ELECTROCHEMICAL MEMBRANE PHENOMENA ASSOCIATED WITH LYMPHOCYTE COMMUNICATION

by

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INTRODUCTION

Cellular communication enables a multicellular organism to respond to external stimuli in an integrated fashion. Cell behavior may be modulated by an exchange of information via the cellular release of soluble factors. Initial contact between the cell and intercellular mediators occurs at the plasma membrane. In this framework the plasma membrane functions as a biological transducer, receiving external messages and relaying the appropriate signals to the cell interior. As such, the membrane plays a critical role in the function of the cell.

Lymphocyte activation by specific antigen graphically illustrates how cell function may be modulated by surface reactants. Upon interaction with appropriate stimulating ligands the lymphocyte is transformed from a metabolically quiescent cell to a proliferating cell involved in executing its physiological function of defending the body against invading foreign organisms. Dormant lymphocytes are triggered to undergo blast transformation not only by specific antigens but also by the phytomitogens (1), bacterial products (2), anti-immunoglobulins (3), trypsin (4,5), the divalent cation ionophore (A23187) (6) and reagents such as sodium periodate (7). Lymphocyte activation is a phenomenon initiated at the cell surface. Mitogenic agents maintain their ability to activate lymphocytes after being coupled to insoluble supports, indicating that transport of mitogen to the cell interior is not obligatory for mitogenic triggering (8,9). Despite this established relationship between mitogen-cell surface binding and transformation initiation, the triggering or transmembrane signaling mechanism remains to be clarified. Salient biochemical and physico-chemical changes are

manifest in the cell membrane minutes after mitogen binding. The means by which a ligand bound to the lymphocyte surface initiates the series of events defining stimulation will not be resolved, however, by the mere cataloging of membrane alterations apparent immediately after mitogen binding. Primary transitions must be distinguished from those which arise secondarily and an integrated description of the properties of the activated lymphocyte must emerge.

1.1 Lectin Lymphocyte Interaction

Studies of lymphocyte activation frequently rely upon lectins as stimulants. Lectins are proteins of nonimmune origin that are quite widely distributed in nature, ranging from concanavalin A of the jack bean to helix pomatia lectin of the snail (10). Lectins share a host of unusual biological and chemical properties, the foremost among these properties being the ability to bind mono- or oligo saccharides with remarkable specificity (11). Mitogenic stimulation arises by virtue of the carbohydrate binding capacity of lectins. The first stage in the mitogenic activation of lymphocytes involves binding of mitogen to the saccharide units of glycoproteins and glycolipids. The lymphocyte response is nonspecific, initiated by a process independent of the immunogenicity of the stimulant. Polyclonal blastogenesis is triggered by mitogens. It is this ability to turn on multiple clones in the lymphocyte population in a manner analagous to specific antigen stimulation which has led to their widespread use in immunological research.

Although the interaction of lymphocytes with mitogenic lectins leads to the expression of a myriad of functional activities, it is generally assumed that only a restricted number of changes originate

directly from interaction between a lectin and its receptor and that an amplifying mechanism must operate within the membrane leading to the other expressed changes. Typically, early events in activation have been analyzed in three relatively discrete stages: 1. Recognition -- ligand-receptor interaction 2. Transmission -- information transfer across membrane 3. Modulation -- redistribution of surface components. For the purposes of this discussion, general conclusions derived from ligand receptor studies will be briefly presented and then followed by a description of the changes documented in the plasma membrane subsequent to mitogen binding. The relation of these membrane changes to the transfer of the mitogenic signal to the cell interior will be addressed.

1.1.1 Ligand-Receptor Interactions

Immunoglobulin, the receptor of B lymphocytes for specific antigen has been the subject of a rigorous structural analysis. In recent years the complete amino acid sequence of membrane bound immunoglobulin has been determined (12). B lymphocytes are presumed to have a receptor distinct from immunoglobulin for mitogenic lectins. Evidence suggesting that B lymphocytes may be activated by at least two different and independent receptors derives from capping (receptor-redistribution) studies. Lectins and anti-immunoglobulins do not co-cap, but rather redistribute independently to different sites on the membrane, implying the involvement of at least two distinct types of receptors in activation (13).

In view of the carbohydrate binding specificity of lectins, it would be expected that they would bind with either of the two major classes of sugar containing molecules on cell surfaces, the

glycoproteins and glycolipids. Inhibition studies demonstrate that glycoproteins but not glycolipids may effectively inhibit lymphocyte transformation (14). Generally, it is therefore accepted that glycoproteins are the critical receptors involved in the process of mitogenesis.

Lectins exhibit binding specificity for a variety of saccharides associated with membrane glycoproteins. Simultaneous interaction with all available cellular receptors for mitogenic lectins fails to stimulate, whereas proliferation is maximal when only 5% of the receptors are occupied (15,16). Evidence from Toyoshima et al. suggests that phytomitogens bind to the cell surface via a common receptor not shared by the non-mitogenic lectins (17). Wynne et al. addressed the question of whether different mitogens trigger cells by affecting different membrane sites (18). This question was approached through the use of a chemical method which involved the insertion of a mitogenic site onto the lymphocyte membrane. The extreme specificity of the interaction between conjugated ligand and the cell surface required to induce blastogenesis prompted the group to infer that the lymphocyte activation site is unique. Prevailing evidence does suggest that a single transformation sensitive receptor molecule exists which mediates activation of lymphocytes by a variety of ligands.

Note that ligand-receptor interaction is not the sole means of initiating lymphocyte activation. Heavy metal ions as well as certain bacterial products are thought to induce mitogenesis by interacting with the lipid bilayer, thereby perturbing the membrane and mimicking the result of receptor-ligand interaction (19,20,21).

1.1.2 Membrane Associated Events in Lectin Lymphocyte Interaction

The cell surface is implicated in the regulation of the lymphocyte activation phenomenon. Subsequent to interaction with mitogenic lectins, lymphocytes undergo metabolic, biochemical, morphological and proliferative changes. These alterations are sequentially expressed, manifest first in the plasma membrane and later in the cytoplasm and nucleus.

Changes in the permeability of the plasma membrane to small molecules is one of the primary functional alterations evident after mitogen binding. Increased uptake of nucleosides (22), sugars (23,24), amino acids (25,26), and cations (27,28) have been measured. Changes in membrane permeability are known to accompany the transition of a resting cell to a state of enhanced activity in a number of other systems. However, a causal relationship has yet to be established and it has been suggested that the permeability change is only an indicator for a more general change in the cell membrane. Nevertheless, one hypothesis has been advanced which relates early changes in the membrane permeability in Ca^{++} to the late biochemical changes associated with blast transformation (27). This hypothesis hinges on the fact that one of the initial alterations induced by mitogen interaction is the stimulation of guanosine 3':5'-monophosphate (cGMP) accumulation (29,30). Membrane alterations are thought to lead to a change in Ca^{+2} permeability with a resultant Ca^{+2} influx. The elevated guanylate cyclase activity is suggested to arise from the increase in cytoplasmic Ca^{+2}, a cation which stimulates the enzyme. These experimental observations are relevant in view of the reported correlation between

cyclic GMP and increases in RNA synthesis and changes in the synthesis, phosphorylation, and DNA binding of nonhistone acidic nuclear proteins (31,32). Cyclic GMP is thus envisioned as an intracellular messenger, a mediator between the cell surface and the nucleus in the mitogen-stimulated lymphocyte.

In an apparent contradiction to these studies, however, is the work reported by Parker et al. (33,34,35). Parker and collaborators have long sought to establish cAMP as a positive effector of mitogenesis. Initially, they reported finding no change in cGMP levels while observing a rapid increase in cAMP levels in PHA stimulated lymhocytes. In later work, the selective activation of a cyclic AMP-dependent protein kinase was demonstrated (36). More recently they have focused their attention on mitogen induced alterations in protein phosphorylation and speculate on a plausible role for these proteins in the intracellular propogation of the mitogenic signal (37).

For years now the literature in this area has continued to expand unwarrantably. Yet a consensus has not been reached on just what role, if any, the cyclic nucleotides play in mitogenesis and the literature remains replete with contradictions. The primary flaw in this work is obvious. The change in intracellular concentration of a biochemical cannot be measured with precision if the cell population is heterogenous. All of these studies have been carried out on heterogenous cell populations which have not been well characterized. It is likely that the range of values reported in the literature reflect to a large extent differences in the cell populations which have been assayed.

Changes in membrane associated function such as

permeability have been linked to concomitant changes in the lipid phase of the plasma membrane. A considerable body of literature now attests to the biological significance of alterations in membrane phospholipids. Resch et al. (38) has implicated the activation of membrane phospholipid metabolism in the induction of mitosis. Subsequent to mitogen binding, the fluidity of the lymphocyte plasma membrane increases. The fluidity change is attributed to alterations in the fatty acids of phospholipids which result in a less constrained packing of the membrane phospholipids. According to Resch, all membrane associated events in lymphocyte activation originate from this alteration in phospholipid hydrocarbon packing.

Phospholipid methylation has also been touted as a critical biochemical signal modulating lymphocyte mitogenesis. Hirata et al. (39) reported increases in phospholipid methylation subsequent to the binding of mitogenic lectins to the lymphocyte cell surface. The increase in phospholipid methylation is short-lived since phospholipase A2 is also activated by mitogen binding and this enzyme then proceeds to degrade the methylated phospholipids.

Phospholipase A2 action results in the release of arachidonic acid. Speculation on the involvement of this process in lymphocyte activation also exists (40). Arachidonic acid is the precursor to a variety of biologically active fatty acid derivatives such as thromboxane and the prostaglandins. To date, however, a specific role for arachidonic acid metabolites in the activation process has not been identified. Moreover, recent work by Hirata et al. (39) has shown that the inhibition of prostaglandin synthesis by indomethacin has little effect on mitogenesis.

In addition to the aforementioned biochemical changes, physico-chemical changes at the cell surface have also been observed in the response of lymphocytes to mitogenic lectins. Cellular surface charge density has been assessed using analytical particle electrophoresis (41,42,43). This biophysical technique is capable of detecting subtle changes in the radial distribution of cell membrane charge. Blume et al. (43) monitored early events associated with lymphocyte stimulation via analytical particle electrophoresis. Increases in the electrophoretic mobility of murine and human derived T lymphocytes subsequent to short term exposure to mitogenic lectins such as Con A or specific antigen were described.

Changes in membrane potential have been identified during mitogenic stimulation of mouse spleen lymphocytes (44). The membrane potential is a reflection of transmembrane ionic concentration ratios. Cone (45) has discussed an experimentally observed correlation between the level of electrical transmembrane potential in somatic cells and the intensity of mitotic activity. Kiefer et al. (44) report that mitogens exhibit the same specificity for depolarization as for mitogenic stimulation. Their group has put forward the notion that depolarization of the plasma membrane initiates the transition of lymphocytes from a resting state to mitotic activity.

Biophysical methodology has been used to also monitor early events in lectin-lymphocyte interaction. Immediately after the addition of Con A, rat thymocytes respond in the presence of luminol with a burst of chemiluminescence which can be measured/monitored in a liquid scintillation spectrometer (46). Chemiluminescence is thought to arise from the generation of reactive products of oxygen (O_2^-, H_2O_2,

$OH^{\cdot}$) and singlet O_2 which emit light in the presence of polyunsaturated fatty acids, polysaccharides or easily oxidizable substances such as luminol. Typically, lymphocyte stimulation is followed experimentally by measuring the rate of incorporation of tritiated thymidine 48 to 72 hr after the addition of a stimulating agent. Chemiluminescence affords the obvious advantage of early detection of lymphocyte stimulation.

In attempting to identify the initial response of the plasma membrane to mitogen binding, a host of functional and physicochemical surface changes have been detected. Unfortunately, most investigators have approached this question from a narrow perspective and have neglected to relate their findings to other published data in this area. As a consequence the literature in this area is difficult to assimilate. At this time it is not possible to define the precise sequence of events initiated by mitogen binding. The emphasis in the majority of the studies described herein has been to establish if indeed the alteration under study is critical to the process of blast transformation. The term critical has been applied to those alterations which when blocked lead to a reduction in tritiated thymidine incorporation. It is highly probable, however, that lymphocyte activation consists of a series of many individual steps for each of which certain conditions must be met if the ultimate outcome is to be realized. Interferring with any biochemical step in the chain of events would then be expected to dramatically affect the net outcome. Hence, the value of demonstrating that the blockage of a specific biochemical event results in a reduction in the incorporation of tritiated thymidine is quite limited in itself. A broader scope is desirable in addressing the subject of lymphocyte activation. Cell surface changes must be

analyzed not only in regard to their influence upon tritiated thymidine incorporation but also in relation to other changes accompanying mitogen binding. Questions meriting consideration include: Does the alteration provide the cell with substrate(s) required for cell activation? Does the membrane associated change contribute to a signal transducing mechanism? Where does the alteration fit into the chronological sequence of other observed changes?

1.1.3 Cellular Communication Involved in Lectin-Lymphocyte Interaction

Since the discovery of T cell growth stimulating factors, pronounced attention has been focused on identifying surface receptors which emerge subsequent to mitogen lymphocyte interaction. Evaluation of lymphocyte surface topography subsequent to lectin interaction has revealed changes in antigen and receptor patterns. McCune et al. (47) report an eleven-fold increase in the availability of HLA antigens twelve hours after incubation with PHA. Interestingly, the receptor changes which are expressed at the cell surface are dependent upon the mitogen which initiated stimulation. For instance, lymphocytes transformed with Con A are reported to exhibit increased numbers of receptors for insulin and Con A, whereas sodium periodate-induced transformation of lymphocytes results in an increase in the number of receptors for PHA but not for Con A (48,49).

Speckart et al. (50) assessed membrane alterations arising from either Con A or PHA stimulation by analyzing the distribution of receptors for six purified plant lectins. Quantitative differences in the density of lectin receptors on resting human lymphocytes and on lymphocytes transformed with Con A or PHA were

reported. From Speckart's later work, it appears that Con A and PHA affect both the multiglycosyltransferase enzyme system and the protein synthetic apparatus differently, thereby leading to the expression of distinct surface changes (51).

The analysis of mitogen induced changes in the cell surface continues to be an area of interest as investigators attempt to understand how mitogens elicit a state of responsiveness to T cell growth factor. It was in 1976 that Morgan et al. (52) reported the presence of a mitogenic factor in conditioned media from lectin stimulated mononuclear cells. This factor, which is referred to in the literature as either T cell growth factor, TCGF, or interleukin 2, was shown to support the continuous exponential proliferation of lectin activated human T cells. Since the sole requirement for maintaining growth was shown to be the availability of T cell growth factor, it was inferred that proliferation was mediated by T cell growth factor (TCGF), mitogen or antigen only being required to initiate the process.

Smith et al. (53) have demonstrated that TCGF production requires not only the interaction of mitogenic lectins with T cells but also the participation of macrophages. Habu and Raff (54) had pointed out in 1977 that lectin stimulation of T lymphocytes required the participation of adherent Ia positive accessory cells identified as macrophages. Macrophages are thought to contribute to T cell activation in a dual fashion. Firstly, macrophages present antigen to T cells in an immunogenic form (55,56) and secondly, macrophages produce soluble factors capable of modulating T cell function (57).

Lymphocyte activating factor (LAF), also referred to as interleukin 1, is a soluble product released by macrophages which has

been shown to potentiate the mitogenicity of lectins in thymocyte culture (58). The cellular requirement for macrophages in TCGF production may be circumvented by the addition of preformed LAF (59). The specific influence which LAF exerts in TCGF production is largely unknown. Preliminary findings of Puri et al. (60) reveal that LAF increases the lipid viscosity of the T cell membrane as measured by fluorescence depolarization. Puri et al. (60) suggest that the increase in microviscosity of the cell membrane may lead to unmasking of membrane bound receptors.

While TCGF production is a cell cooperative response involving both T cells and macrophages (61), the acquisition of responsiveness to TCGF is a direct response of T lymphocytes to the binding of mitogenic lectins (62). TCGF is a selective mitogen for precursors of cytotoxic T cells (63,64) and the initial event in activation involves the binding of mitogen to these precursor cells (Lyt 1^- 2^+ 3^+ cytotoxic T cells). Mitogen binding induces these cells into a TCGF responsive state. TCGF responsiveness is thought to be attributable to the appearance of TCGF-specific membrane receptors, although such receptors have yet to be experimentally identified. Larson's data suggests that the triggering of resting T cells to the expression of growth receptors arises from specific interactions with the cell surface membrane, rather than from nonspecific membrane rearrangements(61,65). Thus far, specific antigen and mitogenic lectins are the only surface ligands which have displayed the ability to promote precursor cells to a TCGF responsive state.

The second event in T cell activation involves production of TCGF. Upon specific interaction with LAF produced by

macrophages, helper cells (Lyt 1^+ T cells) will produce TCGF (59,66). Proliferation commences once TCGF produced by the helper cells (Lyt 1^+ T cells) interacts with the TCGF receptors present on the cytotoxic precursor cells (Lyt 1^- 2^+ 3^+ T cells) (67).

Insight into the means by which cells communicate and cooperate in the immune response is steadily developing. Experimental studies aimed at providing a more definitive description of the mechanism of action of T cell growth factor are in progress.

1.2 Cellular Electrophoresis Applied to Lectin-Lymphocyte Interaction

The peripheral zone denotes the portion of the cell membrane exterior to the lipid bilayer which consists of a matrix of hydrophilic material porous to water and small ions. Initial contact between the cell and activating agents occurs in this region. Surface reagent-induced changes in the constituents and conformation of the peripheral zone may be systematically evaluated through the application of cellular electrophoresis.

The thrust of our work has been directed towards understanding the early response of the plasma membrane subsequent to interaction with mitogenic lectins. We have applied cellular electrophoresis to the study of early events associated with lectin lymphocyte interaction. Analytical particle electrophoresis was considered to be methodology eminently suitable for approaching this problem since it is sensitive to perturbations in the peripheral components of the membrane. Prior to elaborating on our experimental findings, I would like to briefly outline the theory of electrophoresis and describe earlier electrophoretic studies involving lymphoid cells.

1.2.1 Application of Analytical Particle Electrophoresis

Biological cells possess a net negative surface charge density (68). Consequently, when these cells are suspended in an electrophoresis chamber they migrate toward the anode under the influence of an electric field with the rate of migration dependent on the surface charge density of the cell and the electric field strength. An electrophoretic mobility (μ) defined as the electrophoretic velocity (v) of a cell per unit electrical field strength (x) may be calculated in $\mu m\ sec^{-1}v^{-1}\ cm$. Through sequential application of the Helmholtz-Smoluchowski (69) and Gouy-Chapman (70,71) equations the surface charge density of the cell may be calculated from the experimentally determined electrophoretic mobility. Application of these equations involve several underlying assumptions which have been reviewed by Seaman (72). Consequently the accuracy of the values for zeta potential and surface charge density derived from these equations are dependent upon the degree to which the system investigated fulfills the assumptions.

HELMHOLTZ-SMOLUCHOWSKI EQUATION

$$\frac{v}{x} = u = \frac{\zeta\varepsilon}{4\pi\eta}$$

where η is the viscosity in poise, ε the dielectric constant and ζ the zeta potential.

GOUY-CHAPMAN EQUATION

(Uni-univalent Electrolytes)

$$\sigma(\text{statcoul. cm}^{-2}) = 2\left(\frac{NkT}{2000\pi}\right)^{1/2} (\varepsilon I)^{1/2} \sinh\left(\frac{e\zeta}{2kT}\right)$$

where N is Avogadro's number, k the Boltzmann constant, T the absolute

temperature, e the charge on an electron and I the ionic strength.

Only a limited amount of information on the actual surface chemistry of a cell may be deduced from the measured electrophoretic mobility and surface charge density. The net surface charge density of a cell may be multiplied by the surface area of the cell to arrive at an estimate of the net number of electron charges per cell. However, this electrophoretic analysis assumes that the charge is uniformly distributed throughout the peripheral zone. Realistically it must be emphasized that two cell types which have identical electrophoretic mobilities may differ in the nature of the ionogenic groups which contribute to cellular charge and in the manner in which the ionogenic groups are distributed throughout the peripheral zone. The specific ionogenic groups contributing to cellular charge and information about their spatial distribution within the peripheral zone may be ascertained by studying the dependence of the electrophoretic mobility on (a) pH of the suspending medium, (b) ionic strength of the suspending medium, (c) treatment with group-specific reagents, and (d) treatment with enzymes.

Measuring the change in electrophoretic mobility of a cell over an extended range of pH yields a data plot similar to the titration curves of amino acids or proteins. The pH vs mobility relationship provides information on the pK values of the major ionogenic groups present in the peripheral zone. Admittedly the cellular situation is more complex and the analysis not quite as straightforward as in the case of simple acids or proteins. Often the effectiveness of this method of analysis in identifying cellular ionogenic groups is enhanced by comparing the pH vs mobility

relationship of untreated cells with cells that have undergone specific chemical or enzymatic modification.

The peripheral zone extends outward approximately 150 Å from the exterior of the lipid bilayer (73). It is possible to arrive at a relative idea of where specific ionogenic groups are located within the peripheral zone by varying the ionic strength of the suspending medium and measuring the electrophoretic mobility. As the ionic strength is reduced, groups deeper within the cellular peripheral zone begin contributing to the electrokinetic charge. Application of this method of analysis led Heard and Seaman (74) to postulate the existence of basic groups deep in the interior of the peripheral zone of the human red cell from the decrease in net negative electrokinetic charge density they detected when reducing the ionic strength of the suspending medium.

Often through an awareness of cellular surface chemistry and biochemistry it is possible to postulate which functional groups are likely to be found in the peripheral zone of a specific cell type. One means of establishing the presence of a specific functional group within the peripheral zone is to develop a reversible chemical modification scheme specific for the surface group in question. The electrokinetic behavior of the native and modified cell may then be compared. Crucial in determining the effectiveness of this type of approach is knowledge of the specificity and extent of completeness of the modification reaction. If it is not established whether the reaction has solely produced a modification of a specific surface group or if it has led also to a conformational rearrangement within the peripheral zone serious errors in electrokinetic interpretation ensue. The condition of electrokinetic reversibility aids in distinguishing those reactions

which have produced rearrangement within the peripheral zone. To date this method of surface analysis has not been thoroughly exploited. Means and Feeney's (75) review of the experimental schemes for functional group modification in proteins emphasizes the potential of this technique. Successful application of these modification schemes to chemical groups on the cell surface is dependent, however, upon maintaining the stability of the cell which generally requires physiological conditions for the reactions.

Although the versatility associated with use of the chemical modification schemes is appealing, treating functional groups on the cell surface enzymatically provides specificity under physiological conditions. In fact this method of analysis proved to be the potent technique in establishing the identity of the major ionogenic component contributing to the net negative surface charge density of the human red cell (76). Cook et al. (76) obtained a 75% decrease in the surface charge density of the human red cell by treating with neuraminidase. Enzymatic treatment coupled with an examination of the reaction products has since been effectively applied in electrokinetic studies using other enzymes.

A rigorous series of guidelines meriting consideration when interpreting electrokinetic data on enzyme-treated cells has been provided by Seaman (72). Attributing the change in electrophoretic mobility of an enzyme-treated cell solely to enzymolysis is naive. The possible complexities associated with enzymatic treatment are apparent by reviewing the following points of Seaman (72):

1. Has adsorption of the enzyme and/or products of its autolysis occurred?

2. Have other enzymes or components present as impurities in the preparation either adsorbed or acted on the cell?

3. Has the reaction been taken to completion, i.e., will retreatment with a fresh enzyme preparation lead to further changes?

4. Have any components which have leaked from the cell or which have resulted from breakdown of other cells adsorbed on to the cell?

5. Have any of the products of proteolysis either those from the peripheral zone or degraded leakage products adsorbed on to the cell?

6. Is the enzyme able to enter the cell and what effects might result from this?

7. Have any of the parameters which influence electrophoretic mobility changed, for example, position of the plane of shear, conductivity of the cell, radius of curvature of the surface, etc.?

8. Have any changes in the distribution of ions occurred either within the internal (porous peripheral zone) or external electrical double layer, i.e., have non-ionogenic (hydrophobic) regions been exposed by enzyme action or has the porosity of the peripheral zone (α factor) changed?

9. Have any conformational changes occurred at the electrophoretic surface, for instance reorientation or unmasking of groups?

1.2.2 Lymphocyte Electrophoretic Studies

Initial electrophoretic studies in the lymphocyte field focused primarily on the difference in electrophoretic mobilities of lymphocytes from different subpopulations, the final expectation being to separate subpopulations of lymphocytes by preparative electrophoretic techniques (77,78,79). Free-flow electrophoresis represents a physical method of cell separation which is dependent on membrane-specific properties. Murine cells of lymphoid origin have been electrophoretically separated into two distinct subpopulations. In physiological suspending media, murine T cells express a high electrophoretic mobility (-1.33 μm/sec/v/cm) whereas B cells may be distinguished by their lower electrophoretic mobility of -0.90 μm/sec/v/cm (80). Nordling et al. (81) demonstrated that the difference in mobility between T and B cells arose from quantitative differences in neuraminidase-susceptible sialic acid on the surface of the cell membrane.

By means of chemical modification of the cell surface and subsequent measurements of the alteration in electrokinetic behavior, Mehrishi (80,82) identified five different types of chemical groups in the cell periphery of murine B cells. At a physiological pH, Mehrishi (80) detected at least three types of ionizable groups in the cell periphery of murine T cells. Later experimental studies revealed significant differences in the mobility of T cells isolated from mice with varying major histocompatibility haplotypes (83). The difference in cellular electrophoretic mobility is related to different amounts and/or types of surface membrane molecules under the control of the major histocompatibility complex (83). Supplemental electrokinetic

studies on the murine T lymphocyte should lead to a more thorough characterization of the ionogenic moieties in the cell periphery which contribute to cellular charge.

Sundaram's (84) 1967 experimental observation that antigen stimulated lymph node cells had a lower electrophoretic mobility extended the range of experimentation in which cellular electrophoresis proved to be of use. Surface reactions of lymphocytes were assessed using cellular electrophoresis. Alterations in lymphocyte electrophoretic mobility subsequent to treatment with immunosuppressing agents (85), anti-neoplastic agents (86), asparaginase (87), human chorionic gonadotropin (88) and poly A:U (89) were reported. The narrow scope of the experimental design in the majority of these studies allowed only a limited interpretation of lymphocyte cell-surface effects to be made. The generalized experimental scheme employed in these studies consisted of treating lymphocytes with a fixed concentration of a surface reagent and measuring the electrophoretic mobility prior and subsequent to treatment. An alteration in observed electrophoretic mobility was viewed as being indicative of a change in the net surface charge density of the lymphocyte. Rarely did the investigators attempt to discern the exact physicochemical processes occurring at the cell surface which manifest themselves as changes in the electrophoretic mobilities of the cells. For instance, Bert (90) noted that the electrophoretic mobility of human peripheral lymphocytes was reduced after treatment with PHA, anti-immunoglobulin serum and specific antigen. On this basis he suggested that substances which would induce lymphocyte transformation would also reduce the net surface charge density of the lymphocyte with the decrease in charge playing a central

role in the transformation of the lymphocyte.

Reduction in electrophoretic mobility, however, is a nonspecific phenomenon. An alteration in electrophoretic mobility may result from changes in the number or arrangement of ionogenic groups at the cell surface, a shift in the position of the electrophoretic slip plane or ion redistribution within the peripheral zone. Blume and coworkers (43) have reported that incubating 10^6 lymphocytes with Con A at concentrations greater than 5 μg/ml produced a dose-dependent decrease in electrophoretic mobility attributable simply to the adsorption of Con A molecules to the cell surface. Hence, in this instance, it is not a lectin-induced membrane transformation which is being detected but rather the presence of Con A bound to the lymphocyte surface.

Emphasis must therefore be placed on the limitations of experimental interpretations which are made solely on the basis of an alteration in cellular electrophoretic mobility detected subsequent to a reaction at the cell surface. A change in the electrophoretic mobility of a cell does not provide information on the specific changes which have occurred in the peripheral zone of the cell. It is only through a series of exhaustive electrophoretic studies in which the dependence of cellular electrophoretic mobility on pH, ionic strength and chemical and enzymatic modification is established that a characterization of the specific charge alterations within the peripheral zone may be made.

In 1974 Wioland (42) examined the electrokinetic behavior of the mouse thymocyte and reported an 11% increase in electrophoretic mobility upon exposure to Con A. An attempt was then made to systematically identify the alterations in the Con A-stimulated

thymocyte which resulted in the increase in electrophoretic mobility. Prior to this work there had been no attempt to exploit the potential of cellular electrophoresis to probe the ultrastructure of lymphocytes which had undergone cell surface reactions. Wioland's (42) premise was that the increase in thymocyte mobility arose from Con-A induced cap formation and the subsequent endocytosis of the cap. Cap formation would lead to a redistribution of charged groups on the cell surface while endocytosis would yield a change in the number of surface ionogenic groups. Initially, Wioland demonstrated that at low temperatures where cap formation and endocytosis were known to be inhibited, Con A had no effect on the electrophoretic mobility of the thymocyte.

In a later paper Wioland et al. (91) provided evidence of a decreased surface density of amino groups, carboxyl groups of sialic acid as well as alkaline phosphatase-susceptible phosphate groups. In addition the density of unidentified negatively charged surface groups was shown to have increased twentyfold. The decrease in density of specific ionized groups in the cell periphery was considered a reflection of endocytosis or shedding while the increase in density of the unidentified negatively charged surface groups was interpreted as a consequence of the redistribution of membrane receptors and concurrent appearance of new structures in the membrane.

Sainis et al. (92,93) also evaluated the electrophoretic response of murine lymphocytes treated with Con A. Con A was shown to induce biphasic changes in the electrophoretic mobility of murine splenic lymphocytes. Exposure to mitogenic doses of Con A led to an increase in the electrophoretic mobility of the cells, whereas

incubation with higher concentrations of Con A resulted in a reduction in cellular electrophoretic mobility.

Human T lymphocytes also are reported to exhibit an elevated electrophoretic mobility subsequent to interaction with the T cell mitogen, PHA (94). Through the use of cellular electrophoresis combined with specific enzymes and varying ionic strength media, Sato revealed a topological change of acidic sugars in the plasma membrane of PHA stimulated lymphocytes. Both an early translocation of hyaluronic acid to the T-cell periphery and a subsequent elevation in the biosynthesis of chondroitin sulfate was shown to accompany the phytohemagglutinin-induced blast transformation of human T lymphocytes.

The intent of reviewing the preceding experimental work was to emphasize the utility of electrophoretic methods in providing information on cellular surface structure. Moreover, the studies cited demonstrated that the course of chemical, enzymatic, and immunological reactions which change the number of surface charge groups may be followed by cellular electrophoresis.

1.2.3 Initial Description of the Electrochemical Membrane Phenomena

The interaction of mitogens with suitable lymphocyte surface receptors initiates the series of events associated with lymphocyte activation. Although investigators have acquired an increased awareness of the complexity of this process, a complete understanding of the early membrane changes induced by mitogen binding is lacking. Previously, it had been conjectured that mitogen binding induced a redistribution of transmembrane protein (95). The redistribution of transmembrane protein was thought to convey the

mitogenic signal to the interior of the cell and elicit the metabolic and transport changes associated with mitogenesis.

Blume et al. (43) speculated that if such a conformational change in membrane structure occurred at the time of lymphocyte-mitogen encounter, perhaps it could be detected by a biophysical technique. In attempting to identify such an early membrane conformational change, analytical particle electrophoresis, a technique sensitive to perturbations in the charged peripheral components of the membrane, was applied. The electrophoretic mobility of a cell is a reflection of its net surface charge density (70,71). It was hypothesized that given the presence of charged surface groups on the lymphocyte membrane, a conformational change involving these groups would give rise to an alteration in the surface charge density.

Early events associated with the interaction of Con A with murine T lymphocytes were monitored via analytical particle electrophoresis (43) using a nylon wool fractionated preparation (96) suspended in phosphate buffered saline (PBS). Treatment of the murine T lymphocytes with mitogenic concentrations of Con A (1-5 μg/ml) for 5 minutes at 37°C resulted in an increase in electrophoretic mobility by about 30% over control values. The change in mobility was blocked by incorporation of a competitive inhibitor of Con A, α-methylmannopyranoside, into the incubation medium (43). The Con A induced change in electrophoretic mobility of the lymphocytes was consistent with the hypothesis of a membrane configurational change accompanying the initial binding of mitogen to the lymphocytes.

However, when the concentration of Con A was progressively reduced in an attempt to determine the threshold dose that

would induce the observed mobility change, an entirely unexpected observation was made. The maximal response of a 30% increase in mobility was noted at concentrations of Con A as low as 10^{-11} µg/ml (i.e., 10^{-17} g/ml). (Fig 1.1 and 1.2) The electrophoretic response could not be attributed to the direct interaction between lectin molecules and the surface of each lymphocyte in the population in view of the short incubation period and the fact that at the threshold concentration of Con A required to initiate the response only a few hundred molecules of Con A were present. Consequently, a cellular communication mechanism was postulated to mediate the electrokinetic effect. A factor released by a few cells that bound Con A was envisioned to effect the change in electrokinetic properties of the population of cells.

The existence of this factor was demonstrated by a simple experiment involving the use of the effective Con A inhibitor, α-methylmannopyranoside. Splenic murine T cells were incubated with Con A at a concentration of 10^{-6} µg/ml for 10 minutes, the suspension centrifuged and the supernatant fluid removed. This supernatant fluid contained the putative factor as well as an indeterminate amount of residual Con A. An aliquot of this supernatant fluid was mixed with an equal amount of α-methylmannopyranoside and fresh T cells were added. These cells displayed the same 30% increase in mobility as did those cells treated directly with Con A.

A few initial experiments directed toward the characterization of the factor have been carried out (97). Preliminary purification of the factor was achieved using a Pro-di-con (Biomolecular Dynamics, Beaverton, Oregon) negative pressure apparatus. The factor solution was filtered under vacuum through a cellophane dialysis

Figure 1.1 Influence of Con A on the electrophoretic mobility of murine T lymphocytes. White bars refer to cell mobility in the absence of α-MM and black bars refer to cell mobility in the presence of α-MM. The standard error of the mean is given for the number of measurements indicated above each bar.

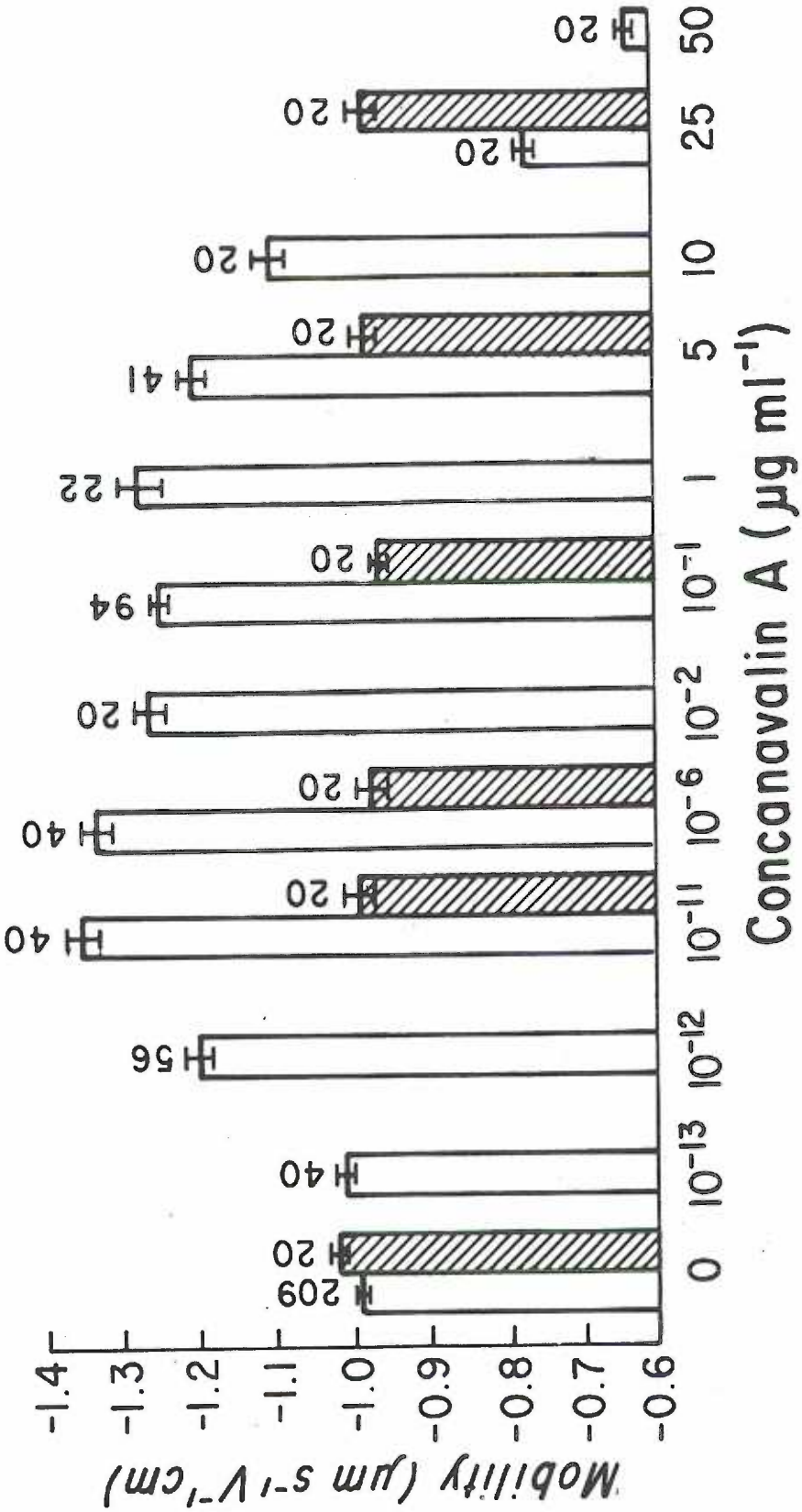
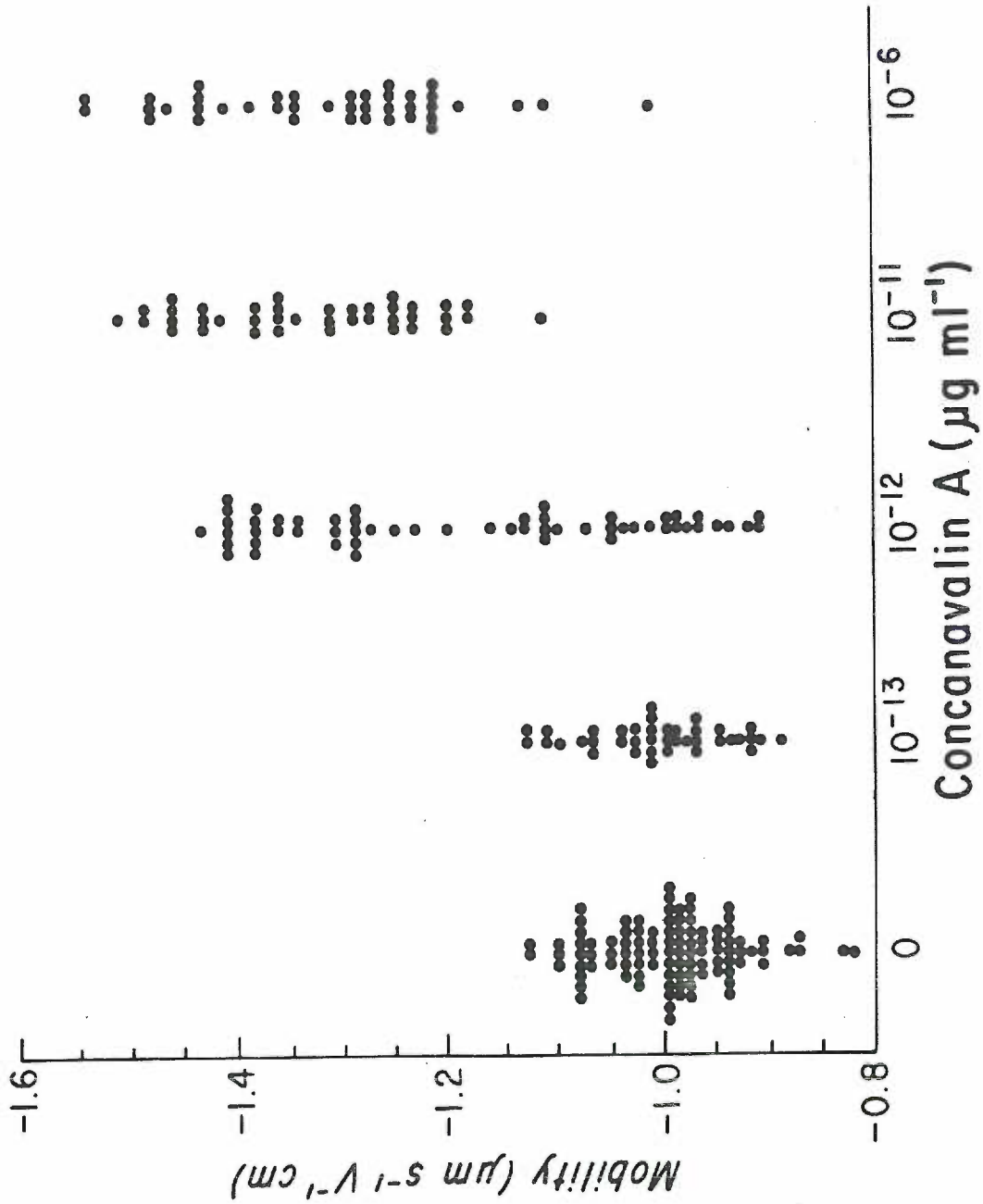

Mobility (μm s⁻¹ V⁻¹cm)
-1.4
-1.3
-1.2
-1.1
-1.0
-0.9
-0.8
-0.7
-0.6
209
20
40
56
40
20
40
20
20
94
20
22
41
20
10
20
25
20
50
0
10⁻¹³
10⁻¹²
10⁻¹¹
10⁻⁶
10⁻²
10⁻¹
1
5
Concanavalin A (μg ml⁻¹)

Figure 1.2 Distribution of mobilities of individual cells treated with various concentrations of Con A. Note that at the threshold concentration of 10^{-12} $\mu g/ml^{-1}$ of Con A the mobility values of individual cells are distinguished from control values to the maximum response values.


Mobility (μm s⁻¹ V⁻¹ cm)
-1.6
-1.4
-1.2
-1.0
-0.8
0
10⁻¹³
10⁻¹²
10⁻¹¹
10⁻⁶
Concanavalin A (μg ml⁻¹)

membrane. High molecular weight components (>12,000) in the supernatant of Con A stimulated T cells will remain inside the dialysis membrane. On the other hand, molecular weight components less than 12,000 will be pulled through the membrane. Upon testing the high (>12,000) and low (<12,000) molecular weight fractions, only the low molecular weight fraction was found to initiate a change in T cell electrophoretic mobility. The low molecular weight fraction was found to be colorless, was heat labile, stable to freeze-thawing, and exhibited the ability to effect T cell mobility at a 1:250 dilution.

Additional purification of the factor was achieved by gel filtration on Bio-gel P-2 in PBS. The elution profile of polypeptides of known molecular weight (insulin, bacitracin, ACTH, and lysozyme) lightly conjugated with trinitrophenol (TNP) was determined. The factor was applied to the same column and elution volumes corresponding to the polypeptides of known molecular weight were collected and analyzed for their ability to alter the mobility of T cells. The fraction eluted with a molecular weight between 2000 and 3000 and appeared to possess all of the activity present in the original factor preparation.

A number of additional observations were made. When unfractionated splenic lymphocytes were treated with Con A, two populations of cells resulted, only one of which displayed an increased electrophoretic mobility. Furthermore, the T cell lectin phytohemagglutinin produced an increase in the mobility of splenic T cells similar to that observed with Con A. On the other hand, the B cell lectin, bacterial lipopolysaccharide, did not produce any change in the mobility of the T cells. Experiments using cultured human leukemic

cells, CCRF-CEM (98) which have been characterized as T cells (99) were also carried out (Table 1.1). The electrokinetic response of these cells was similar to the response of murine T cells when exposed to Con A or the supernatant fluid containing the factor. Moreover, it was noted that the human derived cells responded to the murine factor with an increase in mobility just as the murine cells did.

As a result of these initial studies, several new questions bearing significance to an understanding of the immune response and lymphocyte communication emerged. In view of the biophysical orientation of our laboratory, we were particularly interested in identifying the specific membrane changes which accompany the electrophoretic response. The experimental studies detailed in this thesis represent my attempt to establish the nature of the physicochemical alterations in the peripheral zone of the factor stimulated lymphocyte which are manifest as changes in cellular surface charge.

TABLE 1-1

Behavior of CCRF-CEM Human Derived Cultured Lymphocytes

Treatment	Mean mobility, $\mu m\ s^{-1}\ V^{-1} cm$	No. measure-ments	SEM
None,control cells	-1.01	40	0.010
Con A, 10^{-1} µg/ml	-1.34	20	0.026
Con A, 10^{-6} µg/ml	-1.34	20	0.020
Con A, 10^{-6} µg/ml + α-methylmannopyranoside, 0.05 mol/liter	-0.960	10	0.012
Factor from CCRF-CEM cells + α-methylmannopyranoside, 0.05 mol/liter	-1.45	20	0.052
Factor from murine cells + α-methylmannopyranoside, 0.05 mol/liter	-1.42	20	0.052

Chapter 2

GENERAL METHODOLOGY

This chapter describes the materials and procedures which are common to much of the work presented throughout this thesis. Specific experimental details of individual experiments are outlined in their appropriate section.

2.1 Isolation of Murine T Lymphocytes

Spleens were removed from 8 to 12-week old female LAF_1 mice (Jackson Laboratories) which had been ether-anesthetized and sacrificed by cervical dislocation. Splenic cell suspensions were prepared and washed three times in 0.01M phosphate-buffered saline containing 5% v/v heat-inactivated fetal calf serum (PBS-FCS), pH 7.2, by centrifugation at 490 g for 10 min at 4°C. T lymphocytes were separated by the nylon wool filtration method of Julius et al. (96). Cell suspensions are added to a nylon wool column and the column incubated for 45 min at 37°C. B lymphocytes adhere nonspecifically to the column while the cells which pass through the column are predominately T cells. In the majority of experiments erythrocytes were lysed by treatment with buffered 0.154M ammonium chloride (100). This treatment did not influence the electrophoretic results. The final cell preparation was diluted to a cell concentration of 10^7/ml. When treated with fluorescein-labelled anti-thy serum (Microbiological Associates), 98 to 99% of the cells bound the antibody. When treated with trypan blue, more than 95% of the cells of each preparation excluded the dye.

2.2 Generation of Factor and Factor Treatment of Murine T Lymphocytes

2.2.1 Concanavalin A

Concanavalin A (Miles Laboratories) was obtained as a three-times recrystallized powder. A stock solution (100 μg ml^{-1}) of Con A was prepared by weighing out 10 mg of Con A and diluting to 100 ml with phosphate buffered saline (PBS). Con A solutions of various concentrations were prepared by serial dilution of the stock solution.

2.2.2 Preparation of Factor

Equal volumes of Con A (10^{-6} μg/ml) and murine T lymphocyte suspension (10^{-7} cells/ml) were mixed and incubated for 5 min in a water bath at 37°C. Following incubation the cell suspension was centrifuged (490 g, 10 min, 4°C) and the cell-free supernatant fluid containing the factor was collected.

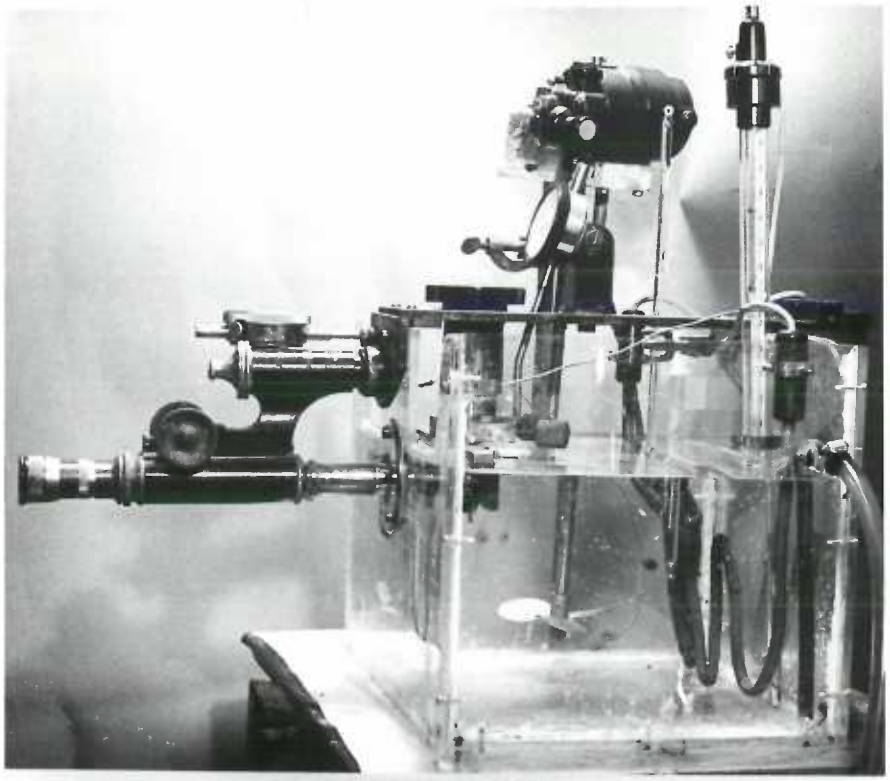
2.2.3 Factor Treatment of Murine T Lymphocytes

The general protocol for these experiments consisted of mixing 50 μl of the factor preparation with 50 μl of 0.2 M α-methylmannopyranoside (α-MM). These experiments are performed in the presence of α-MM to eliminate the effect of residual Con A which is not removed from the factor preparation. To this mixture was added 100 μl of cell suspension. The mixture was incubated for 5 min at 37°C and then subjected to electrophoretic analysis.

2.3 Analytical Particle Electrophoresis

The cylindrical cell electrophoresis equipment originally described by Bangham et al. (101) and subsequently modified by Seaman and Heard (102) was used for the determination of cellular electrophoretic mobilities. The electrophoretic instrumentation is pictured and schematically represented in Fig. 2.1 and Fig. 2.2, respectively. Seaman (103) has carefully described both the construction and operation of the apparatus.

Figure 2.1 Cylindrical chamber microelectrophoresis apparatus employed for the collection of mobility data.



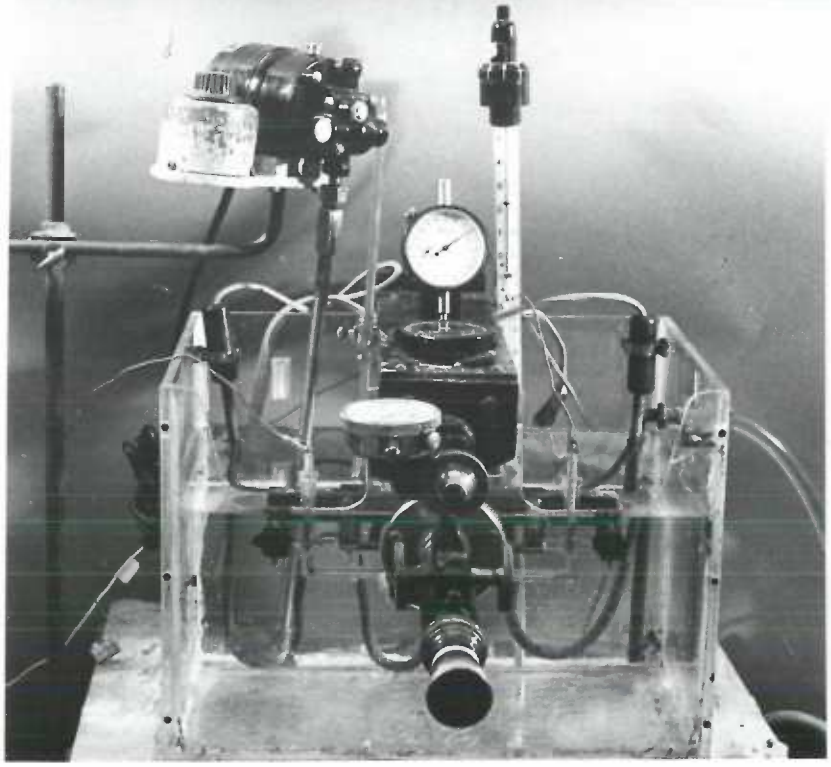
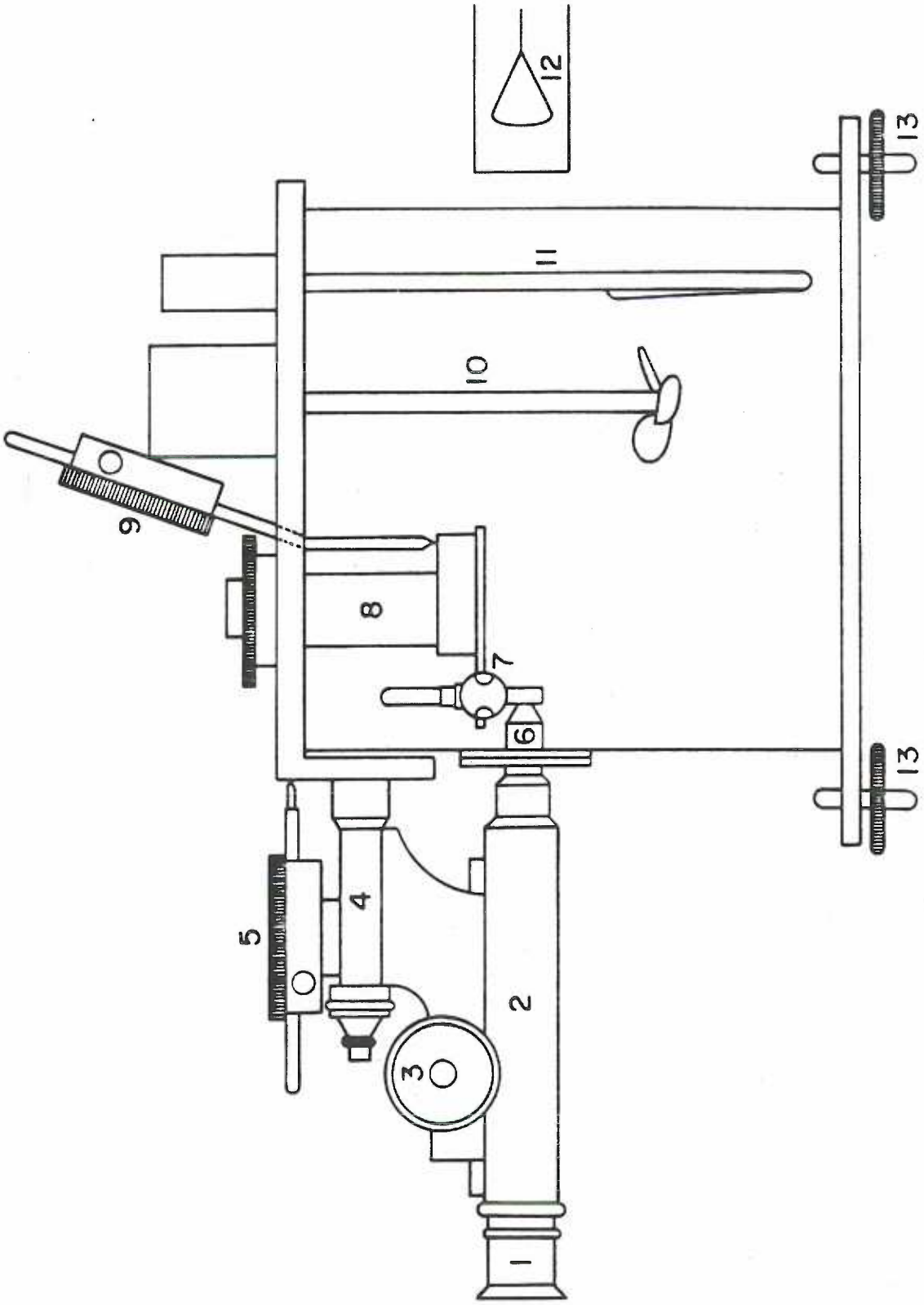


Figure 2.2 Schematic illustration of a cylindrical microelectrophoresis apparatus.

1) ocular with graticule
2) microscope tube
3) coarse adjustment for microscope
4) fine adjustment for microscope
5) dial test indicator for horizontal positioning
6) x 40 objective
7) electrophoresis chamber and chamber holder
8) vertical adjustment screw
9) dial test indicator for vertical positioning of chamber
10) stirrer
11) heater
12) light source
13) tank leveling screws


12
13
11
10
9
8
7
6
13
4
5
2
3
1

Unless stated otherwise, all electrophoretic measurements were collected at 25°C. The temperature of the water bath was controlled to within ± 0.1°C by a 200 watt heating element connected to a relay via a mercury thermostat. The stirrer in the electrophoretic apparatus was incorporated to aid in minimizing temperature gradients. The electrophoretic mobility is a temperature dependent measurement with the primary parameter responsible for the dependence being the change in viscosity of the suspending medium (104). Temperature must be controlled within a range of ± 0.5°C to prevent significant measurement errors.

The electrophoresis chamber which was incorporated in the apparatus pictured in Fig 2.1 is termed a mini-all-glass cylindrical microelectrophoresis chamber (Fig 2.3 and Fig 2.4). This electrophoresis chamber consists of a glass capillary tube which is separated from the electrode compartments by sintered-glass disks. Silver-silver chloride electrodes were used in all experiments and the electrode chambers were filled with 1.0M potassium chloride solution. In most experiments a voltage of 45V was applied across the chamber by a regulated direct-current power supply. Both voltage and amperage were monitored by a digital multimeter (Hewlett Packard 3435A) and manually recorded.

The electrical field strength, x, was calculated according to the equation:

$$x = \frac{V}{I}$$

where V is the electrical potential in practical volts across a cell

Figure 2.3 Mini-all-glass cylindrical microelectrophoresis chamber.

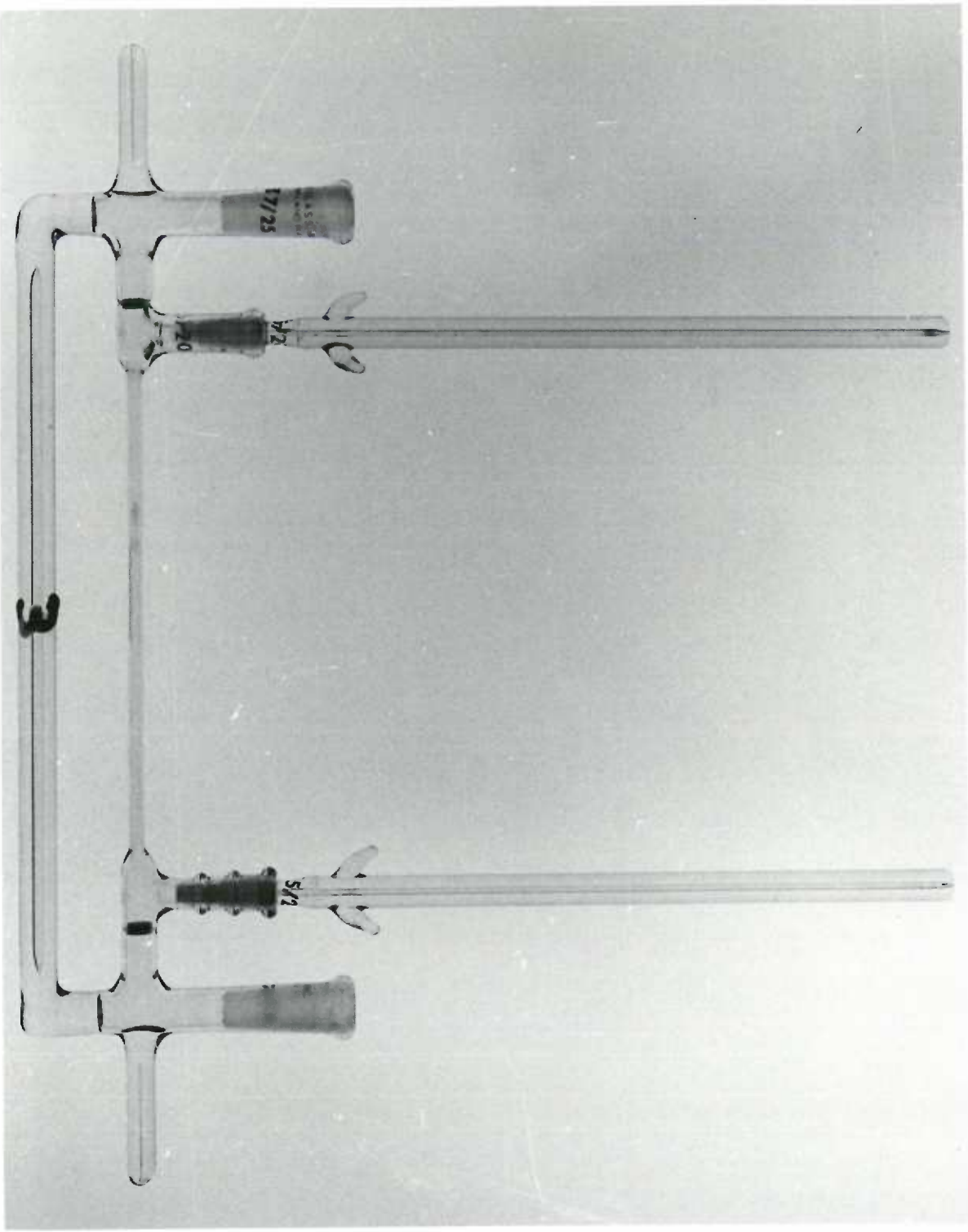
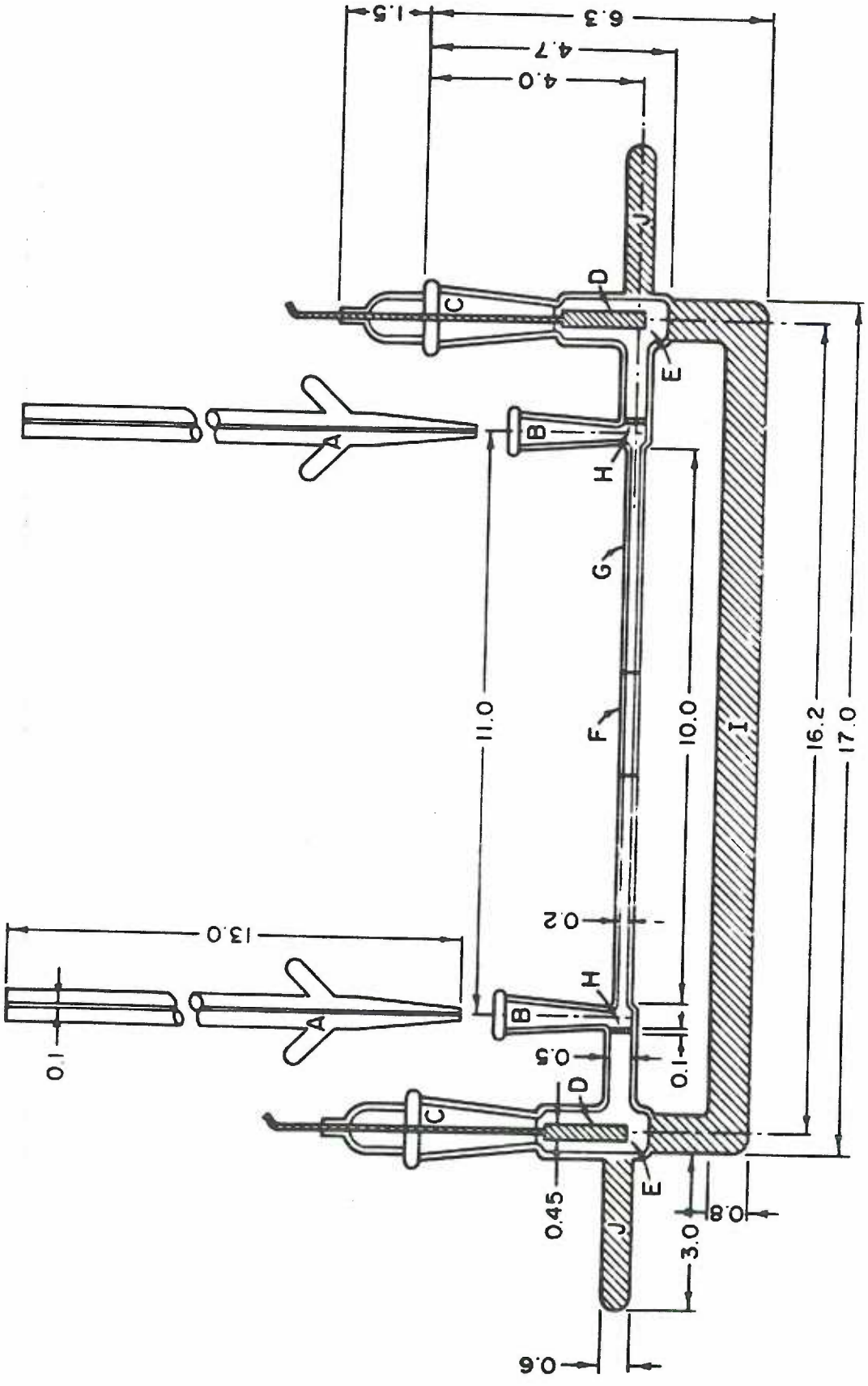

17/25
24/40
24/40

Figure 2.4 Dimensional specifications (cm) for the mini-all-glass electrophoresis chamber incorporating solid Ag/Ag Cl electrodes (D), fused in sintered glass disks (H) and capillary standard taper stoppers (A).


1.5
6.3
4.7
4.0
J
D
C
E
A
B
H
G
F
11.0
10.0
I
16.2
17.0
13.0
0.2
0.1
0.5
0.45
0.8
3.0
0.6

with a length l. In electrophoresis chambers which contain sintered glass discs to separate the electrode and sample chamber, the length is not easily measured directly. The effective electrical length, le, may be experimentally determined and substituted in the electrical field strength equation. The effective electrical length is computed from the measured amperage, I, through the sample chamber of cross sectional area, πa^2, filled with a standard KCl solution when a voltage, V, is applied, i.e.,

$$le = \frac{KV\pi a^2}{I}$$

in which K is the specific conductivity of the KCl solution at the concentration and temperature of the measurement. Analytical reagent grade KCl was always dried at 150°C to constant weight before preparing the standard KCl solutions used in the determination of effective electrical length.

One other additional adjustment that must be made prior to operating the electrophoresis apparatus is the "stationary level" determination. When a direct-current voltage is applied across the chamber, counterions in the suspending media flow in the direction of the cathode with a forced return flow along the center of the tube. This fluid flow, termed electro-osmosis, contributes to the electrophoretic mobility. This complication may be alleviated by presetting the point of focus of the microscope into the electrophoresis chamber to a position where no net flow of fluid occurs. At this position, termed the stationary level, the observed cellular mobility is a reflection solely of the interaction between cellular charge with the

potential field. The stationary level is located at a distance of 0.293 x electrophoresis tube radius from the wall of the chamber. The stationary level adjustment was made by focusing the microscope upon the inside proximal wall of the tube and then advancing the proper distance with the aid of the fine adjustment of the microscope and the horizontal dial test indicator. Only cells which are in focus at this position are electrophoretically measured.

Cell suspensions were introduced into the observation region of the electrophoresis chamber using a microinjection technique. The design of the mini-all-glass cylindrical microelectrophoresis chamber was predicated to a large extent upon reducing the required experimental sample volume. When this chamber is used in conjunction with the microinjection technique, only 0.1 ml of sample (10^7 cells/ml) is necessary to obtain electrophoretic measurements. The following paragraphs provide a thorough description of how samples were microinjected into the electrophoresis chamber.

The electrophoresis chamber was initially filled with particle free buffer (suspending media). A closed stopcock was then placed in one of the filling ports and the suspending media was allowed to equilibrate to the operation temperature of the apparatus. The sample was introduced directly into the region where the electrophoretic measurements are made via a piece of polyethylene tubing, ~15 cm long and having an inner diameter of 0.2mm, which is fitted over a 20 gauge syringe needle attached to a tuberculin syringe. Care must be taken in both filling the syringe and in injecting the sample from the syringe to assure that air bubbles are not introduced into the electrophoresis chamber.

The polyethylene tubing containing the sample was fed through the open filling port into the capillary tube and allowed to equilibrate, typically, for three minutes before the sample was injected. Once the sample was injected a stopcock was placed in the second filling port and a check was made to assure that the cells were not drifting horizontally or sedimenting more or less rapidly than normal.

It is critical that no difference in density exists between the injected sample suspension and the suspending medium. Density differences may arise from differences in temperature or small differences in media composition. Density differences will lead to convective disturbances which prevent the determination of an accurate electrophoretic velocity.

Immediately after injecting the sample into the chamber and establishing that no convections or drifts were present, the electrophoretic velocities of individual cells were measured. Generally, measurements were taken on 20 different cells in focus at the stationary level with a reversal of the polarity of the electric field between measurements. The electrophoretic mobilities are expressed in $\mu m\ s^{-1}\ V^{-1}cm$ and are based on the times of migration of a series of cells across a fixed number of divisions of an eyepiece graticule. While no empirical calibration factors are used with this technique, the performance of the apparatus was monitored by determining that the mobility of human erythrocytes suspended in 0.150M aqueous sodium chloride solution conformed with the generally accepted value of -1.08 $\mu m\ s^{-1}\ V^{-1}cm$.

2.4 Iodination of Cell Membrane Protein

The surface protein of murine T lymphocytes was selectively labeled by the [^{125}I] lactoperoxidase method described by Vitetta et al. (105). In brief, the experimental scheme consisted of adding three pulses of 0.03% H_2O_2 to cells ($\simeq 10^8$) suspended in 1 ml of PBS containing 200 µg of lactoperoxidase and 1 mCi of carrier-free Na^{125}I. Cell suspensions were incubated at room temperature with the reaction being terminated in 10 min by the addition of 10 volumes of cold 5 mM L-cysteine hydrochloride in PBS. Cell viability was determined before and after radiolabeling by trypan dye exclusion. The iodinated cells (10^7 cells/ml) were then either incubated with PBS or Con A (10^{-6} µg/ml) for 10 min, at 37°C as previously described (43). Cell suspensions were centrifuged, 350 g, 10 min, 4°C, the supernatant fluids collected and the cells solubilized in buffer containing 0.05M Tris-hydrochloride (pH 6.8), 1% v/v SDS, 1% v/v β-mercaptoethanol, 20% v/v glycerol and 6M urea.

2.5 Sodium Dodecyl Sulfate (SDS) Urea Polyacrylamide Gel Electrophoresis

Labelled surface protein was examined by gel electrophoresis in the presence of sodium dodecyl sulfate according to the procedure of Laemmli (106). Prior to gel electrophoresis the samples were heated for 2 min at 100°C. Electrophoresis was performed in 10% to 20% polyacrylamide gradient gel containing 0.6% w/v SDS. Gels were then fixed in 12.5% v/v trichloroacetic acid. The fixed gels were dried and covered with Kodak X-omat medical X-ray film and exposed at -70°C. Molecular weights were estimated by comparison to labelled proteins of defined molecular weight. (Lactoglobulin A, 18,637; carbonic anhydrase,

30,000; ovalbumin, 46,000; bovine serum albumin, 69,000; phosphorylase B, 92,500).

Chapter 3

THE RELATIONSHIP OF THE ELECTROCHEMICAL MEMBRANE PHENOMENON TO OTHER EARLY EVENTS IN LECTIN-LYMPHOCYTE INTERACTION

Originally, Blume et al. (43) sought to monitor early membrane changes associated with the lectin stimulation of lymphocytes by biophysical methodology. A rapid change in the surface charge density of murine lymphocytes subsequent to lectin interaction was detected by analytical particle electrophoresis. Further work indicated that the change in cellular charge was mediated by a low molecular weight factor which was released by those cells which reacted directly with Con A.

These preliminary studies provided prospects for the application of analytical electrophoresis to clarification of the mechanisms of cellular cooperation in the response to various stimuli. In addition the marked alterations in cellular electrophoretic mobility established that the method would also be of use in describing the alterations in the peripheral zone of the membrane which are responsible for the changes which occur in the cellular surface charge density. While the precise role of both the low molecular weight factor and cellular mobility change in blast transformation has yet to be defined, we have attempted to establish how these electrophoretic events relate to other membrane associated phenomena known to accompany lectin-lymphocyte interaction.

The experimental work detailed in this chapter describes the effects on lymphocyte electrophoretic mobility of other low molecular weight substances implicated in influencing early events in cell activation. The time course of the release of the low molecular weight factor closely approximates changes in cyclic nucleotide concentrations

in lymphocytes during activation reported by other investigators (29,30,33,35). In order to evaluate the possibility that the mobility changes associated with Con A treatment are mediated by a cyclic nucleotide, and that the factor is such a compound, experiments were performed in which the mobility of T cells was measured in the presence of cyclic nucleotides and theophylline. Theophylline is an inhibitor of cyclic nucleotide phosphodiesterase and thereby increases intracellular cyclic nucleotide levels.

Further insight into the mechanism of action of the low molecular weight factor was obtained by examining the mobility of T cells incubated with the 250-fold-diluted factor. The question addressed was whether factor-modified cells produced more factor or whether only those cells directly interacting with the Con A produce factor.

Finally, experimental studies which define the relationship between the membrane phenomena termed "capping" and the changes in cellular electrophoretic mobility are detailed in this chapter. Approximately one hour after exposure to mitogenic doses of Con A, lymphocytes undergo capping (107). Capping consists of a coordinated migration of ligand-receptor complex to one discrete area of the membrane. It was originally conjectured that the cellular mobility change might be the consequence of an alteration in the distribution of the charged moieties of membrane glycoproteins. In order to establish if this phenomenon of receptor redistribution, capping, contributed to the increase in cellular surface charge, electrophoretic studies were performed both at 0°C and in the presence of sodium azide. Capping does not occur in the presence of the metabolic inhibitor sodium azide nor at

0°C. Reducing the temperature from 37°C to 0°C increases the viscosity of the lipid bilayer of the membrane, thereby markedly reducing the mobility of membrane proteins and inhibiting capping.

Although only a percentage of cells in the lymphocyte population cap in response to mitogenic lectins and despite the fact that this event is known to occur later than the time in which mobility changes are detected, we elected to study what relation, if any, capping has to the change in cellular electrophoretic mobility. Other investigators had previously reported changes in lymphocyte electrophoretic mobility in response to mitogenic lectins during the time frame in which capping is known to occur and attributed these changes to capping of membrane protein and glycoprotein (42,92).

3.1 Materials and Methods

3.1.1 Cyclic Nucleotide Treatment of Murine T Lymphocytes

Cyclic guanidine monophosphate (cGMP), cyclic adenine monophosphate (cAMP), dibutryl cAMP and theophylline (Sigma) were diluted to the appropriate concentrations in PBS. Equal volumes of the murine T lymphocyte suspension (10^7 cells/ml) and the cyclic nucleotide preparation were mixed and incubated for 5 min in a water bath at 37°C. Subsequent to the incubation, the cells were electrophoretically analyzed at 25°C as outlined in section 2.3 of this thesis.

3.1.2 Sodium Azide Treatment of Murine T Lymphocytes

Sodium azide (J. T. Baker Chemical Company) was dissolved in PBS to make a 10 mM solution. The general protocol for these experiments consisted of mixing 50 µl of sodium azide (10^{-2}M) with 50 µl of Con A (10^{-6} µg/ml). To this mixture was added 100 µl of the murine T lymphocyte suspension. The resulting mixture was incubated for

5 min at 37°C and then electrophoretically examined as described in section 2.3 of this thesis.

3.1.3 Low Temperature Electrophoretic Experiments

Electrophoretic mobility measurements of individual cells were determined in a cylindrical chamber apparatus according to the procedure outlined in section 2.3 of this thesis. The sole exception to this previously described procedure was that the electrophoretic mobility measurements were carried out at 0°C. These low temperature experiments were performed by filling the instrument's water bath with ice and water and placing silica gel within the microscope tube to prevent condensation on the optics. The cell suspensions and all of the solutions used were brought to 0°C by placing them in an ice-water bath before mixing and maintaining that temperature during incubation and measurement.

3.2 Results

In order to evaluate the possibility that the mobility changes associated with Con A treatment were mediated by a cyclic nucleotide, and that the factor was such a compound, several experiments were performed in which the mobility of murine T cells was measured in the presence of cyclic nucleotides and theophylline. The conditions of measurement were comparable to those used for the lectin experiments. The data is shown in Table 3-1. It indicates that neither cAMP, cGMP, dibutryl cAMP, theophylline nor cyclic nucleotides in the presence of theophylline produce the increase in mobility observed following treatment with Con A. The fact that these compounds do not influence the electrophoretic mobility of murine T cells suggests that the mobility altering factor is not a cyclic nucleotide.

TABLE 3-1

Effect of Cyclic Nucleotides on Electrophoretic Mobility

of Murine Splenic T Lymphocytes

Treatment	Concentration	Mean Mobility ± S.E.M. ($\mu m\ sec^{-1}\ V^{-1}\ cm$)
Control		-1.05 ± 0.02
cAMP	2.5×10^{-3} M	-1.09 ± 0.02
cAMP	2.5×10^{-6} M	-1.05 ± 0.02
cGMP	2.5×10^{-3} M	-1.06 ± 0.02
cGMP	2.5×10^{-6} M	-1.08 ± 0.03
Dibutryl cAMP	1.0×10^{-2} M	-1.03 ± 0.02
Dibutryl cAMP	1.0×10^{-5} M	-1.04 ± 0.02
Theophylline	2.5×10^{-3} M	-1.04 ± 0.02
Theophylline	2.5×10^{-6} M	-1.08 ± 0.02
Concanavalin A	10^{-6} µg/ml	-1.32 ± 0.02
Dibutryl cAMP + Theophylline	2.5×10^{-3} M 2.5×10^{-3} M	-1.04 ± 0.03

In another series of experiments, the factor preparation was progressively diluted with PBS and tested for its ability to alter the mobility of T cells. The factor preparation was diluted in an attempt to arrive at a factor concentration where the limited amount present would alter the mobility of only a fraction of the cells present. When T cells were incubated with a 250-fold dilution of factor only a fraction of the cells in the population expressed a change in electrophoretic mobility after the routine 5 min incubation. However after a 45-60 min incubation with the diluted factor, the mobilities of all of the T cells present were increased. This preliminary data suggests that the factor may be involved in some kind of cascade amplification system. In other words, factor is produced not only by those cells directly interacting with Con A but also by factor modified cells.

In attempting to discover the nature of the cellular phenomena which give rise to a change in mobility, it was of interest to ascertain whether these phenomena are dependent upon metabolically generated adenosine triphosphate (ATP). As indicated by the data presented in Table 3-2, changes in electrophoretic mobility subsequent to Con A interaction are detected in the presence of the metabolic inhibitor, sodium azide. Cellular metabolic activity may also be inhibited or retarded by lowering the temperature of the cells to 0°C. Persistence of the mobility change effect at 0°C would be consistent with the sodium azide data and support the contention that the mobility change does not require metabolic energy. However it would, in addition, argue against a conformational change mechanism which involved migration of glycoprotein receptors through the lipid matrix of the membrane such as

Table 3-2

Effect of Sodium Azide upon Concanavalin A mediated changes in the Electrophoretic Mobility of Murine Splenic T Lymphocytes

Conditions	Mean Mobility ± S.E.M. ($\mu m\ sec^{-1}\ V^{-1}\ cm$)
Control	-1.04 ± 0.02
Con A 10^{-6} µg/ml	-1.39 ± 0.02
Sodium Azide 10^{-2} M and Con A 10^{-6} µg/ml	-1.31 ± 0.05

Table 3-3

Low Temperature Electrokinetic Experiments

Conditions	Mean Mobility ± S.E.M. ($\mu m\ sec^{-1}\ V^{-1}\ cm$)
Control lymphocytes	-0.39 ± 0.01
Lymphocytes + Con A 10^{-6} µg/ml incubated 5 min, 0°C	-0.49 ± 0.01
Lymphocytes + Con A 10^{-6} µg/ml incubated 5 min, 37°C	-0.50 ± 0.01

capping. The results of electrophoretic experiments conducted at 0°C are presented in Table 3-3. Analysis of these data indicates that even when the entire experiment is performed at 0°C there is a significant increase (23%) in the mobility of T lymphocytes treated with Con A at 10^{-6} µg/ml. When the Con A incubation was performed at 37°C and the cells then cooled to 0°C for measurement, essentially the same mobility measurement was obtained as when the cells were treated with Con A at 0°C. The markedly lower mobilities at 0°C are attributable to the increase in viscosity of the suspending medium at reduced temperature.

3.3 Discussion

The interaction of lymphocytes with mitogenic lectins leads to the expression of a myriad of functional activities. The complexity of of the sequence of events initiated by lectin binding is just now being appreciated. In the past investigators had been somewhat negligent in relating their findings to other published data in the area. Subsequent to identifying the lymphocyte electrochemical membrane phenomena, it was imperative that the relationship of this phenomena to other events known to accompany lectin-lymphocyte interaction be established.

The time frame in which cellular electrophoretic mobility changes are observed is also the one in which changes in cyclic nucleotide levels have been reported. Accordingly, the following questions were addressed: is the low molecular weight factor which is released after Con A lymphocyte interaction a cyclic nucleotide? is the change in cellular charge mediated by an alteration in the level of one of the cyclic nucleotides? The data presented in Table 3-1 suggest that the mobility altering factor is not a cyclic nucleotide as these compounds did not elicit a change in the surface charge of the T

lymphocytes. Furthermore, experiments involving the use of dibutryl cAMP illustrate that the intracellular cyclic nucleotide concentration may be altered while the mobility of the lymphocyte remains unchanged. Although cyclic nucleotides do appear to be involved in mitogenesis (30,35), this data would suggest that the involvement is at a step subsequent to that which our electrokinetic studies are addressing.

Other experimental studies reported in this chapter probe the nature of the surface changes which are manifest as an increase in cellular electrophoretic mobility. As previously emphasized, a change in the electrophoretic mobility of a cell provides only a limited amount of information on the specific changes which have occurred in the peripheral zone of the cell. An alteration in electrophoretic mobility of a cell usually arises from changes in the number or arrangement of ionogenic groups at the cell surface.

Capping is a membrane event which results in the redistribution of membrane protein and glycoprotein. Capping does not occur in the presence of the metabolic inhibitor sodium azide nor at 0°C. Reducing the temperature from 37°C to 0°C increases the viscosity of the lipid bilayer of the membrane, thereby preventing cap formation. Patch formation, however, does occur under the influence of divalent ligands at 0°C and in the presence of sodium azide (108).

In order to establish if this phenomenon of capping contributed to the increase in surface charge, electrophoretic studies were performed at 0°C. Results of these studies have shown that significant changes in the electrophoretic mobility of murine splenic T cells in response to Con A occurs at 0°C. This certainly distinguishes the factor mediated electrophoretic response from capping as well as from

the electrophoretic studies of other groups which failed to detect a change in electrophoretic mobility at 0°C (42,92).

The electrophoretic mobility is a temperature dependent measurement. Temperature dependent changes in the mobility of cells may arise in two ways, either from the result of any changes in the physical parameters relating mobility to surface charge density or from an intrinsic modification in the disposition of charged groups. The relationship between mobility and surface charge density is given in the following equation:

$$\mu = \frac{\sigma}{k\eta}$$

where μ is the electrophoretic mobility, σ is the surface charge density, k is the Debye-Huckel function, and η is the viscosity in poise.

The Debye-Huckel function is a parameter which deals with the relationship between the distribution of counter ions and co-ions near a charged boundary. This term contains an expression $(DT)^{1/2}$, D representing the dielectric constant and T representing the absolute temperature. When temperature is decreased there is a concommitant increase in dielectric constant and these two terms are assumed to compensate for one another at constant surface charge density. Therefore, the primary parameter responsible for the temperature dependence of the electrophoretic mobility measurement is the change in viscosity of the suspending medium (104).

In 1967 Seaman and Mehrishi (104) investigated the relationship between temperature and mobility for quartz particles and human red blood cells in saline. The viscosity corrected mobility was shown to be

independent of temperature from 0°C to 40°C. However, Weiss (108) later presented experimental evidence that this relationship did not hold true for metabolically active cells. Likewise, the lymphocyte electrophoretic mobility data in Table 3-3 cannot be viscosity corrected and directly compared to the electrophoretic measurements taken at 25°C. However the detection of a significant increase in lymphocyte electrophoretic mobility after exposure to Con A at 0°C does, nonetheless, provide final evidence that receptor redistribution, capping, is not involved in the electrophoretic phenomenon.

Chapter 4

ELECTROPHORETIC CHARACTERIZATION OF THE CHARGED MOIETIES CONTRIBUTING TO THE CELLULAR ELECTROPHORETIC MOBILITY

Acquiring an understanding of the early response of the lymphocyte plasma membrane to lectin binding has been the prime motivating force behind these electrophoretic studies. Following the detection of an increase in the electrophoretic mobility of murine T lymphocytes exposed to Con A, experiments were initiated which were aimed at establishing the nature of the physicochemical alterations in the peripheral zone of the Con A treated lymphocytes which are manifest as changes in cellular surface charge. As low temperature experimental results argue against the mobility changes being mediated by a redistribution of proteins in the membrane, it is incumbent upon us to arrive at an alternative mechanism. The experiments detailed in this chapter were designed to identify the specific charge groups within the peripheral zone of the murine T lymphocyte (prior and subsequent to interaction with Con A) which contribute to cellular charge.

Critical to the recognition of the peripheral zone alterations of the Con A treated lymphocyte is knowledge of the electrophoretic characteristics of the T lymphocyte prior to Con A treatment. Obviously, establishing a basis from which change may be assessed must be the initial priority.

The motivation behind the sequential electrophoretic manipulation of a cell is to identify the chemical entities populating the peripheral zone and their spatial distribution within this region. To date, the human red blood cell has been subjected to the most rigorous electrophoretic examination. Complete characterization of the

peripheral zone of the red cell, however, has not been attained. Likewise, the absolute characterization of the peripheral zone of the T lymphocyte is beyond the scope of this thesis. The studies herein were devised with the intent of identifying those constituents of the peripheral zone which contribute to the net electrokinetic charge density. Only a limited amount of information on the spatial distribution of the constituents of the peripheral zone was sought. Biological cells acquire their charge by the ionization of chemical functional groups which are an integral part of the cellular surface structure. The electrophoretic mobility of a cell provides information only on the net electrokinetic charge density and it is assumed that this charge is distributed uniformly over the electrophoretic surface. The nature of the cellular surface charge groups which contribute to the observed electrophoretic mobility may be ascertained, however, by studying the electrophoretic mobility of cells treated with enzymes and group specific chemical reagents.

Valuable information about the specific anionogenic groups contributing to the electrokinetic charge has been obtained by electrophoretically examining enzyme treated cells. The carboxyl groups of acylated neuraminic acids are a dominant anionogenic species present in the peripheral zone of many cell types (110). The specific contribution of sialic acid to the negative charge of a variety of cell types has been evaluated by releasing membrane bound sialic acid with Vibrio cholerae neuraminidase and electrophoretically examining the enzyme treated cells (76,111,112). Differences in the cell membrane structure of murine thymocytes, T and B cells have been revealed by studying the influence of neuraminidase treatment on the cellular

electrophoretic mobility (112). Mehrishi (112) has calculated through application of the Helmholtz-Smoluchowski and Gouy-Chapman equations the number of neuraminidase susceptible sialic acid groups on the cell surface of T and B cells from CBA/J mice. A quantitative difference in the amount of sialic acid exposed at the electrokinetic plane of shear of murine T and B cells was demonstrated (112). This difference in the density distribution of sialic acid accounts for the difference in electrophoretic mobility of the two cell types. In this chapter results of experiments involving the electrophoretic examination of neuraminidase treated lymphocytes, prior and following Con A treatment, are reported. These experiments were performed to ascertain if Con A binding to murine T lymphocytes is associated with a rapid change in the number or distribution of sialyl groups present in the cell periphery.

Although ambiguities in electrokinetic interpretations are minimized by examining only those cells which have been modified in both a specific and reversible manner, the electrokinetic behavior of murine T cells fixed with formaldehyde was also examined and the results are detailed in this chapter. Formaldehyde reacts with amino, imino, guanidino, hydroxyl and thiol groups on the cell surface. An increase in anodic mobility subsequent to reaction with formaldehyde is due to the elimination of positive charge within the peripheral zone of the cell.

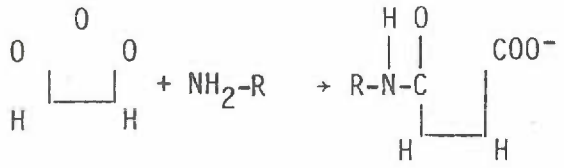
$$HCHO + RNH_2 \rightarrow R\text{-}N(H)(CH_2OH)$$

The utility of aldehyde treatment in identifying positively charged amino groups is limited by its lack of specificity. As a result, a second and more specific chemical modification scheme was

applied to detect the presence of membrane associated amino groups.

Dixon and Perham (113) are credited with first noting the usefulness of 2,3-dimethylmaleic anhydride (DMA) as a reversible blocking agent for protein amino groups in insulin and arginine. Later, Mehrishi (114) investigated and reported on the application of maleic anhydride, citraconic anhydride, and 2,3-dimethylmaleic anhydride in the determination and quantitation of positively charged amino groups by cellular electrophoresis. Most recently, Mehrishi (115) has demonstrated through the use of maleic anhydride a difference in the number of cationogenic amino groups of thymocytes from congenic mice of different major histocompatibility haplotypes.

Positively charged amino groups on the surface of murine T lymphocytes (prior and following Con A treatment) were electrophoretically assessed by chemically modifying the cell surface amino groups with maleic anhydride. The blocking of a cell surface amino group with maleic anhydride results in the introduction of a structure bearing an ionizable carboxyl group. An increase in anodic mobility after reaction with maleic anhydride is therefore due not only to the elimination of positive charge but also to the introduction of an adduct carrying an ionizable negative group at the cell periphery. The anhydride-amino group reaction is represented by the following scheme:



To summarize, the experimental studies described in this chapter are focused on identifying the specific membrane alterations of the Con A treated lymphocyte which contribute to the increase in

cellular surface charge.

4.1 Materials and Methods

4.1.1 Neuraminidase Treatment of Murine T Lymphocytes

The specific contribution of sialic acid to the negative charge of the murine T lymphocyte was assessed by treating the cells with Vibrio cholerae neuraminidase (VCN, acylneuraminyl hydrolase EC 3.2.1.18) and electrophoretically examining the cells subsequent to enzymatic treatment. Vibrio cholerae neuraminidase releases membrane bound N-acetylneuraminic acid (NANA), the main sialic acid constituent of lymphoid cells (116). Neuraminidase isolated from the Vibrio cholerae organism acts by hydrolyzing α 2→3 (117), α 2→4 (118), α 2→6 (117), and α 2→8 (118) linkages of α-O-ketosidically linked sialic acids. Vibrio cholerae neuraminidase, prepared as described by Schick and Zilg (119), was purchased from Calbiochem-Behring Corp. (La Jolla, CA). The enzyme was provided in 0.05 M sodium acetate buffer, pH 5.5, containing 0.15 M NaCl and 0.01 M $CaCl_2$ (enzyme buffer) in 1 ml vials containing 500 units of activity where one unit is the amount of enzyme required to release 1 µg of N-acetylneuraminic acid from human α_1-acid glycoprotein in 15 min at 37°C.

Murine T lymphocytes, either native cells or Con A (10^{-6} µg/ml) treated cells (1 vol) were washed 2X in PBS (9 vol) and centrifuged at 350 g, 10 min, 4°C. Cells were suspended to a final concentration of 10^7 cells/ml and pre-equilibrated to 37°C. Subsequently, VCN (60 µl of 500 units ml^{-1} VCN preparation) in enzyme buffer was added to a 1 ml sample of cell suspension. The suspension was mixed and incubated for one hour at 37°C. Control experiments were performed in which an equivalent volume of pH 5.5 enzyme buffer was

added instead of enzyme. Following incubation, samples were centrifuged for 10 min at 350 g and the supernatant fluid removed. The samples were then washed 2X in PBS (9 vol) and electrophoretically examined according to the procedures outlined in section 2.3 of this thesis. In the initial experiments samples were enzymatically treated a second time and electrophoretically re-examined to assure that all neuraminidase susceptible sialic acid had been released by the enzymatic treatment.

4.1.2 Formaldehyde Treatment of Murine T Lymphocytes

Pure formaldehyde gas was generated by heating paraformaldehyde (Matheson, Coleman, and Bell, lots A5H17 and 11F12) to 203-210°C (121). The generated formaldehyde gas was dissolved in water and the resulting solution assayed by the sodium sulfite method (122) in order to determine the molarity. The solution was then diluted with PBS to give a final ionic strength of 0.150 g ions/μl and a formaldehyde concentration of 0.583 M.

Control lymphocytes or Con A (10^{-6} μg/ml) treated lymphocytes (1 vol) were washed twice in PBS (9 vol) and centrifuged at 350 g, 10 min, 4°C. Cells (1 vol, approximately 10^8 per ml) were then suspended in 0.583 M formaldehyde in PBS (9 vol) and stored at room temperature for at least 48 hr. Formaldehyde treated lymphocytes were washed twice in PBS prior to electrophoretic examination.

4.1.3 Maleic Anhydride Treatment of Murine T Lymphocytes

Maleic anhydride (Pierce Chemicals, Rockford, IL) was furnished in fused briquettes and subsequently re-crystallized in chloroform. Maleic anhydride readily reacts with water to form maleic acid. Prior to each experiment the melting point of maleic anhydride (52.8°C) was determined to assure that it had not been converted to

maleic acid (M.P. 138°C). Re-crystallized maleic anhydride was dissolved in glass distilled p-dioxane (MCB Reagents, Darmstadt, Germany) to a final concentration of 20 mg/ml. Freshly distilled dioxane was always used to ensure that no peroxides interferred with the reaction.

Murine T lymphocytes [native lymphocytes and Con A (10^{-6} µg/ml) treated lymphocytes] were washed twice in 9X their volume of PBS and suspended to a final concentration of 10^7 cells/ml. A 1 ml aliquot of cell suspension was mixed with 3.5 µl of maleic anhydride (20 mg/ml) in a 15 ml glass conical test tube. Control experiments in which cells were incubated with 3.5 µl of dioxane were run to observe the effects of the solvent on the electrophoretic mobility of murine T lymphocytes. Dioxane is reported to be non-toxic for cells at the concentration employed in this experiment (115). Nevertheless, the viability of the lymphocytes before and after maleic anhydride treatment was determined by trypan blue dye exclusion.

Immediately after a 5 min incubation at room temperature with maleic anhydride, the T cells were electrophoretically examined according to the procedures described in section 2.3. The maleic anhydride reaction is quite rigorous and rapidly goes to completion at physiological pH. It is imperative that the reaction be carried out in a buffered media such as PBS to ensure that the addition of maleic anhydride does not produce a significant decrease in the pH of the media which would be deleterious to the cells. The electrophoretic analysis was promptly completed, usually within 20 min after the addition of maleic anhydride to the cells.

4.2 Results

In order to evaluate the possibility that an alteration in the number of sialic acid groups present in the peripheral zone of the cell membrane contributes to the observed increase in electrophoretic mobility of lymphocytes treated with Con A, the specific contribution of sialic acid to the mobility of both control and Con A treated lymphocytes was evaluated. Approximately 30% of the net negative charge of both control and Con A treated lymphocytes arises from neuraminidase susceptible sialic acid as evidenced by the data in Table 4-1. The concentration of neuraminidase required to release the membrane associated sialic acid was determined by consulting previously published studies in the literature (91,111,112). In order to assure that all neuraminidase susceptible sialic acid had indeed been liberated, the cells were enzymatically treated a second time and the cellular electrophoretic mobility measured. No further reduction in the cellular electrophoretic mobility was induced by the second enzymatic treatment. These results indicate that the Con A treatment of T lymphocytes does not significantly change the number of sialic acid residues contributing to cellular charge at physiological ionic strength.

In a second series of experiments the electrophoretic behavior of formaldehyde fixed cells was examined. After reviewing Vassar et al's (123) analyses of the physicochemical effects of aldehydes on human erythrocytes, formaldehyde was selected as the fixative agent of choice. Aldehyde purity was always monitored prior to use by scanning its spectrum from 200 to 340 nm on a Beckman Model 25 spectrophotometer (122). A high degree of aldehyde purity and lack of detectable

Table 4-1

Influence of *Vibrio cholerae* Neuraminidase on the Electrophoretic Mobility of Murine Splenic T Lymphocytes

Treatment	Mean Mobility ± S.E.M. ($\mu m\ sec^{-1}\ V^{-1}\ cm$)
Control lymphocytes	-1.19 ± 0.03
Control lymphocytes *Vibrio cholerae* neuraminidase	-0.79 ± 0.02
Lymphocytes + Con A 10^{-6} µg/ml	-1.40 ± 0.03
Lymphocytes + Con A 10^{-6} µg/ml *Vibrio cholerae* neuraminidase	-0.91 ± 0.03

contaminants was indicated by the presence of a single absorption band at 280 nm.

Murine T lymphocytes prior and subsequent to interaction with Con A were treated with 0.584 M formaldehyde. It was found that the mobility of control cells increased 30% after treatment with formaldehyde, whereas the mobility of Con A treated cells exhibited no detectable change in electrophoretic mobility (Table 4-2). Experiments were performed in triplicate and the electrophoretic mobility measurements reported in Table 4-2 represent the mean values. These experimental results imply that there are more formaldehyde reactive groups in the peripheral zone of control cells than cells which have interacted with Con A.

To investigate the nature of the formaldehyde reactive groups present in the peripheral zone of control lymphocytes but absent in the peripheral zone of Con A treated lymphocytes, the influence of maleic anhydride on cellular electrophoretic mobility was studied. Free amino groups present at the cell surface were detected from the increase in anodic mobility of cells following treatment with maleic anhydride. In these studies maleic anhydride was dissolved in p-dioxane to a concentration of 20 mg/ml. Lymphocytes were treated with $\sim 1 \times 10^{-13}$ moles of maleic anhydride per lymphocyte. Cell viability both before and after maleic anhydride treatment was always greater than 95% as determined by trypan blue dye exclusion. Moreover, dioxane was shown to have no effect on the electrophoretic mobility of the lymphocytes as evidenced by the data in Table 4-3. More importantly however, the data in Table 4-3 reveals that only control lymphocytes exhibited a significant increase in mobility after reaction with maleic anhydride,

Table 4-2

Effect of Formaldehyde Treatment on the Electrophoretic Mobility of Murine Splenic T Lymphocytes

Treatment	Mean Mobility ± S.E.M. ($\mu m\ sec^{-1}\ V^{-1}\ cm$)
Control lymphocytes	-1.05 ± 0.02
Control lymphocytes fixed 1.75% HCHO	-1.33 ± 0.01
Lymphocytes + Con A 10^{-6} µg/ml	-1.35 ± 0.02
Lymphocytes + Con A 10^{-6} µg/ml fixed 1.75% HCHO	-1.36 ± 0.01

Table 4-3

Influence of Maleic Anhydride Treatment on the Electrophoretic Mobility of Murine Splenic T Lymphocytes

Treatment	Mean Mobility ± S.E.M. ($\mu m\ sec^{-1}\ V^{-1}\ cm$)
Control lymphocytes	-1.09 ± 0.02
Control lymphocytes p-dioxane	-1.09 ± 0.02
Control lymphocytes maleic anhydride	-1.63 ± 0.03
Lymphocytes + Con A 10^{-6} µg/ml	-1.30 ± 0.02
Lymphocytes + Con A 10^{-6} µg/ml p-dioxane	-1.29 ± 0.02
Lymphocytes + Con A 10^{-6} µg/ml maleic anhydride	-1.28 ± 0.02

whereas Con A treated lymphocytes expressed no further change in cellular surface charge upon treatment with maleic anhydride. Maleic anhydride induced a 50% increase in the anodic mobility of control lymphocytes. Note that when interpreting this result, it must be remembered that an increase in anodic mobility after reaction with maleic anhydride is due not only to the elimination of positive charge but also to the introduction of an adduct carrying an ionizable negative group at the cell periphery.

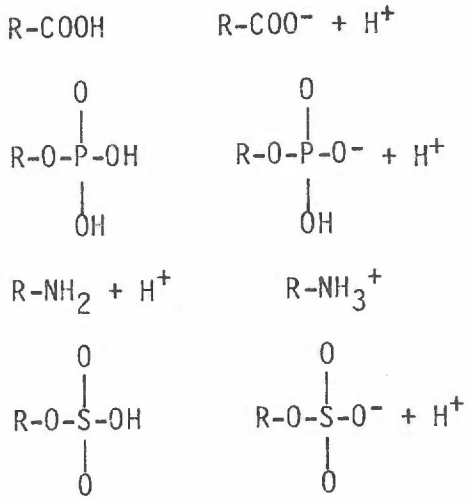
4.3 Discussion

Initial contact between biological cells and environmental stimuli occurs in the region of the cell membrane termed the peripheral zone. The course of any chemical, enzymatic, immunological or viral reaction which occurs in this region and effects an alteration in surface charge density may be followed by electrophoretic methods. The measurement of cellular charge and the examination of the factors which modify cell charge has proven valuable in acquiring an understanding of a variety of biological interactions.

Analytical particle electrophoresis was applied to the study of lectin lymphocyte interaction in an attempt to gain an understanding of the early response of the plasma membrane to mitogenic lectins. Con A was shown to induce a significant increase in the cellular surface charge density of murine T lymphocytes (43). The experiments described in this chapter were performed to establish the nature of the physicochemical alterations in the peripheral zone of Con A treated lymphocytes which are manifest as an increase in cellular surface charge.

The electrical charge at the surface of biological cells is

derived almost exclusively from the ionization of chemical functional groups within the peripheral zone of the cell while ion adsorption or desorption exerts only a minimal influence in determining cellular charge (124). Ionizable groups typically found in the cell periphery include:



The terminal carboxyl groups of sialic acids are known to be a major anionogenic component contributing to the negative charge density of a variety of biological cells (76,111,112). "Sialic acids" refer to a family of compounds which are derived from an unsubstituted nine-carbon chain called neuraminic acid:

$$COOH\text{-}CO\text{-}CH_2\text{-}CHOH\text{-}CHNH_2\text{-}(CHOH)_3\text{-}CH_2OH$$

In most cases sialic acids occupy the terminal nonreducing ends of oligosaccharide chain which are associated with cell surface glycoproteins and glycolipids. The specific contribution of sialic acid to the electrokinetic charge density of a variety of cell types has been assessed by treating the cells with neuraminidase and measuring the cellular electrophoretic mobility (76,111,112). Neuraminidase is a specific α-glycosidase which cleaves the α-ketosidic linkage between the keto group of a terminal N-acetylated neuraminic acid and the

penultimate carbohydrate residue.

Prior to the experimental studies outlined in this chapter, it had been established that sialic acid was a dominant anionogenic species contributing to the electrokinetic charge of both murine T and B cells (82). In physiological suspending media (0.15 M NaCl pH = 7.2), murine T cells express a high electrophoretic mobility (-1.33 μm/sec/V/cm), whereas B cells may be distinguished by their lower electrophoretic mobility of -0.90 μm/sec/V/cm (80). Nordling et al. (81) demonstrated that the difference in mobility between T and B cells arose from quanitative differences in neuraminidase-susceptible sialic acid on the surface of the cell membrane.

Sialic acid is incorporated into the terminal carbohydrate portions of membrane glycoproteins through the action of sialyltransferase. Sialyltransferase catalyzes the addition of sialic acid units from nucleotide sugar donors to incomplete carbohydrate chains attached to protein or lipid (125). Painter and White (126) demonstrated that sialyltransferase activity of mouse thymocytes was enhanced shortly after thymocyte interaction with Con A. After consideration of the preceding experimental findings, experiments were initiated to determine if Con A treatment of T lymphocytes induced a change in the number of sialic acid residues contributing to cellular charge. This contention would be experimentally supported if neuraminidase treatment reduced the electrophoretic mobility of Con A treated lymphocytes to a greater extent than it reduced the mobility of control lymphocytes. The results from the neuraminidase treatment experiments proved otherwise. Analysis of the data in Table 4-1 indicates that neuraminidase treatment of the cell populations produced

a 30% diminution in the electrophoretic mobility of both control and Con A treated lymphocytes. In other words, the contribution of sialic acid to the net electrokinetic charge density of murine T lymphocytes is unaltered by Con A treatment.

In a second series of experiments the possibility that the increase in negative surface charge of Con A treated lymphocytes arose from a loss of positively charged amino groups at the electrophoretic surface was investigated. Initially, the contribution of amino groups to the electrophoretic properties of the cell was evaluated by examining formaldehyde fixed cells. Optimally, the procedure of fixation should maintain cells in their original condition as regards size, morphology, and spatial relationship of organelles and macromolecules. Aldehyde fixed cells have been widely used in electron microscopic research on cells and tissues (127,128), as well as in rheological studies (129) and serological investigations. As previously stated, formaldehyde reacts with amino, imino, guanidino, hydroxyl, and thiol groups on the cell surface. At room temperature formaldehyde reacts with amino groups of proteins to form methylolamines, which then react more slowly to form a methylene bridge between primary or secondary amino groups (131) according to the following reaction schemes:

$$R\text{-}NH_2 + HCHO \rightarrow R\text{-}NH\text{-}CH_2OH$$

$$R\text{-}NH\text{-}CH_2OH + HCHO \rightarrow R\text{-}N\text{-}(\text{-}CH_2OH)_2$$

$$\begin{array}{l} CH_2OH \\ R\text{-}N \\ CH_2OH \end{array} + 2\ R'\text{-}NH_2 \rightarrow \begin{array}{l} CH_2\text{-}NH\text{-}R' \\ R\text{-}N \\ CH_2\text{-}NH\text{-}R' \end{array}$$

An increase in anodic mobility subsequent to reaction with formaldehyde is due to the elimination of positive charge within the peripheral zone

of the cell. Vassar et al. (132) demonstrated that formaldehyde treatment effected a 15% increase in the electrophoretic mobility of human blood lymphocytes. Wiig (133) analyzed the cell membrane structure of murine thymocytes, T and B cells by enzyme and formalin treatment. Aldehyde fixation increased the electrophoretic mobility of all the cell types (thymocytes, T and B cells) to a comparable degree, indicating a similarity in the number of positive amino groups on all three kinds of cells.

Evaluation of the formaldehyde fixation experimental results reported in Table 4-1 reveals that the mobility of control cells increased approximately 30% after treatment with formaldehyde, whereas the mobility of Con A treated lymphocytes exhibited no further detectable change in electrophoretic mobility subsequent to formaldehyde treatment. A loss of formaldehyde reactive groups, putatively amino groups, from the peripheral zone of cells that have been treated with Con A is implied from these experimental results. It should be noted that the increase in anodic electrophoretic mobility produced by either Con A or formaldehyde fixation is a reflection of only those amino groups which are effective at the electrophoretic surface which, under the conditions of physiological ionic strength used for the measurements, represents only those groups in the outermost 8Å or so of the peripheral zone of the cell membrane. The depth of the electrophoretic surface (effective thickness of the double layer, 1/K) is calculated with the aid of the following equation.

$$K = \left(\frac{4\pi e^2}{1000\ \varepsilon\ kT}\right) N \sum_i c_i z_i^2 \Bigg]^{1/2}$$

where

e = the electronic charge

ϵ = the dielectric constant of the suspending medium

k = Botzmann's constant

T = the absolute temperature

N = Avogadro's number

c_i = the molarity of the ionic species of type i

z_i = the valence of charge of the ionic species of type i

It is apparent after studying the above equation that a decrease in ionic valence or concentration (ionic strength) will expand the thickness of the double layer. Working in media of low ionic strength affords the opportunity of characterizing the innermost portion of the peripheral zone. In practice, however, it is not possible to work in media of extremely low ionic strength as the adsorption of macromolecules to the cell surface is enhanced under these conditions and biological cells are frequently electrokinetically unstable in low ionic strength media.

In an attempt to identify those formaldehyde reactive groups present in the peripheral zone of control lymphocytes but absent from Con A treated lymphocytes, both the control and Con A treated cell populations were chemically treated with maleic anhydride and electrophoretically examined. A variety of chemical modification schemes (134) are available for the detection of positively charged amino groups. Several of these modification schemes such as 2,4,6-trinitrobenzene sulfonic acid (TNBS) are not specific for amino groups but also react with sulphydryl groups (134). At physiological pH maleic anhydride does not react with sulphydryl groups and thereby complicate

the electrophoretic detection of positively charged amino groups (134). Hence, maleic anhydride was selected as the optimal chemical modification scheme for detection of amino groups present in the cell periphery.

Analysis of the results of these chemical modification experiments (Table 4-3) indicates that only the control lymphocyte population exhibited an increase in mobility after reaction with maleic anhydride, whereas Con A treated lymphocytes expressed no further change in cellular surface charge upon treatment with maleic anhydride. In electrophoretic terms, the anhydride-amino group reactions are twice as sensitive as the formaldehyde-amino group reaction in which the positive charge is simply blocked. Hence, if all of the formaldehyde reactive groups present in the peripheral zone of control lymphocytes were positively charged amino groups then reaction of these control cells with maleic anhydride would result in an increase in cellular electrophoretic mobility twice as great as the increase produced by formaldehyde treatment. Detection of a 50% increase in cellular mobility of control lymphocytes subsequent to reaction with maleic anhydride would suggest that the majority (~85%) of the formaldehyde reactive groups are indeed positively charged amino groups. In conclusion, there is a reduction in the number of positively charged amino groups present in the peripheral zone of T lymphocytes and contributing to the net negative surface charge density at physiological ionic strength after short term incubation with Con A. This reduction in amino groups might arise from either a loss of amino group containing membrane moieties or from a conformational change of amino group containing proteins such that the amino groups were no longer effective at the electrophoretic surface.

Chapter 5

CHARACTERIZATION OF SURFACE PROTEIN OF MURINE T LYMPHOCYTES AND RELATION TO THE ELECTROCHEMICAL MEMBRANE PHENOMENA

The analytical particle electrophoresis studies described in the preceding chapters were complemented by experimental work involving the selective labeling, separation, and characterization of surface protein and glycoprotein. Aside from identifying and quantitating the specific chemical moieties in the peripheral zone which contribute to cellular charge, experiments were performed to also identify the glycoproteins and proteins present on the surface of the murine T lymphocyte, prior and subsequent to interaction with Con A. This method of examination was utilized in an attempt to discern whether the increase in mobility of the Con A treated lymphocytes arose from the covalent modification of proteins already present in the cell membrane or from either the release of proteins from the cell surface or from the addition of new proteins to the cell surface. Acquiring an understanding of the electrochemical membrane phenomena would ideally involve the identification of both the ionogenic groups which contribute to the increase in surface charge of the Con A treated lymphocyte and the specific membrane proteins or glycoproteins with which these groups are associated at the cell surface.

Lymphocyte membrane protein and glycoprotein had been selectively labeled and characterized by other investigators (135,136). Gahmberg et al. (137) analyzed the surface components of murine lymphoid cells by utilizing the galactose oxidase tritiated sodium borohydride technique to label exposed glycoproteins. Basically, galactose oxidase oxidizes terminal galactosyl and N-acetyl

galactosaminyl residues to the corresponding C6 aldehydes. The oxidized glycoproteins are then labeled by reduction with tritiated sodium borohydride. Since sialic acids are frequently linked to penultimate galactosyl/N-acetyl galactosaminyl residues, pretreatment of the cells with neuraminidase will increase the efficiency of the labeling by leading to the detection of those glycoproteins containing a terminal sialic acid. In these studies Gahmberg et al. (137) demonstrated that murine T lymphocytes which were stimulated by a 72 hour culture with Con A exhibited only weak labeling of the major glycoproteins characteristic of T cells. In addition, new components of lower molecular weights were reported to appear on the cell surface of the stimulated lymphocytes.

Trowbridge et al. (135) also examined the surface protein of mitogen activated murine T cells. The cell surface protein was selectively labeled by means of lactoperoxidase catalyzed iodination. Labeled proteins were then examined by gel electrophoresis in the presence of sodium dodecylsulfate. The major cell surface proteins of murine lymphoid cells with affinity for concanavalin A were isolated and characterized in studies by Nilsson et al. (138). While a number of selective labeling approaches have been applied to characterize membrane protein changes associated with lectin lymphocyte interaction, these studies have not focused on identifying the early membrane changes associated with Con A binding. The experiments which are described in this chapter were performed to ascertain if Con A binding to T lymphocytes induced a rapid change in the cell membrane protein which contributed to the observed increase in cellular electrophoretic mobility.

5.1 Materials and Methods

5.1.1 Cell Surface Labeling Experiments

In the majority of experiments cell surface membrane proteins were labeled by the lactoperoxidase-catalyzed iodination method described in section 2.4 of this thesis. In a few experiments cell populations were iodinated by the chloroglycouril method of Markwell and Fox (139). Scintilation vials were plated with 200 µg of 1,3,4,6-tetrachloro-diphenylglycouril. One milliliter of cell suspension (10^7 cells/ml) was then added to each vial. The iodination reactions were initiated by the addition of 500 µCi of $Na^{125}I$ to each vial and allowed to proceed for 10 min with gentle agitation at room temperature. The iodination procedure was terminated by removing the cell suspensions from the scintillation vial and washing the cells twice in 9 vol. PBS, 350 g, 4°C. The iodinated cells (10^7 cells/ml) were then incubated with either PBS or Con A (10^6 µg/ml) for 10 min, 37°C as previously described (43). Subsequent to incubation the cell suspensions were centrifuged at 350 g, 10 min, 4°C. The supernatant fluids were then collected and the cells solubilized in buffer containing 0.05 M tris-hydrochloride (pH 6.8), 1% w/v SDS, 1% v/v β mercaptoethanol, 20% v/v glycerol and 6 M urea. Labeled proteins were examined by SDS urea polyacrylamide gel electrophoresis as outlined in section 2.5 of this thesis. The chloroglycouril iodination method avoids the introduction of extraneous proteins into the reaction system. Extraneous protein such as the enzyme lactoperoxidase added in the lactoperoxidase catalyzed iodination scheme described in section 2.4 of this thesis may itself become highly labeled during the iodination process and interfere with the analysis of labeled protein.

5.1.2 Concentration of Cell Free Supernatant Fluids

Cell free supernatant fluids collected from iodinated lymphocytes which had been incubated with either PBS or Con A (10^{-6} µg/ml) were concentrated approximately 20 fold by a Pro-di-con (Biomolecular Dynamics, Beaverton, OR) negative pressure apparatus. High molecular weight components (>12,000) in the supernatant fluids were retained inside the dialysis membrane while molecular weight components less than 12,000 were pulled through the membrane. After the supernatant fluids were concentrated to a final volume of ~100 µl, lysis buffer containing 0.05 M tris-hydrochloride (pH 6.8), 1% w/v SDS, 1% v/v betamercaptoethanol, 20% v/v glycerol and 6 M urea was added. Labeled proteins in the supernatant fluids were then examined by SDS urea polyacrylamide gel electrophoresis as described in section 2.5 of this thesis.

5.2 Results

Subsequent to cell surface iodination, T cells were incubated with either PBS, Con A (10^{-6} µg/ml) or factor prepared according to the procedure outlined in section 2.2 of this thesis. Following incubation cell viability was determined by trypan blue dye exclusion and was always greater than 95%. In addition, the cellular electrophoretic mobility was determined prior and subsequent to incubation (Con A, or factor) to assure that the labeled surface protein to be examined by gel electrophoresis was derived from cells which expressed the elevated electrophoretic mobility (-1.35 µm/sec/v/cm). The electrophoretic mobility of control cells incubated with PBS was also checked.

The profiles of the proteins from control and Con A treated murine T cells labeled by lactoperoxidase-catalyzed iodination are shown

Figure 5.1 Polyacrylamide slab gel electrophoresis of LAF, splenic T lymphocyte membrane proteins. A, control cells; B, Con A treated cells.

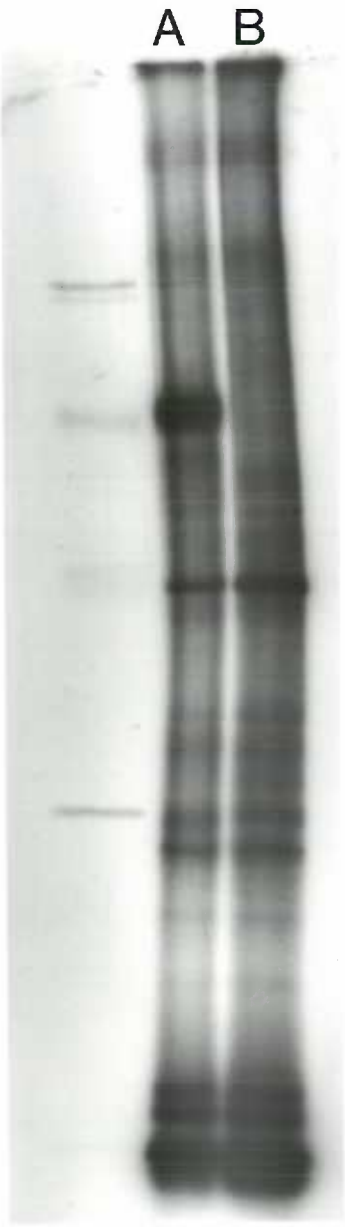

A
B

Figure 5.2 Polyacrylamide slab gel electrophoresis. A, iodinated membrane proteins of Con A treated murine T lymphocytes; B, concentrated supernatant from Con A treated murine T lymphocytes.


A
B

in Fig. 5-1. The salient observation reached by comparing these two profiles of membrane protein is the absence of a 70,000 dalton protein from the autoradiograph of the membrane protein of Con A treated lymphocytes. Similar protein profiles for the two cell populations were obtained when 1,3,4,6-tetrachloro-3α6-diphenylglycouril was used to iodinate membrane protein.

In a later series of experiments the supernatant fluid from iodinated T cells which were incubated with Con A was collected and analyzed. The intent of these experiments was to ascertain if the 70,000 dalton protein absent in the autoradiographic analysis of Con A treated lymphocyte membrane protein was present in the supernatant fluid. SDS polyacrylamide gel electrophoresis analyses of the concentrated supernatant fluid of Con A treated lymphocytes along with the profile of membrane proteins of Con A treated lymphocytes are shown in Fig. 5-2. Only one protein was detected in the supernatant fluid and this protein had a molecular weight of 70,000 daltons.

Before proceeding to a discussion of these experimental results it should be noted that the amount of the 70,000 dalton protein detected on control cell membranes was variable. The cellular electrophoretic mobility, however, was constant and independent of the amount of 70,000 dalton protein present on the control T lymphocytes.

5.3 Discussion

The cell surface of lymphoid cells has been extensively studied in an effort to relate putative chemical differences with known differences in structure and function. A number of experimental approaches of varying degrees of sophistication have been applied to study the lymphocyte plasma membrane. Trowbridge et al. (135) labeled

cell surface protein and examined the labeled protein by gel electrophoresis in an attempt to detect cell surface differences between different subclasses of lymphoid cells. The major cell surface proteins with affinity for specific lectins have been identified and isolated from murine lymphoid cells by an approach involving affinity chromatography (138). In addition, a number of changes in the cell surface glycoprotein and protein of murine T lymphocytes subsequent to interaction with mitogenic doses of Con A have been reported (137).

While the published literature is replete with reports which characterize the cell surface protein and glycoprotein of lymphoid cells, it is difficult to relate the information derived from these studies to the changes in cellular surface charge reported in this thesis. Firstly, the change in cellular electrophoretic mobility is distinguished from the polyacrylamide gel electrophoresis studies of membrane proteins by the fact that the mobility changes are observed within 10 min after incubation with Con A. Secondly, the changes in cellular mobility are detected even when lymphocytes are incubated with very low concentrations of Con A (10^{-11} μg/ml). The gel studies were performed using mitogenic doses of Con A.

The polyacrylamide gel electrophoresis studies reported in this chapter were carried out using experimental conditions directly comparable to the conditions outlined for the cellular electrophoretic studies. Analysis of the autoradiograph presented in Fig. 5-1 indicates that a 70,000 dalton protein present on control cells is absent from Con A treated cells. Initially, we questioned whether this protein was a receptor for Con A which was rapidly shed subsequent to interaction with Con A. After reviewing the literature it was ascertained that this

protein was not a cell surface protein with affinity for Con A.

When the gel analysis of surface protein was repeated several times, it was noted that the quantity of the 70,000 dalton protein present on control cells was variable. It was questioned whether the enzyme lactoperoxidase which catalyzed the iodination reaction was itself labeled and interferring with the analysis of labeled cell surface protein. As a result, experiments were carried out in which the lymphoid cells were selectively labeled by the chloroglycouril method. This procedure did not involve the addition of extraneous protein. The results of these experiments were the same as those results obtained when cells were labeled by lactoperoxidase catalyzed iodination.

The precise relationship between the presence of a protein on the cell surface and the cellular electrophoretic mobility is exceedingly difficult to determine. As discussed in a preceding section of this thesis, at physiological ionic strength only the most peripheral components of the cell membrane (the outer 8 Å) contribute to the cellular electrophoretic mobility. Hence, membrane proteins which are located deeper within the peripheral zone would not directly contribute to cellular charge. The contribution of the 70,000 dalton protein to the cellular electrophoretic mobility is questionable since the quantity detected on control cells varied, yet the cellular mobility was invariant. While the detection of the 70,000 dalton protein (Fig. 5-2) in the supernatant of Con A treated T lymphocytes is an interesting observation, at this time further experiments have not been carried out to determine what the significance of this event may be.

BIBLIOGRAPHY

1. Nowell, P. C. Phytohemagglutinin: An initiator of mitosis in cultures of normal human leukocytes. Cancer Res., 1966. 20, 462-466.

2. Peavy, D. L., Adler, W. H., and Smith, R. T. The mitogenic effects of endotoxin and staphylococcal enterotoxin B on mouse spleen cells and human peripheral lymphocytes. J. Immunol., 1970. 105, 1453-1455.

3. Oppenheim, J. J., and Rosenstreich, D. L. Signals requlating in vitro activation of lymphocytes. Prog. Allergy, 1976. 20, 65-194.

4. Kaplan, J. G., and Bona, C. Proteases as mitogens. The effects of trypsin and pronase on mouse and human lymphocytes. Exp. Cell Res., 1974. 88, 388-394.

5. Vischer, T. L. Stimulation of mouse B lymphocytes by trypsin. J. Immunol., 1974. 113, 58-62.

6. Whitfield, J. F., Macmanus, J. P., Boynton, A. L., Gillan, D. J., and Issacs, R. J. Concanavalin A and the initiation of thymic lymphoblast DNA synthesis and proliferation by a calcium-dependent increase in cyclic GMP level. J. Cell Physiol., 1974. 84, 445-457.

7. Novogrodsky, A., and Katchalski, E. Membrane site modification on induction of the transformation of lymphocytes by periodate. Proc. Natl. Acad. Sci., 1972. 69, 3207-3210.

8. Greaves, M. F., and Bauminger, S. Activation of T and B lymphocytes by insoluble phytomitogens. Nature New Biol., 1972. 235, 67-70.

9. Betel, I., and van den Berg, K. J. Interaction of concanavalin A with rat lymphocytes. Eur. J. Biochem., 1972. 30, 571-578.

10. Hammarstrom, S., and Kabat, E. A. Purification and characterization of a blood-group A reactive hemagglutinin from the snail helix pomavia and a study of its combining site. Biochem., 1969. 8, 2696-2705.

11. Goldstein, I. J., and Hayes, C. E. The lectins: Carbohydrate binding proteins of plants and animals. Adv. Carbo. Chem. & Biochem., 1978. 35, 127-340.

12. Williams, P. B., Kubo, R. T., and Grey, H. M. μ-Chains from a nonsecretor B cell line differ from secreted μ-chains at the C-terminal end. J. Immunol., 1978. 121, 2435-2439.

13. Stackpole, C. W., Jacobson, J. B., and Lardis, M. P. Two distinct types of capping of surface receptors on mouse lymphoid cells. Nature, 1974. 248, 232-234.

14. Allen, D., Auger, J., and Crumpton, M., J. Glycoprotein receptors for concanavalin A isolated from pig lymphocyte plasma membrane by affinity chromatography in sodium deoxycholate. Nature New Biol., 1972. 236, 23-25.

15. Allen, D., and Crumpton, M. J. Phytohemagglutinin-lymphocyte interaction -- characterization of binding sites on pig lymphocytes for ^{125}I labelled phytohemagglutinin. Exp. Cell Res., 1973. 78, 271-278.

16. Novogrodsky, A., and Katchalski, E. Lymphocyte transformation induced by concanavalin A and its reversion by methyl-α-D-mannopyranoside. Biochim. Biophys. Acta, 1971. 228, 579-583.

17. Toyoshima, S., Fukuda, M., and Osawa, T. Chemical nature of the receptor site for various phytomitogens. Biochemistiry, 1972. 11, 4000-4005.

18. Wynne, D., Wilchek, M., and Novogrodsky, A. A chemical approach for the localization of membrane sites involved in lymphocyte activation. Biochem. Biophys. Res. Commun., 1976. 68, 730-739.

19. Peavy, D. L., Shands, J. W., Jr., Adler, W. H., and Smith, R. T. Mitogenicity of bacterial endotoxins: Characterization of the mitogenic principle. J. Immunol., 1973. 111, 352-357.

20. Andersson, J., Melchers, F., Galanos, C., and Luderitz, O. The mitogenic effect of lipopolysaccharide on bone marrow mouse lymphocytes. J. Exp. Med., 1973. 137, 943-953.

21. Berger,, N. A., and Skinner, A. M. Characterization of lymphocyte transformation induced by zinc ions. J. Cell Biol., 1974. 61, 45-55.

22. Peters, J. H., and Hausen, P. Effect of phytohemagglutinin on lymphocyte membrane transport. 1. Stimulation of uridine uptake. Eur. J. Biochem., 1971. 19, 502-508.

23. Peters, J. H., and Hausen, P. Effect of phytohemagglutinin on lymphocyte membrane transport. 2. Stimulation of "facilitated diffusion" of 3-O-methyl-glucose. Eur. J. Biochem., 1971. 19, 509-513.

24. Auerdunk, R. Uber die wirkung von phytohamagglutinin and anti lymphozyten serum auf den kalium-, glucose- and aminosaure-transport bei menschlichen lymphozyten. Hoppe-Seyler's Z. Physiol. Chem., 1972. 353, 79-87.

25. van den Berg, K. J., and Betel, I. Early increase of amino acid transport in stimulated lymphocytes. Exp. Cell Res., 1971. 66, 257-259.

26. Mendelsohn, J., Skinner, A., and Kornfeld, S. The rapid induction by phytohemagglutinin of increased α-aminoisobutyric acid uptake by lymphocytes. J. Clin. Invest., 1971. 50, 818-826.

27. Parker, C. W. Correlation between mitogenicity and stimulation of calcium uptake in human lymphocytes. Biochem. Biophys. Res. Commun., 1974. 61, 1180-1186.

28. Kaplan, J. G. Activation of cation transport during lymphocyte stimulation: The molecular theology of spinning metabolic wheels. Trends Biochem. Sci., 1979. 4, 147-149.

29. Coffey, R. G., Hadden, E. M., and Hadden, J. W. Evidence for cyclic GMP and calcium mediation of lymphocyte activation by mitogens. J. Immunol., 1977. 119, 1387-1394.

30. Hadden, J. W. Cyclic nucleotides and lymphocyte function. Ann. N. Y. Acad. Sci., 1975. 256, 352-364.

31. Johnson, E. M., Inove, A., Crouse, L., Allfrey, V. G., and Hadden, J. W. Cyclic GMP modifies DNA-binding by a calf thymus nuclear protein fraction. Biochem. Biophys. Res. Commun., 1975. 65, 714-721.

32. Johnson, E. M., and Hadden, J. W. Phosphorylation of lymphocyte nuclear acidic proteins: Regulation by cyclic nucleotides. Science, 1975. 187, 1198-1200.

33. Smith, J. W., Steiner, A. L., Newberry, N. M., and Parker, C. W. Cyclic adenosine 3',5'-monophosphate in human lymphocytes. Alterations after PHA stimulation. J. Clin. Invest., 1971. 50, 432-441.

34. Parker, C. W., Sullivan, T. J., and Wedner, H. J. Cyclic AMP and the immune response. Adv. Cyclic Nucleotide Res., 1974. 4, 1-89.

35. Wedner, H. J., and Parker, C. W. Lymphocyte activation. Prog. Allergy, 1976. 20, 195-300.

36. Byrus, C. V., Klimpel, G. R., Lucas, D. O., and Russell, D. H. Implication of type I or type II cyclic AMP-dependent protein kinase as independent positive or negative effectors of lymphocyte mitogenesis. Nature, 1977. 268, 63-64.

37. Chaplin, D. D., Wedner, H. J., and Parker, C. W. Protein phosphorylation in human peripheral blood lymphocytes: Mitogen-induced increases in protein phosphorylation in intact lymphocytes. J. Immunol., 1980. 124, 2390-2398.

38. Resch, K., Ferber, E., Odenthal, J., and Fischer, H. Early changes in the phospholipid metabolism of lymphocytes following stimulation with phytohemagglutinin and with lyso lecithin. Eur. J. Immunol., 1971. 1, 162-165.

39. Hirata, F., Toyoshima, S., Axelrod, J., and Waxdal, M. J. Phospholipid methylation: A biochemical signal modulating lymphocyte mitogenesis. Proc. Natl. Acad. Sci., 1980. 77, 862-865.

40. Parker, C. W., Kelly, J. P., Falkenheim, S. F., and Huber, M. G. Release of arachidonic acid from human lymphocytes in response to mitogenic lectins. J. Exp. Med., 1979. 149, 1487-1503.

41. Currie, G. A. Effect of phytohaemagglutinin on the surface charge of human and murine lymphocytes. Nature, 1967. 216, 694-695.

42. Wioland, M. Electrophoretic mobility of the thymocyte during endocytosis of concanavalin A. Cell. Immunol., 1974. 12, 472-486.

43. Blume, P., Malley, A., Knox, R. J., and Seaman, G. V. F. Electrophoretic mobility as a sensitive probe of lectin-lymphocyte interaction. Nature, 1978. 271, 378-380.

44. Kiefer, H., Blume, A. J., and Kaback, H. R. Membrane potential changes during mitogenic stimulation of mouse spleen lymphocytes. Proc. Natl. Acad. Sci., 1980. 77, 2200-2204.

45. Cone, C. D., Jr. Unified theory on the basic mechanism of normal mitotic control and oncogenesis. J. Theor. Biol., 1971. 30, 151-181.

46. Wrogemann, K., Weidemann, M. J., Peskar, B. A., Stavdinger, H., Rietschel, E. T., and Fischer, H. Chemiluminescence and immune cell activation. 1. Early activation of rat thymocytes can be monitored by chemiluminescence measurements. Eur. J. Immunol., 1978. 8, 749-752.

47. McCune, J. M., Humpherys, R. E., Yocum, R. R., and Strominger, J. L. Enhanced representation of HL-A antigens on human lymphocytes after mitogenesis induced by phytohemagglutinin or Epstein-Barr virus. Proc. Natl. Acad. Sci., 1975. 72, 3206-3209.

48. Krug, U., Krug, F., and Cuatrecasas, P. Emergence of insulin receptors on human lymphocytes during in vitro transformation. Proc. Natl. Acad. Sci., 1972. 69, 2604-2608.

49. Presant, C. A., and Parker, S. Surface alterations in calf lymphocytes oxidized by sodium periodate. J. Biol. Chem., 1976. 251, 1864-1870.

50. Speckart, S. F., Boldt, D. H., and Ryerson, K. L. Cell surface changes in transformed human lymphocytes. 1. Con A and E-PHA induced unique changes in surface topography. Exp. Cell Res., 1978. 111, 385-395.

51. Speckart, S. F., Boldt, D. H., and Ryerson, K. L. Different effects of concanavalin A and E-phytohemagglutinin upon lymphocyte glycosyltransferase activities. Cancer Res., 1978. 38, 4554-4561.

52. Morgan, D. A., Ruscetti, F. W., and Gallo, R. Selective in vitro growth of T-lymphocytes from normal human bone marrows. Science, 1976. 193, 1007-1008.

53. Smith, K. T cell growth factor. Immunological Rev., 1980. 51, 337-357.

54. Habu, S., and Raff, M. C. Accessory cell dependence of lectin-induced proliferation of mouse T lymphocytes. Eur. J. Immunol., 1977. 7, 451-457.

55. Erb, P., and Feldmann, M. Role of macrophages in in vitro induction of helper cells. Nature, 1974. 254, 352-354.

56. Rosenwasser, L. J., and Rosenthal, A. S. Adherent cell function in murine T lymphocyte antigen recognition. II. Definition of genetically restricted and nonrestricted macrophage functions in T cell proliferation. J. Immunol., 1978. 121, 2497-2501.

57. Oppenheim, J. J. Nizel, S. B., and Meltzer, M. S. Biological effects of lymphocyte and macrophage-derived mitogenic "amplification" factors. In S. Cohen, E. Pick, and J. J. Oppenheim (Eds.) Biology of the Lymphokines. New York: Academic Press, 1979. (pages 291-324)

58. Gery, I., Gershon, R. K., and Waksman, B. H. Potentiation of T lymphocyte response to mitogens. II. The cellular source of potentiating mediators. J. Exp. Med., 1972. 136, 143-155.

59. Gronvik, K. O., and Andersson, J. The role of T cell growth stimulating factors in T cell triggering. Immunol. Rev., 1980. 51, 35-59.

60. Puri, J., Shinitzky, M., and Lonai, P. Concomitant increase in antigen binding and in T cell membrane lipid viscosity induced by the lymphocyte activating factor, LAF. J. Immunol., 1980. 124, 1937-1942.

61. Andersson, J., Gronvik, K. O., Larsson, E. L., and Coutinho, A. Studies on lymphocyte activation. I. Requirements for the mitogen-dependent production of T cell growth factors. Eur. J. Immunol., 1979. 9, 581-587.

62. Larsson, E. L., and Coutinho, A. The role of mitogenic lectins in T cell triggering. Nature, 1979. 280, 239-241.

63. Gillis, S., and Smith, K. A. Long-term culture of tumor specific cytotoxic T cells. Nature, 1977. 268, 154-156.

77. Anderson, L. C., Nordling, S., and Hayry, P. Fractionation of mouse T and B lymphocytes by preparative cell electrophoresis. Cell. Immunol., 1973. 8, 235-248.

78. Stein, G., Flad, H. D., Pabst, R., and Trepel, F. Separation of human lymphocytes by free flow electrophoresis. Biomedicine, 1973. 19, 388-391.

79. Chollet, Ph., Chassagne, J., Thierry, C., Sauvezie, B., Serrou, B., and Plagne, R. Isolation and electrophoretic mobility of three lymphoid populations in normal human blood. Eur. J. Cancer, 1977. 13, 333-339.

80. Mehrishi, J. N., and Zeiller, K. T and B lymphocytes: Striking differences in surface membranes. Br. Med. J., 1974. 1, 360-362.

81. Nordling, S., Anderson, L. C., and Hayry, P. Thymus-dependent and thymus independent lymphocyte separation: Relation to exposed sialic acid on cell surface. Science, 1972. 178, 1001-1002.

82. Mehrishi, J. N. Surface molecular components of human lymphocytes. Int. Arch. Allergy, 1972. 42, 69-77.

83. Donner, M., and Mehrishi, J. N. The lymphocyte surface-surface topochemistry of murine thymocytes related to the major histocompatibility complex. Int. Archs. Allergy Appl. Immun., 1979. 59, 173-185.

84. Sundaram, K., Phondke, G. P., Ambrose, E. J. Electrophoretic mobilities of antigen stimulated lymph node cells. Immunology, 1967. 12, 21-26.

85. Bert, G., Lajolo Di Cossano, D., Pecco, P., and Mazzei, D. Effect of A.L.S. and prednisolone on the electrophoretic mobility of lymphocytes. Lancet, 1970. 1, 365.

86. Dumont, F. Electrophoretic mobility and surface immunoglobulin of albumin gradient fractionated mouse spleen cells. Immunology, 1975. 28, 731-739.

87. Lajolo Di Cossano, D., Astaloi, A., Pecco, P., Bert, G., and Astaldi, G. Electrophoretic mobility of human lymphocytes in the presence of E. coli L-asparaginase. Exp. Cell Res., 1970. 60, 458-459.

88. Wrezlewicz, W., Rashkoff, E., Lucas, T., Ramasamy, N., and Sawyer, P. N. Effects of human chorionic gonadotropin and anti-thymocyte globulin on the electrophoretic mobility of human lymphocytes. Transplant. Proc., 1977. 4, 441-448.

89. Hanjan, S. N. S., and Talwar, G. P. The selective action of poly A:U on the electrophoretic mobility and surface charge of a subpopulation of B cells. J. Immunol., 1975. 114, 55-58.

90. Bert, G. Some applications of the cell electrophoresis technique to the study of lymphocytes. Folia. Allerg., 1970. 17, 548-555.

91. Wioland, M., Donner, M., and Neauport-Sautes, C. Modification of the thymocyte membrane during redistribution of concanavalin A receptors. Eur. J. Immunol., 1976. 6, 273-278.

92. Sainis, K. B., Bhisey, A. N., Sundaram, K., and Phondke, G. P. Cell electrophoretic studies on mouse lymphocytes treated with concanavalin A: Possible existence of two types of receptors on cell surface. Immunology, 1979. 37, 555-561.

93. Sainis, K. B., Bhisey, A. N., Sundaram, K., and Phondke, G. P. Time kinetics of the interaction of concanavalin A with splenic lymphocytes of normal AKR mice. Immunology, 1979. 37, 563-568.

94. Sato, C., Miyazawa, T., Nishizawa, K., Kojima, K., and Okayama, M. Changes in the organization and biosynthesis of cell surface acidic sugars during the phytohemagglutinin-induced blast transformation of human T-lymphocytes. Exp. Cell Res., 1979. 124, 285-292.

95. Nicolson, G. L. The interaction of lectins with animal cell surfaces. Int. Rev. Cytol., 1974. 39, 89-164.

96. Julius, M. H., Simpson, E., and Herzenberg, L. A. A rapid method for the isolation of functional thymus-derived murine lymphocytes. Eur. J. Immunol., 1973. 3, 645-649.

97. Blume, P., Malley, A., Knox, R. J., and Seaman, G. V. F. Analytical particle electrophoresis applied to immunologic research. Clin. Chem., 1978. 24, 1300-1304.

98. Foley, G. E., Lazarus, H., Farber, S., Uzman, B. G., Boone, B. A., and McCarthy, R. E. Continuous culture of human lymphoblasts from peripheral blood on a child with acute leukemia. Cancer, 1965. 18, 522-529.

99. Minowaba, J., Tsubota, T., Nakazawa, S., Srivastava, D. I. S., Huang, C. C., Oshimura, M., Sonta, S., Han, T., Sinks, C. F., and Sandberg, A. A. Establishment and characterization of leukemia T-cell lines, B-cell lines, and Null-cell line. In S. Thierfelder, H. Rodt, and E. Thiel (Eds.) Hematology and Blood Transfusion (Vol. 20) Berlin: Springer-Verlag, 1977. (pages 241-251)

100. Agostoni, A., and Ideo, G. Separation of large numbers of lymphocytes from human blood. Experientia, 1965. 21, 82-83.

101. Bangham, A. D., Flemens, R., Heard, D. H., and Seaman, G. V. F. An apparatus for microelectrophoresis of small particles. Nature (London), 1958. 182, 642-644.

102. Seaman, G. V. F., and Heard, D. H. A microelectrophoresis chamber of small volume for use with biological systems. Blood, 1961. 18, 599-604.

103. Seaman, G. V. F. Electrophoresis using a cylindrical chamber. In E. J. Ambrose (Ed.) Cell Electrophoresis. London: J. & A. Churchill, Ltd., 1965. (pages 4-21)

104. Mehrishi, J. N., and Seaman, G. V. F. Temperature dependence of the electrophoretic mobility of cells and quartz particles. Biochim. Biophys. Acta, 1966. 112, 154-159.

105. Vitetta, E. S., Baur, S., and Uhr, J. W. Cell surface immunoglobulin. II. Isolation and characterization of immunoglobulin from mouse spleen lymphocytes. J. Exp. Med., 1971. 134, 242-264.

106. Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 1970. 227, 680-685.

107. Taylor, R. B., Duffus, W. P. H., Raff, M. C., and de Petris, S. Redistribution and pinocytosis of lymphocyte surface immunoglobulin molecules induced by anti-immunoglobulin antibody. Nature New Biol., 1971. 233, 225-229.

108. de Petris, S., and Raff, M. Normal distribution, patching and capping of lymphocyte surface immunoglobulin studied by electron microscopy. Nature New Biol., 1973. 241, 257-259.

109. Weiss, L. Effect of temperature on cellular electrophoretic mobility phenomena. J. Nat. Cancer Inst., 1966. 36, 837-847.

110. Gottschalk, A. The Chemistry and Biology of Sialic Acids and Related Substances. London: Cambridge University Press, 1960.

111. Wiig, J. N. Effect of neuraminidase on lymphoid cells -- Differences in structure of B and T cells and thymocytes of the mouse shown by cell electrophoresis and sialic acid determination. Scand. J. Immunol., 1974. 3, 357-363.

112. Mehrishi, J. N., and Zeiller, K. Surface molecular components of T and B lymphocytes. Eur. J. Immunol., 1974. 4, 478-483.

113. Dixon, H. B. F., and Perham, R. N. Reversible blocking of amino groups with citraconic anhydride. Biochem. J., 1969. 112, 679-689.

114. Mehrishi, J. N. Positively charged amino groups on the surface of normal and cancer groups. Eur. J. Cancer, 1970. 6, 127-137.

115. Donner, M., and Mehrishi, J. N. The lymphocyte surface: Differences in the surface chemistry of murine T lymphocytes of varying major histocompatibility haplotypes. Proc. R. Soc. London, Ser. B, 1978. 201, 271-284.

116. Klenk, E., and Lempfried, H. Uber die natur der zell receptoren fur das influenzavirus. Hoppe-Seyler's Z. Physiol. Chem., 1957. 307, 278-283.

117. Drezeniek, R., Bogel, K., and Ron, R. On the classification of bovine parainfluenza 3 viruses. Virology, 1967. 31, 725-727.

118. Huang, R. T., and Ohrlich, M. Substrate specificities of the neuraminidases of Newcastle disease and fowl plague viruses. Hoppe-Seyler's Z. Physiol. Chem., 1972. 353, 318-322.

119. Drezeniek, R. Wechselwirkungen zwischen myxovirus und zelloberflachen I. Veranderungen der zelloberflache durch myxovirus. Z. Med. Mikrobiol. Immunol., 1970. 155, 315-334.

120. Schick, H. J., and Zilg, H. Molecular properties of Vibrio cholerae neuraminidase. Proceedings of the Tenth International Congress of Biochemistry, 1976, 232. (Abstract)

121. Heard, D. H., and Seaman, G. V. F. The action of the lower aldehydes on the human erythrocyte. Biochim. Biophys. Acta, 1961. 53, 366-374.

122. Walker, J. F. Formaldehyde. New York: Reinhold, 1967. (pages 483-510)

123. Vassar, P. S., Hards, J. M., Brooks, D. E., Hagenberger, B., and Seaman, G. V. F. Physicochemical effects of aldehydes on the human erythrocyte. J. Cell Biol., 1972. 53, 809-818.

124. Seaman, G. V. F. The surface chemistry of the erythrocyte and thrombocyte membrane. J. Supramol. Struct., 1973. 2, 437-447.

125. Rosenberg, S. A., and Einstein, A. B., Jr. Sialic acid on the plasma membrane of cultured human lymphoid cells. J. Cell Biol., 1972. 53, 466-473.

126. Painter, R. G., and White, A. Effect of concanavalin A on expression of cell surface sialyltransferase activity of mouse lymphocytes. Proc. Natl. Acad. Sci., 1975. 73, 837-841.

127. Chambers, R. W., Bowling, H. C., and Grimley, P. M. Glutaraldehyde fixation in routine histopathology. Arch. Pathol., 1968. 85, 18-30.

128. Marikovsky, Y., and Danon, D. Electron microscope analysis of young and old red blood cells stained with colloidal iron for surface charge evaluation. J. Cell Biol., 1969. 43, 1-7.

129. Seaman, G. V. F., and Swank, R. L. The influence of electrokinetic charge and deformability of the red blood cell on the flow properties of its suspensions. Biorheology, 1967. 4, 47-59.

130. Brooks, D. E., Goodwin, J. W., and Seaman, G. V. F. Interactions among erythrocytes under shear. J. Appl. Physiol., 1970. 28, 172-177.

131. Fraenkel-Conrat, H., and Olcott, H. S. The reaction of formaldehyde with proteins. V. Cross-linking between amino or primary amide or guanidyl groups. J. Am. Chem. Soc., 1948. 70, 2673-2684.

132. Vassar, P. S., Hards, J. M., and Seaman, G. V. F. Surface properties of human lymphocytes. Biochim. Biophys. Acta, 1973. 291, 107-115.

133. Wiig, J. N. Electrophoresis of lymphoid cells: Differences in the cell membrane structure of murine thymocytes, T and B cells revealed by enzyme and formalin treatment. Clin. Exp. Immunol., 1975. 22, 91-101.

134. Mehrishi, J. N. Molecular aspects of the mammalian cell surface. In J. A. V. Butler and D. Noble (Eds.) Progress in Biophysics and Molecular Biology (Vol. 25). Oxford: Pergamon Press 1972. (pages 3-70).

135. Trowbridge, Z. S., Ralph, P., and Bevan, M. J. Differences in the surface protein of mouse B and T cells. Proc. Natl. Acad. Sci., 1975. 72, 157-161.

136. Andersson, R. E., Standefer, J. C., and Scaletti, J. V. The phospholipid and glycoprotein composition of T and B cells. Lab. Inv., 1977. 37, 329-337.

137. Gahmberg, C. G., Hayry, P., and Anderson, L. C. Characterization of surface glycoproteins of mouse lymphoid cells. J. Cell Biol., 1976. 68, 642-653.

138. Nilsson, S. F., and Waxdal, M. J. Isolation and characterization of lectin binding proteins from murine lymphoid cells. Biochemistry, 1978. 17, 903-910.

139. Markwell, M. A. K., and Fox, C. F. Surface-specific iodination of membrane proteins of viruses and eucaryotic cells using 1,3,4,6-tetrachloro-3α,6α-diphenylglycouril. Biochemistry, 1978. 17, 4807-4817.