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Tschismadia, Irene

SEPARATION AND PARTIAL CHARACTERIZATION OF POLYMORPHONUCLEAR NEUTROPHIL PLASMA MEMBRANE COMPONENTS

The Ohio State University

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SEPARATION AND PARTIAL CHARACTERIZATION
OF POLYMORPHONUCLEAR NEUTROPHIL
PLASMA MEMBRANE COMPONENTS

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of The Ohio State University

By
Irene Tschismadia, B.S., M.S.

* * * * *

The Ohio State University
1982

Reading Committee:
G.A. Ackerman, M.D., Ph.D.
T.G. Hayes, Ph.D.
R.L. St. Pierre, Ph.D.

Approved By

G.A. Ackerman
Adviser
Department of Anatomy
ACKNOWLEDGMENTS

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VITA

NAME: Irene Tschismadia

DATE OF BIRTH: October 29, 1950

PLACE OF BIRTH: Villach, Austria

U.S. CITIZENSHIP: 1965

EDUCATION:

1972 B.S. in Biology, John Carroll University
Cleveland, Ohio

1975 M.S. in Biology, John Carroll University
Cleveland, Ohio

1982 Ph.D. in Anatomy, The Ohio State University,
Columbus, Ohio

HONORS:

Graduate Student Alumni Research Award
The Ohio State University Nov. 1980

Graduate Committee Representative
Department of Anatomy
The Ohio State University 1980-1981

Association of Anatomy Graduate Students
The Ohio State University
President 1979-1980
Vice-President 1977-1979

John Huntington Fund Scholarship 1968-1972

John Carroll University Grant 1968-1969

Jesuit Honor Society for Women
Vice-President 1971-1972
EMPLOYMENT:

Graduate Teaching Associateship  
Department of Anatomy  
The Ohio State University  
1977-1981

Graduate Assistantship  
Department of Biology  
John Carroll University  
1972-1975

Instructor  
Department of Anatomy  
The Ohio State University  
Summer, 1981

Instructor  
Department of Biology  
John Carroll University  
Summer, 1975-1980

PROFESSIONAL MEMBERSHIPS:

International Research Group on Acetabularia  
American Association for the Advancement of Science  
The Ohio Academy of Science

PRESENTATIONS:

Nov. 1975: Biosynthesis in isolated chloroplasts of Acetabularia mediterranea.  
IV. Plastoquinones; The Society for Cell Biology Meeting, San Juan, Puerto Rico.

Apr. 1977: The biosynthesis of plastoquinones in isolated chloroplasts of Acetabularia mediterranea; The Ohio Academy of Science Meeting, Columbus, Ohio.

FIELD OF STUDY: Anatomy, membrane composition of white blood cells.  
Professor G.A. Ackerman, Adviser
PUBLICATIONS


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INTRODUCTION

The major role of the polymorphonuclear neutrophil (PMN) is to protect the body against infection. It accomplishes this task by phagocytizing and destroying invading bacteria or foreign substances. When an infection occurs, the neutrophil is among the first cells to respond and migrate to the site of infection. The migratory response is a result of an interaction between signal molecules (proteins, chemicals, drugs, foreign substances) sent from the infection site and receptor molecules on the surface of the neutrophil. The plasma membrane of the neutrophil is one of the cell's means of communication with its environment.

Many external membrane proteins of mammalian cells are linked with various carbohydrate residues which play a major role in cell membrane structure and function. Glycoproteins act as cell surface receptors and modulate membrane processes such as cell motility (1), cell metabolism (2, 3, 4), membrane transport (2), antigenicity (5, 6) and other functional activities of the cell. They have a major influence on cell recognition, intercellular adhesion and communication (5, 6, 7) and serve as specific binding sites for various extracellular substances (e.g. viruses, bacteria, drugs) (1).
Cell surface properties are dependent on the type and orientation of specific carbohydrate residues which form branching chains on the cell surface (5, 6). The type and orientation of carbohydrate residues depend on the cell's specific function and metabolism (5, 7, 8, 9, 10, 11). For example, surface carbohydrates of lymphocytes allow normal recirculation via complementary recognition between the endothelial cells surface of post-capillary venules and lymphocyte surface (5). Another example is the insulin receptor in fat cells, in which removal of sialic acid from glycoproteins stops all physiological effects except binding (9).

Plant lectins, or agglutinins, have been used extensively to investigate membrane structure and function and to isolate specific membrane constituents. Lectins are a class of site-specific lab gels, not directed to an active site but to specific oligosaccharide conformations. The use of a wide variety of lectins with different sugar specificities has facilitated the localization of different specific oligosaccharide residues on the cell surface and has contributed much to the current view of membrane structure (12, 13). Lectins are extracts of plants and invertebrates (8) whose general properties include mitogenisis, cell agglutination and stimulation of chemotactic response (14). Specifically, lectin binding to cells can induce an initial increase in adenyl cyclase-enhanced uptake and transport of small molecules, acetylation of histones, phosphorylation of nuclear proteins and modification of lipid and carbohydrate metabolism (8, 15).

Lectins are excellent surface probes since they attach reversibly and do not enter the cell during short periods of contact. Attachment
and detachment do not cause changes to either lectin or cell surface (8).

Lectin-induced agglutination is due to formation of multiple lectin cross-bridges. Cross-bridging occurs because of the complex structural relationship of agglutinable molecules and their numbers on the cell surface, and also on properties of the cell such as rigidity or deformability, metabolic state, surface charge and distribution, and mobility and number of lectin receptor sites on the membrane (16). Clustering of receptor sites plays an important role in agglutination of the cell. It occurs at room temperature and can be prevented if cells are labeled at 0–4°C (8).

Lectins selectively bind various terminal carbohydrate residues. Concanavalin A (Con A) (M.W. 108,000) (17, 18) is a globular hemagglutinating protein (tetramer) with four equivalent reaction sites (18, 19). It is known to bind to simple sugars and polysaccharides with terminal, non-reducing \( \alpha \)-D-glucopyranosyl and mannopyranosyl and \( \alpha \)-D-fructofuranosyl residues (8, 16, 20, 21). Wheat germ agglutinin (WGA) (M.W. 36,000) (18, 22) is a hemagglutinating protein. It has two identical chains each with two binding sites for N-acetyl-D-glucosamine (11), its beta (1-4)-oligomers, N-acetyl neuraminic acid and N-acetyl-D-galactosamine (8, 21, 23, 24).

Similarity of binding of different lectins could be due to binding to a common set of major glycoproteins or glycolipids which contain all the carbohydrate residues recognized by these lectins. It may also reflect a common glycosylation sequence of different glycoproteins and glycolipids on the cell surface (11). It is not
clear if the different degrees of binding to different cells reflect relative abundance of common glycoproteins on different cells or relative activities of glycosyl transferases (enzymes which mediate transfer of sugar residues between glycoproteins and/or glycolipids) (25, 26) on different glycoproteins. Available evidence suggests that both factors contribute to the pattern of lectin binding (11, 27).

Con A and WGA have been used in many studies to aid in identification of surface receptor structure and function in blood cells.

The structure and function of several receptor molecules of the PMN plasma membrane have been tentatively identified. Using fluoresceinated Con A, Baehner and Boxer (2) showed that prevention of microtubule assembly by colchicine permitted fluoresceinated Con A complexes to move from an inherently random distribution to a more polarized area of the cell surface known as the cap. Conversely, PMN exposure to cytochalasin B prevented the redistribution of Con A into caps. They concluded that membrane associated microtubules may serve as an anchor for PMN cell-surface Con A receptors.

Cell surface stimulation, via Con A binding, of PMNs induces phagocytosis, secretion and an increase in oxidative metabolism. PMNs which are stimulated with Con A undergo an increase in oxidative metabolism which results in a marked enhancement of cyanide-insensitive oxygen consumption leading to the univalent (O$_2^-$, superoxide) and divalent (H$_2$O$_2$) reduction products of oxygen. This superoxide generating system is associated with PMN cell surface (4). Ultrastructural studies using human PMNs show a PMN-NADH oxidase to be the
ectoenzyme activated by Con A (2). Depierre and Karnovsky (15) indicate this ectoenzyme to be AMPase in guinea pigs.

Certain vital functions of these cells, i.e. secretion and oxidative metabolism, may be modulated independently by receptors on the cell surface for immunoglobulins and complement (4). Plasma membranes of human PMNs, erythrocytes, monocytes and B-lymphocytes have receptors for C3b, the major cleavage fragment of C3 complement, that mediates attachment to these cells of complexes that bear this complement protein (28). Studies of the function of the C3b receptor on these cells have defined its participation in phagocytic and secretory reactions. Trypsin sensitivity suggests it is a protein (3). Immunoprecipitation and electrophoretic studies indicate the C3b receptor is a glycoprotein with a molecular weight of 205,000 daltons (3).

PMN membrane receptors for complement and immunoglobulins may also play a role in directed motility and in recognition of particles to be ingested. They may send signals that initiate membrane fusion and metabolic events that accompany phagocytosis and that are essential for microbial killing (4). Additional evidence that human PMN functions are determined by cell surface recognition of and stimulation by specific ligands has been provided by studies of the independent effects upon these cells of IgG and complement C3b. C3b receptor is primarily involved in recognition and attachment of particles and only inefficiently promotes ingestion of these particles. IgG receptors, on the other hand, are necessary for induction of optimal phagocytosis (3, 4).
The mechanisms whereby binding of specific ligands, i.e. IgG, C3b, to PMN stimulates membrane fusion (degranulation) and enhances oxidative metabolism are unknown. Con A stimulation can also induce these functional alterations perhaps through membrane perturbation (4, 29). This may provoke either association or disassociation of membrane protein subunits leading to activation of ectoenzymes and to interactions with subplasmalemmal structural proteins (4). The enzymes and structural proteins can then initiate or regulate cell motility, particle engulfment or metabolic activity (4, 30).

An actin-binding protein has been identified with immunologic stains and shown to be associated with the PMN plasma membrane (2, 31). This protein plays a role in PMN movement and phagocytosis (2).

Abnormal PMN membrane structure and function have been implicated in Chediak-Higashi Syndrome (autosomal recessive) and in chronic granulomatous disease (sex linked or autosomal recessive) (2). Greatly reduced surface activity was observed in intact PMNs from patients with chronic granulomatous disease (4, 32).

Madyastha et al. (33) showed neutrophil agglutination with Con A to be similar both quantitatively and qualitatively in normal and leukemic cells. Although transformed cells were more agglutinable they showed less capping indicating restriction of receptor mobility.

Andersson and Gahmberg (34, 35) employed polyacrylamide gel electrophoresis (PAGE) to identify five major and six minor glycoprotein bands in PMN plasma membranes that were labeled with NaB\(^3\)H\(_4\) after neuraminidase and galactose oxidase treatment. This allowed identification of surface proteins with galactose residues covered by sialic
acid. Molecular weights of these glycoproteins ranged from 42,000–245,000. Removal of sialic acid residues effects mobility of proteins. This may be due to artifactual changes in physical or chemical properties of sialoglycoproteins, or their organization at the cell surface.

When surface sialic acid residues were labeled after periodate treatment (periodate did not enter the cell) a fluorography pattern similar to that seen after neuraminidase and galactose oxidase treatment was obtained. The main difference seemed to be the absence of glycoprotein, GP 130, after periodate treatment (M.W. 130,000). Since most major surface proteins were labeled after treatment with galactose oxidase only and since similar patterns were obtained after neuraminidase and galactose oxidase, and periodate treatment, the investigators concluded that most PMN surface glycoproteins, with the exception of GP 130, were relatively poorly substituted with sialic acid residues (34, 35).

Membrane studies on human erythrocytes using PAGE reveal ten major and one minor protein band (24, 36, 37, 38, 39, 40) with molecular weights ranging from 240,000 to 29,000 daltons (36). Glycophorin has been identified as the major sialoglycoprotein (PAS 1) (M.W. 55,000) (36) of the erythrocyte membrane. It is a transmembrane protein with its carbohydrate portion externally located (41). This major sialoglycoprotein is known to strongly bind WGA (8, 39, 42, 43, 44). It binds little, if any, Con A (37, 38, 42, 43, 45). Neuraminidase treatment stops WGA but not Con A binding to glycophorin (38). Con A binding to mammalian erythrocytes has been attributed to Band 3 glycoprotein (M.W. 90,000) (37, 38, 43, 46, 47) whose major carbohydrate residues
exposed to the cell surface are mannose, galactose and N-acetyl-
glucosamine (25, 37). WGA binding to band 3 is weak (39).

Electrophoretic studies done on PMN plasma membranes have only
shown that most PMN surface glycoproteins are relatively poorly sub-
stituted with sialic acid residues (34, 35). The object of this study
was to further characterize membrane proteins and specific glycoprotein
membrane receptors of the PMN. This was accomplished using specific
radiolabeled cell surface markers and separating plasma membrane com-
ponents on SDS-polyacrylamide gels. Lactoperoxidase-catalyzed radio-
iodination of surface proteins was the method employed to detect cell
surface proteins. Tritiated lectins (WGA and Con A) were used to
identify specific plasma membrane glycoprotein receptors of the PMN.
Comparison of relative mobilities of the protein bands to those of
molecular weight standards allowed determination of molecular weights
of these proteins. Banding patterns and relative mobilities of bands
in different gels were compared and used to identify the same protein
band in different gels.

In view of the important role of the neutrophil in the body, a
knowledge of the type of proteins that make up receptor sites in its
plasma membrane is basic for the understanding of how the neutrophil
functions in normal and pathological situations. If the biochemical
composition of the plasma membrane (i.e. identification of its protein
and glycoprotein constituents) can be elucidated, then experiments can
be designed in an attempt to further understand how the neutrophil
functions upon antigenic introduction (i.e. viral, bacterial, various
drugs or other foreign materials), in disease states such as leukemias,
other blood pathologies, infections or immune responses. When the specific species of protein or proteins responsible for contact inhibition and control of migratory response is identified, then perhaps the control of these processes in disease states such as cancer can be better approached. The information obtained for the PMN may potentially be extrapolated to other cell types, with varying degrees of modification, since various proteins may have similar functions in all membranes.
MATERIAL AND METHODS

Chemicals

Seligman's Balanced Salt Solution (SBSS) and Dulbecco's Phosphate Buffered Saline (DPBS) were purchased from Grand Island Biological Company (Grand Island, New York). Bromphenol blue, dextran (M.W. 200,000-275,000, clinical grade), dimethyl sulfoxide (DMSO), 2,5-diphenyloxazole (PPO), glycine, histopaque, 2-mercaptoethanol, cross-linked SDS molecular weight markers (M.W. 16,000-280,000), phenylmethyl sulfonylfluoride (PMSF) and sodium dodecyl sulfate (SDS) were obtained from Sigma Chemical Company (St. Louis, Mo.). Acrylamide, ammonium persulfate, Brilliant Blue R (Coomassie Blue), N'N'-Bis methylene acrylamide (BIS) and N',N',N',N'-tetra methylethylenediamine (TEMED) were purchased from Bio Rad Laboratories (Richmond, Ca.). Lactoperoxidase was obtained from Calbiochem (La Jolla, Ca.) and Nonidet P40 (NP 40) from BDH Chemicals (Poole, England). New England Nuclear (Boston, Ma.) supplied Na\textsuperscript{125}I (Sp. Act. 551.191 mC/ml), \textsuperscript{3}H-Concanavalin A (Con A) (Sp. Act. 25.0 Ci/mM) and \textsuperscript{3}H-Wheat Germ Agglutinin (WGA) (Sp. Act. 60.0 Ci/mM).
Polymorphonuclear Neutrophil (PMN) Isolation

In the 45 experiments performed, 50 cc blood was obtained from 10 healthy male (6) and female (4) volunteers. The blood was introduced into a flask containing 0.5 ml heparin (0.1 m/10 cc blood) and swirled gently to prevent coagulation. The blood was diluted 1:2 with 1X SBSS. This solution was layered onto a histopaque cushion and centrifuged for 25 min at 2 g. Three layers were obtained. Layer 1 contained serum and platelets; layer 2, histopaque; interface between layers 1 and 2 contained lymphocytes and monocytes; and layer 3 was the red blood cell (RBC) pellet with a white button of neutrophils on the surface. Layers 1 and 2 were aspirated. Layer 3 was resuspended with 30 ml SBSS and 5 ml 4% dextran. The cells were then incubated at 37°C for 75 min. The neutrophils formed a layer on top of the red blood cell layer and were drawn off and pelleted (10 min, 1.8 g). Red blood cell contaminants were lysed with distilled water for 20 sec. Cells were centrifuged again for 10 min at 1.8 g. This procedure was repeated until all red blood cells were removed (48). Microscopic slides made of the final pellet had only intact neutrophils present with a viability greater than 90% as determined by trypan blue exclusion.

Tritiated Lectin Labeling of Surface Receptors

This procedure was a modification of Nicola et al. (11). The PMN pellet was resuspended in 0.1 ml phosphate buffered saline-bovine serum albumin-sodium azide (PBS-BSA-azide) (pH 7.3) to which tritiated lectin ($^{3}$H-Con A, 35 µCi; $^{3}$H-WGA, 50 µCi) was added. The
reaction was allowed to proceed on ice for 25 min. The solution was then centrifuged at 1.8 g for 10 min. The pellet was washed 3 times with 2 ml phosphate buffered saline (PBS) (0.02 M, pH 7.3), with intervening centrifugation (10 min, 1.8 g), to remove excess label. The final pellet was resuspended in 0.5 ml PBS (0.02 M, pH 7.3) to which 0.5 ml lysing solution was added (Appendix A). The solution was incubated on ice for 40 min followed by a 5 min centrifugation at 1 g. An aliquot (100 µl) of the supernatant was mixed with 500 µl of sample buffer and heated on a boiling water bath for 2 min (Appendix C). A 10 µl sample was counted in a Beckman LS 8100 liquid scintillation counter (Beckman, Irvine, Ca.) to determine percentage of radioactive material bound. Sixty microliter samples were applied to 9% SDS-polyacrylamide gels and electrophoresed at 40 V, constant voltage, for 16 hours. The gel was stained and photographed (Appendix C). It was then impregnated with PPO for fluorography, dried and incubated with Kodak X-Omat AR film for 4 weeks (Appendix D).

Cell Surface Radioiodination

Lactoperoxidase-catalyzed cell surface iodination was performed essentially as described by Andersson and Gahmberg (34). The neutrophil pellet was resuspended in 0.5 ml PBS (0.02 M, pH 7.3). The following was then added to initiate the reaction: 200 µCi Na$^{125}$I, 75 µl 0.1% lactoperoxidase and 20 µl 0.001% hydrogen peroxide. The reaction mixture was incubated 5 min at room temperature followed by the addition of 75 µl lactoperoxidase and 20 µl hydrogen peroxide.
for 5 min at room temperature and then 20 µl hydrogen peroxide for 15 min at room temperature.

The cells were washed 3 times with 2 ml cold DPBS, with intervening 10 min centrifuging at 1.8 g, to remove excess label. The final pellet was resuspended in 0.5 ml DPBS and 0.5 ml solubilizer was added. The solution was incubated on ice for 40 min then centrifuged at 1 g for 5 min to remove nuclei and cell debris. The supernatant was mixed 1:5 with sample buffer (Appendix C) and heated on a boiling water bath for 2 min. Twenty-five microliter samples were applied to 9% SDS-polyacrylamide gels and electrophoresed at 40 V, constant voltage, for 16 hours. Gels were stained with Coomassie blue, photographed, dried and incubated with Kodak X-Omat AR film for 3 months (Appendix D).

**SDS-Polyacrylamide Gel Electrophoresis**

Nine percent SDS-polyacrylamide slab gels were prepared according to a modified Laemeli procedure (Bio Rad Instruction Manual, 1977) (49). Samples and cross-linked molecular weight marker proteins (Sigma Chemical Company) were electrophoresed at 40 V constant voltage overnight. Gels were removed and migration of marker dye from top of gel was noted. Gels were stained with Coomassie blue and photographed. Gels were then either dried for autoradiography or processed with subsequent drying for fluorography.
Autoradiography

Gels containing $^{125}I$-labeled molecules were dried on a slab gel dryer (Bio Rad Laboratories). A dried gel and a sheet of Kodak X-Omat AR film were sandwiched together between two boards and then wrapped in aluminum foil. Clamps were placed over the foil to insure contact for best resolution. The gel and film were incubated for 3 months.

Fluorography

Tritium labeled gels were prepared for fluorography according to the method of Bonner and Laskey (50). The fixed gels were soaked in DMSO for 1 hour to remove water, then in DMSO containing PPO for 3 hours. The PPO was precipitated in the gel by soaking it in water for 1 hour. The gels were then dried and placed in direct contact with Kodak X-Omat AR film as described above. The gel and film were incubated 4 weeks at $-70^\circ C$.

Film Developing Procedure

Kodak X-Omat AR films were developed in Kodak liquid x-ray developer for 5 min, stopped for 30 sec and fixed in Kodak rapid fixer for 2-4 min. Films were rinsed with water and hung up to dry.
RESULTS

Separation of Polymorphonuclear Neutrophil Plasma Membrane Components.

Twenty-four bands were visualized in Coomassie blue stained gels of isolated polymorphonuclear neutrophil (PMN) plasma membrane components (Plate I). Of these, 10 stained with moderate intensity (components 12-21). The remaining 14, although distinct, stained less prominently (Table 1). Apparent molecular weights were determined from relative mobilities of molecular weight standards (Figure 1) and ranged from 32,000-300,000 (Table 1).

Separation of Radioiodinated PMN Plasma Membrane Components.

Plasma membrane components from lactoperoxidase (LPO)-catalyzed radioiodinated PMNs were separated and 22 bands were detected with Coomassie blue (Plate I). Of these, 12 bands (components 1, 2, 4, 5, 7-10, 12, 20, 21, 24) stained very intensely and the remaining 9 bands were moderately stained. Each band was discrete. Bands 1 and 2, 4 and 5, 7 and 8, 9 and 10 migrated very close together. Radioautography showed 16 bands (components 4, 5, 7, 8, 11-21, 24) were labeled (Plate II). Apparent molecular weights of these proteins ranged from 32,000-300,000 (Table 1).
Comparison of Banding Patterns of PMN Plasma Membrane and Radioiodinated PMN Plasma Membrane Components.

The Coomassie blue banding pattern of PMN plasma membranes was identical to that observed after iodination. Proteins of the iodinated plasma membrane showed decreased mobility compared to non-iodinated plasma membrane components. Components 1-11, while faintly stained in the iodinated plasma membrane gels were intensely stained in the iodinated plasma membrane gels. Components 1 and 2, 4 and 5, 7 and 8, and 9 and 10 migrated very close together in iodinated plasma membrane gels while in PMN plasma membrane gels, these components migrated further apart, which showed the presence of two bands more distinctly.

PMN plasma membrane gels showed the presence of three bands near the dye front, components 22, 23 and 24, whereas only one broad, heavily stained band was seen in iodinated gels.

Separation of $^{3}$H-WGA-Labeled PMN Plasma Membrane Components.

Fluorography of $^{3}$H-WGA-labeled gels showed components 16a, 20 and 21a to be radioactive (Plate III). Component 20 was intensely labeled. Apparent molecular weights of $^{3}$H-WGA-labeled glycoproteins were 90,000, 55,000 and 40,000 respectively (Table 1). A spectrophotometric scan of the x-ray films incubated with these gels gave three peaks at 35.0, 54.5 and 81.3 mm from the origin (Figure 2).

A 10 μl aliquot of the sample applied to the gels was taken for counting on a Beckman Liquid Scintillation Counter to determine the amount of label present. Counts obtained were 62,775 cpm.
Fourteen plasma membrane components from WGA-labeled PMNs were identified with Coomassie blue protein stain (Plate I). Component 12 was the most intensely stained band. Seven bands (components 15-16a, 18-21) stained moderately while six (components 11, 13, 14, 17, 21a, 23) were very faint. The area of the gels above band 12 was stained very lightly with Coomassie blue but distinct bands were not visible. Apparent molecular weights of these proteins ranged from 32,000-185,000 (Table 1).

Separation of $^3$H-Con A-Labeled PMN Plasma Membrane Components.

Fluorography of $^3$H-Con A-labeled gels showed components 19a and 23, M.W. 70,000 and 34,000 respectively, to be radioactive (Plate IV). A spectrophotometric scan of the x-ray films incubated with the gels showed two small peaks 27.3 and 61.6 mm from the origin (Figure 2).

A 10 µl aliquot of the sample applied to the gels was taken for counting on a Beckman Liquid Scintillation Counter to determine the amount of label present. Counts obtained were 17,682.4 cpm.

Twenty-five plasma membrane components from Con A-labeled PMNs were visualized with Coomassie blue stain (Plate I). Components 12 and 16 were very intensely stained compared to the other components present. Six bands, components 14, 17-21), stained with moderate intensity while the remaining bands were less prominent. Components 1-11 were very faint but bands were distinct. Apparent molecular weights of proteins bands ranged from 32,000-300,000 (Table 1).
Comparison of Banding Patterns of PMN Plasma Membrane and $^{3}H$-WGA-Labeled PMN Plasma Membrane Components.

The Coomassie blue banding pattern of the plasma membrane components of WGA-labeled cells was essentially the same as the PMN plasma membrane banding pattern beginning with component 12 (Plate I). In PMN-plasma membrane gels, the upper one quarter of the gels contained bands (components 1-11) which stained faintly, but were nevertheless distinct. The same area in WGA-labeled gels stained lightly with Coomassie blue, but distinct bands could not be discerned. An obvious difference in staining intensity was seen in band 12 which was much darker in the WGA-labeled gels. In general, the bands in the WGA-labeled gels stained lighter than the bands in PMN plasma membrane gels. Radiolabeled bands 16a and 21a found in $^{3}H$-WGA-labeled gels were absent in PMN plasma membrane gels.

Comparison of Banding Patterns of PMN Plasma Membrane and $^{3}H$-Con A-Labeled Plasma Membrane Components.

The Coomassie blue banding pattern of Con A-labeled cells was essentially the same as that obtained for PMN plasma membranes (Plate I). Components 12 and 16 were more intensely stained in the Con A-labeled gels than in PMN plasma membrane gels. Radiolabeled band 19a, present in $^{3}H$-Con A-labeled gels, was not seen in PMN plasma membrane gels.
Comparison of Banding Patterns of $^3$H-WGA- and $^3$H-Con A-Labeled PMN Plasma Membrane Components.

The Coomassie blue banding patterns of WGA- and Con A-labeled PMN plasma membrane components were essentially the same (Plate I). Band 16 stained much more intensely in Con A-labeled gels than did the corresponding band in WGA-labeled gels. Bands 14 and 15 were slightly darker in Con A-labeled gels. Bands 1-11 were faint but distinguishable in Con A-labeled gels, whereas only a diffuse light blue stain was seen in this region in WGA-labeled gels. Radioactive bands 16 and 21a seen in $^3$H-WGA-labeled gels were absent in $^3$H-Con A-labeled gels, whereas radioactive bands 19a and 23 were seen only in $^3$H-Con A-labeled gels.
TABLE 1

Apparent Molecular Weights of Membrane Proteins
of Polymorphonuclear Neutrophils

<table>
<thead>
<tr>
<th>Component</th>
<th>PMNs</th>
<th>Iodinated PMNs</th>
<th>(^3^H)-WGA-Labeled PMNs</th>
<th>(^3^H)-Con A-Labeled PMNs</th>
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<tbody>
<tr>
<td>1</td>
<td>300,000</td>
<td>300,000 &quot;</td>
<td>-</td>
<td>300,000</td>
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<tr>
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<td>275,000</td>
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<tr>
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<td>-</td>
<td>-</td>
<td>40,000*</td>
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<tr>
<td>22</td>
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<td>23</td>
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<tr>
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<td>32,000*&quot;</td>
<td>32,000</td>
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</tbody>
</table>

*indicates radioactive bands
'indicates moderate Coomassie blue staining
"indicates very intense Coomassie blue staining
Figure 1: Log molecular weight versus relative mobility plot for marker proteins of known molecular weights; 9% SDS-polyacrylamide gels. Each circle is a mean value of relative mobility determinations from ten different gels.
Figure 1
Figure 2: Spectrophotometric tracing of x-ray of fluorograph of $^3$H-WGA-labeled gels (A) and $^3$H-Con A-labeled gels (B) showing positions of radioactive bands relative to one another.
Absorbance in mm

Distance in mm

Figure 2
Plate I: Banding patterns of Coomassie blue stained PMN plasma membrane proteins separated on a 9% SDS-polyacrylamide gels. Plasma membrane proteins from (A) PMN, (B) radioiodinated PMNs, (C) $^3$H-WGA-labeled PMNs, and (D) $^3$H-Con A-labeled PMNs.
Numbers of components are indicated.
Plate II: Autoradiography pattern (left) of lactoperoxidase-catalyzed iodinated PMN cell surface proteins separated on 9% SDS-polyacrylamide gels (right) showing positions of radioactive bands.
Numbers of components are indicated.
Plate III: Fluorography pattern (left) of $^3$WGA-labeled surface glycoproteins separated on 9% SDS-polyacrylamide gels (right) showing positions of radioactive bands.
Numbers of components are indicated.

*Indicates radioactive band in Coomassie Blue stained gels.
Plate IV: Fluorography pattern (left) of $^3$H-Con A-labeled surface glycoproteins separated on 9% SDS-polyacrylamide gels (right) showing positions of radioactive bands.
Numbers of components are indicated.

*Indicates radioactive band in Coomassie Blue stained gels.
Plate V: Fluorography pattern of $^3$H-WGA-labeled (left) and $^3$H-Con A-labeled surface glycoproteins separated on 9% SDS-polyacrylamide gels showing positions of radioactive bands relative to one another.
DISCUSSION

The study of the chemical nature of membrane proteins falls into two categories. First, the isolation and separation of the individual proteins. Second, the characterization of these proteins for heterogeneity and to elucidate the structure of the component. Classical methods of protein fractionation exploit differences in size, shape, solubility and electric charge between different molecular species. Gel electrophoresis is by far the most widely used technique and has the advantages of high resolving power and sensitivity.

Polyacrylamide gel electrophoresis (PAGE) in the presence of SDS was the method used to separate PMN plasma membrane proteins based on molecular size. SDS is a strong anionic detergent which allows solubilization of more than 90% of total cell proteins. The interaction of proteins with SDS results in conversion of all protein to prolate ellipsoid shapes that have a constant diameter and a length proportional to the length of the polypeptide chain (49). A discontinuous buffer system was used to concentrate the protein sample into a thin band in the stacking gels. This allowed separation of proteins (in the separating gels) from a very thin starting zone (~1 mm) instead of a thicker unstacked sample.
Electrophoresis in the presence of SDS enables separation of whole cell preparations. If the surface is selectively labeled with a radioactive marker, then autoradiography or fluorography can be used to detect surface components of whole cell samples. Plasma membrane preparations of PMNs, separated on 9% SDS-polyacrylamide gels, showed the presence of 24 protein bands after Coomassie blue staining. Twenty-two protein bands were seen in gels containing plasma membranes of radioiodinated PMNs. The number and positions of protein bands in the two gels were the same except for the absence of components 22 and 23 in the iodinated gels. This band was very broad and intensely stained. It is possible that three bands were present and migrated very close together and, therefore, were not resolvable.

Lactoperoxidase-catalyzed iodination of surface proteins is a semi-quantitative method of detecting cell surface proteins by labeling exposed tyrosine residues (49, 51, 52). Lactoperoxidase (LPO) (M.W. 78,600) catalyzes the formation of a C-halogen bond in the presence of H₂O₂, a halide and a nucleophilic acceptor. The following reaction occurs at pH 7.4 at 4°C:

\[
H₂O₂ + I^- + \text{[Tyrosine]} \rightarrow H₂O + OH^- + \text{[Iodotyrosine]}
\]

The H₂O₂ and I⁻ complex with the haeme prosthetic group of the enzyme. Tyrosine must be bound by the enzyme to be iodinated. This suggests
that LPO catalyzes the electrophilic substitution of an H⁺ for an I⁺ on the phenyl residue of an enzyme bound tyrosine and is not merely oxidizing iodide to a reactive form which diffuses away and reacts non-enzymatically. The high molecular weight of LPO precludes its entry into the cell, therefore, all labeled proteins are on the cell surface. Carrier free ¹²⁵I is used to increase specific activity of binding (49, 51, 52).

Iodinated proteins showed a decreased mobility compared to PMN plasma membrane components due to ¹²⁵I, M.W. 125, binding. Radioautography of the gels determined bands 4, 5, 7, 8, 11-21 and 24 to be radioactive. These bands represented proteins, containing tyrosine residues, of the cell surface. Molecular weights of these proteins and those of PMN plasma membrane gels were determined by comparing their relative mobilities to those of molecular weight marker proteins. Molecular weights ranged from 32,000 to 300,000.

Bromphenol blue, the tracking dye, was included with each protein sample to serve as a visual marker during electrophoresis. The dye was electrophoresed to the bottom of the gels and its position used in determining relative mobilities of sample proteins. Best resolution was obtained when the mobility of the protein in question was between 20-80% of the tracking dye measured from the top of the separating gels. A series of cross-linked proteins of known molecular weights between 16,000-280,000 were used to construct a log molecular weight versus relative mobility plot for the 9% gels.

Protein banding patterns of both WGA- and Con A-labeled PMN plasma membranes were essentially the same as the PMN plasma membrane
banding pattern. The area of bands 1-11 was lightly stained in WGA-labeled gels. This indicated that protein was present in small amounts but it was beyond the detection limits of Coomassie blue stain. Distinct bands, therefore, were not seen. Differences in staining intensities of some bands, especially components 12 and 16, was observed between the lectin labeled gels and PMN plasma membrane gels. These differences result from a modification in protein components due to membrane perturbation upon lectin stimulation. Changes in surface proteins are attributed to increased amidation of glutamate and aspartate residues (49, 50). These alterations would not change molecular weight appreciably and, therefore, band migration would not change. Increased glycosylation of proteins can decrease Coomassie blue staining ability which accounts for the lighter staining intensity seen in some bands in the lectin labeled gels.

Fluorography of tritiated lectin labeled gels revealed three radioactive bands in $^3$H-WGA-labeled gels and two in $^3$H-Con A-labeled gels. Labeling density of the bands observed on the x-ray films was much heavier in the $^3$H-WGA-labeled bands. Absorbance values obtained from scanning the film at 500 nm were 0.294, 0.785 and 0.93 nm for $^3$H-WGA bands 16a, 20 and 21a respectively, and 0.014 and 0.003 nm for $^3$H-Con A-labeled bands 19a and 22 respectively. Several factors accounted for this increased labeling intensity. Specific activity of $^3$H-WGA with respect to $^3$H-Con A was 2.4:1. Each WGA molecule was labeled with two $^3$H atoms while Con A molecules bound only one $^3$H atom (New England Nuclear, personal communication). Both, however, had small amounts of unlabeled molecules present. The quantity of
$^3$H-WGA used was 100 $\mu$C/ml and the quantity of $^3$H-Con A was 70 $\mu$C/ml and Con A was 1.2 times more diluted than WGA. Counts of the samples applied directly to the gels showed the WGA sample was 8.5 times as radioactive as the Con A sample. When samples were corrected for dilution and differences in specific activity, the WGA/Con A labeling ratio was approximately 2:1. This agrees with the mean labeling values obtained from ultrastructural WGA and Con A labeling studies performed by Ackerman (44, 45) and Zinsmeister (25).

Proteins are broken down to their monomeric forms when denatured in the presence of SDS. The WGA and Con A monomers have molecular weights of 17,000 and 27,000 respectively. These molecular weight species would migrate to an area below component 27, near the dye front. Some radioactivity was seen on the x-ray films at the dye front indicating the presence of either free monomer or its breakdown products.

Binding of WGA and Con A to a glycoprotein species will cause a decrease in mobility of that glycoprotein proportional to the molecular weight of the lectin monomer. Molecular weight of $^3$H-WGA-labeled glycoproteins (components 17, 22, 24) can, therefore, be corrected for the monomer weight to 73,000, 38,000 and 23,000 respectively. Proteins of these molecular weights are expected to be absent or very faintly stained in the gel. Component 20, M.W. 73,000, was present in the WGA-labeled gels but very faint. A component of molecular weight 38,000 was not seen and the 23,000 molecular weight component was beyond resolution of the gels.
Molecular weights of $^3$H-Con A-labeled components, 21 and 26, corrected for monomer weight were 43,000 and 7,000. Component 23, M.W. 44,000, was seen on these gels, but it was very faintly stained. The 7,000 molecular weight component was beyond the resolution of the 9% gels.

The $^3$H-WGA-labeled bands, therefore, represented surface glycoprotein WGA receptors of PMN plasma membranes containing terminal N-acetyl-D-glucosamine, N-acetyl-D-galactosamine, and/or N-acetyl neuraminic acid residues. The $^3$H-Con A-labeled bands represented surface glycoproteins Con A receptors of PMN plasma membranes containing terminal, non-reducing $\alpha$-D-glucopyranosyl, mannopyranosyl and/or $\alpha$-D-fructopyranosyl residues.

Comparison of $^3$H-WGA-labeled and $^3$H-Con A-labeled bands indicated separate and distinct labeling of surface glycoproteins by WGA and Con A. Spectrophotometric scans of films confirmed this. These substantially different binding patterns indicate major differences in membrane carbohydrate residues that are sterically available for WGA and Con A binding. This finding was substantiated by ultrastructural binding studies using WGA-ovomucoid-gold and Con A-horseradish peroxidase-gold performed by Ackerman (44, 45) and Zinsmeister (25).

Band 3, M.W. 90,000, protein of red blood cell membranes strongly binds WGA and glycophorin, M.W. 55,000, binds Con A. The binding data and apparent molecular weights obtained for WGA and Con A glycoprotein receptors indicated these glycoprotein lectin receptors were different molecular species in PMN plasma membranes.
A basic biochemical profile of PMN plasma membrane proteins and glycoproteins has been established using normal cells. The data from this study can be extrapolated to other experiments using PMNs from patients with various pathological conditions, such as leukemias. Any changes observed in the biochemical profiles may aid in the understanding of structure/function relationship of cell surface moieties and their role in disease states.
APPENDIXES
APPENDIX A

Protocol for Separating Human Blood Cells

Solutions

Seligman's Balanced Salt Solution (SBSS), 1X, filter sterilized
(GIBCO, \(-\text{Mg}^{2+}, -\text{Ca}^{2+}, -\text{phenol red}\))

Histopaque (Sigma Chemical Company)

Dextran, M.W. 20,000-250,000, 4% (Sigma Chemical Company)

1. All solutions are sterile and kept on ice.

2. Fifty milliliters of blood is drawn, added to a flask containing
   0.5 ml heparin (0.1 ml/10 cc blood) and swirled gently.

3. Blood is diluted 1:2 with SBSS and layered on a 15 ml histopaque
   cushion in a 50 ml centrifuge tube.

4. Solution is centrifuged for 25 min at 2 g.

5. Three layers result:
   1 = serum and platelets;
   2 = histopaque;
   1-2 interface = monocytes and lymphocytes; and
   3 = red blood cells (RBCs) and polymorphonuclear neutrophils
      (PMNs).

6. Layers 1 and 2 are aspirated. Third layers are serially combined
   into one tube with 30 ml SBSS and placed into a 50 cc plastic
   syringe.
7. Dextran, 1 ml/10 cc blood, is added and solution is brought up to original volume with SBSS.

8. Cells are incubated in an upright position at 37°C for 75 min.

9. Upper layer containing PMNs is removed, diluted to 50 ml with SBSS and centrifuged for 10 min at 1.8 g.

10. Supernatant is aspirated leaving a soft, white PMN pellet.

11. If RBCs are present in pellet, they are shock-lysed with 1 ml distilled water for 20 sec. Cells are then diluted to 50 ml with SBSS and centrifuged for 10 min at 1.8 g. This procedure is repeated until all RBCs are removed.

12. Pellet is resuspended with 1 ml SBSS.

13. A small drop of suspension is placed on a hemocytometer for cell count. Normal yield is 5 X 10^7 cells/ml.

14. Cell viability is tested by adding a drop of trypan blue to cells on the hemocytometer and counting the number of cells that have extruded the dye. Viability ranges from 90-95%.

**Protocol for Lysing Neutrophils**

**Lysing Solution, pH 6.8**

- 0.5 % Nonidet P40 (NP 40)
- 2.0 mM Phenylmethyl sulfonyl fluoride (PMSF)
- 150.0 mM Sodium chloride (NaCl)
- 50.0 mM Trizma base (Tris)

Store solution at 4°C.

1. Final PMN pellet is resuspended in 0.5 ml phosphate buffered saline (PBS) (0.02 M, pH 7.3) to which 0.5 ml lysing solution is added.
2. Reaction mixture is incubated on ice for 40 min then centrifuged at 1 g for 10 min to remove nuclei and cell debris.

3. A 100 µl aliquot of the supernatant is mixed with 500 µl of sample buffer (see Appendix C) and heated on a boiling water bath for 2 min.

4. Samples are now applied to SDS-polyacrylamide gels and electrophoresed (see Appendix C).
APPENDIX B

Tritiated-Lectin Cell Labeling Procedure

Tritiated Lectins.

Concanavalin A (Con A) (\(^{3}H\) (G)) New England Nuclear
Specific Activity 25.0 Ci/mM
0.05 mCi in 1.0 ml 0.01 M NaPO\(_4\) buffer, pH 6.8
Lot #1469-164
M.W. 106,000; 0.21 mg/ml

Wheat Germ Agglutinin (WGA) N(acetyl-\(^{3}H\)) acetylated
Specific Activity 60.0 Ci/mM
0.05 mCi in 0.5 ml 0.01 M NaPO\(_4\) buffer, pH 6.8
Lot #1453-059
M.W. 34,000; 0.0058 mg/ml

Solutions.

Phosphate Buffered Saline (PBS), 0.02 M, pH 7.3

Phosphate Buffered Saline-Bovine Serum Albumin-Sodium
Azide (PBS-BSA-azide)

0.02 M Phosphate buffer, pH 7.3
0.12 M Sodium chloride (NaCl)
1.0 % Bovine serum albumin (BSA) fraction V
0.02 % Sodium azide

This procedure is a modification of Nicola et al. (11).

1. Neutrophil pellet is resuspended in 0.2 ml PBS-BSA-azide to
   which 0.5 ml tritiated lectin is added.

2. Reaction is allowed to proceed on ice for 20 min.

3. Solution is centrifuged for 10 min at 1.8 g.
4. Pellet is washed 3 times with 1.0 ml PBS, with intervening 10 min centrifugation at 1.8 g, to remove excess label.

5. Pellet is resuspended in 0.5 ml PBS, then 0.5 ml lysing solution is added.

6. Reaction mixture is then processed as described in Appendix A.

Lactoperoxidase Method for Cell Surface Iodination

Isotope.

$^{125}$I, New England Nuclear
Specific Activity 551.191 mCi/ml
Carrier free

Solutions.

Solubilizer

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<th>0.10 ml</th>
<th>Nonidet P40 (NP 40)</th>
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<tbody>
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<td>3.48 mg</td>
<td>Phenylmethyl sulfonyle fluoride (PMSF)</td>
</tr>
<tr>
<td>100.00 mg</td>
<td>Soybean trypsin inhibitor (STI)</td>
</tr>
<tr>
<td>200.00 mg</td>
<td>Episolon amino caproic acid (EACA)</td>
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<tr>
<td>9.90 ml</td>
<td>Distilled water</td>
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</table>

Store at 4°C.

Dulbecco's Phosphate Buffered Saline (DPBS), 1X

Hydrogen peroxide ($H_2O_2$), 0.1 ml/10 ml DPBS

Lactoperoxidase (LPO), 1 mg/ml in DPBS, store at 0°C

$Na^{125}I$, 200 μCi

1. Neutrophil pellet is resuspended with 0.5 ml DPBS.

2. The following are added in order, then mixed well

   a. 20 μl $^{125}I$

   b. 75 μl LPO

   c. 20 μl $H_2O_2$
3. Solution is incubated 5 min at room temperature, then 75 μl LPO and 20 μl H$_2$O$_2$ is added.

4. Solution is incubated 5 min at room temperature, then 20 μl H$_2$O$_2$ is added.

5. Solution is incubated 15 min at room temperature, the centrifuged at 1.8 g for 10 min.

6. Pellet is washed 3 times with 2 ml cold DPBS, with intervening 10 min centrifugations at 1.8 g, to remove excess label.

7. Pellet is resuspended in 0.5 ml DPBS to which 0.5 ml solubilizer is added.

8. Solution is mixed well and incubated 30 min on ice, followed by a 5 min centrifugation at 1 g.

9. Supernatant is mixed with sample buffer in a 1:5 ratio and processed as described in Appendix A.
Protocol for SDS-Polyacrylamide Gel Electrophoresis

All procedures are done according to Bio Rad Model 220 and 221 Vertical Slab Electrophoresis Cell Instruction Manual (49).

Solutions.

Acrylamide Stock Solution

30.0 g acrylamide
0.8 g N'N'-bis methylene acrylamide (BIS)

Bring up to 100 ml with distilled water and store at 4°C in the dark.

Buffer Solutions.

1.5 M Tris-Cl, pH 8.8

18.15 g Trizma base (Tris)
50.00 ml Distilled water

Adjust to pH 8.8 with 1N Hcl. Bring up to 100 ml with distilled water and store at room temperature.

0.5 M Tris-Cl, pH 6.8

3.0 g Trizma base (Tris)
40.0 ml Distilled water

Adjust to pH 6.8 with 1N HCl. Bring up to 50 ml with distilled water and store at room temperature.
Separating Gel Composition

9% gels, 0.375 M Tris, pH 8.8

Sufficient for two 0.75 mm X 10 cm X 28 cm slabs:

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<td>22.5 ml</td>
<td>1.5 M Tris-Cl, pH 8.8</td>
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<tr>
<td>0.9 ml</td>
<td>10% (2/v) sodium dodecyl sulfate (SDS)</td>
</tr>
<tr>
<td>27.0 ml</td>
<td>acrylamide stock solution</td>
</tr>
<tr>
<td>0.3 ml</td>
<td>10% ammonium persulfate (fresh)</td>
</tr>
<tr>
<td>0.0225 ml</td>
<td>N',N',N',N'-tetra methylethlenediamine (TEMED)</td>
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Stacking Gel Composition

3% gels, 0.125 M Tris, pH 6.8

Sufficient for two 0.75 mm thick stacking gels:

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<th>Component</th>
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<td>6.3 ml</td>
<td>Distilled water</td>
</tr>
<tr>
<td>2.5 ml</td>
<td>0.5 M Tris-Cl, pH 6.8</td>
</tr>
<tr>
<td>0.1 ml</td>
<td>10% (w/v) SDS</td>
</tr>
<tr>
<td>1.0 ml</td>
<td>acrylamide stock solution</td>
</tr>
<tr>
<td>0.1 ml</td>
<td>10% ammonium persulfate (fresh)</td>
</tr>
<tr>
<td>0.005 ml</td>
<td>TEMED</td>
</tr>
</tbody>
</table>

Sample Buffer

<table>
<thead>
<tr>
<th>Quantity</th>
<th>Component</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.7 ml</td>
<td>Distilled water</td>
</tr>
<tr>
<td>1.0 ml</td>
<td>0.5 M Tris-Cl, pH 6.8</td>
</tr>
<tr>
<td>1.0 ml</td>
<td>Glycerol</td>
</tr>
<tr>
<td>1.0 ml</td>
<td>10% (w/v) SDS</td>
</tr>
<tr>
<td>0.1 ml</td>
<td>2-mercaptoethanol</td>
</tr>
<tr>
<td>0.2 ml</td>
<td>0.05% (w/v) bromphenol blue in 40% sucrose</td>
</tr>
</tbody>
</table>

Electrode Buffer, pH 8.3

<table>
<thead>
<tr>
<th>Quantity</th>
<th>Component</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.0 g</td>
<td>Trizma base</td>
</tr>
<tr>
<td>28.8 g</td>
<td>Glycine</td>
</tr>
<tr>
<td>10.0 ml</td>
<td>10% (w/v) SDS</td>
</tr>
</tbody>
</table>

Bring to 1 liter with distilled water. Four liters are needed for Model 221 Vertical Slab Gel Electrophoresis Cell (Bio Rad Laboratories).
Gel Preparation

Separating Gels:
1. Prepare all solutions on ice. Add all reagents, except TEMED, in order and deaerate under 25 lbs vacuum for 15 min.
2. To initiate polymerization, add TEMED and swirl gently to mix.
3. Allow separating gels to polymerize overnight.

Stacking Gels:
1. Prepare all solutions on ice. Add all reagents, except TEMED, in order and deaerate under 25 lbs vacuum for 15 min.
2. To initiate polymerization, add TEMED and swirl gently to mix.
3. Gently layer distilled water over stacking gel with a microliter syringe to aid polymerization better well formation.
4. Stacking gels polymerizes in 60 min.
5. After stacking gels are polymerized, add electrode buffer to upper and lower buffer reservoirs. Gently wash out each well with buffer to remove any unpolymerized reagents using a pasteur pipette.

Preparation of Sample and Electrophoresis
1. Dilute sample 1:5 with sample buffer and heat on a boiling water bath for 2 min.
2. Apply sample to gel with a microliter syringe, 25-60 μl depending on well size.
3. Electrophorese at constant voltage, 40 V, for 16 hours (ISCO Constant Power Source, Model # 460); set current and wattage dials at 100.

4. Remove gel from cell and measure marker dye migration from top of gels.

**Gel Staining Procedure**

**Solutions**

**Coomassie Blue Stain**

**STAIN I:**

- 27.0 % Ethyl alcohol
- 10.0 % Acetic acid
- 0.50 % Copper sulfate
- 0.05 % Coomassie blue

**STAIN II:**

- 25.0 % Ethyl alcohol
- 10.0 % Acetic acid
- 0.01 % Coomassie blue

**Destain**

- 10 % Ethyl alcohol
- 10 % Acetic acid

**Storage Solution**

- 10% Acetic acid

Gels are placed in Stain I for 3 hours, then in Stain II for 2 hours. Gels are destained overnight in a diffusion destainer (Bio Rad Laboratories).
Gel Drying Procedure

1. To prevent gel cracking, leave gels in destain or storage solution for 3-4 days before drying.

2. Soak gels in 1% glycerol in 10% acetic acid for one hour, then float gels onto filter paper.

3. Place filter paper with gels onto the metal screen of the gel slab dryer (Bio Rad Laboratories) and dry under vacuum overnight.
Autoradiography

Gels containing $^{125}$I-labeled proteins are dried then placed in direct contact with Kodak X-Omat AR film. The film and gels are sandwiched between two boards, wrapped in aluminum foil then clamped to assure maximum contact for best resolution. The gels are incubated for 3 months, at room temperature.

Fluorography

Gels containing tritium labeled molecules are prepared for fluorography using the method of Bonner and Laskey (50) to enhance detection.

The gels are soaked in two changes of 500 ml DMSO, for 30 min each. If the gels are stained with Coomassie blue, prolonged exposure to DMSO will remove stain from gels. The gels are then soaked in 500 ml of 20% PPO dissolved in DMSO for 3 hours followed by a 1 hour immersion in 1% glycerol. The gels are dried overnight and placed in direct contact with Kodak X-Omat AR film as described above. The gels are incubated for 4 weeks at $-70^\circ$C.

Kodak X-Omat AR Film Developing Procedure

X-ray film is developed in Kodak liquid x-ray developer for 5 min, stopped for 30 sec and fixed in Kodak rapid fixer for 2-4 min. The film is rinsed in water and hung up to dry.


48. O'Dorisio, S. Personal communication.


