixed and then left on ice for 10 min.
8) Cell debris and nuclei were then pelleted by spinning at 400g (2000 rpm) in an MSE 'Superspeed', precooled to 4°C.

9) The supernatant was then decanted into labelled black-cap vials and stored at -70 ^{o}C.

2.12.3 Determination of amount of label covalently bound

Covalently bound material was determined by precipitation with TCA (trichloacetic acid). 10ul samples were spotted onto glassfibre discs (Whatman, GF1) and allowed to dry for 5 minutes. The discs were then transferred to ice-cold 5% TCA for 10 minutes and then washed twice in 5% TCA for 5 minutes each. The last TCA wash was decanted, and the discs were washed in absolute alchohol before being dried and counted, in a Pharmacia 'multi-rack' automatic gammacounter. Samples were generally counted for 60 secs.

For acetone precipitation, acetone (90%) was added to protein solution in the ratio 5:1 (v/v) with addition of BSA as carrier (.1mg/ml). After 30 min at -20^{o}C, the pellet was recovered by centrifugation in an Eppendorf 'microfuge' at 12,000 rpm at 4^{o}C. After removal of acetone the pelleted material was dried under nitrogen.

Section 2.13 Plasma membrane Isolation

The method of Willinger and Frankel (1979) with various modifications was followed. Neutrophils were purified by the standard method and then washed in homogenization buffer. They were resuspended in the same medium at a concentration of 7 x 10^{7} cells/ml on ice. Cells were disrupted with a motor-driven Potter-Elvehjem homogenizer with a Teflon pestle. Homogenization was carried out at full speed and consisted of 6-8 up and down strokes within a one minute sequence. This operation was carried out using a precooled vessel, on ice.

Cell breakage was monitored by phase contrast microscopy, homogenization being stopped when approx. 80% cell breakage had occurred.

The homogenate was centrifuged at 2000 rpm (400g) in a MSE benchtop centrifuge in order to pellet nuclei and unbroken cells. The supernatant was carefully removed and routinely stored at -70° C prior to further use. Sucrose discrete step gradients were prepared immediately prior to use and kept at 4 °C. Samples in homogenization buffer were then carefully layered onto the gradient using a syringe. Overlay buffer was then added to fill the tube. Beckmann polyallomer 'Quickseal' tubes were routinely used. For separation of homogenized material on sucrose gradients, a Beckmann SW 28 was used in a Beckmann L4 Ultracentrifuge. Samples were spun at 25K (approx. 100,000g) for 60' with the brake off at 4 °C. Rotors were precooled prior to use. For pelleting material for e.m. analysis a Ti 70 rotor was used, spun at 25K for 30'. Fractions were removed from the bottom of the tube using an MSE stand/puncturing device and collected in plastic tubes (Sarsdedt).

Section 2.14 Immunoprecipitation

2.14.1 Staph. A Method

The method used was that of Dr. A. Cross as demonstrated in an EMBO course entitled, 'Teratomas' held in Glasgow, March, 1980. Washed, radiolabelled cells in PBS A + PMSF + 1% NP-40 were whirlimixed thoroughly and then incubated on ice for 30 minutes. Insoluble material was then removed by centrifugation at 2500 rpm for 30 min. at 4°C. The supernatant was carefully removed to form the antigen preparation. Equal volumes (40ul) of antigen preparation and antibody were mixed in a conical centrifuge tube, capped with foil and incubated for 1hr at 37°C in a water bath. 0.4 ml of prepared Staph. A (see Materials Section 2.6) was added to each antigen-antibody tube and mixed by flicking. Incubation for a further 30 min. at 37°C was then performed. 4 ml of STN buffer was then added to the mixture and spun down at 1500 rpm for 10 min. The

supernatant was poured off and the pellet was resuspended again in 4 ml STN by whirlimixing. The preparation was then spun down again and the pouring off of the supernatant and resuspension in STN was repeated. After a third spin the supernatant was poured off and the tubes were inverted and as much as possible of the residual liquid was allowed to drain off. Samples were then counted or prepared for SDS-PAGE as required.

2.14.2 Second antibody method

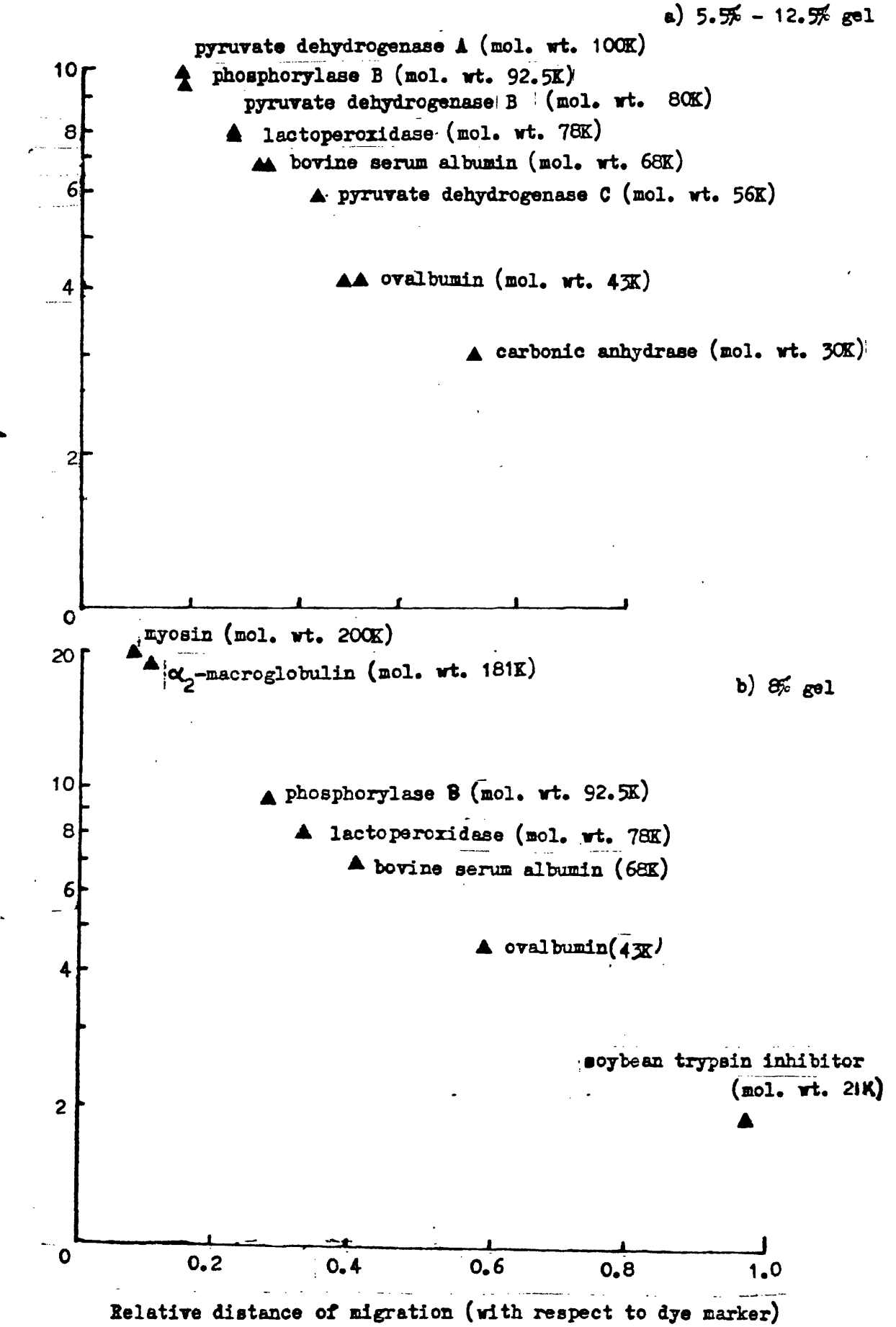
25ul of NP-40 labelled extract (approx. 10^7 cells in 200ul) was incubated for 30 min. at room temperature with 12.5 ul of F10-44-2. 5 ul of normal mouse serum and 100 ul of rabbit anti-mouse IgG was added and the incubation continued overnight at 4 ^{o}C. A control was performed without F10-44-2. The samples were centrifuged at in an Eppendorf 'microfuge' (12,000 rpm) at $4^{o}C$ for 15 min. Supernatant was removed and the precipitates counted before being dissolved in SDS-sample buffer and run on SDS-PAGE.

Section 2.15 Protein Separation and Identification on SDS-PAGE

2.15.1 Protein determination

Protein determination was by the method of Lowry, (1951). Test tubes used for protein were 'repelcoted' before use. Samples (10ul) or BSA standards (20ul) were dispensed into tubes containing 4.8ml of solution C. Tubes were briefly vortex mixed and allowed to stand for 5 minutes. 200ul of Folin's solution was added to each tube and vortexed immediately. The tubes were then left for 30 minutes in the dark. Absorbance was read at 660nm within 4 hours. Each sample was duplicated and read twice.

Fig. 2.2. Calibration curves of molecular weights for polyacrylamide slab gel electrophoresis.



The abcissa was the same on both graphs. Values for molecular weights are in daltons. For sources of markers see Section 2.16.1.

2.15.2 SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis **See section 2.7.2**

SDS-PAGE was carried out as described by Marsden et al. (1976) and Buultjens (personal communication). Routinely samples were treated with sample buffer -DTT, -bromophenol blue, and boiled. Protein determinations and counts were then performed if required before addition of DTT and bromophenol blue, boiling and application to the gel.

Gels were made up as required using glass plates, teflon 'spacers' and tape (3M company). The gel mixture for single concentration gels (8%) were: acrylamide (30%) 12.8mls, running gel buffer 12.0mls, deionised H_2O 23.2mls, ammonium persulphate (10% w/v) 150ul, TEMED 10ul. For gradient gels the relative volumes of acrylamide solution and **deionised** water were adjusted accordingly. For gradient gels mixing chambers connected to a peristaltic pump (Pharmacia) were used. A 5% spacer gel was overlayed on top of the running gel in which a toothed teflon comb was set.Gels were run at constant current, 40mA (approx. 200V at start) until the dye front (bromophenol blue (BioRad) reached the bottom of the gel, (approx. 3hrs). The gel was then stained or fixed, (45 min.) and then destained overnight before drying down under vacuum.

2.15.3 Molecular weight determination by SDS-PAGE

The approximate molecular weights of polypeptides of unknown molecular weight were determined by their relative mobility (M_r) on SDS-PAGE with respect to protein markers. Routinely the following markers were run on gels: $_2$-macroglobulin, (molecular weight, 181K, Sutcliffe et al. 1980); lactoperoxidase, (mol.wt. 78K approx., Rombouts et al. 1967, and see fig. 2.2); bovine serum albumin, (mol. wt. 68K, Weber and Osborn, 1969); and ovalbumin, (mol. wt. 43K, Weber and Osborn, 1969) and β-lactoglobulin, 18.4K, Weber and Osborn, 1969, 5.5%-12.5% gels only).

In order to check the relationship between relative mobility and moolecular weight and to check the molecular weight of the markers

routinely used a further series of markers were run on single concentration gels (8%) and gradient gels (5.5%-12.5%). The results confirm that the relationship between log. molecular weight (ordinate) versus migration (abcissa) is sigmoidal in nature, tending to be linear in the molecular weight range 15,000 to 100,000, (Takacs, 1979).

The sources of the markers used for fig. 2.2 are as follows: myosin, phosphorylase b, carbonic anhydrase, (Amersham) a gift from Dr. E Buultjens and pyruvate dehydrogenase of E. coli (chains A, B and C), (Sutcliffe et al., 1980) and Soybean Trypsin Inhibitor (Sigma) were a gift from Dr. R. Sutcliffe.

Section 2.16 Chromatographic Techniques

2.16.1 Gel Filtration

Gel Filtration (desalting) was routinely performed on radiolabelled samples in the following experiments:

1) Assessment of amount of iodine covalently bound to protein.
2) Removal of unbound ^{125}I prior to affinity chromatography experiments.
3) Removal of unbound ^{125}I after radiolabelling of lectins.

Columns were prepared in batches and stored at $4^{o}C$, see Section 2.8. Before use columns were placed at room tempperature for 30 minutes and presaturated with 5 ml, 50mM phosphate buffer, pH 7.5 + 10mg/ml BSA. The column was then washed through with 25ml 50mM phosphate buffer + .1% BSA.

2.16.2 Lectin Affinity Chromatography

Preswollen gel was loaded onto a P10 BioRad 'econocolumn in a cold room (4°C), to give an approximate bed volume of 2 ml. The gel was thoroughly washed with 25ml of starting buffer before the sample was applied. The sample in 200ul was applied and allowed to penetrate the gel and then was incubated for 10 minutes. 25ml of starting buffer was then added and fractions, (.5ml approx.), collected in plastic tubes (Sarsdedt) and immediately placed on ice.. Counts were monitored and when the rate returned to background, elution buffer was added to the gel and incubated for 2 hrs. Fractions were again collected and counted. Fractions were counted for 20 secs in a Pharmacia, multirack counter.

See Section 2.8.2 for details of buffers etc.

Section 2.17 ^{125}I-Lectin Overlay on SDS-PAGE

2.17.1 Chloramine-T iodination of lectin

The reaction was carried out in a plastic test tube (Sarsdedt) inside a small lead pot within a fume hood.

The following reagents were added in order: 10 ul 0.25M phosphate buffer, 5 ul ^{125}I (500uCi), 10 ul lectin (2mg/ml), 10 ul chloramine-T. The reaction was allowed to proceed for 30 secs and then stopped by the addition of: 10 ul sodium metabisulphite and 100 ul KI. The tube was counted using a desk top gamma counter with a plastic extension tube.

The sample was then desalted by adding the solution with a glass pipette to a prepared column. The solution was absorbed under gravity. Washing solution was then added and the flow rate of the column controlled applying pressure to the top of the column with a rubber teat. In this way samples could be efficiently desalted very quickly. Routinely, 5 drop fractions were collected in Sarsdedt tubes and counted. High activity fractions (4 or 5 fractions) were pooled and diluted 1:1 (v/v) with 0.1m Tris-Hcl, + 1 mg/ml BSA, pH 7.0 (for Con A, 1 mM $CaCl_2$ and 1 mM $MgCl_2$ were added). Routinely a specific activity of between 5-15 uCi/ug of protein were obtained. This solution was then stored at -70°C until further use.

2.17.2 Overlay on SDS-PAGE of ^{125}I-lectin conjugates

Acrylamide solutions were deionized by pretreatment with Amberlite MB 1 resin. After electrophoresis gels were fixed for 45 mins, and then destained for 3 hrs. They were then placed in Equilibration Buffer for 24 hrs, on a 'shaker', set at a low speed. The buffer was changed several times. The gel was then transferred to a plastic sandwich box encased in lead and 50 ml of Incubation Buffer was added, containing the appropriate lectin. The gel was then left for 24 hrs on a shaker. Finally the gel was washed copiously with Washing Buffer, for a further 24hrs.

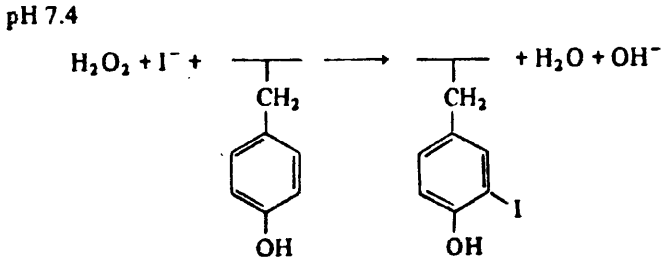
Gels were then stained with Coomassie Blue solution for 45 mins., destained for 24 hrs, photographed, dried down and exposed to Kodak XH-1 film with an intensifying screen, (DuPont-'Lightning Plus') at -70^{o}C.

Chapter 3 Labelling of human neutrophils using ^{125}I/lactoperoxidase

Section 3.1. Introduction

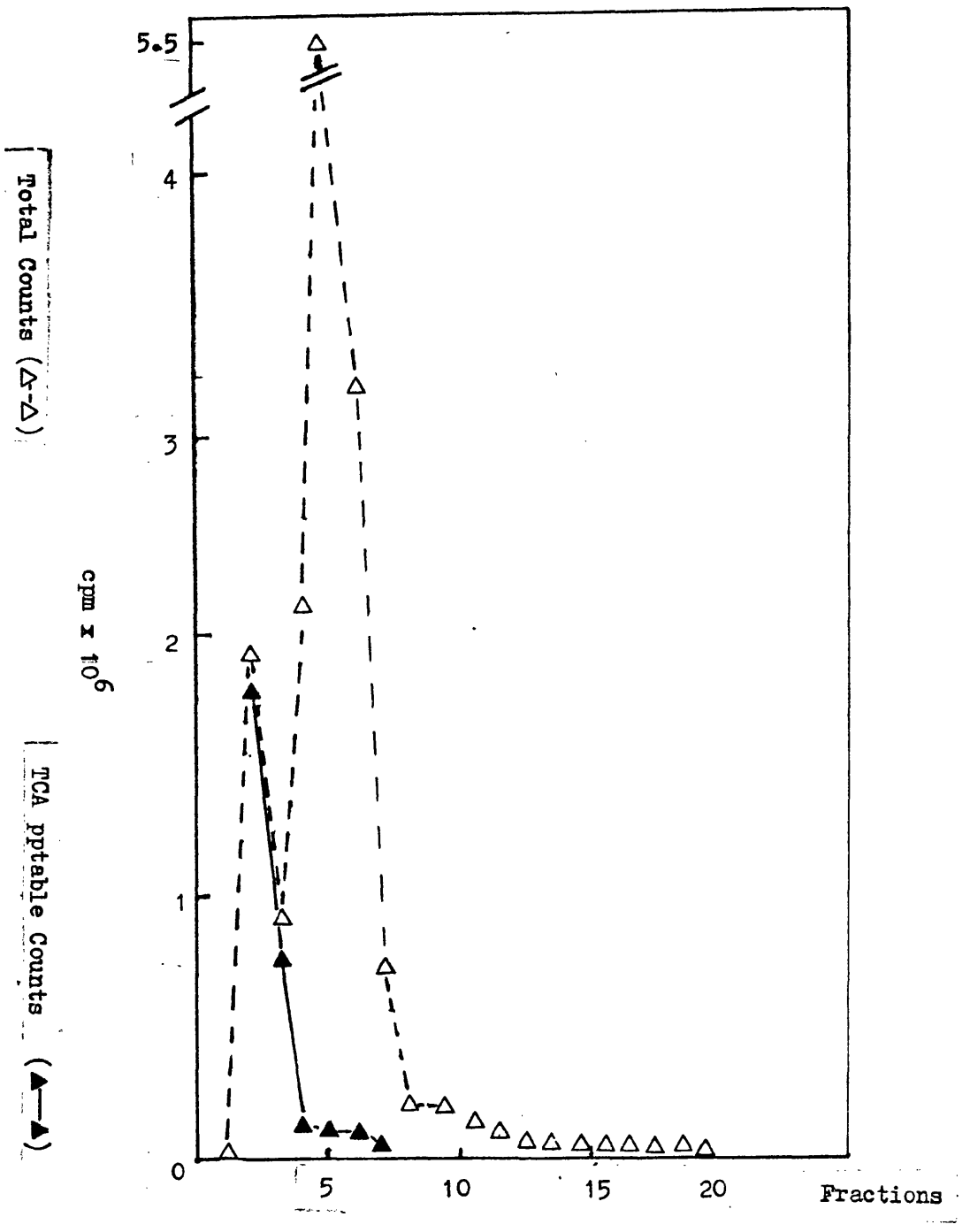
Labelling methods involving the use of macromolecular enzymatic probes were developed on the assumption that the large size of the labelling enzyme would prevent its penetration of the membrane and entry into the cell, (see Hynes, 1975 and Hubbard and Cohn, 1976 for review). There is evidence that lactoperoxidase forms an activated complex with iodide and that this is the entity which reacts with tyrosine, and to a lesser extent with histidine, residues. The reaction, in which the peroxidase catalyses the formation of a carbon-halogen bond in the presence of hydrogen peroxide, a halide and a nucleophilic receptor is outlined in Fig. 3.1 after Morrison et al. (1970).

Fig. 3.1. ^{125}I-lactoperoxidase labelling reaction



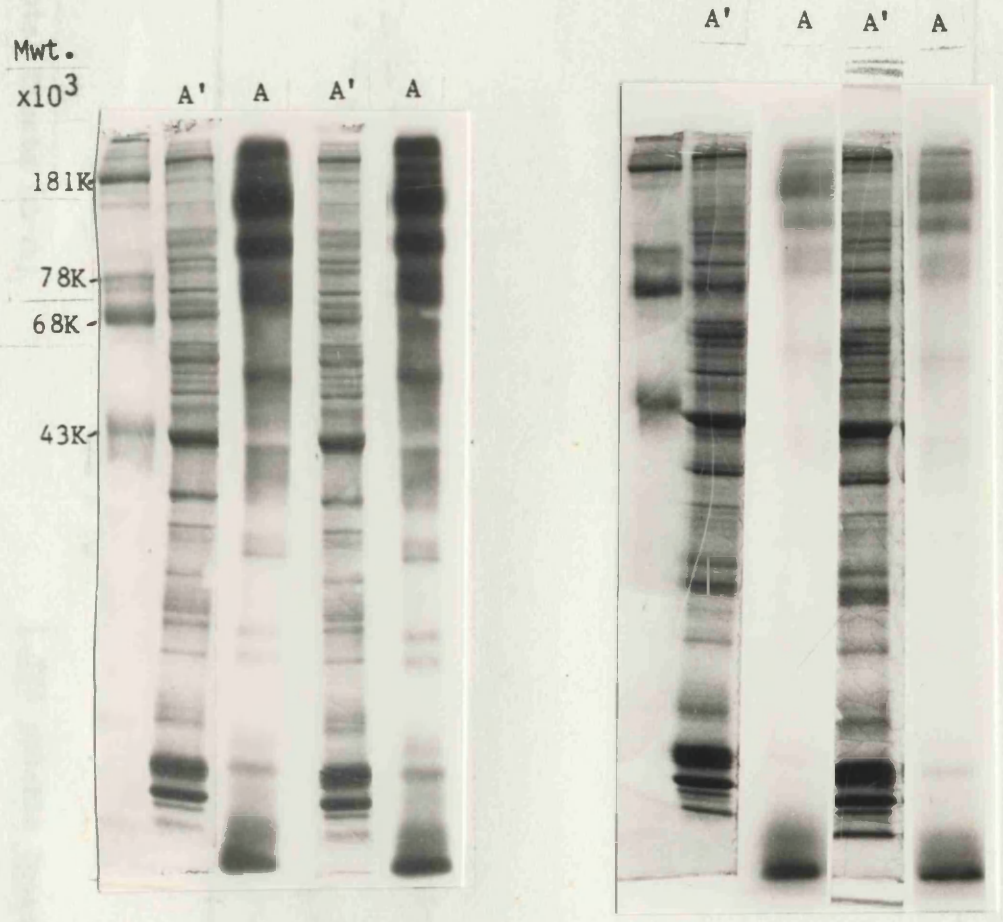
The label incoporated then is small and as a result protein

Fig. 3.2. Gel filtration of detergent solubilised, radiolabelled cell surface protein.



Gel filtration was performed with G-25 Sephadex (Coarse).

Fig. 3.3. Triton X-100 extraction of labelled polypeptides (gradient gels)

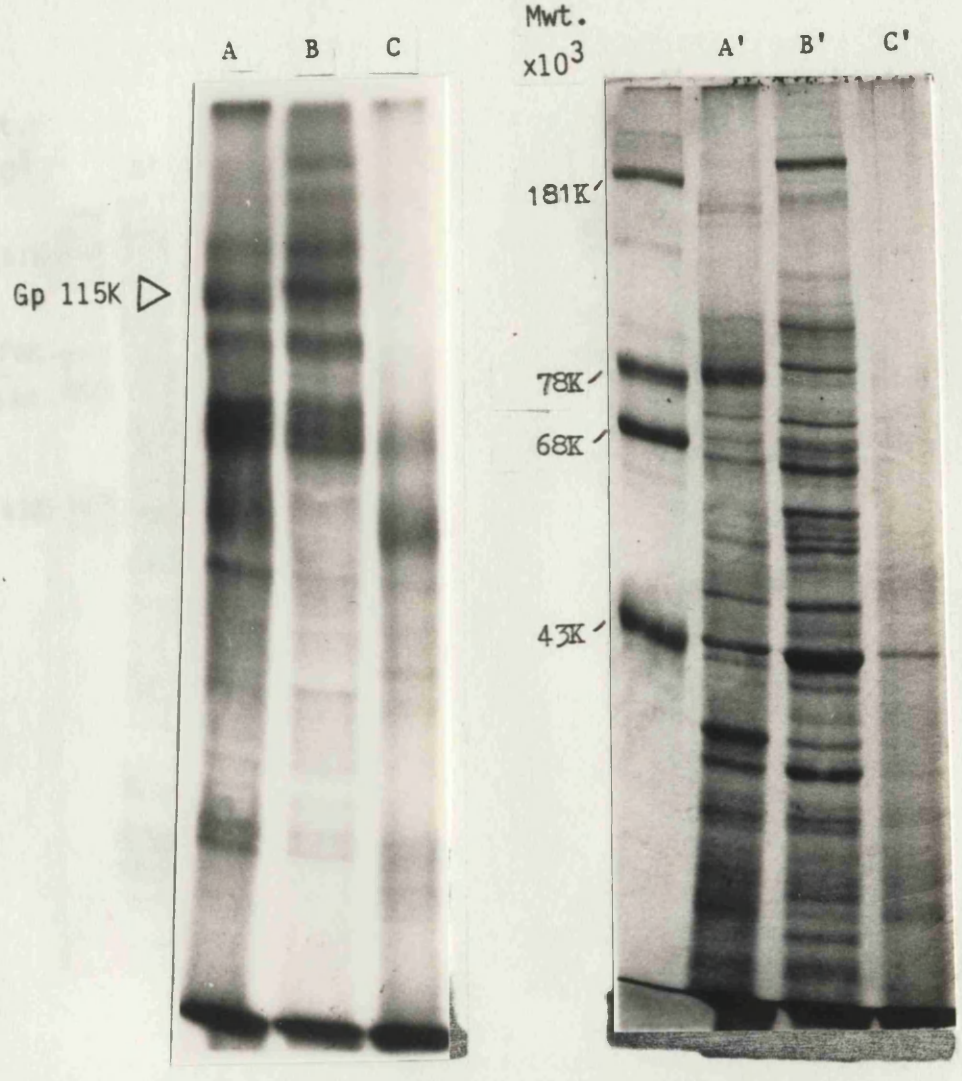


A - Labelled polypeptides A' - Whole cell proteins (TX-100 extract) coomassie blue stained

The autoradiographs were exposed for 10 and 15 days respectively.

Fig. 3.4. Triton X-100 extraction of labelled polypeptides

(Comparison of whole cell, detergent extract and residue)



A-C autoradiograph; A'-C' coomassie blue stained protein.

A-A' solubilised Whole cell; B-B' TX-100 extract; C-C' residual pellet after TX-100 extraction.

Equal counts were loaded in slots A and B. Residual pellet material (C-C') was too viscous to accurately quantify counts.

The autoradiograph was exposed for 11 days

Table 3.1

Fate of ^{125}I used to Covalently Label Proteins

Input Counts cpm x 10^6	Absorbed Counts cpm x 10^6	% Input	Bound Counts TCA ppt. cpm x 10^6	% Input	% Bound/ Abs.
300-370 (n=9)	2.9-8.9 (n=2)	2.5-8	.31-.88 (n=9)	.09-.25	9-17

Table 3.2

Lipid Extraction of Acid Precipitable Counts

	Counts Remaining Bound (x)	%
Control	22404 (n=29)	
+CH_3Cl/Methanol	18933 (n=2)	85
+Acetone	16041 (n=2)	72
+CH_3Cl/Methanol +Acetone	11978 (n=2)	53

conformation should not be drastically disturbed. The major disadvantage from enzyme mediated labelling derives from the large size of the catalyst. Only those reactive amino acid residues accessible to a 30-40nm. diameter enzyme will form an enzyme-substrate complex and hence become labelled; steric hindrance then may lead to the failure to label some proteins, Hynes (1975).

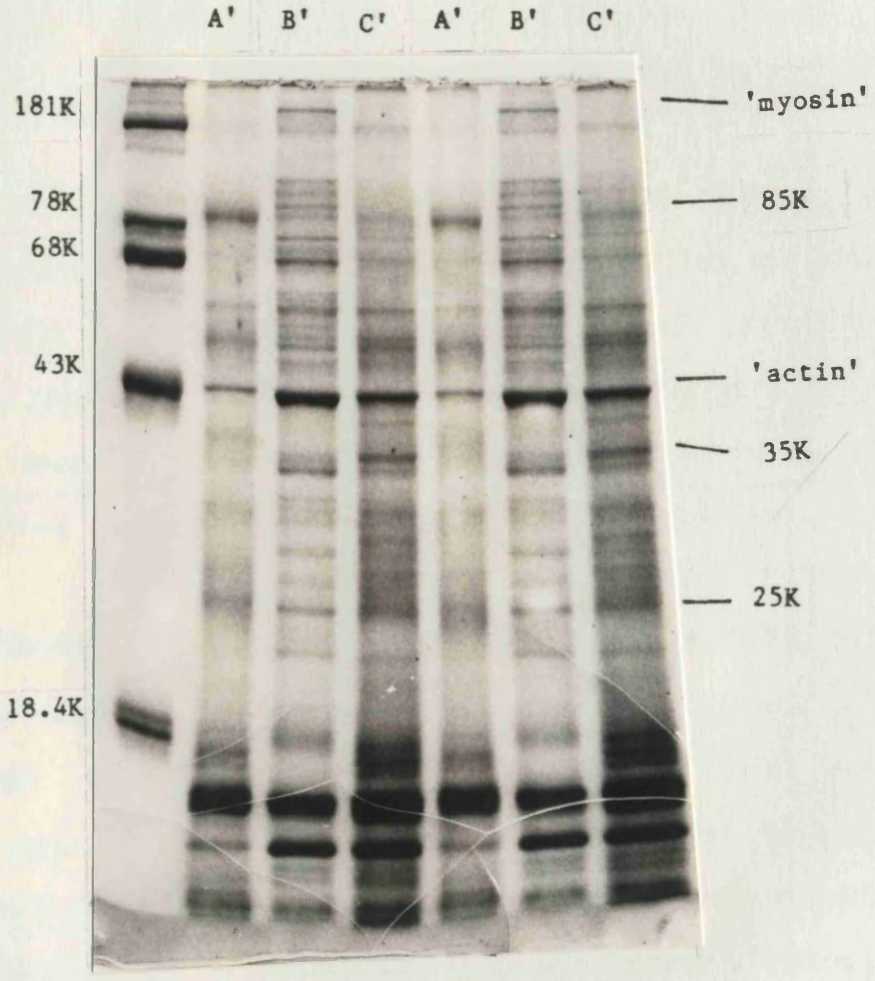
Results and Discussion

Section 3.2 Covalent labelling of polypeptides

The experiments demonstrated that approximately 10% of ^{125}I incorporated into the cells after extensive washing was covalently bound to proteins and lipids, (Table 3.1). This was determined by gel filtration (Fig. 3.1) and TCA precipitation with or without lipid extraction, (Table 3.2). The low proportion of ^{125}I that is covalently bound after extensive washing reflects the ability of neutrophils to accumulate iodine, (Klebanoff, 1975).

Radiolabelled cells were detergent extracted and run on SDS-PAGE. The results clearly suggest that a subfraction of high molecular weight polypeptides are being labelled, (fig. 3.3 and fig. 3.4.). Apparent molecular weights of labelled polypeptides were determined, (see Table 3.4.). High molecular weight polypeptides which are stained with Coomassie Blue, and are probably present in large quantities in the cell are not labelled. Plasma membrane proteins probably make up between 1-3% of total cell protein, (Bretscher and Raff, 1975) and since they may all be glycoproteins, (Lotan and Nicolson, 1979), which stain poorly if at all with Coomassie Blue, Fairbanks et al. (1971), there is a lack of overlap between the profiles.

Fig. 3.5. Comparison of cell protein: whole cell, TX-100 solubilised material, residual pellet.



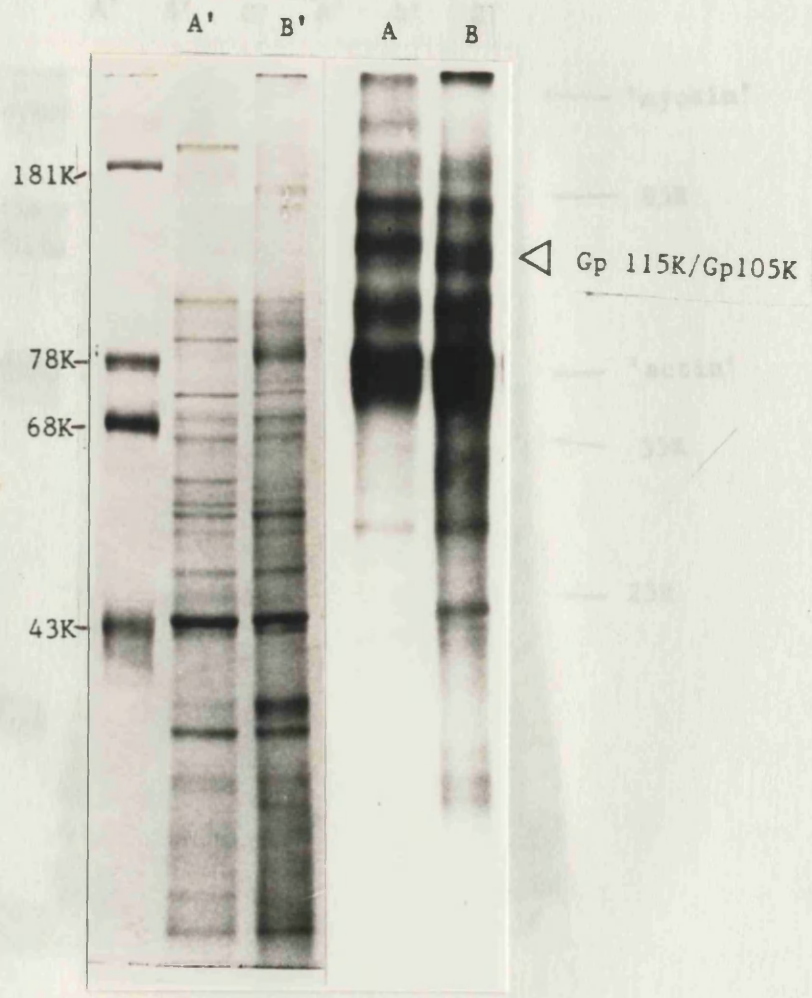
Samples were run on a 7.5%-12.5% gel. Equal protein was applied per slot.

A' - Residual pellet after detergent extraction

B' - Triton X-100 extract

C' - Whole cell

Fig. 3.6. Nonidet P-40 detergent extraction of labelled polypeptides (single concentration gel)



A-A' NP-40 detergent extract; B-B' Whole cell

A,B autoradiograph; A'B' coomassie blue stained protein

Equal volumes loaded per slot. Autoradiograph exposed for 9 days.

Table 3.3

Experiments to determine the proportion of ^{125}I-lactoperoxidase labelled counts soluble in 1% Triton X-100 in PBS A

	Cell Number $x10^7$	cpm TCA ppt. $x10^3$	% Whole Cell	Protein mg/ml	% Whole Cell
Expt.1					
Whole Cell	2	800		2.8	
TX-100 Extract	2	700	80	2.2	79
Expt.2					
Whole Cell	1	480		1.2	
TX-100 Extract	1	350	88	0.8	66

TABLE 3.4

Identification of ^{125}I-lactoperoxidase labelled polypeptides

Polypeptides	Apparent mol.wt. x10^3 Daltons	Presence or absence in: Whole Cell	Triton X-100 Soluble Extract	Residual) Insoluble Material
1	>200	-	+	
2	>200	-	+	
3	<200	-	+/-	
4	130	+++	+++	
5	115	+++	+++	
6	95	+++	+++	
7	78	+++	++	
8	70-75	+++	++	++
9	70-75	+++	++	++
10	55-60	+++	+	++
11	50	+++	-	+
12	45	+		
13	42	+		

In Table 3.6. the results tabulated in Table 3.4 are compared with comparable results from other workers. The radiolabelled bands were ranked according to molecular weight. As may be seen on rabbit neutrophils a comparable number of bands were labelled with some overlap between the results. The more selective periodate-Na^3HB_4 labelling technique (see Section 1.7) again produced a more limited but comparable profile.

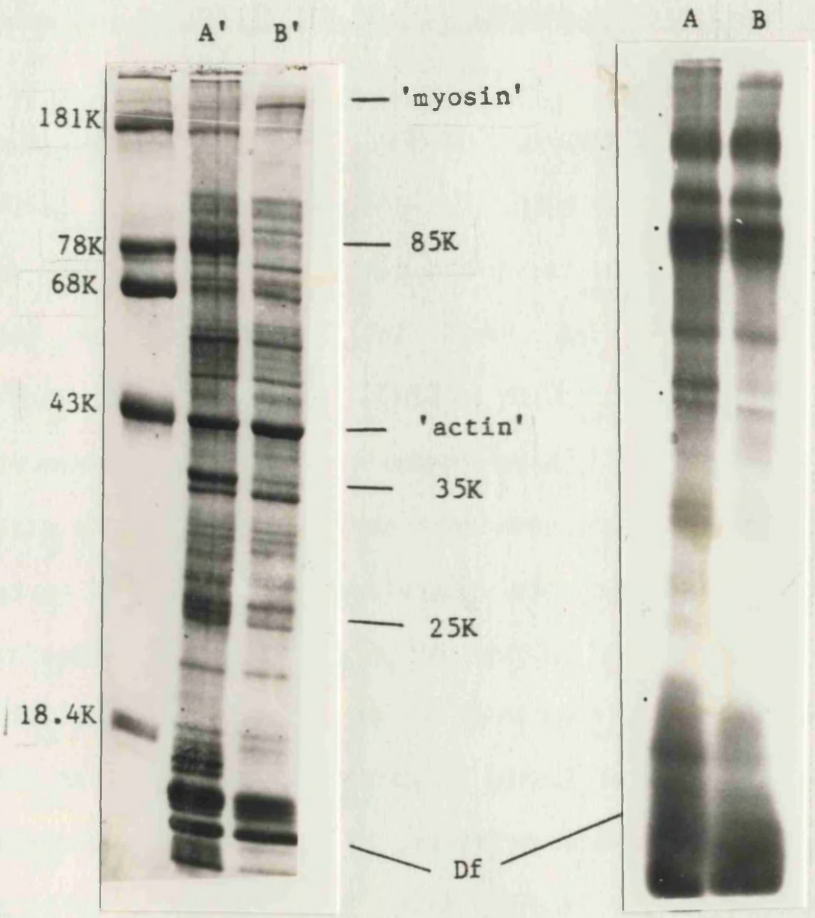
Section 3.3 Evidence for the selective extraction of polypeptides in non-ionic detergent, TX-100 and NP-40.

Following Andersson and Gahmberg (1978), samples were routinely extracted with a non-ionic detergent, Triton-X 100 prior to further analysis, (see Section 3.2). The tacit assumption being that all cell surface radiolabelled polypeptides would be extracted under these conditions. However Flanagan and Koch (1978), Sheterline and Hopkins (1981) and Mescher et al. (1981) have demonstrated that transmembrane linkages between microfilaments and some cell surface proteins are stable in the presence of non-ionic detergents. Therefore it was decided to test the possibility that selective extraction of polypeptides, as visualized by coomassie blue stain or autoradiography in the case of labelled cells, was occurring. Experiments were performed to compare: coomassie blue stain profiles from whole cells and TX-100 extracts, (fig. 3.5.); radiolabelled polypetides from whole cells and TX-100, (fig. 3.3) and whole cells and NP-40, (Nonidet P-40), (figs. 3.6 and 3.7).

The comparison of protein profiles from whole cells with those from detergent extracted cells (TX-100), fig. 3.5., show striking differences which are consistently observed, (see figs. 3.4, 3.6 and 3.7) and are tabulated in Table 3.5.

Two major cell proteins were tentatively identifiable on the basis of their relative mobility on SDS-PAGE. These are: myosin (apparent mol. wt. 200K), and actin (apparent molecular weight 42K), (see Willinger and

Fig. 3.7. Nonidet P-40 extraction of labelled polypeptides (gradient gel)



A-A', Whole cell; B-B', Nonidet P-40 extract.

A,B - autoradiograph; A',B' - coomassie blue stain

Samples were run on a 5.5%-12.5% gel. The autoradiograph was exposed for 11 days. Df-Dye Front.

Table 3.5

Identification of Putative Cytoskeletal Elements

Polypeptides	Apparent mol.wt. $x10^3$daltons	Presence or Absence in: Whole cell	Triton x-100 Soluble Material	Residual Insoluble Material
Myosin*[1]	200	-	++	-
85K*[2]	85	++	-	+
Actin*[1]	44	++	+++	+
35K	35	++	+	-
25K	25	++	-	+

-/+/++/+++ denotes qualitative assessment of relative abundance

*-Denotes evidence from other workers of presence in neutrophils

[1]-Amrein and Stossel(1980),Baehner and Boxer(1979)

[2]-Sheterline(1980)

Table 3.6.

Comparison of Labelling Patterns of Cell Surface Proteins of Human Neutrophils

Band No.	^{125}I-LPO labelling (Section 3.)	^{125}I-LPO labelling of rabbit neutrophils (Willinger and Frankel 1979)	Sodium periodate-Na^3H_4 labelling (Andersson and Gahmberg, 1979)
1	>200	>200	245
2	>200		230
3	<200	150-180	165
4	130		155
5	115	120	105**
6	95	90	97
7	78*	-	-
8	70-75	66-68	85
9	70-75	(2 bands)	80
10	55-60	60-62	62
11	50	(2 bands)	50
12	45	45	42
13	42	35	

*-This polypeptide may contain autolabelled lactoperoxidase, (See Section 4.)

**-This polypeptide after neuraminidase pretreatment appears to have a molecular weight of 130K approx., (See Section 1.7.)

Frankel, 1979; and Amrein and Stossel, 1980). The identity of myosin was tentatively confirmed by demonstration that this band had the same M_r (relative mobility) of 0.08 (with respect to dye front), on SDS-PAGE under these conditions as a commercially obtained ^{14}C-myosin (see Section 2.2). Another major polypeptide of molecular weight 85K appeared may be homologous to an 83K cytoskeletal polypeptide noted by Sheterline and Hopkins (1981) in pig neutrophils. Two other minor bands of 35K and 25K also appeared to show anomalous behaviour with respect to detergent extraction.

As noted in Table 3.5, 85K appears to be resistant to detergent extraction and is a major component of the residual insoluble pellet. In contrast myosin is strongly stained in detergent extracts but is absent in whole cell or residual pellet tracks, (see fig. 3.5 and 3.4). The apparent absence of 'myosin' from the whole cell track suggests that proteolysis may be taking place. The relative degradation of high molecular weight labelled polypeptides, (see figs. 3.4 and 3.6 lends support to this possibility.

Sheterline and Hopkins (1981) have demonstrated that whole cell lysates from pig neutrophils contain considerable proteolytic activity at neutral pH. Amrein and Stossel (1980) noted that the use of the protease inhibitor PMSF, (a irreversible but relatively slow-acting serine protease inhibitor, (Sheterline and Hopkins, 1981) retarded the loss of the actin band in human neutrophils after solubilisation in SDS, but was only minimally effective in preventing the degradation of Actin Binding Protein (mol. wt. >200K) or myosin. Amrein and Stossel (1980) also noted that SDS released granule-associated proteolytic enzymes from human neutrophils, during solubilisation, faster than it inactivated them. The apparent increased degradation of protein which occurs in whole cell extracts may then be due to tthe release of proteases from lysosomes in SDS. The relative absence of degradation, of these bands in TX-100 extracts and the

apparent degradation of proteins in the low-speed pellet (Fig 3.5 and 3.4) suggests that intact lysosomal granules may be removed intact in the low-speed pellet following detergent extraction.

Work by Mescher et al.(1981) suggests that much of the actin in non-muscle cells is maintained in a soluble pool. 'Actin' appears to be relatively soluble in detergent extracts. Actin may then occurr in two forms the polymerised form only being associated with the cytoskeleton, Mescher et al.(1981). It is possible that In Fig.3.5. two forms of 'actin' may then be present.

The results of experiments to determine the amount of ^{125}I-labelled material solubilised in TX-100 are shown in Table 3.3. 80-90% of ^{125}I labelled material and 65-80% of total cell protein is solubilised. These figures compare with comparable figures of 95% and 70-80% respectively obtained by Mescher et al. (1981) using lymphoid cells.

Qualitative comparisons of radiolabelled polypeptide profiles are shown in fig. 3.4, (TX-100) and figs. 3.6 and 3.7, (NP-40). The results are tabulated in Table 3.4.

A comparison of the results suggests that degradation of high molecular weight bands is apparently occuring in the whole cell extracts with respect to the detergent extracts. High molecular weight polypeptides are particularly suitable for the detection of proteolysis using SDS-PAGE since their disappearance is easily noted and degradation of higher molecular weight bands does not complicate the interpretation by producing overlapping bands. Interpretation of low molecular weight bands must be done with caution for this reason.

In figs. 3.4 and 3.6 a slight shift in mobility of a polypeptide may be seen (band 5, Table 3.4, apparent mol. wt. 115K). This polypeptide, of apparent mol. wt. 115K, appears to be extremely susceptible to partial degradation, producing a cleavage product, of apparent mol. wt. 105K, (see Section 4.2).

The results also demonstrate that some labelled polypeptides are apparently relatively insoluble in TX-100 and NP-40. Three bands, two of them very broad ones are consistently seen in the low speed pellet after detergent solubilisation. The bands, or groups of bands, labelled 10-13 fall into this category. It is of interest to note that Schneider et al. have recently identified the C_3b receptor on human macrophages as being a glycoprotein that migrates as a broad band on SDS-PAGE of 58-68K, (cf. Band 10, 55-60K). Human neutrophils express this receptor, (Playfair, 1981).

There are a number of possible explanations for the finding of apparently relatively insoluble labelled polypeptides:

1) Under mild lysis conditions many labelled cell surface proteins may be retained in the extracted cells, Ben-Ze'ev et al. (1979).

2)Specific interactions between intrinsic membrane proteins and cytoskeletal elements may occur, Koch and Smith (1978), Flanagan and Koch (1978), Sheterline and Hopkins (1981) and Mescher et al. (1981). This would result in selective retention of some labelled cell-surface polypeptides in the insoluble pellet.

3)Non-specific binding of labelled polypeptides to nuclei or chromatin, or selective trapping of some labelled polypeptides may occur, Willinger and Frankel (1979).

4)The labelling of cytoskeletal elements or nuclei would result in the presence of labelled polypeptides in the insoluble pellet, Gahmberg et al (1979).

Willinger and Frankel (1979), using rabbit neutrophils, describe experiments which demonstrate that during plasma membrane preparation three labelled polypeptides of mol. wt. (approx) 120K, 66-68K and 60-62K are concentrated in a low speed pellet designed to remove whole cells and nuclei after homgenization. It is possible that the latter two bands are homologous to Bands 10 and 11, (approx. mol. wts. 55-60K and 50K,

respectively). The major putative cytoskeletal elements are known to contain lactoperoxidase accessible tyrosine residues, Willinger and Frankel(1979). The fact that they were not labelled argues against the fourth possibility that the detergent-insoluble labelled polypeptides are themselves cytoskeletal, or nuclear membrane proteins. Thus some preliminary evidence has been provided for maintenance of attachment of cytoskeletal elements and possible interactions between such elements and some radiolabelled proteins.

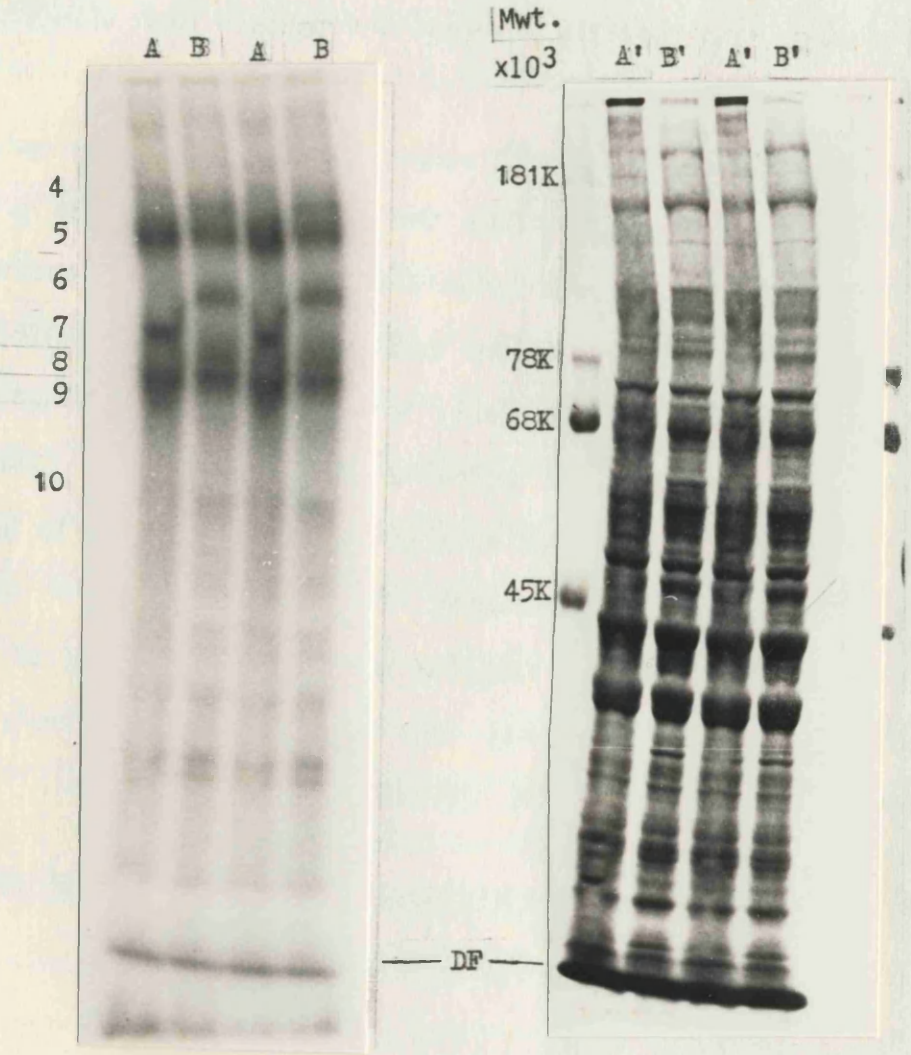
Despite the fact that at least with respect to lower molecular weight labelled polypeptides (see Table 3.4) the detergent extracted profile resulted in loss of label this was outweighed by the ease of handling of the samples and the apparent reduction, under my experimental conditions, of proteolytic degradation, particularly with respect to high molecular weight radiolabelled polypeptides including band 5, Gp 115K, (see Fig. 3.4 and 3.6). Whole cell extracts were viscous and difficult to handle, even in the presence of SDS detergent and after boiling for several minutes at 100°C, resulting in sampling error . The ability of whole cell extracts to form a gel may be due to the relatively high concentration of DNA in these cells, (Amrein and Stossel, 1980).

Section 3.4 Evidence for the presence of intra-chain disulphide bonds in some labelled polypeptides.

Introduction

The nature of covalent linkages within and between polypeptide chains may be important in relating structure to function in membrane proteins. Examples of membrane proteins which contain interchain covalent linkages are few. The best studied examples are membrane bound immunoglobulins e.g.: IgG, (Roitt, 1979) and two T cell surface antigens recognised by the monoclonal antibodies, Anti-T5 and Anti-T9, (Reinherz and Schlossman,

Fig. 3.8. Effect of reducing agent (DTT) on mobility on SDS-PAGE of radiolabelled proteins, (TX-100 extracts)



A - -DTT (autoradiograph) B - +DTT (autoradiograph)
A'- -DTT (coomassie blue) B'- +DTT (coomassie blue)
Equal counts were loaded per slot.
The autoradiograph was exposed for 10 days.

Numbered bands on left of autoradiograph are identified in Table 3.4

1981).

Intrachain covalent linkages (consisting of disulphide bonds between cysteine residues) are much more common. They are present in probably all transmembrane proteins which contain extracellular domains, with not more than two disulphide bonds per domain e.g.: Thy 1, (Cohen et al., 1981), Ig, (Anzel and Poljak, 1979) and H-2, (Coligan et al., 1981). Thus transmembrane proteins may require intrachain disulphide bonds to maintain or to stabilise tertiary structure. Molecules which contain extracellular domains appear to contain exclusively N-linked carbohydrate residues, (see fig. 1.7.). Gibson et al. (1980) have suggested that these residues may also be involved in determining and maitaining the correct tertiary structure of the extracellular portion of the molecule. In contrast transmembrane glycoproteins which contain largely or entirely O-linked carbohydrate residues e.g.: Glycophorin A, Marchesi and Furthmayr (1976) and Rat sialoglycoprotein, Brown et al. (1981) appear to have little or no secondary or tertiary structure and contain no intrachain disulphide bonds.

Results and Discussion

The results of this experiment are shown in Fig.3.8. Polypeptide bands identified as 4, 6 and 7 appear to undergo moderate alteration of mobility which may be consistent with the presence of interchain disulphide bonds. The apparent biased curvature of these 3 bands under non-reducing conditions may be due to diffusion of excess DTT from '+' tracks to '-' tracks, during the running of the gel. Clearly the results must be interpreted with caution. By running samples on the same gel under reducing or non-reducing conditions and comparing polypeptide mobility it may be possible to determine the presence of disulphide bonds. Clearly cleavage of interchain disulphide bonds will bring about a large alteration in mobility. Cleavage of intrachain disulphide bonds may bring

about a small increase or decrease or no apparent change in mobility of a given polypeptide on SDS-PAGE. However the interpretation of overlapping bands is a problem and does not allow firm conclusions to be drawn even within the limitations of the experimental technique.

Nevertheless the available data suggests that there is no evidence for the presence of interchain disulphide bonds between labelled polypeptides, and that only 3 bands, identified as 4, 6 and 7, of apparent molecular weights, 130K, 95K and 78K, (see Table 3.4.), appear to show a marked alteration in mobility in this experiment.

Chapter 4 Evidence for the cell surface localization of ^{125}I-labelled polypeptides

Section 4.1 Introduction

Claims for the specific labelling of cell surface proteins require the use of methods of assessing the cell surface localization of the label. These approaches may conveniently be divided into two types. There are those that concern the labelling method itself such as: does omission of one component of the labelling system eliminate labelling. The other type are those methods which demonstrate that a particular subfraction of total cell protein is labelled. These methods, (see Hubbard and Cohn, 1976) are listed below:

1) Preparation of a high speed pellet and demonstration of pelleting of labelled material.
2) Whole Cell Autoradiography.
3) Comparison with other labelling methods known to selectively label the cell surface.
4) Isolation of plasma membrane and demonstration of segregation of label with it.
5) Use of proteolytic enzymes in concert with an external labelling agent.

The methods used in this study to determine the specificity of the labelling reaction for cell surface components are:

A)The dependence of the labelling reaction on components of the labelling system.

B)The sensitivity of labelled polypeptides on intact cells to the protease, trypsin.

C)The preparation of a membrane enriched fraction and the segregation of label with it.

The first two methods have the advantage of reltive simplicity and have been used extensively by other workers, (see review of Hubbard and Cohn, 1976). Despite the apparent difficulties it was also decided to try and obtain a plasma membrane preparation, to allow further studies on cell surface membrane proteins.

Section 4.2 The dependence of labelling on components of the labelling system

Introduction

Lactoperoxidase-catalysed iodination is an important tool for the study of membrane components of end cells such as neutrophils which are not capable of being labelled **metabollically** using appropriate radiolabelled precursors. However, in theory, neutrophils are not ideal cells for labelling with this method for a number of reasons, (Willinger and Frankel, 1979):

a)the presence of lysosomal myeloperoxidase, a peroxide dependent halogenating enzyme.

b)the presence of peroxide generating, cyanide insensitive, oxidases at the cell surface and in lysosomes.

c)the ability of neutrophils to actively accumulate iodine, (Klebanoff, 1975).

d)the presence of ectoproteases and lysosomal proteases active at neutral pH.

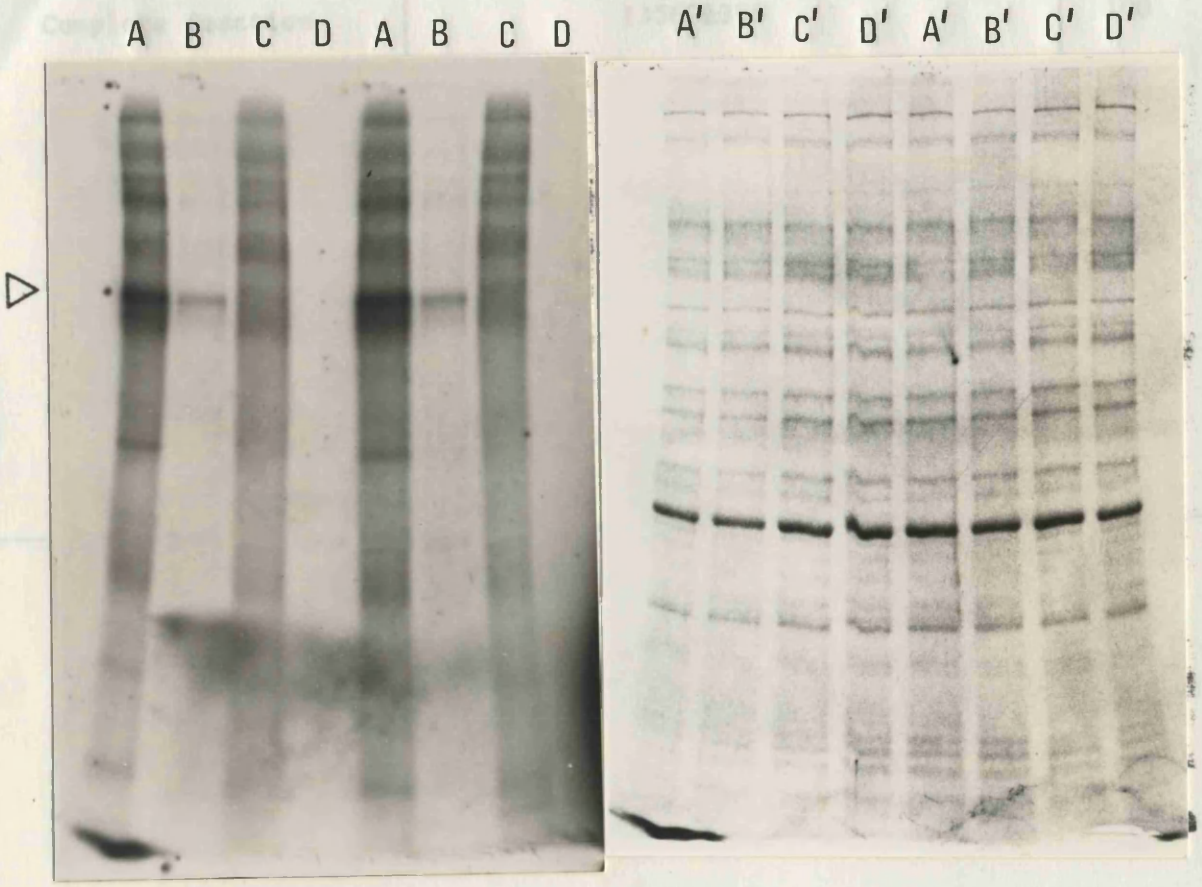
e)the capacity of the cell for bulk membrane internalization by phagocytosis.

Table 4.1

Dependence of ^{125}I labelling on enzyme components of the labelling system

	TCA precipitate of TX-100 solubilised protein cpm/mg	%
Complete Reaction Mix	13560±396 (n=2)	100
Minus Lactoperoxidase	4000±256 (n=2)	29
Minus Gluccose Oxidase	2390±502 (n=2)	18
Minus both enzymes	1140 (n=1)	8

Fig. 4.1. Demonstration of the dependence of the labelling reaction on the presence of exogenous enzymes.



The samples on the autoradiograph are labelled A-D while the corresponding slots on the gel are labelled A'-D'. (A,A') Control; (B,B') glucose oxidase omitted; (C,C') lactoperoxidase omitted; (D,D') glucose oxidase and lactoperoxidase omitted.

Equal protein was applied per slot and the gel was exposed for 8 days.

N.B. Molecular weight markers weere not run with this gel. M_r Values were taken from gels run under similar coditions.

Most of these functions are activated under conditions which stimulate phagocytosis. Labelling at 4°C inhibits this process but intracellular accumulation of iodine may still occur, (Willinger and Frankel, 1979).

Other workers have reported successful cell-surface labelling of neutrophils in other animals using this method. These are: in rabbits, (Hawkins and Sauve, 1978; Willinger and Frankel, 1979; and Thrall et al., 1980); and in pigs, (Sheterline and Hopkins, 1981). A variety of assessment criteria were used for cell surface labelling. To my knowledge the only attempt to selectively label membrane proteins in this cell in humans was reported as unsuccessful, (Segal and Peters, 1976).

Results and Discussion

The results of these experiments are shown quantitatively in Table 4.1. The results suggest that labelling in the absence of lactoperoxidase was quite large. The possibility of intracellular labelling by endogenous peroxidases was apparent. A direct comparison of labelled polypeptides in Fig 4.1 reveals, as in Section 3.2, that the labelled polypeptide profile is substantially different from the total detergent soluble protein profile. Major cell proteins are not labelled.

In Track B a single labelled band is seen, (arrowed), of apparent mol.wt. 80K. Evidence suggests that this band may contain lactoperoxidase (LPO), which may become autolabelled under these conditions, (Hynes, 1975 and Hubbard and Cohn, 1976), since:

a) lactoperoxidase comigrates with this band on SDS-PAGE under reducing conditions, (See Figs. 3.3-3.4). The apparent molecular weight of lactoperoxidase is is 78K, (Rombauts et al., 1967) and see Section 2.16 and fig. 2.2. for confirmation.

b) the band is considerably reduced in Track C (fig. 4.1), where some labelling occurs in the absence of exogenous lactoperoxidase.

c)the polypeptide band binds to immobilised Con A (See Fig 5.5) and may therefore be glycosylated. The marker lactoperoxidase binds Con A by the radiolabelled-lectin overlay method, (see fig. 5.1). This confirms the finding of Rombauts et al. (1967) that lactoperoxidase is glycosylated.

The other notable feature of the autoradiograph is the absence of a band denoted * in Track C, fig. 4.1. This band is particularly trypsin sensitive, (See Fig.4.2, *), and is probably present on the cell surface.

It would appear then that the reaction may be driven by endogenous peroxidases in the absence of LPO. These may be available at the cell surface from the lysis of damaged cells. A major labelled band (80K) may contain autolabelled lactoperoxidase.

Given the inability of LPO to enter cells, The results suggest that even in the absence of exogenous peroxidase, labelling is confined to a unique subfraction of polypeptides, poorly represented in the coomassie blue profile, are labelled which are localized on the cell surface.

Section 4.3 The trypsin sensitivity of radiolabelled proteins on whole cells.

Introduction

A number of enzymes have been used, before or after labelling of intact viable cells, to demonstrate that the radiolabelled proteins are accessible to enzymes which don't penetrate the cell membrane, (Hubbard and Cohn, 1976). The most widely used, on several cell types, are: Pronase, (Gahmberg and Hakomori, 1973a; Willinger and Frankel 1979); Neuraminidase, (Gahmberg and Hakomori, 1973a) and Trypsin, (Hynes, 1973; and Pearlstein et al. 1976).

In order for this type of experiment to be useful in providing evidence for the cell surface localization of labelled proteins two

Table 4.2

Trypsin sensitivity of ^{125}I-labelled proteins

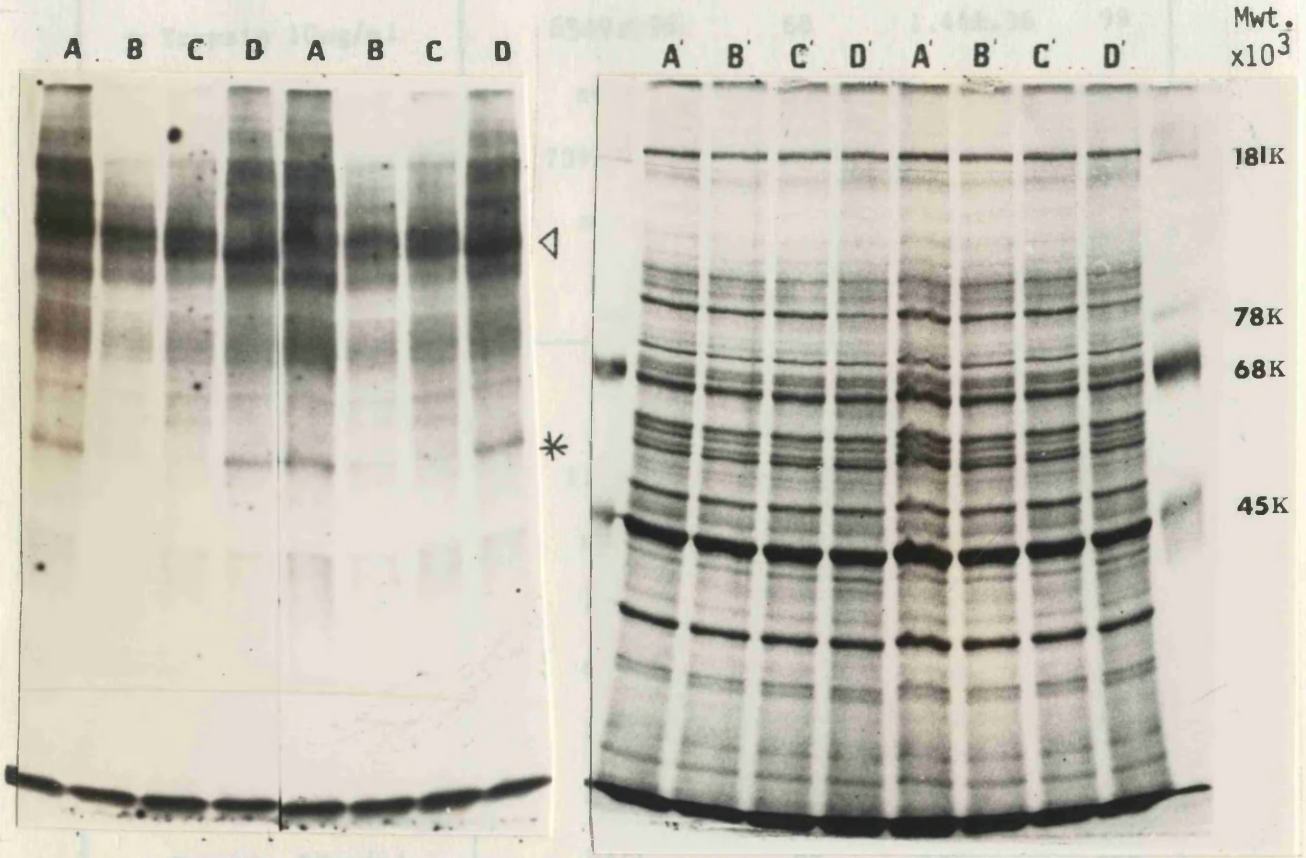
	cpm/mg*	%	mg/ml**	%
Incubation Control	9532±396 n=3	100	1.45±.45	100
+ Trypsin 10ug/ml	6549±996 n=3	68	1.44±.36	99
+ Trypsin 50ug/ml	7392±634 n=3	79	1.39±.41	96
Incubation Control	11008 n=1	100	1.9	100
+ Trypsin 10ug/ml	7337 n=1	67	1.8	95
+ Trypsin 50ug/ml	7807 n=1	71	1.8	95
+ Trypsin 50ug/ml + Soybean Trypsin Inhibitor 60ug/ml	9771	89	1.9	100

*Triton X-100 soluble TCA precipitable counts

**Triton X-100 soluble total protein.

n-number of separate experiments.

Fig. 4.2. Trypsin sensitivity of Triton X-100 extracted, I^{125}-labelled, cell surface proteins of neutrophils.



The samples on the autoradiograph are designated A-D while the corresponding slots on the gel are labelled A'-D'. (A,A') Incubation Control; (B,B') Trypsin 10ug/ml in PBS - 20'; (C,C') Trypsin 50ug/ml in PBS - 20'; (D,D') Trypsin 50ug/ml + Trypsin Inhibitor (Soybean) 60ug/ml in PBS - 20'.
Double arrow indicates a band M_r 115K (approx.) and a partial cleavage product M_r 105K '(approx.)

Equal protein was applied per slot and the gel was exposed for 11 days.

criteria must be satisfied. Selectivity of degradation of putative membrane protein and proteolytic sensitivity of radiolabelled proteins, must be shown.

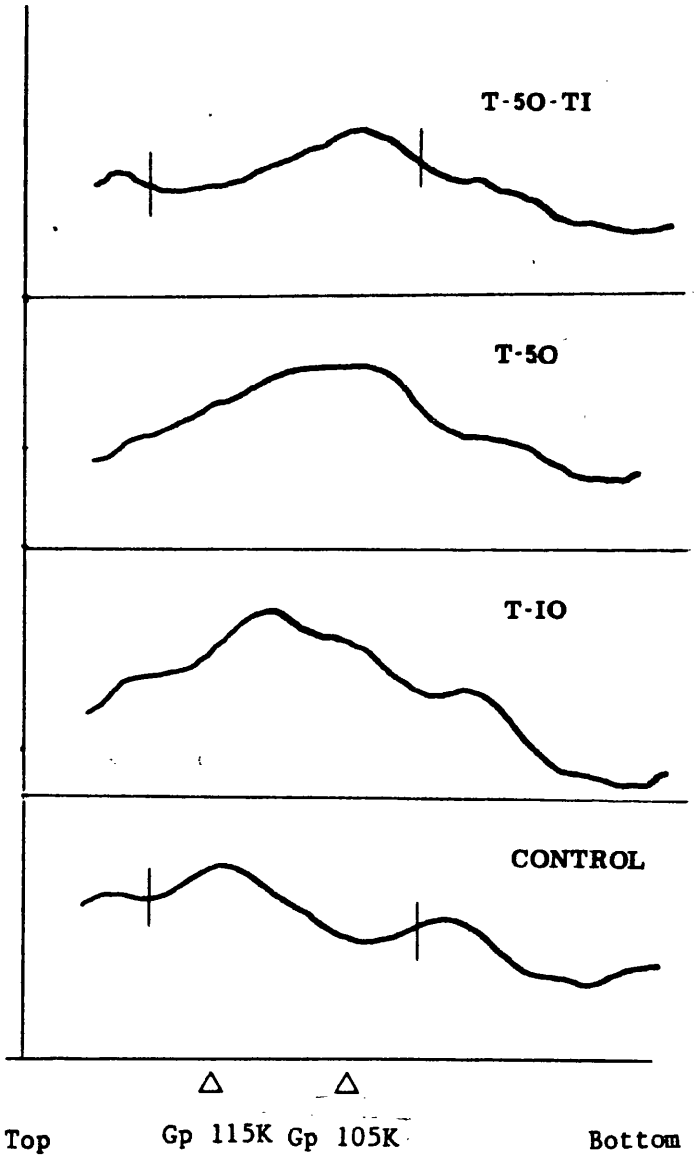
Results and Discussion

The results are expressed quantitatively in Table 4.2. The results of running the samples shown in Table 4.3 on SDS-PAGE are shown in Fig.4.2.

The results expressed in Table 4.2 suggest that the labelled polypeptides are trypsin sensitive. ~~trypsin-specific since it was almost completely reversed by addition of Soybean Trypsin Inhibitor~~. The lack of visualizable cleavage products suggests that trypsin cleavage tends to occur proximally, with respect to the lipid bilayer, to iodinated sites on the protein. Quantitatively my results are similar to those found by Willinger and Frankel (1979) for rabbit peritoneal neutrophils: 66% of activity remaining after incubation at $20^{o}C$ with trypsin (10ug/ml) for 20 minutes. Work on erythrocytes using much higher concentrations of trypsin resulted in a maximum release of 45% of label, (Hubbard and Cohn, 1976). The lack of greater removal of label is then not surprising. Trypsin is a fairly limited protease, resulting in hydrolysis of peptide bonds that follow positively charged sidechains of lysine or arginine residues. Cleavage sites may be unavailable or hidden on some labelled species.

The apparent increased effectiveness of the lower concentration of trypsin, (see Table 4.2 and Fig.4.2), is difficult to explain, but may be related to the increased clumping of cells seen at the higher trypsin concentration. The results also suggest that there was no appreciable overall proteolysis (See Tracks A'-D', Fig.4.2), thus

Fig. 4.3. Densitometry of part of autoradiograph in fig. 4.2. demonstrating apparent shift in mobility of Gp 115K.



The vertical lines denote the area of the autoradiograph, containing the two polypeptides Gp 115K and Gp 105K, which was scanned.
The markers refer to the position of the two polypeptides
See fig. 4.2 for key to T5O-Ti/Control legend

satisfying our criteria that proteolytic degradation is selective and was limited to radiolabelled polypeptides.

Fig.4.2, Tracks A-D, suggest that the high molecular weight polypeptides together with a polypeptide of approx. 50K, (denoted by *), are particularly trypsin sensitive. High molecular weight polypeptides, (e.g. fibronectin), have been found by other workers to be particularly trypsin sensitive in fibroblasts, (Hynes, 1973 and 1976) and in macrophages,(Pearlstein et al., 1978). A band of mol. wt. 115K appears to be partially hydrolysed at 20 C to form a cleavage product of 105K, (denoted by 2 arrows). This hydrolysis appears to be independent of trypsin activity. Evidence for a relationship between these two bands is suggested by the results of densitometric scanning results are shown in Fig. 4.3. Willinger and Frankel noted a similar phenomenom in what is probably the equivalent band in rabbit peritoneal neutrophils.

These experiments then appear to satisfy our criteria that a particular subfraction, of proteins, which are radiolabelled, are trypsin sensitive. Since proteolytic degradation of intracellular proteins does not appear to occur, these experiments provide further evidence for the cell surface localization of the radiolabelled polypeptides.

Section 4.4 Isolation of Plasma Membrane Enriched Fractions from Neutrophils

Introduction

In contrast to the red cell, isolation of pure plasma membrane from PMN cytolysates has been complicated by technical difficulties, due in part, to the complex organelle structure of the cell. A large number of hydrolytic and peroxidative enzymes and cationic proteins are released upon separation and disruption of the organelles which leads to enzyme

inactivation and protein destruction, (Baehrer and Boxer, 1979).

Nevertheless, a number of workers have attempted to prepare plasma membrane enriched fractions from neutrophils, using a variety of markers, e.g. Hawkins and Sauve, (1980) and Willinger and Frankel, (1979).

Use of the classical membrane marker 5'-nucleotidase was not possible since this is not a membrane marker in human neutrophils, (De Jong et al., 1979). Two confirmatory methods were therefore used to 'follow' the radiolabelled polypeptides, transmission electron microscopy (T.E.M.), and tagging of whole cells with radiolabelled lectin prior to further treatment. Intact cells were labelled with trace amounts of ^{125}I-WGA to tag cell surface receptors under conditions which do not induce receptor redistribution, (Chang et al., 1975). WGA was chosen for this study because it does not dissociate during **fractionation** in sucrose gradients, (Chang et. al. 1975). It binds to N-acetylglucosamine and sialic acid, common plasma membrane components, and it does not have the extensive membrane perturbing effects of lectins such as Con A, (Willinger and Frankel, 1975). Con A causes rapid clustering of receptors in pig neutrophils, (Sheterline and Hopkins, 1981). Stabilization, and if possible, selective removal of organelles, after homogenization, is required to minimize binding of membrane to nuclei and to minimize rupture of lysosomes which contain proteases, (Willinger and Frankel, 1979). The addition of 500ug/ml BSA to the homogenization buffer helps to stabilize subcellular organelles. A low speed centrifugation step immediately following homogenization removes intact cells and nuclei.

Results and Discussion

Plasma membrane fractionation experiments were carried out according to the method of Willinger and Frankel (1979). Briefly this involved homogenization followed by pelleting at low speed of whole cells, nuclei

Table 4.3.

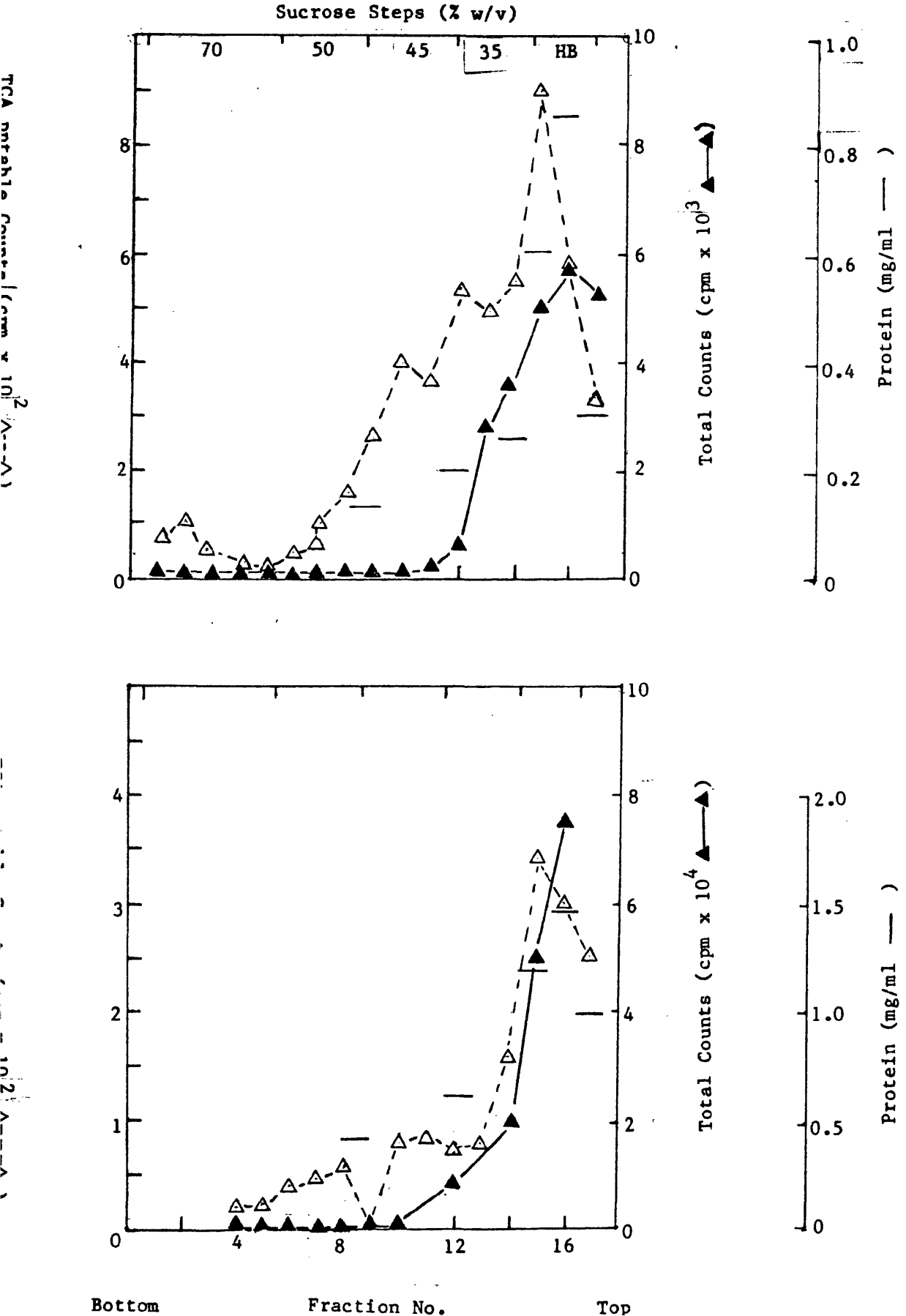
Recovery of labelled plasma membrane during cell fractionation

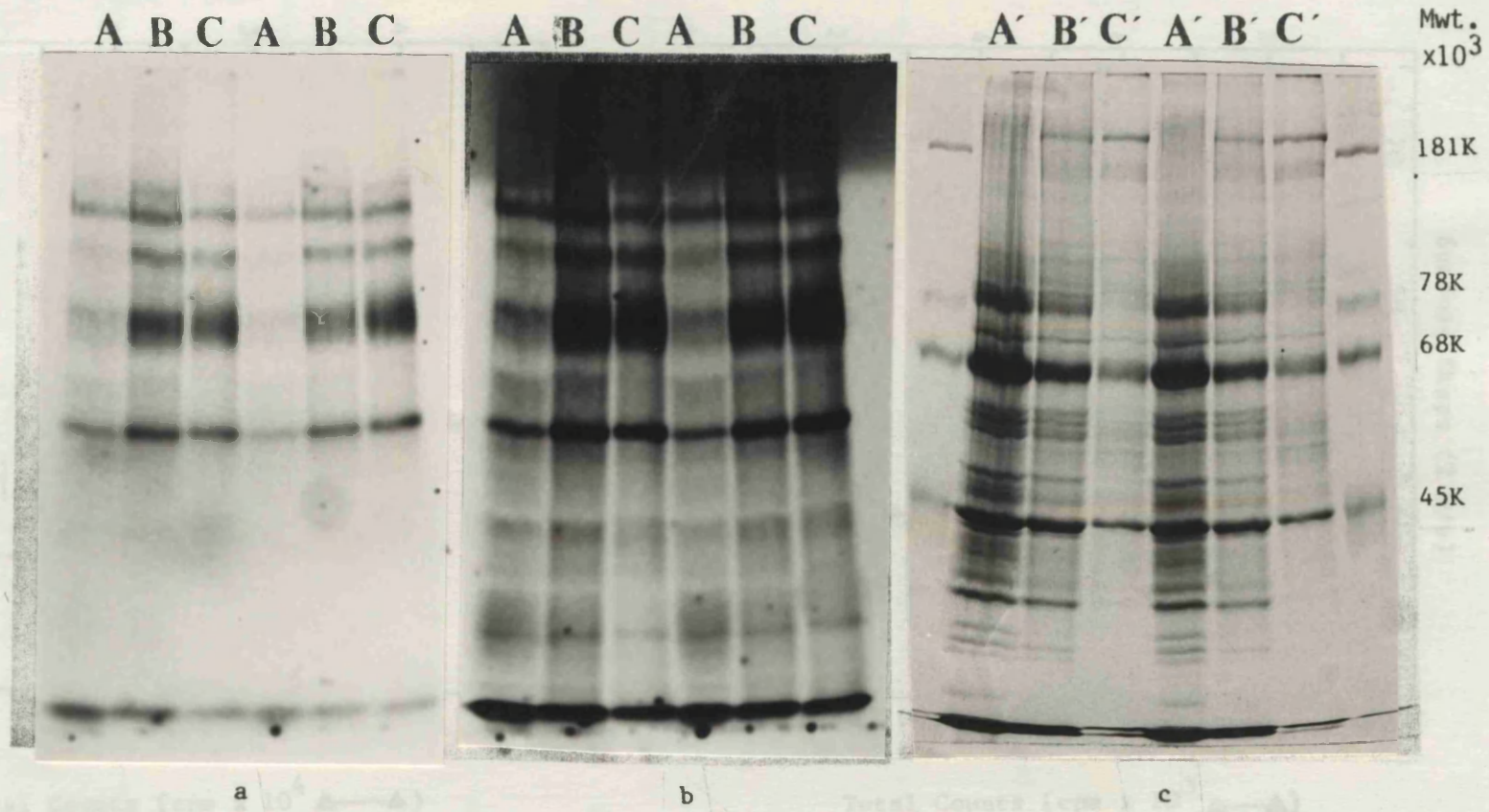
	^{125}I-LPO cpmx10^3*		^{125}I-WGA cpmx10^3		^{125}I-Con A cpmx10^3	
	A	B	A	B	A	B
Cell Homogenate	58.5	38.5	166.2	115.2	20.6	22.5
Low Speed Pellet	26.3	16.6	67.6	45.8	8.3	10.1
%	45	43	41	40	45	42
Low Speed Supernate	37.3	24.3	ND		ND	
%	60	60	-		-	

* TCA pptd. counts

Fig. 4.4. Fractionation of labelled protein on sucrose step gradients

The results of two separate experiments are shown.
HB - Homogenization Buffer
TCA pptable counts were for 60 secs.

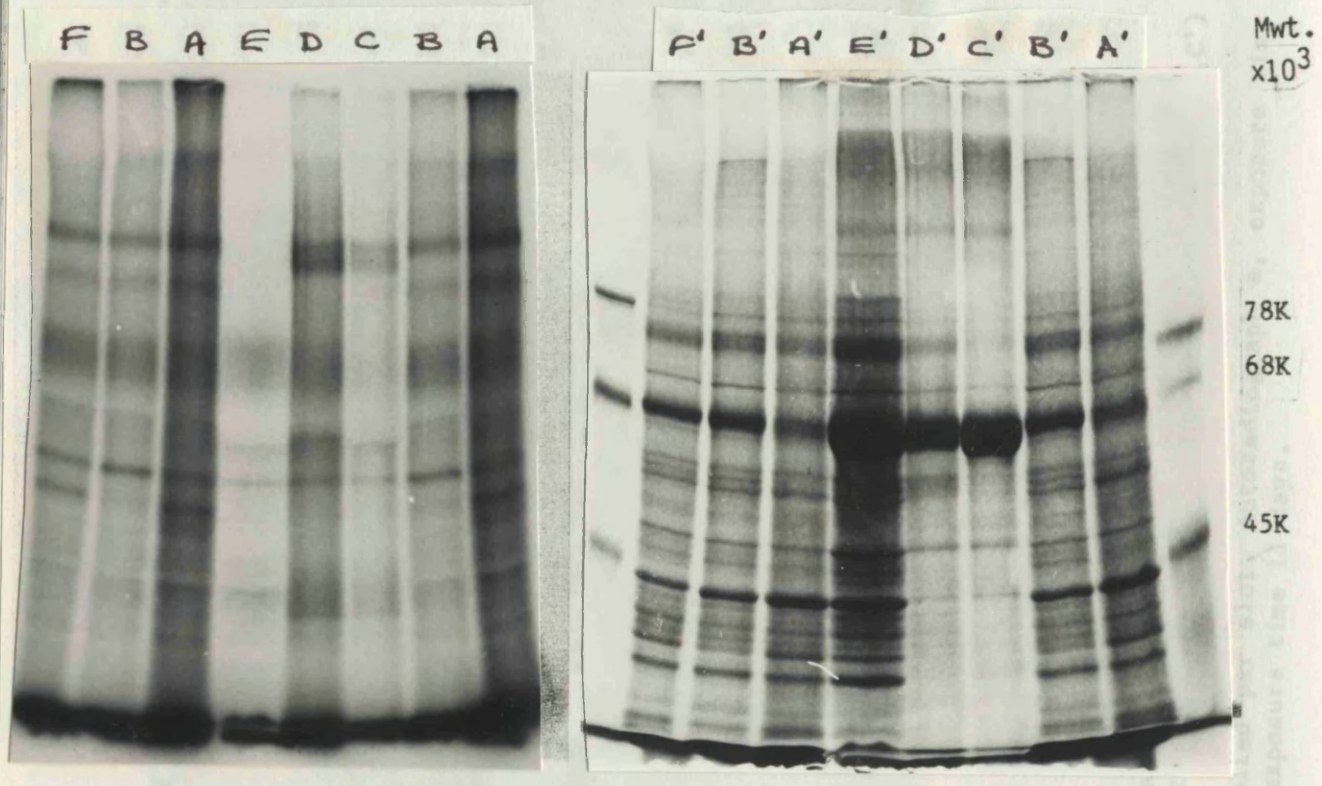




A-C - Autoradiograph; A'-C' - Coomassie blue stained gel
(A,A') Homogenization Buffer; (B,B') 35%/45% sucrose step; (C,C') 45%/50% sucrose step.
Equal volumes applied per slot. Autoradiograph a, exposure time 7 days, autoradiograph b exposure time 11 days.

Fig. 4.5. SDS-PAGE of fractions of homogenized radiolabelled whole cells from a sucrose step gradient

Fig 4.6 SDS-PAGE of fractions of homogenized radiolabelled whole cells taken from a sucrose step gradient, (15%/35% step inserted)



A-F - Autoradiograph; **A'-F'** - Coomassie blue stained gel
(A,A') Low speed pellet; (B,B') Supernatant; (C,C') 35%/45% sucrose step; (D,D') 15%/35% sucrose step; (E,E') Homogenization buffer; (F,F') Whole cell.
Equal volumes per slot. autoradiograph exposed for 15 days.

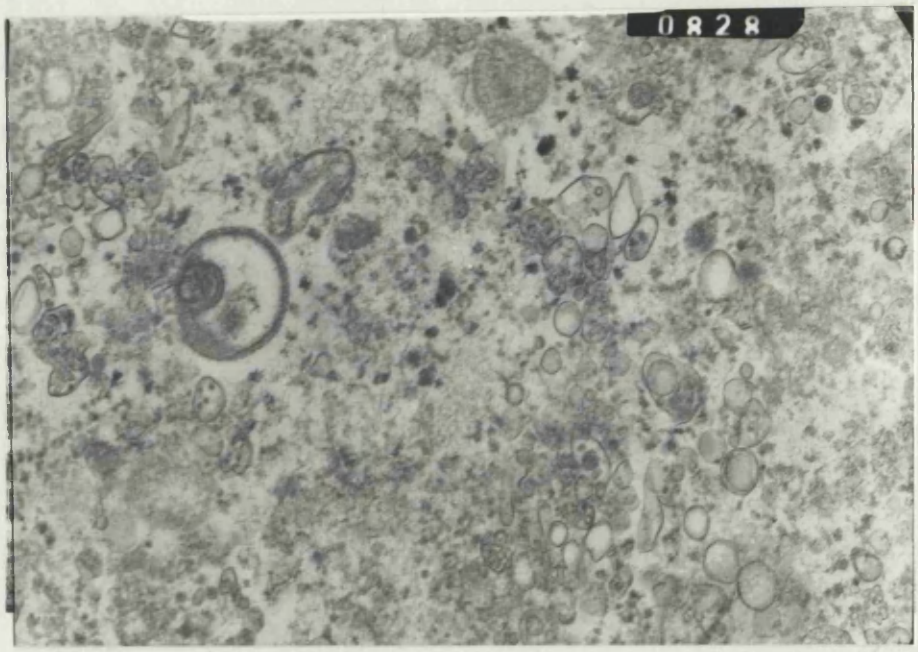
and other organelles. The supernatant was then layered onto a sucrose step gradient and the preparation was subjected to high speed centrifugation. Subsequently the procedure was modified by alteration of the 'steps' to produce a plasma membrane fraction.

Experiments were performed in order to determine the fate of ^{125}I-labelled material during homogenization and fractionation on the sucrose step gradient. These results are shown in Table 4.3. and Fig. 4.4. Some 40% of ^{125}I-labelled material was pelleted at low speed prior to membrane fractionation. Homogenization of cells was carried out until approximately 80% breakage had occurred in order to minimize damage to intracellular organelles. Thus the low speed pellet would be expected to contain a large amount of label for the above reason as well as due to binding of membrane to nuclei and trapping of labelled material. In Fig. 4.6., SDS-PAGE analysis reveals no significant differences between whole cell homogenate and low speed pellet. Experiments using ^{125}I-lectin tagging produced a similar proportion of counts in the low speed pellet, (see Table 4.3.), again suggesting that substantial internal labelling was not taking place using the ^{125}I-LPO system. The results from **Fig. 4.4** suggest that a large proportion of the <u>total</u> counts do not enter the gradient but remain in the homogenization buffer layer, (HB). This was expected since in Section 3.2., Table 3.1., it was demonstrated that only about 10% of label incorporated into washed cells is covalently bound.

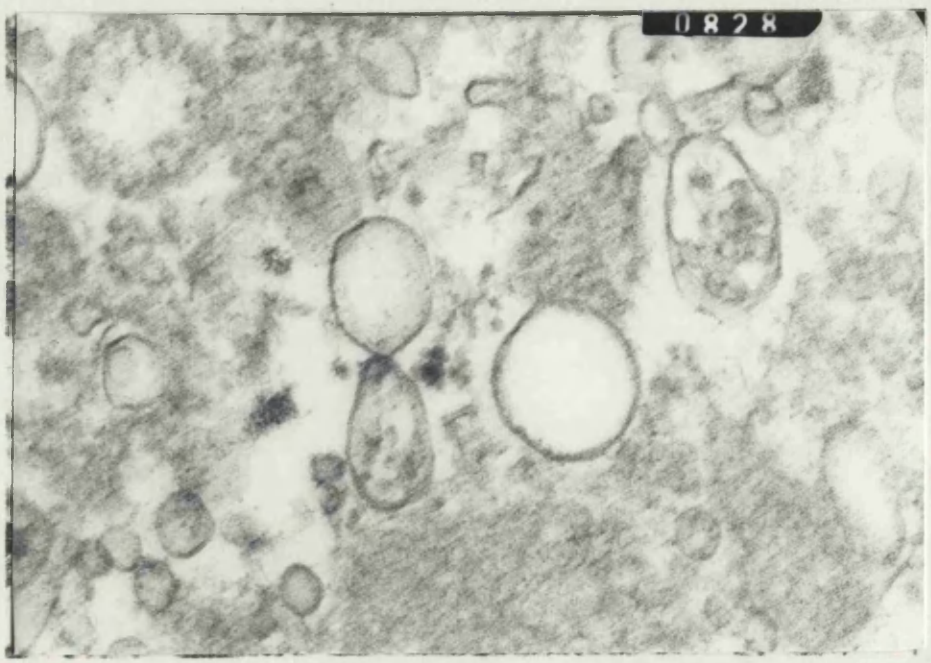
Labelled material precipitable with TCA gave a slightly skewed counts peak with respect to Total counts and protein values. Labelled lipid aggregates, probably in the form of micelles, may contribute substantially to the TCA ppt counts values obtained for the HB fraction, (labelled lipid may contribute up to 30% of covalently labelled material as judged by chloroform extraction, see Table 3.2.). These results suggest then that a large amount of labelled material either does not enter the gradient or enters the 35% sucrose layer.

Fig. 4.7 Electron micrographs of putative plasma membrane sucrose gradient fraction, (35%/45% boundary)

a



b



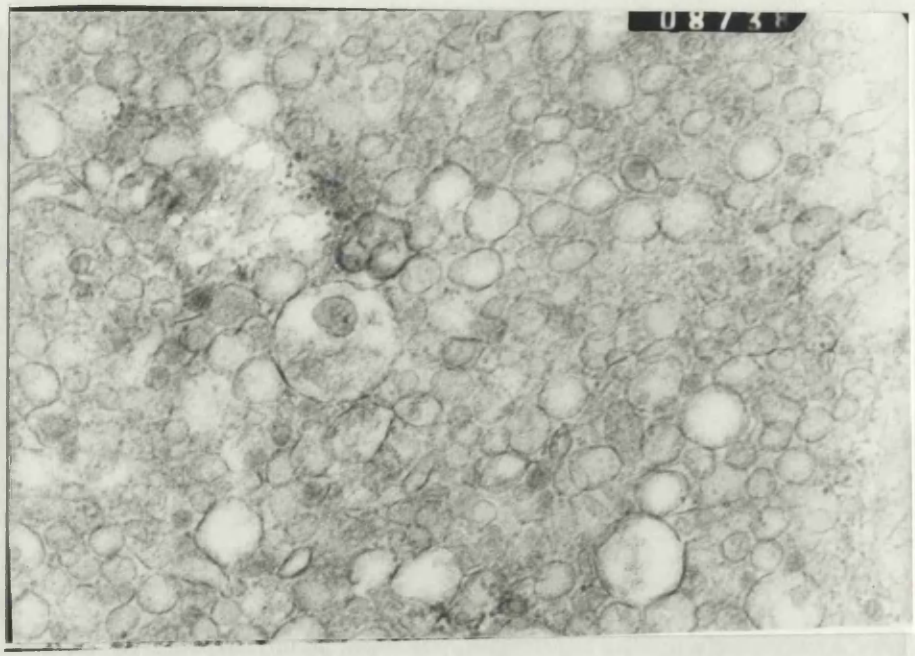
a 75×10^3 approx. magnification

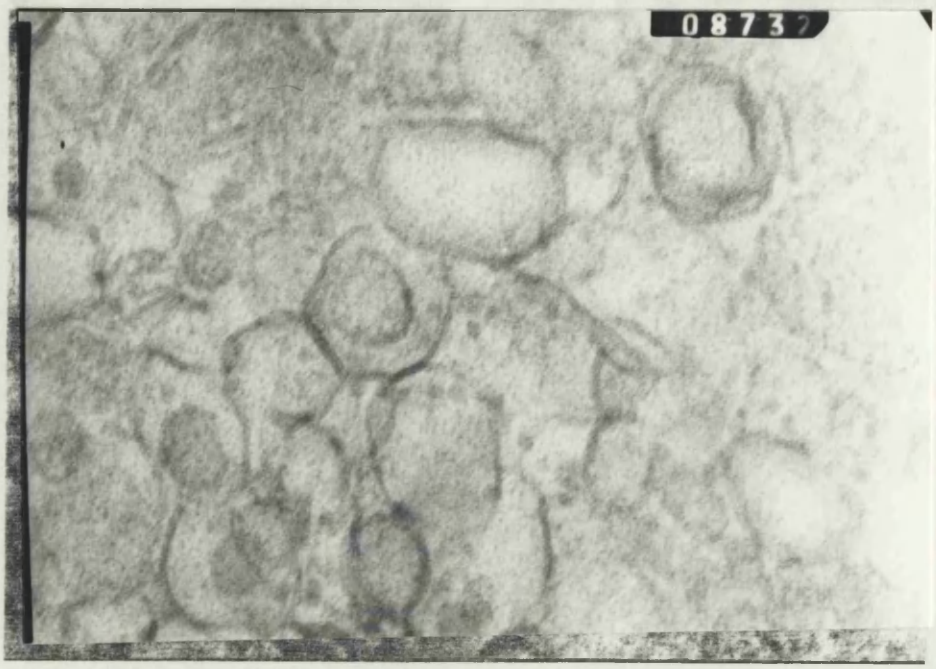
b 200×10^3 approx. magnification

(micrographs courtesy of Mr. J. Parry)

Fig. 4.8 Electron micrographs of putaive plasma membrane sucrose gradient fraction, (15%/35% boundary)

a





a 80×10^3 approx. magnification

b 250×10^3 approx. magnification

(micrographs courtesy of Mr. J. Parry) –

Labelled material is however present throughout the gradient. Some whole nuclei may sediment with bound membrane despite the presence of BSA. Homogenization of cells is also likely to produce a great deal of size heterogeneity among membrane vesicles.

These results were investigated further by 1) running fractions on SDS-PAGE, (Fig. 4.5.), examining the 35% sucrose fraction using transmission electron microcopy, (T.E.M.) and tagging whole cells with ^{125}I-WGA lectin followed by homogenization and fractionation, see Fig 4.9a.

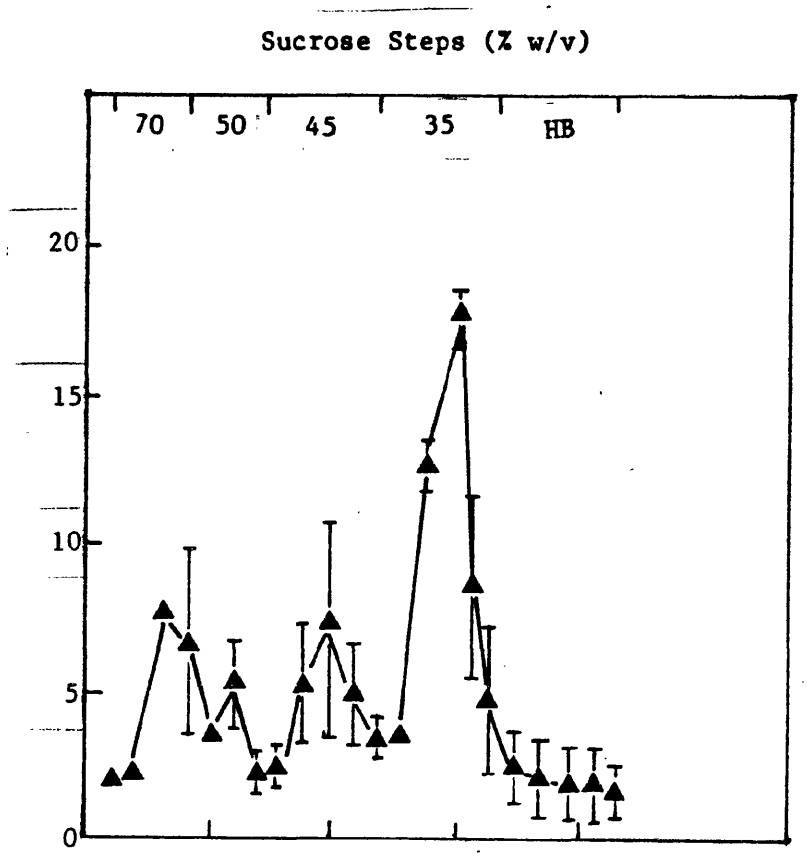
The SDS-PAGE analysis (Fig. 4.5.) confirms that while more protein is found in the HB fraction than in the 35% and 45% sucrose fractions, relatively little labelled protein remains in the HB fraction, most of it entering the gradient in this particular experiment. This result then tends to confirm that a subfraction of labelled material can be detected which enters the gradient but remains within or near the boundary of the first sucrose step. To confirm whether this material was or contained plasma membrane further fractionation experiments were undertaken and the 35% fraction was examined under T.E.M. (courtesy of J. Parry). The results show that the material in this fraction is heterogenous although some membrane may be present, (fig. 4.7).

The results of tagging cells with ^{125}I-WGA before homogenization and fractionation are shown in Fig. 4.9a. These results may more accurately reflect the distribution of membrane in the labelled membrane in the gradient than ^{125}I-LPO labelled material. However there is only one major receptor on the cell surface for WGA, Gp 115K, (see Section 5.3.) Gp 115K may be slightly enriched with respect to other labelled polypeptides in the HB fraction (see Fig. 4.5.), but otherwise appears to fractionate with the rest of the labelled material. The results again suggest that most of the ^{125}I-WGA bound material enters the lightest fraction of the gradient. Dissociation of labelled lectin and/or binding of 'free' ^{125}I-WGA may also occur. In order to see whether the 35% step could be

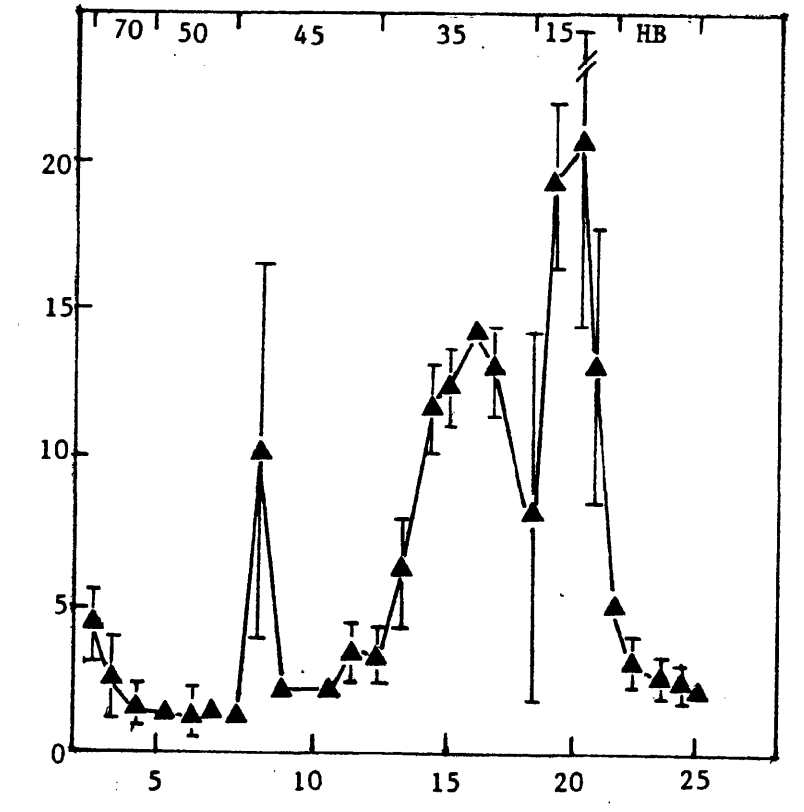
Fig. 4.9 ^{125}I-WGA tagging of whole cells prior to fractionation on sucrose step gradients

The lower abcissa (Fraction No.) is the same for both experiments.

a



b



partitioned and a better yield of putative plasma membrane obtained a 15% (w/v) sucrose step was inserted. SDS-PAGE, T.E.M. and ^{125}I-WGA tagging experiments were performed.

The results of the SDS-PAGE analysis are shown in Fig. 4.6. The 15% fraction appears to be significantly enriched in labelled material, most of the cellular protein remaining in the homogenization buffer as judged by this technique. T.E.M. of material in this fraction, (courtesy of J. Parry) suggests that substantial enrichment of membrane material, largely in vesicular form has been achieved. The lipid bilayers in Fig. 4.8. were measured and found to be 65A wide which is a typical value for mammalian plasma membranes, (M. Davies, personal communication).

The results of ^{125}I-WGA tagging experiments are shown in Fig. 4.9b. These results which represent two seperate fractionations from the same homogenization suggest that it may be possible to obtain an enriched fraction for bound ^{125}I-WGA at the 15%/35% interface although clearly many counts do not enter the gradient. These results may be compared with those in Fig. 4.9a where a single large peak at the 35%/HB interface is seen.

It appears then that partial purification of a putative 'plasma membrane' fraction may be possible, as judged by transmission electron microscopy and ^{125}I-WGA membrane tagging and that a proportion of labelled polypeptides are co-purified. Thus further evidence is provided that labelled polypeptides are localized at the plasma membrane.

Chapter 5 Identification of the Glycosylation of Membrane Proteins

Section 5.1 Introduction

The availibility of purified lectins in conjunction with methods outlined in Section 2 allows a study of the glycosylation of membrane proteins.

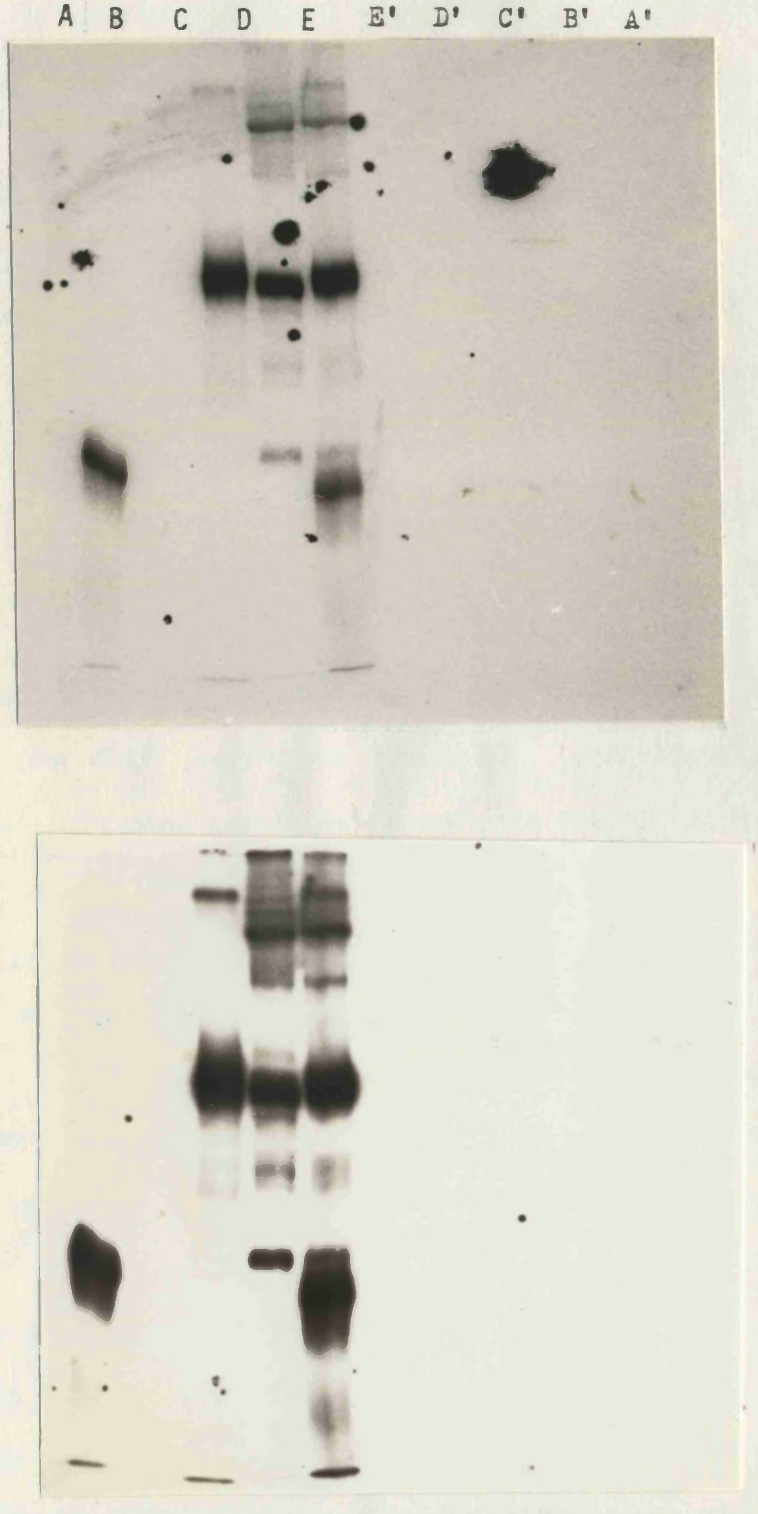
Concanavalin A (Con A) and Wheat Germ Agglutinin (WGA) were used in this study. The reason for the choice of these particular lectins in this study are outlined below.

Con A has a specificity for the following sugar residues in decreasing order of affinity: α-D-mannose > α-D-glucose > α-D-glucosamine, (Lotan and Nicolson, 1979). It interacts preferentially through extended saccharide binding sites, in multivalent interactions with internal mannose residues of complex oligosaccharides, (Goldstein and Hayes, 1978 and Lotan and Nicolson, 1979). Most, if not all, mammalian plasma membrane glycoproteins possess N-linked oligosaccharide side-chains, (see fig. 1.8), containing internal mannose residues, (reviewed in Harrison and Lunt, 1980 and Gibson, Kornfeld and Schlesinger, 1980). Con A then, may be useful to 'scan' a large number of polypeptides simultaneously.

WGA, in contrast, reacts with terminal N-acetyl neuraminic acid residues and terminal and internal N-acetyl-D-glucosamine residues, Bhavanandan and Katlic (1979).

Preliminary control experiments established that Con A binding to polypeptides on SDS-PAGE was effectively inhibited by the addition of 0.2M -D-mannose to the incubation buffer, (fig. 5.1), and to a lesser extent in binding to whole cells, where some non-specific binding appears to be taking place, (see fig. 5.2). The preliminary data here suggests that ^{125}I-Con A may compete less effectively for binding sites on the

Fig. 5.1. Effect of 0.2M α-D-Mannose on binding of ^{125}I-Con A to polypeptides after SDS-PAGE

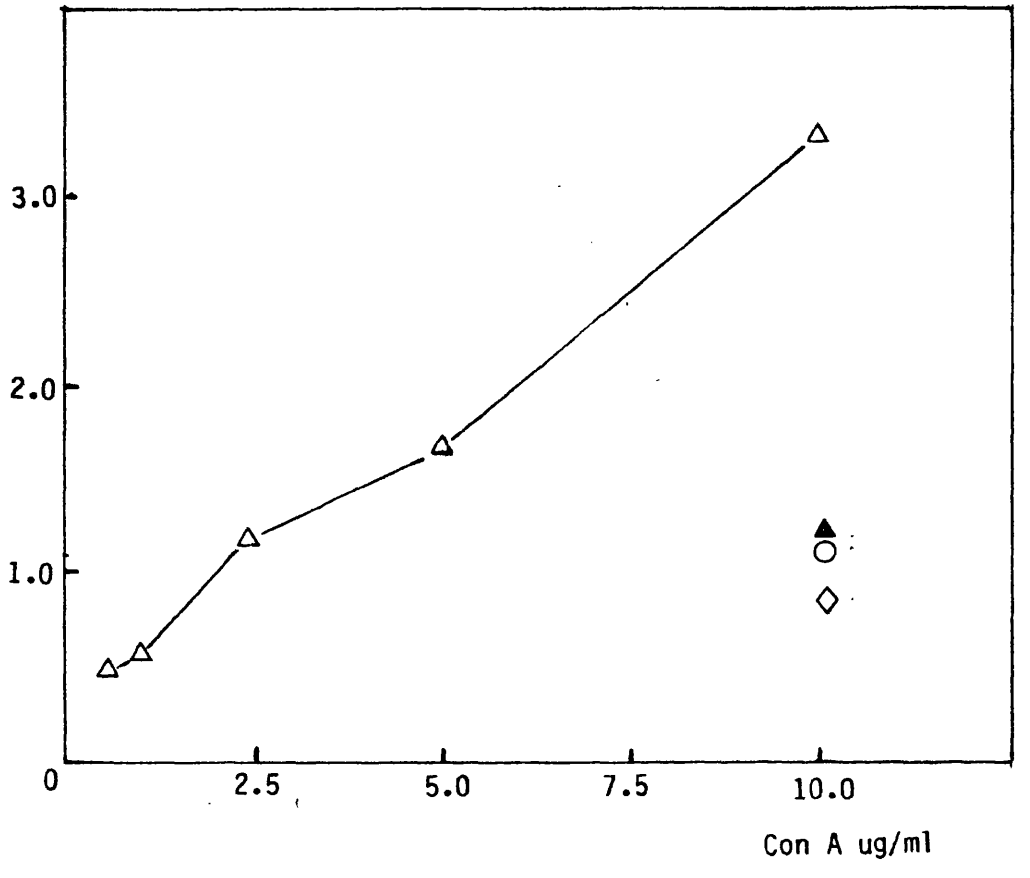


A-E, -α-D-Mannose; A'-E' +α-D-Mannose.

A,A' Ovalubmin; B,B' bovine serum albumin; C,C' lactoperoxidase
D,D' α_2-macroglobulin; E,E' marker mix.

2 autoradiographs of the same gel are shown exposed for 12hrs and 36hrs

Fig. 5.2. Comparison of Binding to whole cells of ^{125}I-Con A and cold Con A

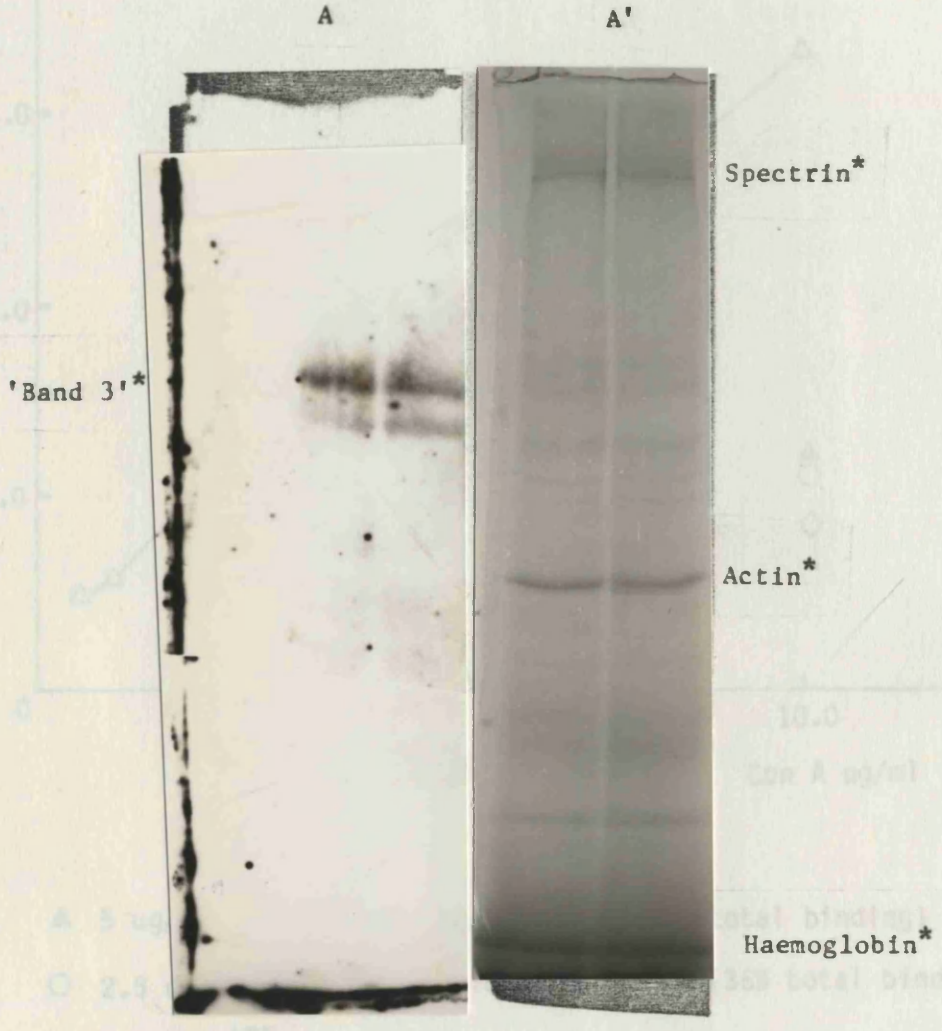


▲ 5 ug/ml ^{125}I-Con A/ 5ug/ml Con A (36% total binding)

○ 2.5 ug/ml ^{125}I-Con A/ 7.5 ug/ml Con A (35% total binding)

◇ 10 ug/ml ^{125}I-Con A + 0.2M α-D-Mannose (25% total binding)

Fig. 5.3 ^{125}I-Con A overlay of lysed red cells on SDS-PAGE



A- autoradiograph A'- Coomassie Blue stain

* Lux S.E. (1979)

** Robinson et al. (1976) and Tanner and Anstee (1976)

Fig. 5.4.

Con A affinity chromatography of Triton X-100 detergent extracted radiolabelled polypeptides from neutrophils

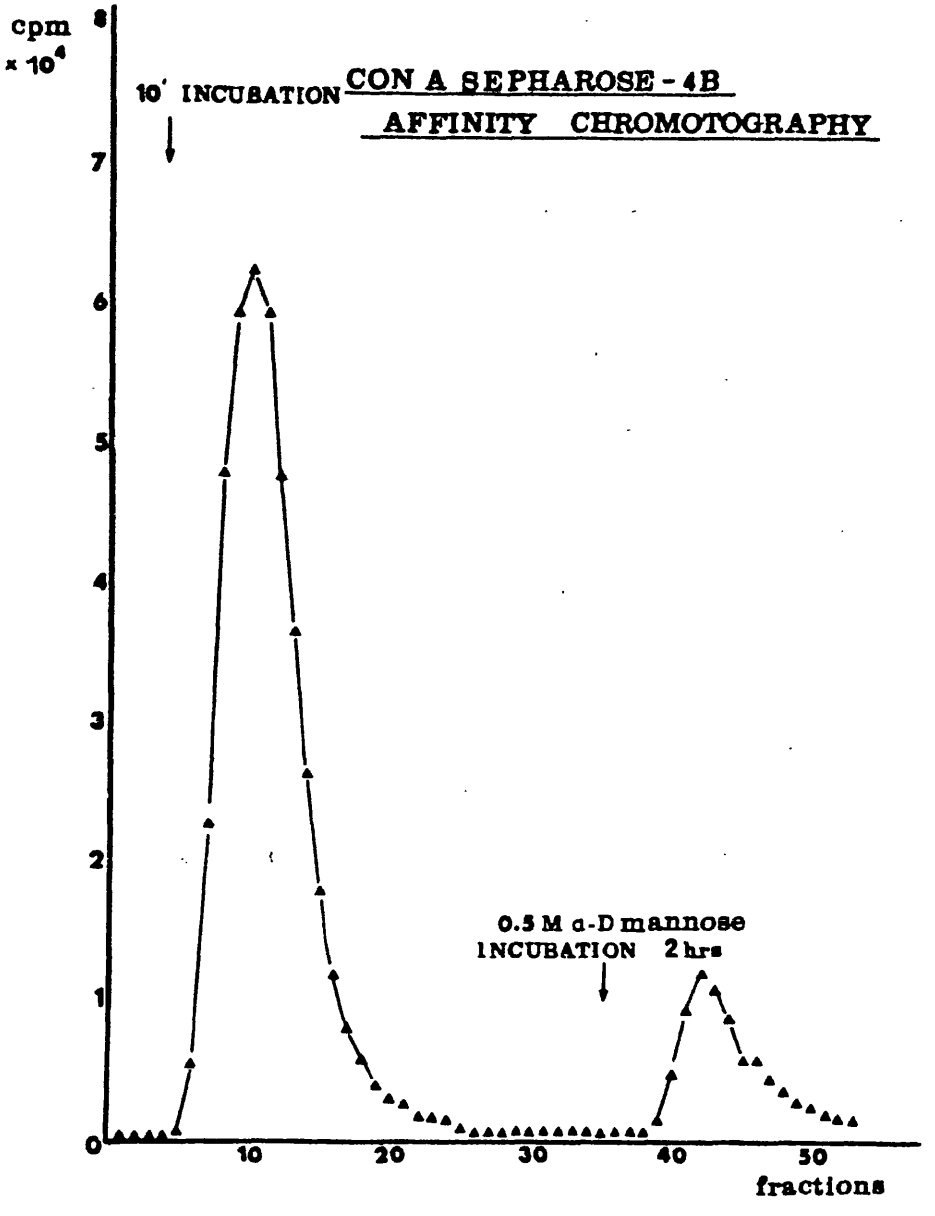
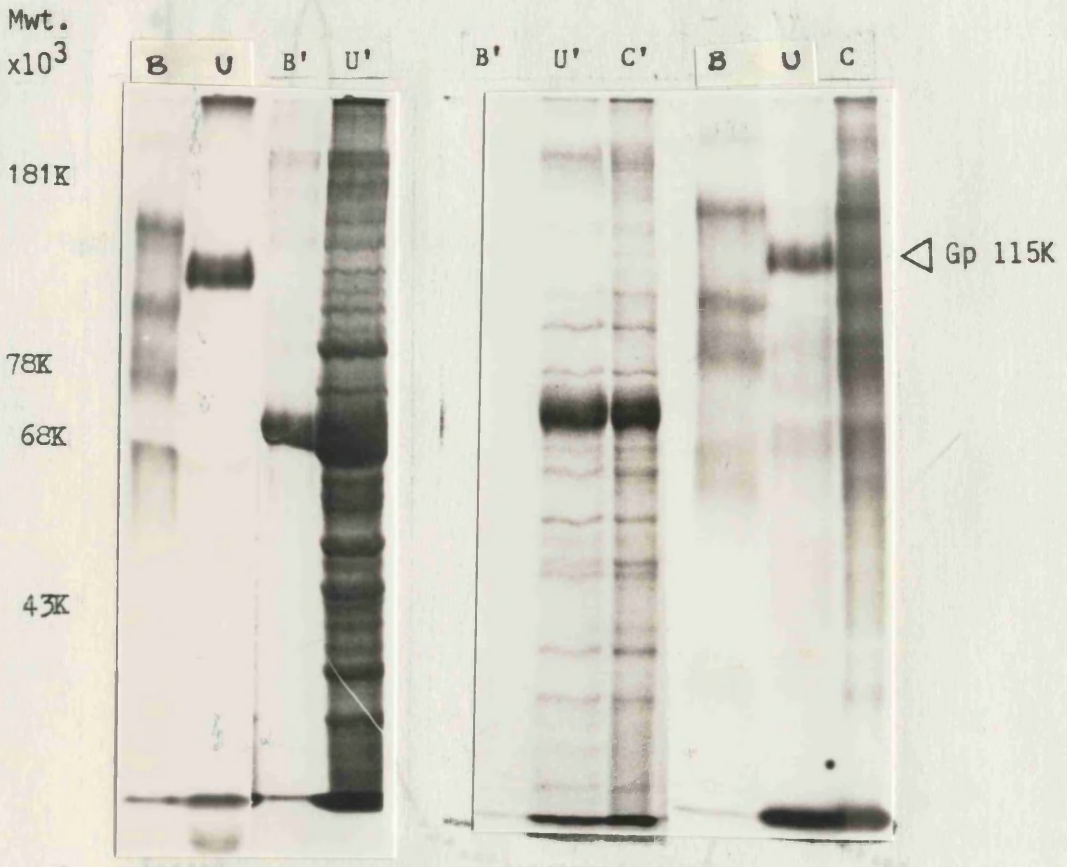


Fig. 5.5. Con A Affinity Chromatography of labelled polypeptides



U,B,C - autoradiography; U',B',C' Coomassie blue stained gel. (U,U') Unbound material; (B,B') Bound material (eluted with 0.5M α-D-mannose); (C,C') Control. Autoradiographs were exposed for 7 days (a) and 12 days (b).

cell surface than 'cold' Con A. Con A overlay of solubilised red cell revealed a similar pattern, (fig. 5.3), to that found by other workers, (Robinson et al., 1975 and Tanner and Anstee, 1976). ^{125}I-WGA overlay on SDS-PAGE revealed a binding pattern substantially different from that of Con A binding, (see fig. 5.5).

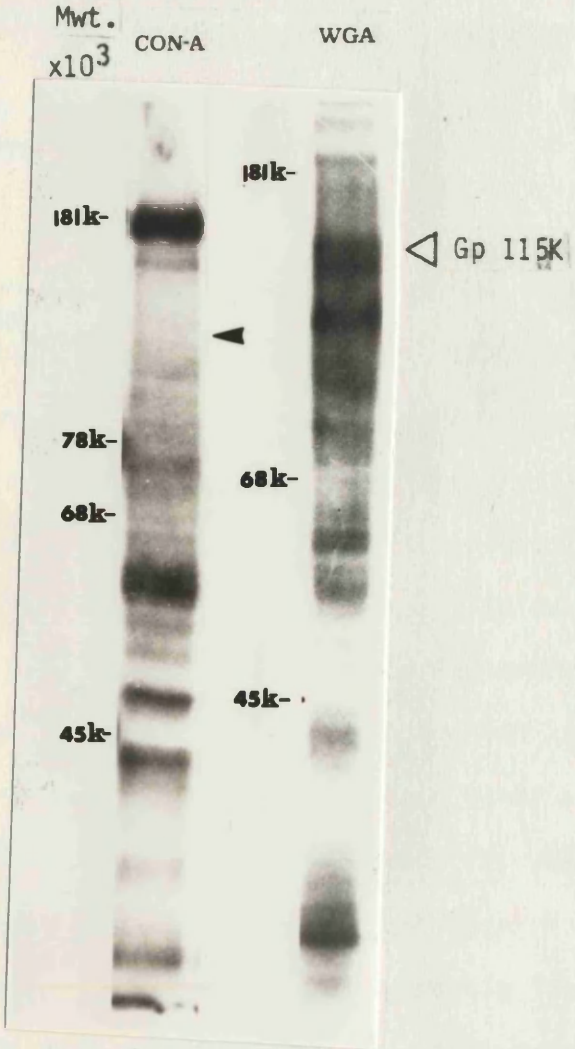
Section 5.2 Evidence that cell surface membrane proteins of human neutrophils are glycosylated

Results and Discussion

The results of Con A Sepharose-4B affinity chromatography of radiolabelled detergent extracts are shown in fig. 5.4. Material from two such experiments was run on SDS-PAGE, (fig. 5.5). The results suggest that all but one of the major radiolabelled polypeptides bind to immobilized Con A under these conditions. Some minor labelled polypeptides also appear to only partially bind or not bind at all under these conditions. Further experiments to determine whether these polypeptides, including the major non-binding polypeptide Gp 115K, would bind to immobilised Con-A under altered conditions were not performed.

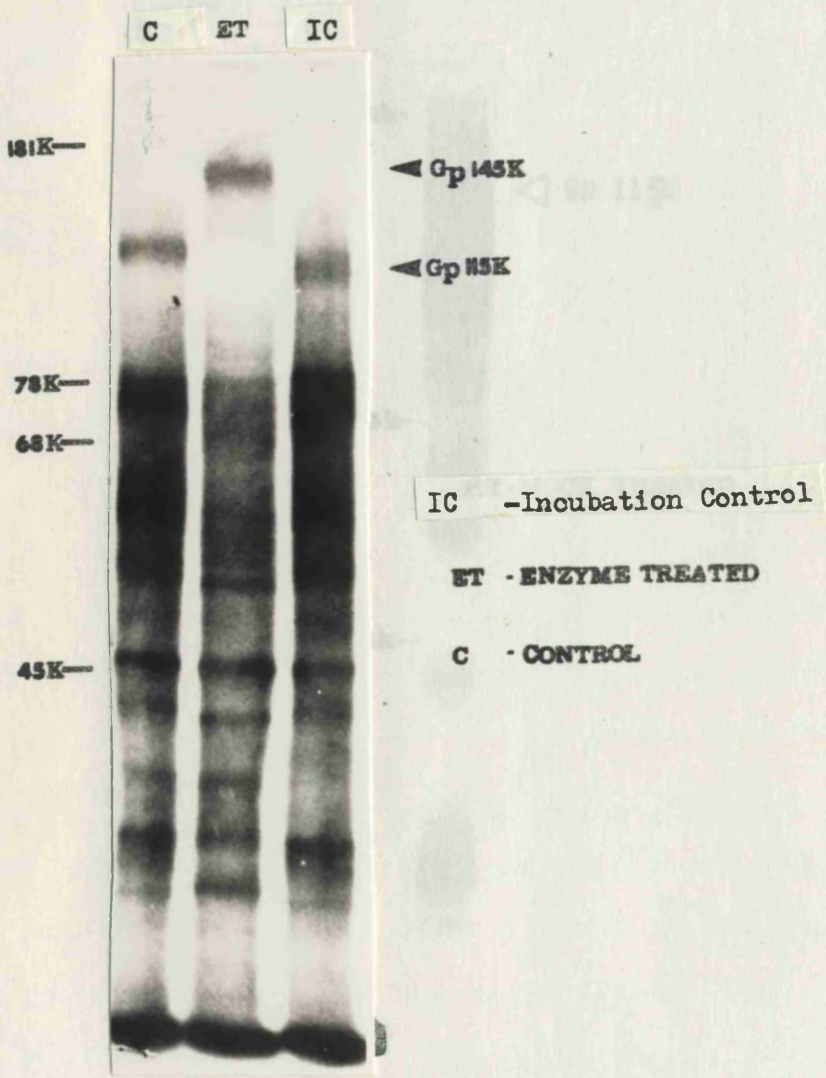
The finding, then, that all but one of the radiolabelled polypeptides binds to immobilized Con A is perhaps unsurprising. The polypeptides which do appear to bind, are all eluted with the appropriate sugar hapten, α-D-Mannose. In Fig. 5.5b examination of the coomassie blue stained protein profile, (Track B'), suggests that most major cellular proteins do not bind to the column. The results of the experiment shown in Fig 5.5a, are complicated in this respect by the use of Bovine Serum Albumin (BSA) as a carrier in the concentration of labelled polypeptides before SDS-PAGE. The major labelled band, Gp 115K, which does not bind to immobilized Con A under these conditions, migrates as a doublet on SDS-PAGE, (Gp 115K/ Gp

Fig. 5.6. ^{125}I-lectin (Con A and WGA) overlay on SDS-PAGE



Equal protein (35ug) was loaded per slot.

Fig. 5.7 Effect of neuraminidase treatment on radiolabelled whole cells



Equal counts were loaded per slot and the autoradiograph was exposed for 11 days.

105K), as noted in Chapter 3 and Chapter 4, Section 4.3. These bands are relatively broad and this may reflect microheterogeneity of carbohydrate residues, which appears to be the case for many cell surface glycoproteins, (Stanley and Sudo, 1981). However given that Gp 115K appears to be particularly susceptible to partial proteolysis, which appears to occur under these experimental conditions, there is no evidence for altered lectin binding due to partial hydrolysis. The results from the ^{125}I-WGA overlay experiments appear to confirm this finding as judged by the equal binding of ^{125}I-WGA to the doublet, (fig. 5.7).

Supporting evidence for the presence of Con A receptors at the cell surface of human neutrophils comes from studies involving the agglutination of whole cells with lectins, (Taub et al., 1980) and binding of fluoroscein- or rhodamine-Con A conjugates to whole cells, (Weinbaum et al., 1980). The latter authors noted that binding was completely inhibited in the presence of 50mM α-D-mannose and suggest that the clustering of Con A receptors at the leading edge of the activated cell, may be important in its abilty to function as an efficient phagocyte. The results in Fig.5.2 also suggests that binding of ^{125}I-Con A is occurring, although here some non-specific binding is apparent.

Evidence has been presented then, that some surface membrane proteins of the neutrophil are glycosylated. The generalization that all mammalian external plasma membrane proteins are glycosylated has been proposed, Gahmberg (1973) and Lotan and Nicolson (1979). A number of points must be taken into account to allow us to support this conclusion, from the evidence presented here, in the neutrophil. Firstly not all cell surface proteins may be labelled using this technique (see Section 3). Secondly I have shown in Section 3.3. that not all labelled proteins are solubilised in TX-100, and those that are not may not be glycosylated. Thirdly Gp 115K binds with a low affinity or not at all to Con A under these conditions. The results of ^{125}I-Con A overlay experiments (see Fig 5.6) confirm the

finding of non-binding of Con A to polypeptides of 105K-115K apparent molecular weight. Other possibilities to explain the observation include the absence of accessible binding sites for Con A, glycosylation of the molecule not involving internal mannose residues or the complete absence of glycosylation of the molecule.

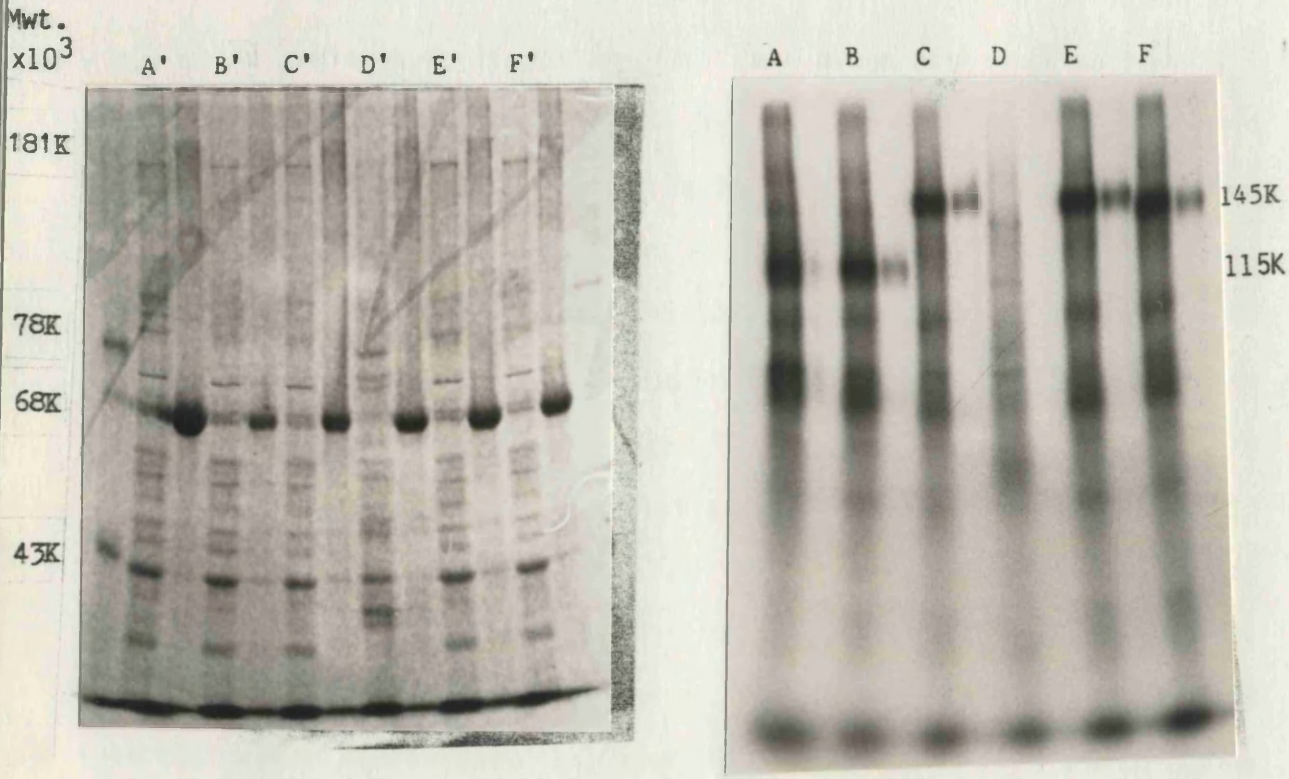
Section 5.3 Evidence that Gp 115K is a major cell surface sialoglycoprotein

Introduction

Other authors have shown that cell surface glycoproteins, which don't bind Con A, are heavily sialylated and may be described as sialoglycoproteins. The terminal sugar residue is commonly N-acetylneuraminic acid, the sialic acid generally found in cell surface glycoproteins, (Hughes, 1976). Thus, Robinson et al. (1975) and Tanner and Anstee (1976), using a radiolabelled lectin overlay technique demonstrated that Con A does not bind to the human red cell sialoglycoprotein, Glycophorin A. Brown et al. (1980) have identified a major sialoglycoprotein on the rat thymocyte (mol.wt.95K) which doesn't bind to immobilized lentil lectin. Lentil lectin has a similar range of specificity to Con A but binds with a lower affinity. Glycophorin A, probably in common with most sialoglycoproteins, binds strongly to Wheat Germ Agglutinin (WGA).The lectin has an affinity for terminal sialic acid as well as internal N-acetylglucosamine residues, (Bhahanvadan and Katlic, 1980, and Greenaway and Levine, 1973).

To test the hypothesis that Gp 115K was indeed a sialoglycoprotein the effect of neuraminidase (which cleaves terminal sialic residues (Hughes, 1976), and the ability to bind WGA under various conditions was investigated.

Fig. 5.8. Effect of neuraminidase and α-D-N-acetyl-oligosaccharidase on the mobility of Gp 115K



A'-F' Coomassie Blue stain; A-F autoradiograph

A-A' control; B-B' incubation control; C-C' 1U neuraminidase; D-D' 1U neuraminidase + oligosaccharidase; E-E' .1U neuraminidase; F-F' .01U neuraminidase.

Equal counts were loaded per slot

Tracks to the right of each lettered track are the same sample after Con A affinity chromatography. Equal volumes per slot.

Table 5.1.

Effect of Neuraminidase Pretreatment on Binding of Radiolabelled Cell Surface Proteins to Con A-Sepharose 4B Affinity Columns

	Control	Incubation Control	Neuraminidase
Total Counts Applied to Column	139.6	91.2	56.0
Unbound Counts	71.7	48.1	28.9
% Unbound	51	53	52
% Unbound acetone ppt.	19	15	20

All values x 10^4cpm

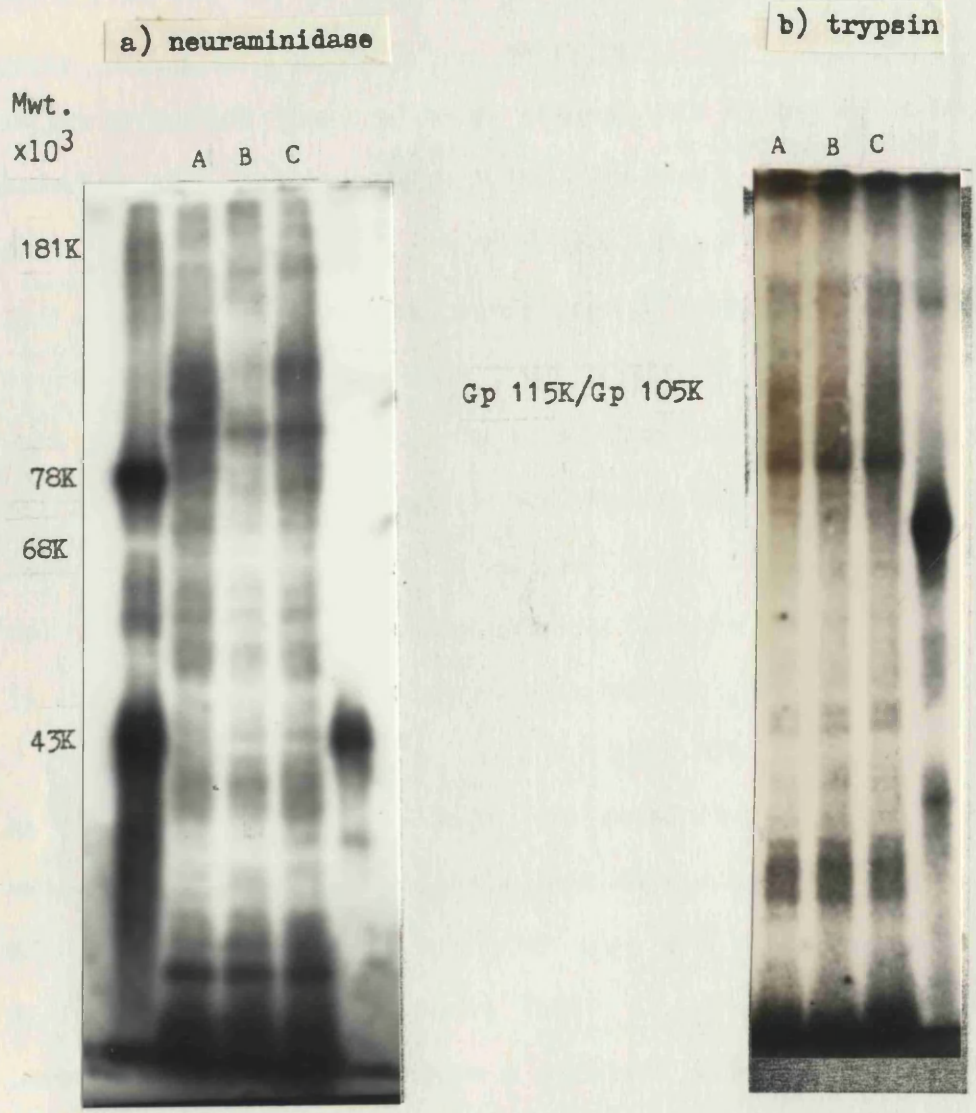
Section 5.3.1 Effect of neuraminidase on the mobility of Gp 115K on SDS-PAGE

Fig. 5.7 (Whole cells) and Fig. 5.8 (TX-100 extracts) show the effect of neuraminidase treatment on prelabelled whole cells and detergent extracts. The most striking observation is the apparent decrease in mobility of Gp 115K after neuraminidase treatment. Other small differences are apparent in Fig 5.8, notably the slight increase in mobility of the 94K band after neuraminidase treatment. This observation would be more expected, given that the molecular weight of the protein would be lower following enzyme treatment. This result is found for serum glycoproteins with N-linked carbohydrate residues e.g. α_2-M (Sutcliffe et al., 1978). The apparent lack of alteration of mobility of most other bands suggests that Gp 115K may be relatively abundant in sialic acid residues. Evidence that these bands are one and the same polypeptide is provided in Fig. 5.8 and Table 5.1, where pretreatment of the protein by elution through immobilized Con A removed interference from other bands. It also suggests that after neuraminidase treatment no internal mannose residues were avaiable for Con A binding. Attempts to find intermediates using various concentrations of neuraminidase were unsuccessful (Fig 5.8).

A precedent for this phenomenom was noted by Brown et al.(1980) on treating rat sialoglycoprotein with neuraminidase. The apparent increase in molecular weight was of the same order of magnitude. Andersson and Gahmberg (1978) using NaB^3H_4 to label human neutrophils noted that when sialic acid residues were labelled a major band of 105K was seen. After neuraminidase treatment and labelling galactose residues this band disappeared and a major band of 130K was seen. They suggest that these bands are identical. For Glycophorin A, however, the situation is rather more complicated, (see Gahmberg and Andersson, 1977) since the dimeric form, PAS 1 shows no alteration in mobility following neuraminidase treatment whereas the monomeric form, PAS 2 shows a slight apparent decrease in mobility.

Fig. 5.9

Binding of ^{125}I-WGA to polypeptides on SDS-PAGE: the effect of enzymatic pretreatment of whole cells.



A incubation control;
B +1U neuraminidase;
C control.

A incubation control; B +10ug/ml trypsin;
C control

Equal protein (25ug) was applied per slot.

Section 5.3.2 Evidence that Gp 115K is the major cell surface receptor for WGA

Fig. 5.6. allows a comparison of Con A and WGA binding to whole cell extracts run on SDS-PAGE. The results suggest that a doublet of approx. mol. wt. 105-115K bound WGA strongly, but not Con A. In order to determine whether this band was cell surface localized, whole cells were pretreated with neuraminidase or trypsin. The results of these experiments are seen in Fig 5.9.

The results in Fig.5.6 suggest that a more limited number of total cell proteins bind WGA compared with Con A. Other workers using different cells have also noted this e.g. Burridge (1976).

The results of the experiments shown in Fig. 5.9. suggest that Gp115K is the major cell surface receptor for WGA. Neuraminidase treatment appears to abolish WGA binding to this molecule suggesting that terminal sialic acid residues bind WGA. The result also suggests that no internal N-acetylglucosamine residues are now accessible for binding on this molecule.

Trypsin pretreatment appears to have no effect on the binding ability or the mobilty of WGA-binding polypeptides run under these conditions, see Fig. 5.9. The results strongly suggest that other WGA-binding proteins are present on internal membrane systems (see Hughes, 1976 for review). Another possibility is that they are trypsin insensitive cell surface membrane proteins which contain internal N-acetylglucosamine residues accesible to WGA binding. The major erythrocyte cell surface sialoglycoprotein, Glycophorin A, binds strongly to WGA under similar experimental conditions, (Robinson et al., 1976). This binding was also completely abolished by neuraminidase.

Bhavandan and Katlic (1979) have suggested, on the basis of their work

and from previous studies on sialoglycopeptides isolated from mouse and human melanoma cells, that glycopeptides having clustered O-glycosydically linked sialyl oligosaccharides are capable of interacting specifically with WGA. They noted that the interaction was through sialic acid residues (rather than through internal N-acetylglucosamine residues) since removal of sialic acid residues abolished the interaction.

The evidence presented in this section suggests that Gp 115K (and its partial cleavage product 105K) is the major WGA receptor on the cell surface, and that since binding is abolished by neuraminidase treatment binding occurs via terminal sialic acid residues. Like Glycophorin A on erythrocytes, (Anstee, 1980) and rat thymocyte 95K sialoglycoprotein on thymocytes, (Brown et al., 1981), Gp 115K may contain the bulk of the net negative surface charge on human neutrophils.

Section 5.3.3. Evidence for O-linkage of Oligosaccharide Sidechains on Gp 115K

O-linked oligosaccharide sidechains are rarely found in mammalian plasma membrane proteins. Of those investigated to date only 'glycophorin' a major membrane component in man, (Anstee, 1980) and cattle, (Kornfeld (1976) and a 'glycophorin-like' molecule described on rat thymocytes, (Brown et al., 1980) contain predominantly this linkage, (see Section 1.6., Table 1.2 and fig. 1.8.). These proteins apparently share a number of other biochemical properties which they have in common with Gp 115K, as outlined in Section 5.2. It was then of some interest to see whether Gp 115K contained largely O-linked oligosaccharide residues. In contrast to other workers who exploited the relative alkali-lability of O-linked residues, I attempted to use a specific endoglycosidase, α-N-acetyl-galactosamine oligo-saccharidase which cleaves specifically at this linkage.

Results and Discussion

In order to be certain that we could identify any cleavage products of Gp 115K, Con A lectin affinity chromatography with appropriate controls was used, (see Section 5.2.). Endoglycosidase treated radiolabelled cells were also pretreated with neuraminidase as recommended by the manufacturers (BRL). Because the enzyme was very expensive a 'no neuraminidase pretreatment' control was not run for this experiment.

The results are shown in Fig 5.8. Examination of the total cell protein profiles reveals differences in mobility of major components including the band tentatively identified as actin on the basis of relative mobility. This throws doubt on the ability to draw conclusions from this experiment since major cell proteins are not thought to be glycosylated. Examination of the autoradiograph reveals that the mobility of other polypeptides has altered in the endoglycosidase track with respect to controls.

Section 5.3.4 Identification of an antigenic determinant on the cell surface of human neutrophils recognised by the monoclonal antibody, F10-44-2.

Introduction

Dalchau et al. (1980b) and Morstyn et al. (1981) describe a mouse

Table 5.2.

F10-44-2 Staph A/HT-2 Immunoprecipitation Experiments

	Actual Counts (50 secs)	'Corrected' Count Rate* cpm	
Staph A			
Background	1296		
PMN +	3429	2570±83	$P \leqslant 0.025$
PMN -	2981	2031±79	
Mono+	2191	1079±71	n.s
Mono-	2004	853±69	
HT-2			
Background	1222		
PMN +	4138	3514±91	n.s.
PMN -	4027	3380±90	
Mono+	1639	503±68	n.s.
Mono-	1632	494±68	

PMN - neutrophils (polymorphonucleocytes)

mono- mononuclear cells

* 'corrected' count rate = $\frac{N_t}{t_t} - \frac{N_b}{t_b} \pm \sqrt{\frac{N_{t_2}}{t_t} + \frac{N_b}{t_b^2}}$

standard deviation (σ) = N

monoclonal antibody, F10-44-2, which recognizes an antigen found predominantly on brain, granulocytes and T lymphocytes. It was established that on mononuclear cells the antigen was a major glycoprotein of the leukocyte membrane of mol. wt. 105K. The authors suggest that there is a striking similarity in biochemistry and tissue distribution to the W3/13 antigen of the rat, (Dalchau et al., 1980 and Brown et al., 1981), and suggest that this is likely to be a human homologue of this antigen. I have shown that the rat 95K polypeptide recognized by W3/13, (Brown et al., 1981) has biochemical features in common with Gp 115K (see Section 5.2.) It would be of some interest then, to be able to show that F10-44-2 recognizes an antigenic determinant on Gp 115K.

Results and Discussion

The results of a search for an antigenic determinant recognised by F10-44-2, are given in Table 5.2 and Fig.5.13. Two different approaches were used to for the immunoprecipitation studies. The results from the Staph. A immunoprecipitation experiment (see Methods, 2.14), Fig 5.13 suggest that some interaction between F10-44-2 and labelled proteins is taking place. When the immunoprecipitates were run on SDS-PAGE gels no bands could be detected, even after prolonged exposure of films (eight weeks). The specificity of this interaction remains unclear. The results are disappointing in that they provide no evidence for recognition by F10-44-2 of an antigenic determinant on Gp 115K and equivocal evidence at best for any antigen recognition on neutrophils or mononuclear cells. There may be the possibility that this particular antibody may not cross-link antigenic determinants, thus not allowing precipitation in the absence of Staph. A, (a partially purified IgG fraction was used- see Section 2). Morstyn et al. (1981) have shown that the antibody binds antigen on cells in the myeloid series in the order:

blast > promyelocytes > myelocytes > granulocytes

Thus the concentration of the antigen diminishes as the committed progenitor cells differentiate to form granulocytes. Morstyn et al. (1981) also show that the antigen is more abundant on the other peripheral blood cell for which it shows reactivity, the T lymphocyte. The % of T lymphocytes in the mononuclear layer population Table 6.13 is not known but was probably in the normal range of 20-45%. Taken together then it appears that mature neutrophils express relatively few antigenic determinants and the mononuclear control population contained only a subfraction of potentially reacting cells. These two points may explain the results obtained.

Chapter 6 A comparison of the cell surface proteins of neutrophils in chronic myeloid leukaemia and controls

Section 6.1 Introduction

Leukaemic neutrophils may differ in a number of different ways from controls all of which may have a direct or indirect effect on the labelling and visualization of glycosylated polypeptides including cell surface glycoproteins. Differences in cell maturation, mean age of circulating cells or metabolism, associated with CML, as well as intrinsic defects in gene expression of cell surface glycoproteins, might be revealed in altered labelling of cell surface polypeptides.

Cell surface heterogeneity of neutrophils and monocytes due to the presence of functional subpopulations of these cells has recently been postulated by Barrett et al. (1979). If such subsets exist there is the possibility that relative expansion of one of these subsets in any given CML may be possible. Barrett et al. (1979) point out that these apparent differences between cells may merely reflect differences in cell maturation within the myeloid cell lineage.

Altered glycosylation of a given polypeptide may accompany cell differentiation (e.g. Thy-1, Hoessli et al. 1980). These differences might be revealed by altered mobility on SDS-PAGE.

Differences in cell metabolism may have a quantitative effect on labelling. The neutrophil contains endogenous peroxides and peroxidases, (Odeberg et al., 1974 and Clark and Klebanoff, 1980). In Section 3 it was demonstrated that these endogenous enzymes, released from cells, may contribute to the labelling of cell surface proteins.

CML neutrophils contain normal amounts of myeloperoxidase, (El-Maallem and Fletcher, 1976) but are defective in H_2O_2 production, (El-Maallem and Fletcher, 1979). Therefore a reduction in the efficency of endogenous

Table 6.1a

Clinical Information on CML Blood Samples used in this Study

Sample No.	Patient	Sex	Clinical Diagnosis	wbc x 10^9 litre	Drug Therapy	Cytogenetic Analysis
17. 4.80	G.McD	M	CML	30	-	+
29. 4.80[a]	J.N	M	CML-ALL	2-10	+	+
29. 4.80[b]	T.H	M	prv/mp	50	+	-[c]
9. 5.80	I.W	F	tcy/mp	10-50	-	-
13. 5.80	E.F	F	CML	100	NA	+
22. 5.80[a]	W.R	F	mp-ALL	2-10	+	-
22. 5.80[b]	E.S	F	CML	>50	-	+[c]
23. 5.80	J.O'D	M	anaemia	10-50	-	-
27. 5.80	J.H	F	mp	10-50	+	-
30. 5.80	R.H[1]	M	CML	NA	+	+/-[c]
5. 6.80	-.P	F	CML	NA	NA	NA
11. 6.80	A.J	M	CML	10-50	-	-[c]
24. 7.80[a]	W.R	M	leuk	15	NA	NA
24. 7.80[b]	R.H[2]	M	CML	<9	NA	+/-
31. 7.80	R.H[3]	M	CML	10-50	-	NA
14. 8.80	A.McI	F	CML	>100	NA	+/-
25. 8.80	H.B[1]	F	CML	>50	-	NA
5. 9.80	H.B[2]	F	CML	>50	+	NA
16. 9.80	M.McC	F	CML	>50	-	+
17. 9.80	R.L	M	CML	NA	NA	NA
19. 9.80	M.McC	M	CML	>50	-	+
23.10.80	C.H	F	prel/mp	11	NA	NA
18.11.80	J.A	M	CML	150	-	+

Table 6.1b

21.11.80	E.E[1]	F	CML	>50	-	+
25.11.80	W.C	M	CML	>50	-	NA
28.11.80	W.S	M	CML	>50	-	+
8. 1.81	R.McC	M	CML	10-50	-	NA
10. 2.81	M.MacQ	F	CML	NA	NA	NA
13. 2.81	S.M	F	NA	NA	NA	NA
17. 2.81	H.B[3]	F	CML	10	+	+/-
23. 2.81	J.J	M	CML	>50	+	+
7. 4.81	J.F	M	CML	19	+	-
23. 4.81	J.P	F	CML	11.9	+	+c
27. 4.81	M.H	F	CML	177	NA	+
13. 5.81	J.F	M	CML	2-10	+	+
28. 5.81	E.E[2]	F	CML	4-7	+	+

Key to abbreviations used in Table 6.1

29.4.80[a]- designation of sample when more than one sample processed on same date

H.B[2] - designation of sample when more than one sample received from same patient

Clinical Diagnosis - CML :Chronic Myeloid Leukaemia

ALL :Acute Lymphocytic Leukaemia

mp :myeloproliferative disorder

prel :'preleukaemia'

thc :thrombocythaemia

prv :polycythaemia ruba vera

Cytogenetic Analysis - NA :Analysis not done / not possible

\+ :Philadelphia (Ph') chromosome present

\- :Ph' chromosome absent

+/- :Ph' chromosome mosaicism

+c :Additional chromosomal abnormalities

Table 6.2 Sample Data

	^{125}I-LPO	^{125}I-WGA	^{125}I-Con A
A		17. 4.80	17. 4.80
B		29. 4.80[a]	29.4.80
C		13. 5.80	13. 5.80
D		27. 5.80	27. 5.80
E		30. 5.80	30. 5.80
F		5. 6.80	5. 6.80
G		25. 7.80	25. 7.80
H		14. 8.80	14. 8.80
I		5. 9.80	5. 9.80
J		17. 9.80	17. 9.80
K		19. 9.80	19. 9.80
L	18.11.80	18.11.80	18.11.80
M	21.11.80	21.11.80	21.11.80
N	25.11.80	25.11.80	25.11.80
O	28.11.80	28.11.80	28.11.80
P	8. 1.81	8. 1.81	8. 1.81
Q		29. 1.81	
R	10. 2.81	10. 2.81	
S		13. 2.81	
T		17. 2.81	
U	23. 2.81		
V	7. 4.81	7. 4.81	
W	23. 4.81	23.4.81	
X		27. 4.81	
Y		13. 5.81	
Z	28.5.81		

H_2O_2-dependent myeloperoxidase catalysed iodination could be reflected in altered levels of cell surface labelling, since labelling does occur in the absence of exogenous enzymes, (see Section 4.2).

It should be emphasised that a group of CML patients were made use of in this study. Samples from those patients who had significantly raised levels of other granulocytes (eosinophils or basophils) were effectively eliminated since these cell types were co-purified with neutrophils, as were samples in which there were insufficient numbers of cells for analysis, ($<10^7$). Some 20% of samples were discarded for this reason. No attempt was made to remove these contaminating cells since to do so would have made the experiments too unwieldy and in many cases would have resulted in too few cells for analysis. Patient data and sample data is provided in Tables 6.1 and 6.2.

In order to try and detect any alterations in the glycosylation of molecules at the molecular level, a slightly modified form of the ^{125}I-lectin overlay method of Robinson et al. (1975) and Burridge (1976), was used, (see Section 5). This method was chosen for its convenience, including the ability to compare a number of samples directly. Differences in glycosylation might be expected to result in alteration of the amount of radiolabelled lectin bound to a given polypeptide and also to result in an alteration of mobility on SDS-PAGE. This method may be regarded as being more 'democratic' than affinity chromotography, (Hughes, personal communication) since, given that there is an excess of lectin, competition effects between glycoproteins with different affinities for the same lectin binding sites, may be reduced. Thus there may be a more direct relationship as visualized by autoradiography between the amount of lectin bound and the number of binding sites available.

WGA (Wheat Germ Agglutinin) and Con A (Concanavalin A) were the lectins used in this study. Con A binds to monosaccharides known to be available for binding on most cell surface glycoproteins of the human neutrophil,

Table 6.3

Specific activity of labelled proteins from neutrophils after Triton X-100 Extraction

	Date	Input	TX-100 TCA ppt.	Protein	Specific Activity
		cpm	cpm x 10^6	mg	cpm x 10^6 $\cdot$ ug^{-1}
Controls					
1	31. 7.80	300	.32	.15	2.1
2	27.10.80	340	.61	.15	4.1
3	29.10.80	350	.88	.29	3.0
4	29.10.80	350	.81	.20	4.1
5	31.10.80	360	.31	.11	2.8
6	31.10.80	360	.75	.14	5.4
7	7. 4.81	360	.43	.20	2.2
8	24. 4.81	370	.71	.23	3.1
CML Samples					
C	13. 5.80	360	.76	.23	3.3
F	5. 6.80	350	.49	.16	3.1
L	18.11.80	410	.82	.24	3.4
M	21.11.80	410	.38	.44	0.9
N	25.11.80	410	1.31	.34	3.9
O	28.11.80	400	.95	.18	5.3
P	8. 1.81	420	.56	.43	1.3
R	10. 2.81	360	.26	.21	1.2
U	23. 2.81	340	.92	.25	3.7
V	7. 4.81	360	.62	.27	2.3
W	23. 4.81	370	.27	.09	3.0
Z	28. 5.81	410	.64	.20	3.2

Fig. 6.1. Comparison of the specific activity of radiolabelled protein from Control and CML samples

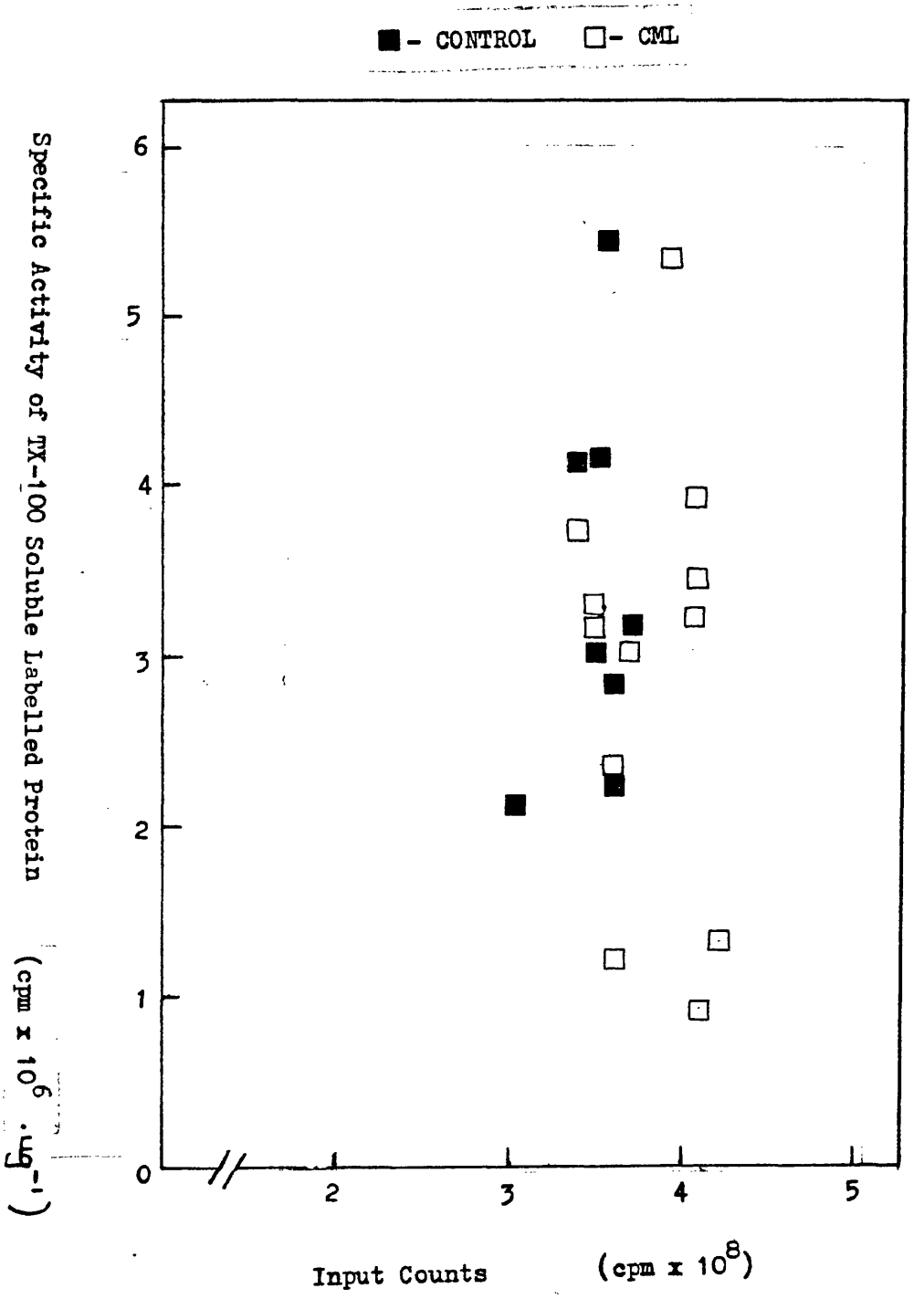
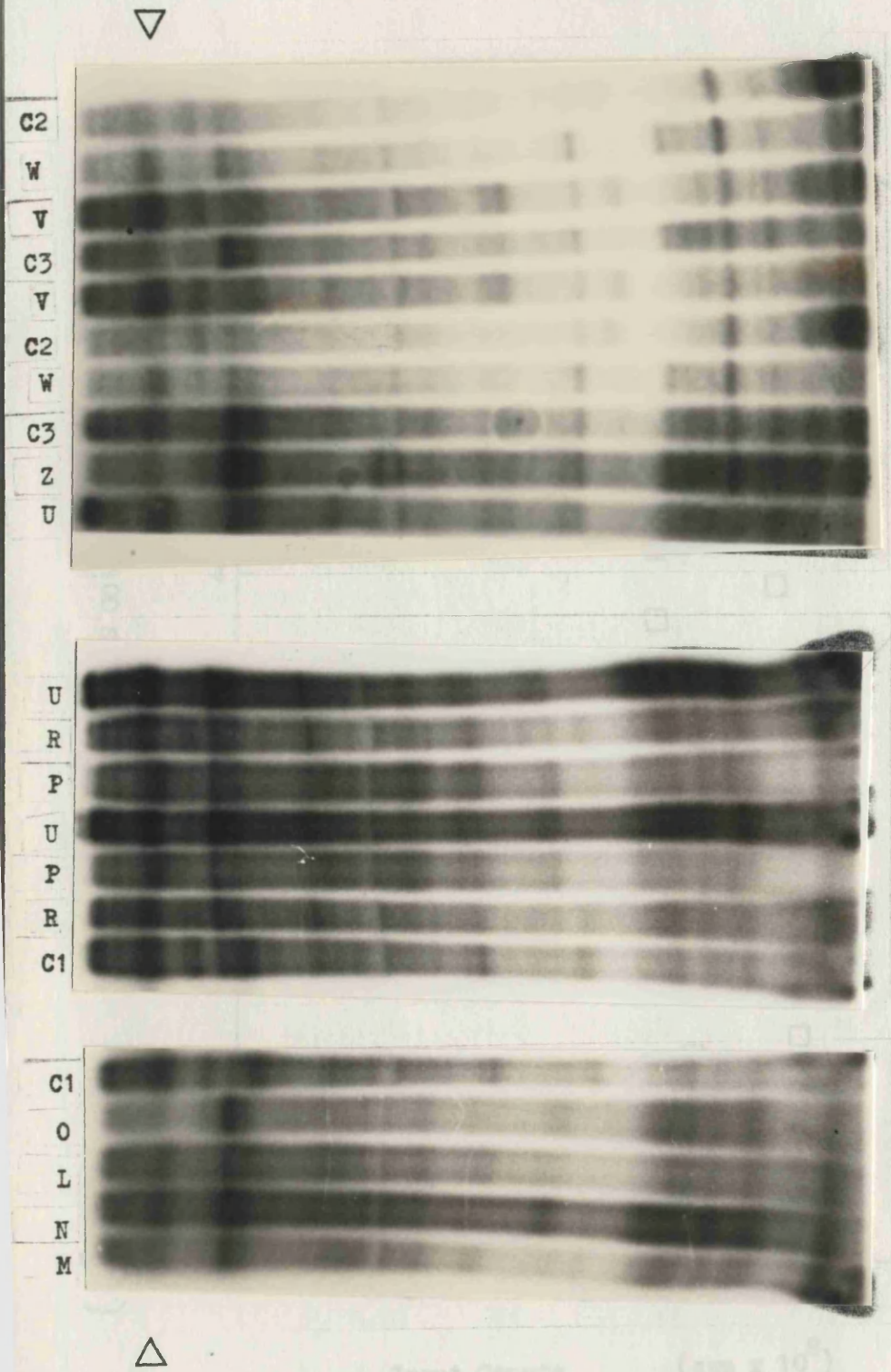


Fig. 6.2. SDS-PAGE of lactoperoxidase ^{125}I-labelled, TX-100 solubilised protein from CONTROL and CML samples



Samples were run on a 5.5%-12.5% gel. Equal counts were loaded per slot.

The autoradiographs a, b and c were exposed for 12, 8 and 14 days respectively.

for letter code see patient list, Tables 6.2. and 6.1.

C1 - C3 Controls

(see Chapter 5). Using Con A it is therefore possible to 'scan' a large number of glycoproteins simultaneously, for alterations in glycosylation. WGA was chosen because of interest in the possible variability of expression of sialic acid residues and in particular variability of expression of Gp 115K.

In assessing any apparent difference in glycosylation it needs to be borne in mind that whole cell extracts are being 'screened' and the assignment of any given glycoprotein to the plasma membrane must be established by other methods as has been done for Gp 115K, (see Section 5).

Possible differences due to sample handling and storage were allowed for. Control samples used in the experiments had been stored for the same length of time as CML samples in any given experiment.

Taken together then the two complementary techniques of direct labelling of surface polypeptides and lectin-binding to polypeptides on SDS-PAGE provide a powerful method for looking for alterations in glycosylation of surface glycoproteins. Alterations may well be reflected in both systems by alterations in mobilty. Each system also provides a different set of controls, i.e. other labelled cell-surface polypeptides, and other suitably glycosylated polypeptides in the whole cell extracts.

Section 6.2 Direct labelling of the polypeptide chain using the ^{125}I-lactoperoxidase method

Results

The results of these comparative labelling experiments are shown quantitatively in Table 6.3 and Fig 6.1 and qualitatively in Fig 6.2.

The results expressed in Fig. 6.1 suggest that their is no quantitative

Table 6.4.

Statistical Analysis of Quantitative Data (Table 6.3. and Fig 6.1.)

	Controls	CML Patients
Sample No.	8	12
Regression Analysis(from Fig 6.3)		
correlation coefficient	-0.22	-0.01
Specific Activity of labelled protein ($cpm \times 10^6 \cdot ug^{-1}$)		
mean	3.35	2.88
variance	1.09	1.48
F-Test	1.48 (df=11) = 1.36 (95% confidence level)	
	1.09 (df=7) F=3.64	
t-Test (df=18)	Difference between means = 0.47 [Confidence Interval(95% level)= +1.044- -1.984]	

difference in labelling of neutrophils from controls and CML patients. This finding is confirmed in Table 6.4 where Regression analysis reveals no correlation between Input Counts and Specific Activity of labelled protein in detergent extracts from both Control and CML cells over the range measured. Thus we can be reasonably sure that the label is in excess compared with the number of available iodinatable sites.

Although reaction conditions were standardised Table 6.3 shows that there was some variation in the amount of ^{125}I added to the reaction mix and considerable variation in the amount of solubilised protein recovered. Inaccuracies in cell counting prior to labelling may account for some of this variation.

analysis of variance, (Table 6.4) does not allow us to reject the null hypothesis that the Control samples and the CML samples are taken from the same population. Thus the results suggest that the same number of iodinatable sites on the cell surface are available on neutrophils from Controls and CML patients.

The qualitative results obtained from autoradiography of SDS-PAGE gels are shown in Fig. 6.2. 5.5%-12.5% gels were used in order to obtain maximum clarity of bands but this has resulted in the minimising of differences in mobility between tracks of high molecular weight bands.

The results demonstrate the large variation in labelling intensity of individual bands. The doublet Gp 115K/105K (arrowed), which on these gels appears as a single 'block' appears to show a particularly large variation in mobility compared to polypeptides of higher or lower molecular relative mobility. However this does not relate to whether the sample is from a CML patient or not. Gahmberg and Andersson (1979) who noted that Gp 130, (see Sections 1.7 and 1.8) which is equivalent to Gp 115K, was more diffuse in 'leukaemic' cells from CML patients than controls. No evidence for this is provided in this experiment.

The 95K band in these experiments, shows considerable variation in

intensity, but no or little variation in relative mobility. The reason for this result is not clear.

Section 6.3 Labelling of oligosaccharide residues of glycoproteins using ^{125}I-lectin overlay on SDS-PAGE.

Introduction

Taub et al. (1980), on the basis of lectin agglutination studies with whole cells, (granulocytes) have suggested that in CML, sialic acid residues or hypersialylated glycoproteins may mask cell surface receptors for lectins resulting in altered agglutination properties of these cells, (for summary of results see Section 1.7.). They noted that the alteration of agglutination of CML granulocytes was partially reversed by neuraminidase treatment.

I have shown in Section 5.3. that the major WGA receptor on the neutrophil cell surface is Gp 115K. The lectin binds largely to terminal sialic acid residues, (Bhavanandan and Katlic, 1979), the binding being largely abolished by neuraminidase treatment, (see Fig.5.9). Demonstration of alteration in glycosylation of the molecule, particularly with respect to sialic acid residues, might go some way to provide a molecular basis for the results of Taub et al, (see Section 1.8.). Alterations in glycosylation of the molecule might affect molecular conformation in such a way that agglutination of cells was inhibited possibly by interference with clustering of suitably glycosylated molecules. Alternatively Taub's observations may involve more subtle changes possibly involving the ability of appropriate membrane proteins and cytoskeletal elements to interact to allow clustering of receptors to take place.

In order to maximise possible variations in mobility of high molecular weight polypeptides samples for ^{125}I-lectin overlay were run on single concentration 8% gels.

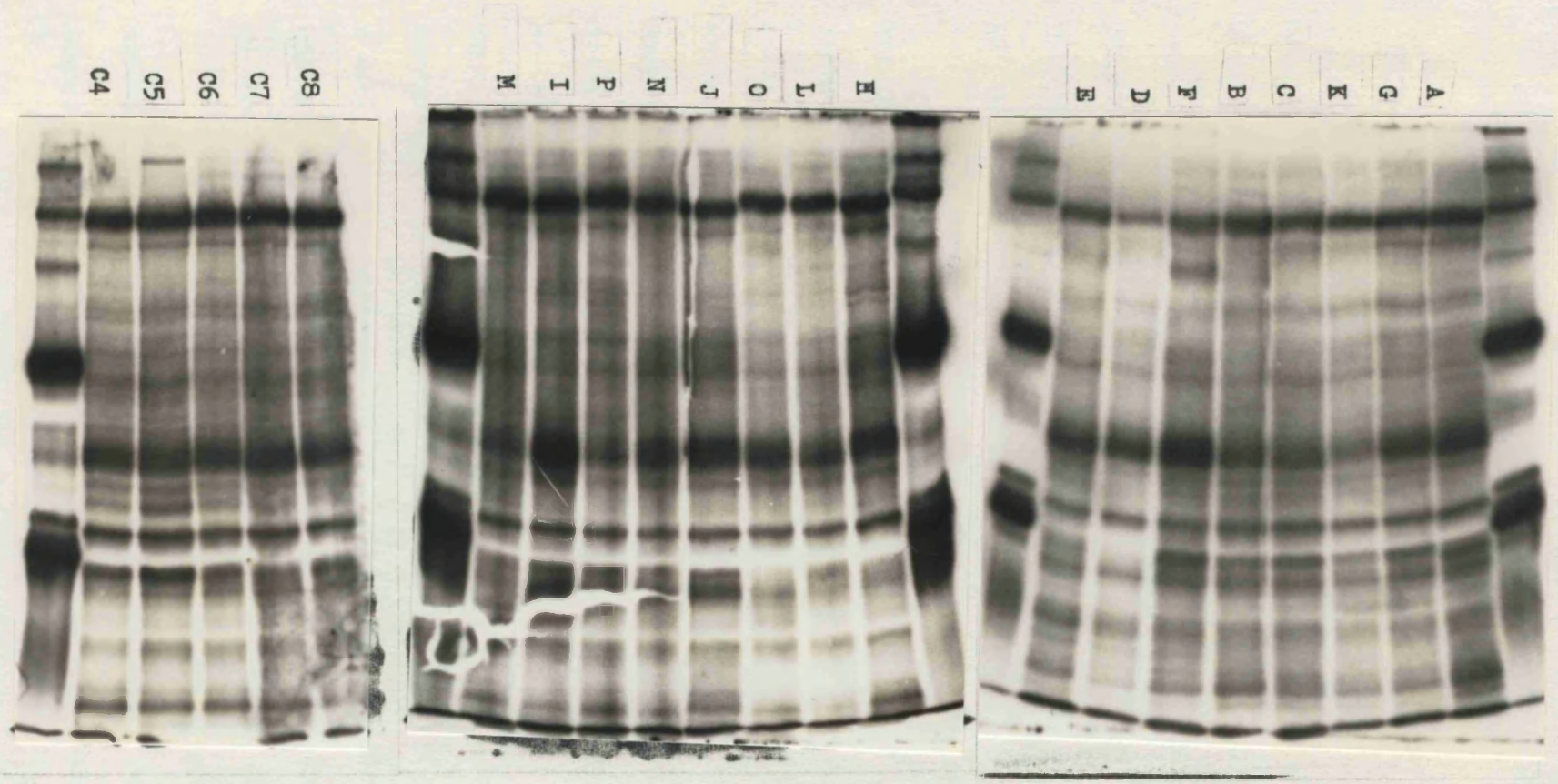


Fig. 6.3. ^{125}I-Con A overlay of SDS-PAGE of CONTROL and CML samples

For letter code see Tables 6.1. and 6.2. Samples were run on an 8% gel. Equal protein (33ug approx.) were laoded per slot.

The autoradiographs were exposed for 3 days

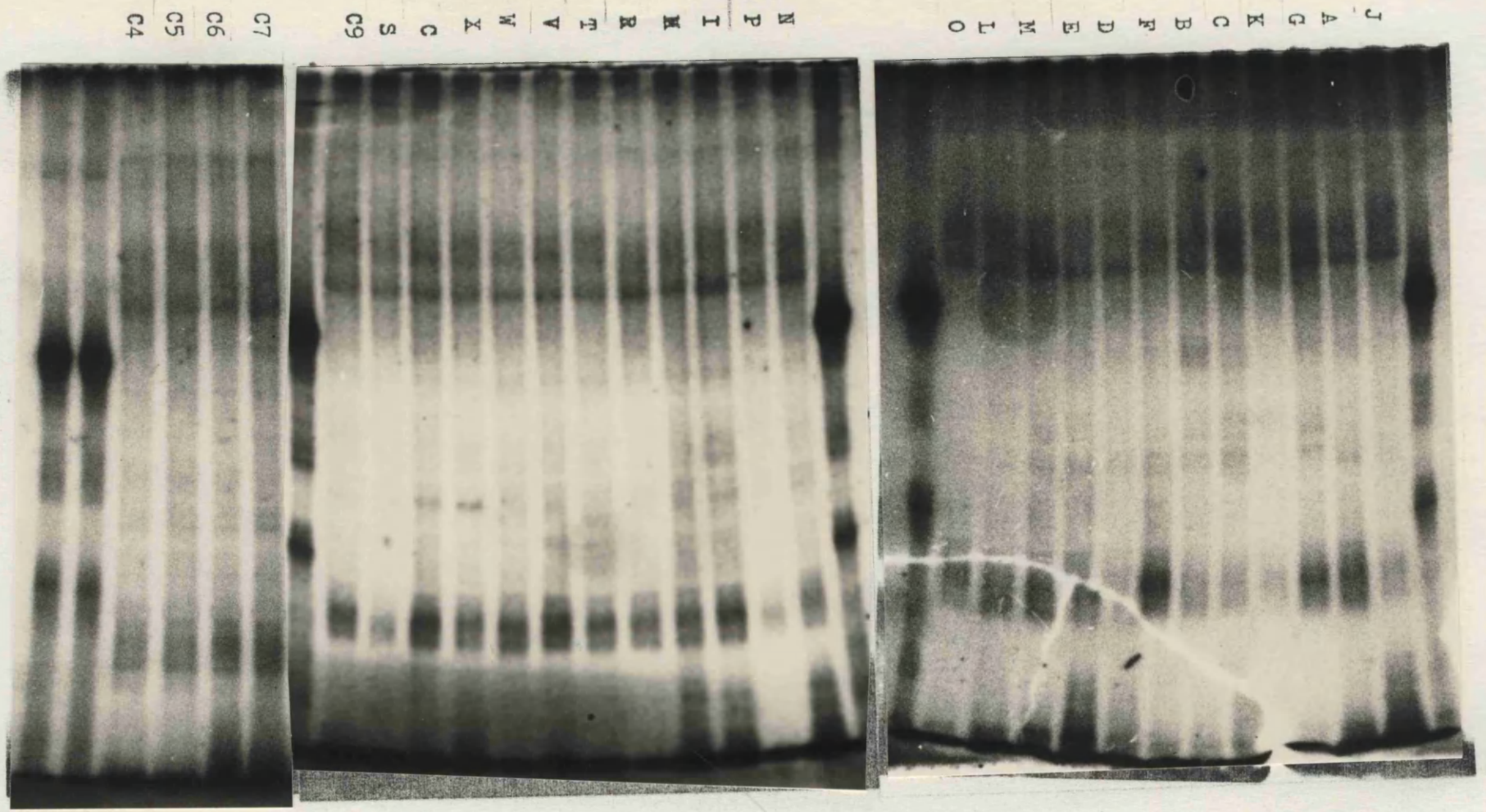


Fig. 6.4. ^{125}I-WGA overlay of SDS-PAGE of CONTROL and CML samples

Equal protein (25ug) was loaded per slot.

The autoradiographs were exposed for 8 days

Results

Controls for the ^{125}I-Con A overlay experiments, ^{125}I-Con A overlay of red cells and control polypeptides, are described in Chapter 5, (fig. 5.1 and 5.3). The qualitative results of the comparative experiments are shown in Fig.6.3.

Control C5 bound ^{125}I-Con A particularly strongly to a high molecular weight band (gel a, Fig. 6.3). The significance, if any, of this observation was not investigated further. CML samples in general show more variability in Con A binding but no consistent differences either in amount of Con A bound to any given band nor the mobility of any bound polypeptide is apparent.

The results of the ^{125}I-WGA overlay experiments are shown in Fig. 6.4. These results are technically of poor quality compared with fig. 6.3. Nevertheless it is possible to discern a diffusely staining region corresponding to the doublet Gp115K/105K. Again no real differences are discernible between CML samples and Controls. Within the limits of the gels then, Gp 115K/105K does appear to show relative variation in mobility in relation to a reference band running in front of it on the gel.

Section 6.4. Conclusion

The above results suggest that glycosylation of polypeptides and in particular the expression of cell surface proteins in CML neutrophils is remarkably stable. Differences in the degree of variation and intensity of various bands were noted, but cannot be related to CML.

Binding of ^{125}I-WGA in general, and to the major receptor for WGA on the cell surface, Gp 115K, in particular does not appear to be altered in CML. Andersson and Gahmberg, (1979) noted that Gp 130K, (equivalent to Gp 115K, see Table 3.6 and Section 1.7.) was more diffuse in CML.

The apparent diffuseness of the polypeptide in fig. 6.3 in all samples would appear to reflect its relative susceptibility to partial hydrolysis, (see Section 4.2).

Taub et al. (1980) demonstrated that a sub-population of non-nylon wool adherent neutrophils showed altered lectin agglutination properties and suggested that hypersialylated cell suface glycoproteins might mask receptor sites for lectins, or that novel gene products were being expressed at the cell surface. Since my results, (fig. 6.1 and 6.3), suggest that Gp 115K shows no alteration in mobility in CML neutrophils, and yet neuraminidase treatment, at low concentrations, results in an alteration in the mobility if this band, one might expect differences in sialylation of this polypeptide to be reflected in alteration in mobility of Gp 115K. Thus, in order to reconcile my results with Taub et al's. a more subtle explanation for the altered whole cell lectin agglutination properties they noted must be sought.

Fishman et al. (1981) noted that in neuroblastoma cells, Con A receptors become immobilized during differentiation. Although interestingly no parallel effect was noted for WGA receptors in this cell. Since it seems reasonable to assume that receptor mobility and cell agglutination are linked, (see Hughes, 1976, for review), the ability of CML neutrophils to agglutinate may be a product of their degree of differentiation. Since CML neutrophil samples may contain a proportion of not fully differentiated cells this may result in altered lectin agglutination properties. Taub et al. (1980) claim that this effect is partially reversible by treatment of the neutrophils with neuraminidase. This result is less easy to reconcile with the apparent lack of difference in the expression of Gp 115K in CML. This result suggests that there are differences at the cell surface involving sialylated species. This is not immediately reconcilable with my results. Thus the significance of the observations made in this Section with respect to Taub's findings remain

unclear, and further study is required.

The results presented in this chapter then provided no evidence for the alteration of the expression of cell surface proteins nor for any significant alterations in the glycosylation of molecules in these cells, (as judged by Con A and WGA binding.). Instead the results demonstrate the remarkable stability of gene expression in these leukaemic cells compared with control cells.

This study was undertaken to determine whether simple consistent differences in the expression of cell membrane proteins exist between neutrophils isolated from patients suffering from chronic myeloid leukaemia and those from controls.

The aims of the study were threefold. Firstly, biochemical identification and characterisation of the cell surface proteins of the human neutrophil, particularly with respect to the glycosylation of these molecules, is intrinsically interesting and of course provides a basis for comparison of controls with leukaemic cells. While much attention has been paid to the characterisation of lymphoid cell surfaces information about myeloid cell surfaces is comparatively scarce.

Secondly, it would be useful to determine whether there are differences in the expression of cell surface proteins of the neutrophil between CML patients and controls. Such information might be of diagnostic and even of therapeutic interest.

Thirdly, it was hoped that the study would provide some gene mapping information about the cell surface membrane proteins under study.

Initial studies concentrated on establishing adequate information from controls. This work provided useful information about the organizatiion of membrane proteins of the cell chosen for study: the neutrophil.

Cell surface proteins of human neutrophils, obtained from peripheral blood samples, were labelled using the ^{125}I-lactoperoxidase technique. SDS slab gel electrophoresis revealed at least 13 labelled polypeptides of mol. wt. >200K - mol. wt. >40 K, one of which comprised or contained auto-labelled lactoperoxidase, (see Table 3.4). These labelled polypeptides did not appear to correspond with any of the Coomassie blue staining bands. The results suggest that membrane proteins exposed to the external milieu represent a very small fraction of the total cell protein in common with

other cells, (Bretscher and Raff, 1975).

As the cells contain a peroxidase-dependent, halogenating system, it was important to demonstrate that the labelled polypeptides were present on the cell surface. This was confirmed by three independent methods: labelling in the presence and absence of exogenous enzymes, trypsin sensitivity and plasma membrane isolation.

Extraction with the non-ionic detergent, Triton X-100 considerably reduced problems of handling and proteolytic digestion associated with this cell. A comparison of Coomassie blue staining bands and labelled bands from whole cell extracts and detergent extracts suggests that non-ionic detergents, (TX-100 and NP-40), extract polypeptides selectively. Thus for Coomassie blue stained proteins (Figs 3.4 and 3.5 and Table 3.5) a major band of mol. wt. 85K and to a lesser extent a band of mol. wt. 25K are selectively retained in the residual material after nonionic detergent extraction. Labelled bands apparently selectively retained, particularly a relatively broad band polypeptide, mol. wt. 55-60K, are polypeptides 9-12 (see Table 3.4 and Figs. 3.4., 3.6. and 3.7.). It is of interest to note that Schneider et al. (1981) have recently isolated a biologically active human macrophage receptor for the third component of complement. This was a cell surface, LPO-iodinatable polypeptide of mol. wt. 64K which migrated as a broad band. Mature neutrophils also express this receptor at the cell surface, (Ross et al., 1978). Such selective retention of polypeptides in a low speed pellet after detergent extraction could be due to selective trapping of soluble proteins or relative resistance of such proteins to proteolytic attack from disrupted lysosomal granules in the residual pellet. Willinger and Frankel, (1979), working with labelled rabbit neutrophils noted that three bands of apparent molecular weight, 120K, 66-68K and 60-62K were enriched in low speed pellets and by disrupting nuclei and observing the same selective retention concluded that these polypeptides were selectively binding to chromatin. It is

possible given the apparent similarity of labelling of rabbit and human neutrophils using this technique, (see Table 3.6), that the polypeptide of apparent mol.wt. 55-60K is the homologue of one of the lower molecular weight bands described above.

Other workers have made similar observations and suggest that a detergent insoluble matrix may exist consisting of cytoskeletal elelments including actin, and that linkages between this matrix and certain transmembrane proteins are retained following detergent solubilisation, which may have functional significance, (Flanagan and Koch, 1978; Mescher et al, 1981; and Sheterline and Hopkins, 1981)

These experiments then, established that the human neutrophil in common with other cells so far studied has a small number of relatively high molecular weight proteins present at the cell surface. These observations confirm and extend those of Andersson and Gahmberg, (1979) who labelled these cells with a more selective technique, (see Section 1.7 and Table 3.6). The results presented here compare closely with similar studies on rabbit neutrophils (Willnger and Frankel, 1979) and pig neutrophils (Sheterline and Hopkins, 1981), (see Section 1.7), confirming the specificity of the technique and emphasing the apparent evolutionary conservation of these cell surface proteins.

The role of glycosylation of membrane proteins is still not clear but it was important to establish whether differences in glycosylation of membrane proteins existed between neutrophils from CML patients and controls. Such differences might reflect differences in maturation or defective gene expression in mature cells. Alteration of glycosylation of Thy-1 antigen, (resulting in increased sialic acid content), is known to accompany T-lymphocyte differentiation (Hoessli et al., 1980) and it was hoped that the study of the glycosylation of neutrophil membrane proteins might be a particularly sensitive means of assessing the maturity of these cells.

Experiments using Concanavalin A (Con A) affinity chromatography showed, as predicted, that most of the labelled polypeptides were glycosylated. A noteworthy result was that one major band showed no binding for Con A either by affinity chromatography or by the use of ^{125}I-Con A overlay on SDS-PAGE. This polypeptide, Gp 115K/105K, runs as a doublet on SDS-PAGE, as a result of partial hydrolysis of the Gp 115K band. This band appears to be particularly sensitive to partial hydrolysis and in experiments examining the trypsin sensitivity of radiolabelled proteins the hydrolysis was particularly clear, (see Section 4.3). Willinger and Frankel, (1979) noted a similar partial hydrolysis in a polypeptide of similar relative mobility which is probably equivalent to Gp 115K.

In Chapter 5 evidence was produced that Gp 115K is the major cell surface receptor for Wheat Germ Agglutinin (WGA) which binds to terminal sialic acid residues. Such sialylated glycoproteins have been shown to be of great biological significance on other circulating cells. Woodruff and Gesner (1969) have shown that neuraminidase treated peripheral lymphocytes do not home to lymphoid organs when injected intravenously into syngenic hosts but are quickly trapped in the liver. Thus changes in the structure of glycoprotein carbohydrates may be as important for cellular function as quantitative changes which occur in the amounts of cell surface protein expressed.

In order to study this polypeptide more closely it was hoped to make use of a monoclonal antibody, F10-44-2, which recognises a brain/thymocyte/granulocyte antigen. This antigen is expressed on 100% of granuloctyes, has a molecular weight of 105,000 daltons and is probably the human homologue of the rat W3/13 antigen, (Dalchau and Fabre, 1980). Since W3/13 shares a number of biochemical characteristics with Gp 115K it seemed reasonable to expect that F-10-44-2 would recognise Gp 115K. As may be seen in Chapter 5 it was not possible to establish that the

antibody recognised Gp 115K.

When labelled cells were treated with neuraminidase there was an apparent decrease in the mobility of Gp 115K, (other labelled bands showed no significant alteration following neuraminidase treatment). This unexpected observation, that a decrease in molecular weight produces a decrease in mobility on SDS-PAGE (and therefore an apparent increase in molecular weight) obviously has important consequences for the interpretation of results from SDS-PAGE gels. Neuraminidase treatment of the rat W3/13 antigen produced a similar effect resulting in a proportional, apparent increase in molecular weight of the desialylated polypeptide, (Brown et al., 1981). Gahmberg and Andersson (1977) noted that after neuraminidase pretreatment labelling of red cells, PAS-2, (Periodic acid Schiff stain-glycophorin A monomer) but not PAS-1 (glycophorin A, dimer) and 'Gp 9' (a glycoprotein from T cells) showed a similar effect. It is interesting to note that PAS-2 (consisting largely of glcophorin A monomer) showed this apparent decrease in mobility following neuraminidase treatment whereas PAS-1 (glycophorin A dimer) did not, (for a summary of glycophorin nomenclature see Harrison and Lunt, 1981). Gahmberg et al. (1979) suggest that such an effect may be peculiar to glycoproteins with predominantly O-linked residues. It is interesting to note that to my knowledge this effect has only been observed in plasma membrane glycoproteins.

Binding of WGA to Gp 115K was abolished by neuraminidase treatment of whole cells. Thus Gp 115K was unequivically localized to the cell surface by two independent methods. After neuraminidase treatment the polypeptide still did not bind to Con A affinity columns. This suggests that the oligosaccharide chains do not contain internal mannose residues. There was the possibility that the carbohydrate residues in common with other glycophorin-like molecules, (Brown et al., 1981) might consist of short tetrasaccharide sidechains, (see Section 1, Fig. 1.7). To test the

implication that in common with other sialoglycoproteins, Gp 115K contains largely O-linked oligosaccharide residues, the polypeptide was treated with an endoglycosidase, N-acetyl-galactosamino-oligosaccharidase, that specifically recognises the usual O-linkage, (Serine-galactosamine). The result of this experiment was inconclusive and so the question remains open on this point.

The mobility of this band was apparently not affected by running samples on SDS-PAGE with or without reducing agent. This suggests that no inter- or intrachain disulphide bonds were present and therefore the molecule may lack secondary and tertiary structure.

From the above evidence, the polypeptide appears to be a major sialoglycoprotein at the cell surface of the neutrophil in humans. This finding may be of general interest since it shares a number of features, described above, with other 'glycophorin-like' sialoglycoproteins described in the literature. The function of this unusual class of cell surface glycoproteins is not clear, but they appear to make a significant contribution to the net negative charge at the cell surface and to lack secondary and tertiary structure in the extracellular portion. 'Glycophorin-like' membrane proteins may be a feature of circulating cells, although no molecule with these features has been demonstrated on B cells.

In summary then the first aim of this work, to characterise the cell surface proteins of the neutrophil was achieved. Clearly this part of the study could be extended in a number of ways. 2-D gel electrophoresis could be used to investigate the homogeneity of the labelled polypeptides. The use of radiolabelled lectin overlay in conjunction with 2-D gel electrophoresis might provide a powerful method of detecting carbohydrate microheterogeneity within broad bands such as Gp 115K. Purification of Gp 115K could be achieved with WGA affinity chromotography. This would result in the copurification of Gp 105K. The apparent relationship between the

two species might be determined by peptide mapping. Again using peptide mapping techniques in conjunction with WGA overlay it may be possible to determine whether the supposition that hydrolysis of Gp 115K is occurring at the extracellular terminal portion of the polypeptide. Preparative electrophoresis or affinity chromatography of Gp 115K, coupled with specific endoglycosidase treatment might allow the type of the protein-carbohydrate linkages to be determined. Other approaches might use beta-elimination experiments to exploit the alkali lability of O-linked carbohydrate residues, (see Fig. 1.8 and Brown et al., 1981) or by the use of lectins such as that derived from Maclura aurantica, (Tanner and Anstee, 1976), which are putatively specific for O-linked carbohydrate residues.

Two complementary techniques, allowing the partial biochemical characterisation of the cell surface proteins, were used to compare CML cells with controls. Comparisons were made by direct labelling of the polypeptide chain of cell surface proteins using the ^{125}I-LPO method and indirectly by lectin overlay of whole cell detergent extracts run on SDS-PAGE in order to try and detect alterations in glycosylation. It should of course be noted that the latter technique provides no evidence in itself for cell surface localization of labelled proteins. Although it has been shown in this study that Gp 115K may be visualized using this method.

The results suggest that large scale alterations in the expression of glycoproteins, and in particular cell-surface glycoproteins, do not occur in neutrophils in CML. Andersson et al., (1979), suggest that Gp 130 (the desialylated equivalent of Gp 115K) is relatively more intensely labelled and at the same time more diffuse. No such differences were found in this study.

Gp 115K can be visualized using both experimental approaches, both by direct labelling of the polpeptide and by ^{125}I-WGA overlay. In the first

part of this study this polypeptide was partially defined biochemically, as a major cell surface sialoglycoprotein. No significant variation in this polypeptide may be seen using direct labelling of the polypeptide. or with respect to ^{125}I-WGA overlay. Gp 115K (and its partial product Gp 105K) may show an increased variability in expression, compared with other bands, although the autoradiographs are of poor quality, but given the particular susceptibility of this band to partial hydrolysis, (see Section 4.2) this result is unsurprising.

Taub et al. (1980) observed increased agglutination of CML neutrophils, with respect to controls and patients with benign leukocytosis, using Limulin, a lectin specific for sialic acid residues. They also noted a reduction of reactivity with Con A and Peanut Agglutinin (PNA) which recognises galactosyl residues. They suggested that in CML, sialic acid or hypersialylated glycoproteins may mask other cell surface receptors and that these alterations may reflect the synthesis of a unique glycoprotein by these cells in CML, a relative increase in normal sialylated glycopeptides or synthesis of glycoproteins containing more sialyl groups per polypeptide chain. Whether such variation merely reflects the relative immaturity of neutrophils in CML is not clear. The results of this work suggest that CML cells show no differences in glycosylation with respect to polypeptide binding using the WGA and Con A overlay method and therefore provide no evidence for any simple alteration in the expression of cell surface proteins which would account for the altered agglutination proerties.

Cell agglutination however is a complex phenomenom (see Hughes, 1976 for review) and it is quite possible that some more subtle alteration in the interactions between cell surface protein and cytoskeletal elements may be responsible for Taub et al.'s results. In neuroblastoma cells there is increasing immobilization of Con A receptors during differentiation although interestingly no change in the mobility of WGA receptors was

noted, (Fishman et al., 1981). However Taub et al. noted that the altered lectin agglutination properties of CML cells were partially reversible by neuraminidase treatment which suggests that simple removal of sialic acid residues from cell surface molecules, (proteins or lipids) reverses the effect. Thus since our results are not immediately reconciliabe further work is required to determine whether apparent differences in lectin agglutination of neutrophils in CML are related directly to altered sialylation of cell surface molecules.

The lack of major differences between controls and CML cells is interesting for a number of other reasons. Many alkaline phosphatases have been shown to be glycosylated and Firestone and Heath (1981) have demonstrated that in mouse L-cells, alkaline phosphatase is a cell-surface localized glycoprotein, (mol. wt. 76K) which binds to both Con A and WGA-Sepharose columns. Willinger and Frankel (1979) also found that 22% of alkaline phosphatase activity in human neutrophils was present in a purified plasma membrane fraction.

In view of the fact that CML neutrophils have a markedly reduced level of alkaline phosphatase this might have been expected to have resulted in the absence of one or more autoradiographic bands, (representing alkaline phosphatase from the CML tracks). It is quite possible of course that leukocyte alkaline phosphatase is not glycosylated and is not accessible to labelling with ^{125}-I-LPO.

In order to look at the expression of cell surface glycoproteins during neutrophil differentiation use has been made of a human promyelocytic leukaemia cell line, HL-60, which has been shown to undergo morphological and functional differentiation 'in vitro', after induction with DMSO, (Gahmberg et al., 1979). By labelling of this cell line during induction Gahmberg et al. (1979) have been able to demonstrate the sequential expression of small number of cell surface proteins. There results suggest that the expression of Gp 110K, (equivalent to Gp 115K, see Table 3.6),

shows no alteration in mobility during induction and therefore presumably no increase in sialic acid content. However by labelling the cells using the neuraminidase/galactose oxidase/Na^3HB_4 method which labels desialylated carbohydrate residues in galactose residues they noted a difference in mobility between 160K (uninduced cells) and 130K (induced cells), (equivalent to Gp 145K, see Chapter 5). The reason for such differences is not clear nor has a precursor product relationship between these two polypeptides been demonstrated. Purified HL-60 granulocytes resemble normal human granulocytes with respect to their expression of Gp 110K, (Gp 115K equivalent).

The third aim of this work was to see whether any alterations in the expression of membrane proteins might be related to the chromosomal abnormalities found in this disease. The number of chromosomally abnormal cases (apart from the 9/22 translocation) that arose while this study was being undertaken was small. One case in which monosomy 7 was detected unfortunately involved neutropaenia and therefore insufficient neutrophils were available for study. Since no consistent differences were found in the expression of membrane proteins and no alteration of glycosylation of cell glycoproteins was noted, this study provides no evidence for the direct involvement of the Ph' chromosome in gene expression of glycoproteins and cell surface proteins.

Since most if not all cancers are thought to result from abnormal levels of expression of normal gene products, (Klein, 1981 and Rigby, 1982) it is possible that subtle but consistent differences in the levels of expression of glycosylated proteins are present but have not been detected.

Conclusion

In conclusion then this study has provided some useful information

about the organisation of cell surface proteins of the neutrophil. In common with other circulating cells the neutrophil expresses a relatively small number of high molecular weight glycoproteins at the cell surface.

Many intriguing questions about the organization of these proteins and the relationship between structure and function have been left unanswered. Some of the ways in which this part of the study could be extended are outlined in the discussion.

The results of the comparison of normal and CML neutrophils suggests that glycosylation of proteins in the neutrophil in CML is not disturbed and that there are no significant differences in the expression of cell surface proteins.

Therefore although maturation of granulocytic cells in CML may be disturbed or even partially arrested, and a large increase in the number of commited myeloid precursor cells results in marked overproduction of neutrophils, nevertheless the differentiation of this cell still shows remarkable fidelity with respect to the gene products studied in this thesis.

The role of the Ph' chromosome in CML remains elusive and may well have to await studies now in progress ~~level~~ using recombinant DNA technology.

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