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# Rat Lymphocyte Surface Glycoproteins Mediating Mitogenesis by Periodate or Neuraminidase Plus Galactose Oxidase

Richard Neal Mitchell


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Richard Neal Mitchell

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"There's no use trying," Alice said, "one can't believe impossible things."

"I daresay you haven't had much practice," said the Queen. "When I was your age I always did it for half an hour a day. Why sometimes, I've believed as many as six impossible things before breakfast."

Lewis Carroll
Through the Looking Glass

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Finally, I wish to thank my wife Diane for her love and support in the last few years, and for all the love we will share in the next seventy-five.

## Abstract

The objectives of this thesis work involved the identification and characterization of rat lymphocyte surface components involved in mitogenic stimulation by the oxidative agents periodate and neuraminidase plus galactose oxidase (NGO). Lymph node cell blastogenesis induced by these oxidative agents was inhibited by reduction with borohydride or reaction with cysteine methyl ester (CME) without affecting the ability of lymph node cells (LNC) to respond to concanavalin A (Con A). $^{35}$S-CME was synthesized, and oxidized LNC were reacted with either the radioactive CME or $^{3}$H-borohydride. The radioactively-tagged LNC were then solubilized in sodium dodecyl sulfate (SDS) and electrophoresced on SDS-polyacrylamide gradient slab gels. Fluorography of these gels demonstrated that $^{35}$S-CME- and $^{3}$H-borohydride-labeled LNC had identical radioactive banding patterns. Differential centrifugation and density gradient isopycnic centrifugation indicated that the radioactive components distributed with the plasma membrane markers: 5'-nucleotidase, $\gamma$-glutamyl transpeptidase, and alkaline phosphodiesterase. The labeling was shown to be specific and uniform for all oxidized components; radioactive Schiff base adducts could not be detected following $^{3}$H-borohydride reduction. Similar sets of molecules were labeled after periodate or NGO reaction. However, some molecules showed a differential sensitivity to periodate or NGO oxidation, or migrated somewhat differently in SDS-polyacrylamide gradient slab gels depending on the oxidizing agent. A heavily-labeled low molecular weight component was shown to be

glycolipid.

A comparison of the labeling of thoracic duct lymphocytes, thymocytes, cortisone-resistant thymocytes, and LNC indicated that lymphoid populations capable of responding to periodate or NGO stimulation (LNC, thoracic duct lymphocytes, and cortisone-resistant thymocytes) had similar radioactive banding patterns. Non-responsive populations (thymocytes) had somewhat different surface labeling. Rat erythrocytes had labeled glycoproteins and overall banding patterns distinct from those seen with any of the lymphoid preparations examined.

Periodate and NGO have been shown by other workers to be T-cell mitogens, and the induction of the stimulation by these agents has been shown to require the interaction of two distinct cell types: the oxidized, responding lymphocyte, and an accessory cell that need not be mitogen-treated. Analysis of nylon wool-purified T-cells and Ficoll gradient-separated responder lymphocytes (both from LNC) showed that these cells had the same labeling patterns as intact LNC populations.

Papain was found to inhibit the stimulation by periodate or NGO in a dose-dependent fashion without affecting the kinetics or magnitude of a Con A response. Treatment with the protease either before or after oxidation had the same result. Heat inactivation of the papain abrogated its inhibitory effect. Trypsin, chymotrypsin, and thermolysin were also found to inhibit oxidative mitogenesis (stimulation by periodate or NGO) in a dose-dependent fashion without reducing the magnitude of the Con A

stimulation. However, the Con A response was delayed by 12-24 hours. None of the proteases affected the immediate or long-term viability of LNC in culture, and none was directly mitogenic for LNC. Protease treatment of Ficoll gradient-separated responder lymphocytes caused an inhibition of oxidative mitogenesis comparable to protease treatment of the entire LNC population.

Radioactive labeling of protease-treated, oxidized LNC gave rise to banding patterns which were unique for each enzyme and distinct from non-digested LNC. Papain-treated responder lymphocytes had the same banding pattern as papain-treated whole LNC populations. Membrane-bound, radio-labeled fragments and protease-resistant molecules differed for each of the four enzymes; glycolipid labeling was not affected. Despite the variable effects of the enzymes on the labeling patterns, levels of the proteases which were inhibitory for stimulation by periodate or NGO consistently caused the loss of four labeled components representing sialoglycoproteins of molecular weights 175,000, 170,000, 160,000, and 155,000 daltons (designated $t_1$-$t_4$).

A monoclonal mouse immunoglobulin (IgG class) originally raised against a protease fragment of the 155,000 dalton molecule on thymocytes, was found to cross-react with all four of the high molecular weight components on LNC as assessed by immunoprecipitation and gel analysis. Moreover, the monoclonal antibody, designated MRC OX1, was also found to be stimulatory for LNC. Both immunoprecipitation and blast transformation required the use of second antibody specific for mouse immunoglobulin; either

$F(ab')_2$ or intact immunoglobulin (Ig) second antibody were found to work. In LNC depleted of B-cells by anti-Ig plus complement lysis, neither MRC OX1 nor anti-Ig second antibody were stimulatory. Combined treatment with optimal concentrations of both antisera (generally in a 1:5 to 1:10 ratio of first:second antibody) gave rise to a blast response with kinetics identical to a periodate response and with a magnitude approximately 1/5 to 1/10 as strong. Efforts to increase the magnitude of the response by using protein A, neuraminidase treatment, heat-aggregated MRC OX1, additional accessory cells, or adding non-mitogenic lectins were not successful. It is not currently clear whether the relatively weak response is caused by the known low affinity of the monoclonal antiserum, or is due to the stimulation of only a sub-population of LNC with a high density of the relevant $t_1$-$t_4$ molecules.

Taken together, the results strongly implicate a role for the high molecular weight $t_1$-$t_4$ tetrad of sialoglycoproteins in oxidative mitogenesis, and indicate that direct cross-linking of these molecules results in blast transformation. The mechanism of stimulation via this cross-linking, however, remains at best speculative.

## Abbreviations

The abbreviations used in this thesis are: NGO, neuraminidase plus galactose oxidase; CME, cysteine methyl ester; LNC, lymph node cells; Con A, concanavalin A; SDS, sodium dodecyl sulfate; MRC OX1, monoclonal antisera directed against the rat leucocyte-common antigen; Ig, immunoglobulin; DNP, dinitrophenyl; LC-antigen, leucocyte-common antigen; EDTA, (ethylenedinitrilo)-tetraacetic acid tetrasodium salt; PBS, phosphate-buffered saline; PPO, 2,5-diphenyl oxazole; BPA, bovine plasma albumin; HBSS, Hanks' balanced salt solution; TDL, thoracic duct lyphocytes; HIHS, heat-inactivated horse serum; RBC, erythrocytes; $^{3}$H-dT, $^{3}$H-thymidine; PMSF, phenyl methyl sulfonyl fluoride; TCA, trichloroacetic acid; DMMA, dimethyl maleic anhydride; other abbreviations and nomenclature are explained in the Legends to Figures 15 (pg. 69) and 34 (pg. 120), and in Tables V (pg. 107) and VI (pg. 123).

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# Introduction

The central issue of immunology concerns the phenomenon of lymphocyte activation. The mechanism by which otherwise quiescent lymphocytes may be stimulated by external agents (in this case foreign antigens) to proliferate and express specific genes is relevant not only to the immune response, but also has a number of counterparts in hormonal, regulatory, and developmental systems. Successive advances in immunological methodology and understanding have revealed progressively increasing levels of complexity in the functioning of lymphocytes. Thirty years of investigation and discovery have contributed to our current concepts of lymphocyte activation and have identified a primary role for certain plasma membrane molecules in facilitating the complete range of immune responses. The work presented in this thesis deals primarily with a small group of surface molecules which mediate a non-antigenic lymphocyte activation induced by mild chemical or enzymatic oxidation. Thus, it is pertinent at the outset to describe the framework of current immunological knowledge, and indicate how the work presented here fits into the overall context.

Historical perspective. The long suspected involvement of the lymphocyte in immune phenomena (e.g., disease resistance and graft rejection; Murphy, 1926) was confirmed by a number of *in vivo* experiments in the 1950's and early 60's. Ablation and reconstitution systems provided convincing evidence that delayed hypersensitivity, tumor immunity, allograft rejection, and anti-

body production were effector functions associated with lymphocytes (reviewed by Gowans and McGregor, 1965). Although there was some controversy concerning a direct role for the small lymphocyte in some inflammatory processes (McCluskey et al., 1963), the graft-versus-host reaction (Simonsen, 1962; Elkins, 1971) and improving techniques of lymphocyte preparation (Gowans and McGregor, 1965) clearly indicated a central role for lymphocytes in the immune response. In these in vivo model systems, the characteristics of specificity (Schlossman and Levin, 1971), immunologic memory (Cochrane and Dixon, 1962), and tolerance (Hasek et al., 1961) were detailed and correctly ascribed to lymphocytes. In addition, the dual systems of T and B lymphocytes with discrete and complementary hapten/carrier activities was also worked out in a number of elegant experiments involving in vivo extirpation and reconstitution, and immune deficiency states (Claman et al., 1966; Cooper et al., 1966; Mitchell and Miller, 1968; Raff, 1970; Good et al., 1971).

With Nowell's discovery that small lymphocytes could be induced to proliferate in culture (1960), a new set of in vitro analogues of the in vivo experimental systems became possible. The development of the mixed lymphocyte response (reviewed by Wilson, 1971), and experimental protocols to examine cell-mediated cytotoxicity (reviewed by Cerottini and Brunner, 1974) and antigen-specific proliferation and antibody production in vitro (Dutton, 1967) led to the discovery of progressively more complex levels of immune interactions. Lymphocyte-determined and serologically-determined antigens in immune responses (Bach et

al., 1976), and histocompatibility requirements for cellular interaction (Rosenthal and Shevach, 1973; Katz et al., 1975; Zinkernagel and Doherty, 1975) were described and characterized largely via the in vitro systems. In addition, the discoveries of functional helper and effector T-lymphocyte subsets (reviewed by Cantor and Boyse, 1976), the requirement for adherent accessory cells in lymphocyte responses (Mosier, 1967; Wagner et al., 1972; Rosenthal et al., 1978; Steinman and Witmer, 1978), and suppressor cells (reviewed by Gershon, 1974), have demonstrated the considerable cellular interaction involved in the generation of the immune response. Finally, a wide variety of soluble factors (Feldman, 1972; Armerding et al., 1974; Takemori and Tada, 1975; Taussig et al., 1974; Ryser et al., 1978) and lymphokines (reviewed by Bloom, 1971), were also found to modulate and mediate immune activity. The concepts of single (Coutinho and Möller, 1975) or double (Cohn and Blomberg, 1975) signals, and the involvement of factors as opposed to direct cell contact (Rosenthal et al., 1978) in mediating immune responsiveness, are issues which are amenable to testing by an in vitro approach.

Mitogen stimulation. The antigen-specific in vitro models closely parallel the more physiologic in vivo systems, and are therefore probably relatively accurate representations of natural processes. However, they do have a significant general disadvantage in that their antigen specificity severely limits the investigation of select molecular events. Since only a small percentage of any given lymphocyte population will interact with and respond to a specific antigen (Burnet, 1959), biochemical

phenomena relevant to lymphocyte stimulation can be easily overlooked in such *in vitro* systems that contain predominantly non-proliferating cells. Thus, the development and use of a considerable number of antigen non-specific polyclonal stimulator mitogens (reviewed by Möller, 1972) which induce the proliferation of large numbers of cells (Greaves and Janossy, 1972; Janossy *et al.*, 1973), have provided a more powerful tool in investigating lymphocyte activation.

In Table I is presented a partial list of currently available mitogens, as well as their lymphocyte sub-population selectivity, and relevant references for each. Figure 1 shows a glycopeptide isolated from rabbit liver (Kawasaki and Ashwell, 1976) to indicate how such a structure could be recognized by a variety of the lectin mitogens. The denoted core and terminal sugars in Figure 1 correspond to the sub-classifications of lectin saccharide binding specificities listed in Table I. The determination of T or B selectivity is in most cases through the use of partially purified sub-populations of lymphocytes, or by use of appropriate surface markers to identify the proliferating cell (Greaves and Janossy, 1972; Greaves *et al.*, 1974b; Roy *et al.*, 1976). The latter technique is complicated in mixed T and B cultures because some recruitment of initially non-responsive cells may also occur (Lohrmann *et al.*, 1974). It should also be emphasized that T and B lymphocyte populations are not homogeneous in their responses; distinct, mitogen-specific subsets may exist (Shortman *et al.*, 1973; Stobo and Paul, 1973) and it is clear, for example, that the cortisone-resistant population of

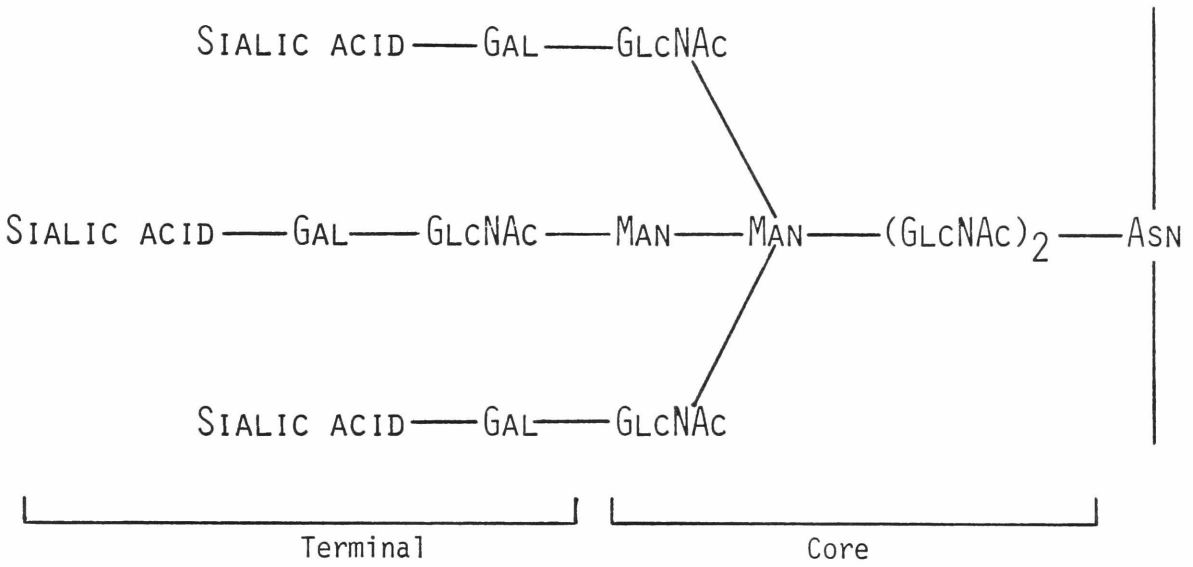



Figure 1. Oligosaccharide structure from rabbit asialoglycoprotein-binding protein (Kawasaki and Ashwell, 1976). Indicated core and terminal sugars correspond to the saccharide binding specificities in Table I. In general, galactose-specific lectin mitogens bind only after the terminal sialic acid is removed by treatment with neuraminidase (Novogrodsky et al., 1977).

TABLE I

Lymphocyte Mitogens and Sub-class Specificity

| Mitogen | Responsive Lymphocyte T | B | Reference |
|---|---|---|---|
| **Lectins** | | | |
| *Terminal Saccharide Specificity* | | | |
| Soybean Agglutinin (Gal) | + | | Novogrodsky et al., 1977 |
| Hepatic Binding Protein (Gal) | + | | Novogrodsky and Ashwell, 1977 |
| Peanut Agglutinin (Gal-GlcNAc) | + | | Novogrodsky et al., 1977 |
| *Core Saccharide Specificity* | | | |
| Concanavalin A (Man) (mono-, di-, and tetravalent) | + | | Wang and Edelman, 1978 |
| Phytohemagglutinin (GlcNAc) | + | | Greaves and Janossy, 1972 |
| Pea Lectin (P. sativum; Man) | +(?) | | Trowbridge, 1973 |
| W. floribunda (GlcNAc) | +(?) | | Toyoshima et al., 1971 |
| Lima Bean Lectin (P. lunatus; GlcNAc) | +(?) | | Reichert et al., 1973 |
| Wheat Germ Agglutinin (GlcNAc) | + | | Brown et al., 1976 |
| Favin (V. faba; Man) | +(?) | | Sela et al., 1975a |
| Insolubilized Concanavalin A or Phytohemagglutinin | + | + | Andersson et al., 1972a<br>Greaves and Bauminger, 1972 |
| Pokeweed Mitogen | + | + | Janossy et al., 1973 |
| **Bacterial Products** | | | |
| Lipopolysaccharide (Lipid A) | | + | Peavy et al., 1973 |
| Staphylococcal Enterotoxin B | + | + | Greaves et al., 1974a |
| Streptolysin S | ? | | Hirschhorn et al., 1964 |
| Aggregated Tuberculin (PPD) | | + | Nilsson et al., 1973 |
| Pneumoccocal Polysaccharide SIII | | + | Coutinho and Möller, 1973 |
| **Antibodies** | | | |
| Anti-lymphocyte Sera | + | | Decker et al., 1977 |
| Anti-immunoglobulin Sera | | + | Sell and Gell, 1965<br>Weiner et al., 1978 |
| Anti-α-macroglobulin | ? | | Sell, 1970 |
| Anti-$\beta_2$-microglobulin | | + | Möller and Persson, 1974 |
| Carbohydrate-specific Antibody (divalent and monovalent) | ? | | Sela et al., 1975b and 1976 |
| Anti-ganglioside $G_{M1}$ | + | | Sela et al., 1978 |
| **Other Reagents** | | | |
| Phorbol Esters | + | | Wang et al., 1975 |
| A-23187 | + | | Maino et al., 1974<br>Wang et al., 1975 |
| Cyclic GMP and derivatives | + | + | Hadden et al., 1972<br>Weinstein et al., 1976 |
| Glycosphingolipids | | + | Ryan and Shinitzky, 1979 |
| Levan | | + | Coutinho and Möller, 1973 |
| Dextran Sulfate | | + | Rühl et al., 1974 |
| Metal Cations ($Zn^{+2}$, $Hg^{+2}$) | ? | | Berger and Skinner, 1974 |
| Proteases (trypsin, pronase, papain, chymotrypsin) | | + | Girard and Fernandes, 1976<br>Hart and Streilen, 1976 |
| Fetal Calf Serum | ? | | Johnson and Russell, 1965 |
| Periodate | + | | Novogrodsky and Katchalski, 1972 |
| Neuraminidase plus Galactose Oxidase | + | | Novogrodsky and Katchalski, 1973 |
| Galactose Oxidase | + | | Dixon et al., 1976 |

thymocytes is responsive to a number of T-cell mitogens, while the cortisone-sensitive, immature thymocytes proliferate only during exposure to Con A (Greaves and Janossy, 1972). Nevertheless, various mitogens can be used to determine the functional purity of T- or B-lymphocyte preparations, or to clinically assess T and B immune capability (Gajl-Peczalska et al., 1973).

Aside from the phorbol esters, $Ca^{+2}$-ionophore, and cyclic nucleotides, which may act internally at the level of "second messenger" (Wang et al., 1975a; Weinstein et al., 1976), the mitogens bind and act at sites on lymphocyte cell surfaces (Greaves and Janossy, 1972; Andersson et al., 1972a). With the exception of anti-immunoglobulin, these sites are probably distinct from the receptors typically associated with antigen recognition, the implication being that lymphocyte activation may proceed via a number of different surface interactions all capable of triggering the same set of secondary events (Greaves and Janossy, 1972; Andersson et al., 1972a). It is worth mentioning that not all agents that can be shown to bind to and agglutinate lymphocytes are mitogenic (Axelsson et al., 1979). Similarly, lectins which are stimulatory for lymphocytes from one species may not cause proliferation in another (Sharon, 1976), and lectins like Con A and phytohemagglutinin, which clearly bind and agglutinate B-lymphocytes as the free lectin, are not stimulatory for the B-cells unless they are presented as an insolubilized matrix (Greaves and Bauminger, 1972; Andersson et al., 1972b).

In general, there is a requirement for cross-linking in

order for the mitogens to be stimulatory (Greaves and Janossy, 1972). Thus, although there are reports of specific monovalent antibody being stimulatory (Sell, 1967; Sela et al., 1976), more frequently, divalent antibody is required to elicit mitogenesis, and monovalent Fab or Fab' fragments (unless cross-linked with a second antibody) are in fact inhibitory for the response obtained with intact immunoglobulin (Woodruff et al., 1967; Fanger et al., 1970; Sela et al., 1978). Likewise, although some Con A derivatives which are monovalent for the saccharide-binding site have been reported to be mitogenic (Beppu et al., 1976; Wang and Edelman, 1978), others have reported monovalent Con A derivatives that are not stimulatory (Wands et al., 1976). Indeed, the stimulation by monovalent Con A may be attributable to a functional cross-linking via the saccharide binding site and a hydrophobic binding pocket at another position on the lectin (Edelman and Wang, 1978). Schecter et al. (1976) have also concluded that multivalency is a requirement for the mitogenesis induced by soybean agglutinin.

Commitment experiments with competing saccharides (Novogrodsky and Katchalski, 1971a; Gunther et al., 1974) or colchicine (Wang et al., 1975b; Gunther et al., 1976) have demonstrated that the lectins must be present for long periods in order to be mitogenic. These results, in conjunction with experiments demonstrating that supra-optimal doses of mitogen are inhibitory (Chauvenet and Scott, 1975; McClain and Edelman, 1976), and interfere with microtubule-mediated surface mobility (Edelman et al., 1973), have led to the formulation of a theory which

suggests that cell surface recognition sites, growth, and movement are integrated via a sub-membranous fibrillar association called the surface modulating assembly (Edelman, 1976).

Membrane modulation following the appropriate mitogenic surface cross-linking presumably then induces a cascade of events leading to highly-elevated protein, RNA, and DNA synthesis and ultimately to lymphocyte proliferation. Elevations in $Ca^{+2}$ influx (Allwood et al., 1971; Maino et al., 1974), membrane fluidity (Ferber et al., 1974), and cyclic GMP levels (Hadden et al., 1972), as well as increased activities of phospholipid metabolism (Resch et al., 1972), polyamine synthesis and carbohydrate metabolism (Ling and Kay, 1975), Na+/K+-ATPase (Novogrodsky, 1972), and transglutaminase (Novogrodsky et al., 1978) have been reported as early events of lymphocyte activation. However, these phenomena display peak activity within the first six hours, and more commonly within the first hour. Thus, since commitment to mitogenesis is incomplete until about 24 hours (Gunther et al., 1976), these early events are probably only necessary but not sufficient processes in the pathway to activation. It is interesting to note, in light of the possible stimulatory role played by cyclic GMP (Weinstein et al., 1976), that mitogenesis can be suppressed by prostaglandins via elevated cyclic AMP (Novogrodsky et al., 1979).

Despite the fact that mitogens are obviously non-physiologic, they have many of the same characteristics of and induce the same effector functions as specific antigenic stimuli.

Thus, multiple cell types (usually macrophages or adherent cells are implicated) and cell-cell interactions are general requirements for mitogenic stimulation (Peters, 1972; Keller, 1975; McClain and Edelman, 1976; Mills et al., 1976; Rosenstreich et al., 1976; Kondracki and Milgrom, 1977; Pilarski et al., 1977; deVries et al., 1979). However, as opposed to the antigen-specific in vitro models, there may not be an absolute histocompatibility restriction for cell-cell interaction in mitogenesis (Greineder and Rosenthal, 1975; Lipsky et al., 1976; deVries et al., 1979). In conjunction with direct cellular co-operation, soluble factors have also been implicated (Gery and Handschumacher, 1974; Waksman and Wagshal, 1978). In addition to proliferation, many of the typical immune effector functions are also promoted by mitogens. For example, cytotoxic T-cells are induced (Waterfield et al., 1976; Pilarski et al., 1977), and non-specific (Gately and Martz, 1977) or specific (Bonavida, 1977) cytotoxicity of specifically-activated lymphocytes may be observed in the presence of various lectins. Suppressor cells are also induced (Rich and Pierce, 1973), and the classical lymphokines are elaborated (David, 1976). Polyclonal B-cell mitogens induce B-lymphocytes to synthesize and secrete antibody (Coutinho and Möller, 1975). The observation that this can occur in the absence of helper T-cells has been prominent in the development of a controversial one-signal theory of lymphocyte triggering and tolerance (Coutinho and Möller, 1975). Certain of the mitogenic systems have also been demonstrated to have features which are reminiscent of immunologic memory; that is,

increased magnitude and accelerated kinetics in a secondary response with the same agent (Pauli and Strauss, 1973; Kalina, 1976).

On the basis of all such results, it seems reasonable to conclude that the pattern of lymphocyte response is innate, and that the programmed behaviors may be elicited by any of a number of non-antigenic mitogens interacting at the lymphocyte cell surface. The properties described above, and the polyclonal triggering potential of such agents strongly suggest that mitogens are valid and useful models of lymphocyte activation.

Mitogenesis by periodate or neuraminidase plus galactose oxidase. Of the mitogens that have been described, periodate (Novogrodsky and Katchalski, 1971b and 1972) or neuraminidase plus galactose oxidase (NGO) (Novogrodsky and Katchalski, 1973) have a number of advantages. Since these mitogenic reagents induce covalent modifications of surface components during a brief treatment prior to culturing (see below) and can then be washed away, they are useful for examining cell-cell interactions in a more defined fashion. Thus, unlike mitogenesis induced by lectins or most other agents, where the polyclonal stimulator binds reversibly to the lymphocyte cell surface and is present for the duration of the culture, specific cell populations may be treated with periodate or NGO, washed, and cultured with untreated cells in the complete absence of exogenous agent. In addition, the covalent modifications may be "followed" by radioactive reagents, and the nature of the surface triggering

molecule(s) may be thus elucidated.

At optimally mitogenic levels of periodate, aldehydes are generated at the carbon-7 position of terminal sialic acids by oxidation of the vicinal hydroxyls on the exocyclic carbons (Van Lenten and Ashwell, 1971; Blumenfeld et al., 1972; Liao et al., 1973; Presant and Parker, 1976)(see Fig. 2). These concentrations of periodate have negligible effects on other exposed saccharides or amino acids on surface glycoproteins (Presant and Parker, 1976). At $0^{o}C$, the periodate anion penetrates the cell membrane very slowly and therefore oxidizes only externally exposed sialic acids (Gahmberg and Andersson, 1977). As a final piece of evidence relevant to the specificity of mitogenic levels of periodate, it is notable that removal of the terminal sialic acids by treatment with neuraminidase prior to periodate oxidation (but not after) renders the lymphocytes unresponsive to the periodate stimulation (Novogrodsky and Katchalski, 1972).

Treatment with NGO removes terminal sialic acids (neuraminidase activity) and creates aldehydes (galactose oxidase activity) at the carbon-6 position of existing terminal, or of newly-exposed penultimate galactose or N-acetyl galactosamine residues (Avigad et al., 1962; Morell et al., 1966)(see Fig. 2). Treatment with neuraminidase alone is not mitogenic (Novogrodsky and Katchalski, 1973), although it may enhance lymphocyte responsiveness to other agents via loss of charge (Beyer and Bowers, 1978). Galactose oxidase treatment alone is variably stimulatory, being relatively weak for rat lymph node cells (Kielian et al., 1977)

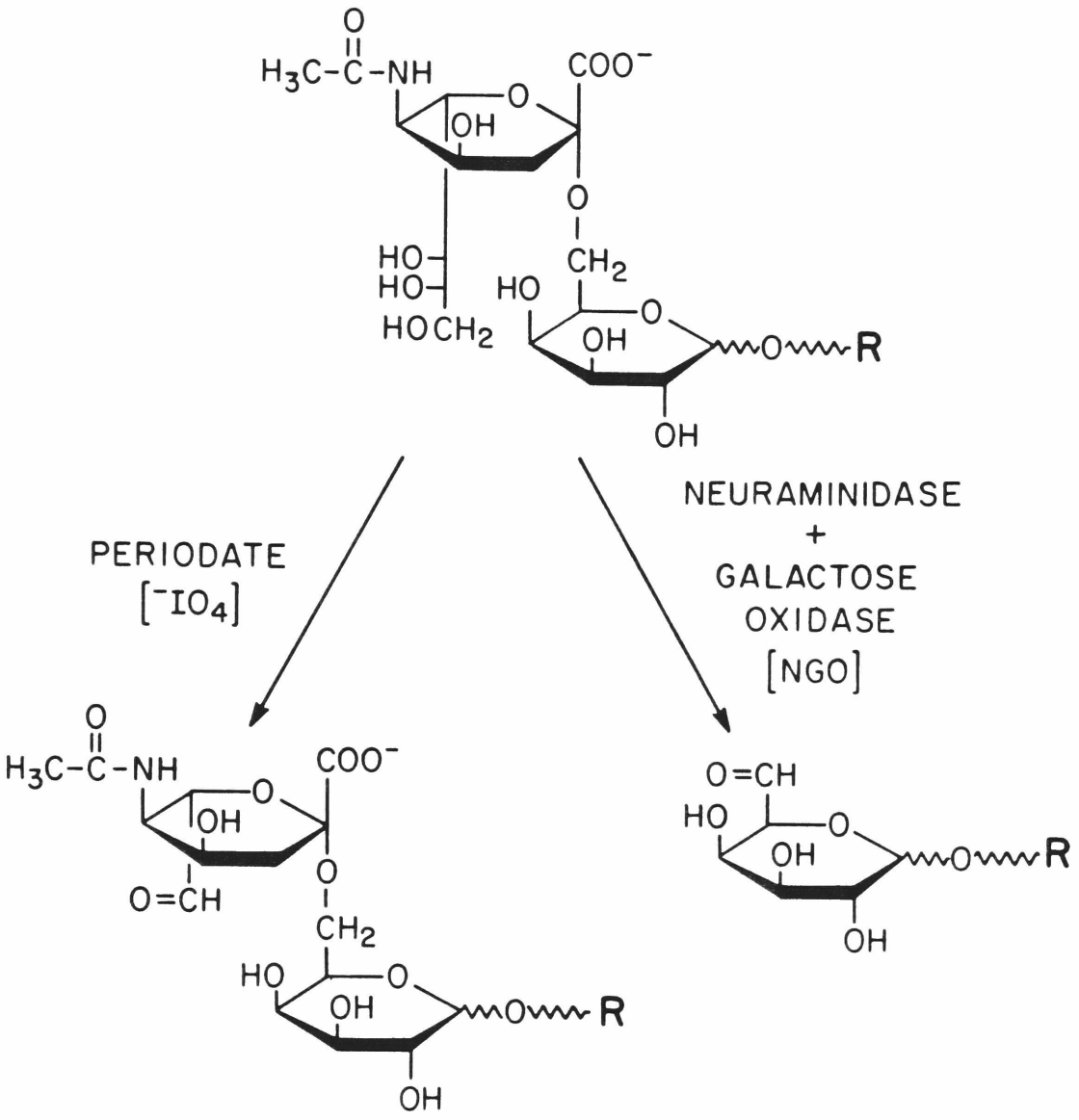



Figure 2. Modification of terminal sialic acid or penultimate galactose on glycosylated molecules by treatment with periodate or neuraminidase plus galactose oxidase. The sialic acid shown is N-acetyl neuraminic acid, although N-glycolyl neuraminic acid would be similarly affected. The galactose residue may also be N-acetyl galactosamine. 'R' can be glycoprotein or glycolipid.

or mouse spleen cells (Novogrodsky and Katchalski, 1973), but approximately equivalent to an NGO stimulation for human peripheral blood lymphocytes (Dixon et al., 1976). Experiments performed with insolubilized galactose oxidase indicate that the action of NGO is strictly limited to the cell surface (Heatley et al., 1977).

It has been shown that the aldehyde group generated either by periodate or NGO oxidation is critical for stimulation, since treatment with any of a number of aldehyde-reactive reagents, including borohydride (Novogrodsky and Katchalski, 1972 and 1973; Dixon et al., 1975), hydroxylamine, thiocarbohydrazide, dimedone (Dixon et al., 1975) or cysteine (Ravid and Novogrodsky, 1976) inhibit mitogenesis after periodate or NGO treatment (but not before).

Experiments where biotin or dinitrophenyl (DNP) groups were grafted onto periodate-oxidized cell surface components and were subsequently reacted with either avidin or anti-DNP antibodies in culture, have indicated that specific cross-linking of the oxidizable groups is required for oxidative mitogenesis (Wynne et al., 1976; Ravid et al., 1978)(see Fig. 3). In those experiments, multivalent avidin (specific for biotin) and divalent anti-DNP antibodies (or divalent fragments) were stimulatory for the biotin- or DNP-modified cells, respectively. In comparison, monovalent Fab anti-DNP antibody was not stimulatory unless it was cross-linked by anti-immunoglobulin second antibody (Ravid et al., 1978). In addition, anti-DNP antibody which cross-linked

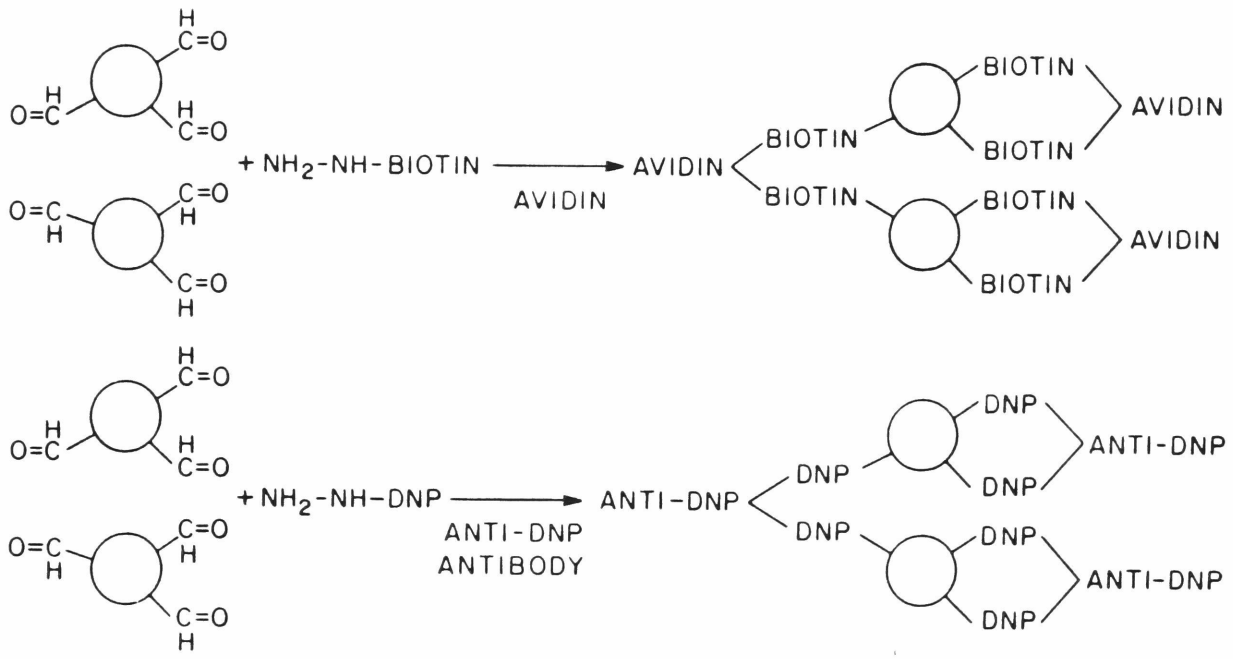



Figure 3. Schematic diagram of experiments performed by Wynne et al. (1976) and Ravid et al. (1978)(see text), demonstrating that specific cross-linking of oxidized surface molecules is required for stimulation of periodate-treated, and DNP- or biotin-grafted lymphocytes. Cross-linking may be either intra- or inter-cellular. DNP = dinitrophenyl. The reagents used for the coupling reactions to the aldehydes were biotin- or DNP-hydrazide.

trinitrophenyl groups coupled to amino functionalities was not mitogenic, indicating that specific cross-linking of only the specifically-oxidizable surface molecules was required (Wynne et al., 1976; Ravid et al., 1978). From the results of these types of experiments, it has been suggested that the periodate- or NGO-generated aldehydes promote mitogenesis by participating in Schiff base cross-links with primary amino groups on proteins of the same or different cells (Novogrodsky and Katchalski, 1973; O'Brien and Parker, 1976)(see Fig. 4), or perhaps by forming cross-links with alcohol or thiol side-chains (Novogrodsky, 1975a). Thus, oxidative mitogenesis may proceed via a surface cross-linking which is functionally analogous to that proposed for stimulation by lectins or anti-immunoglobulin antibody (Greaves and Janossy, 1972).

Periodate and NGO have been shown to be T-cell mitogens (Novogrodsky, 1974; Thurman et al., 1974; McClain et al., 1975), although not all lymphoid populations which contain T-lymphocytes (e.g., thymocytes) are responsive to the oxidative treatments (Bowers and Beyers, 1977). Since papain inhibits the responses to either periodate or NGO (Novogrodsky and Katchalski, 1972 and 1973), it is likely that the oxidative mitogenesis is mediated by glycoproteins as opposed to glycolipids. Both mitogens induce generally only a single round of division (Norin and Strauss, 1975). Experiments which looked at the ability of borohydride (Dixon et al., 1975) or cysteine (Ravid and Novogrodsky, 1976) to inhibit oxidative mitogenesis at various times after the initiation of the culture, indicated that oxidized lymphocytes have a

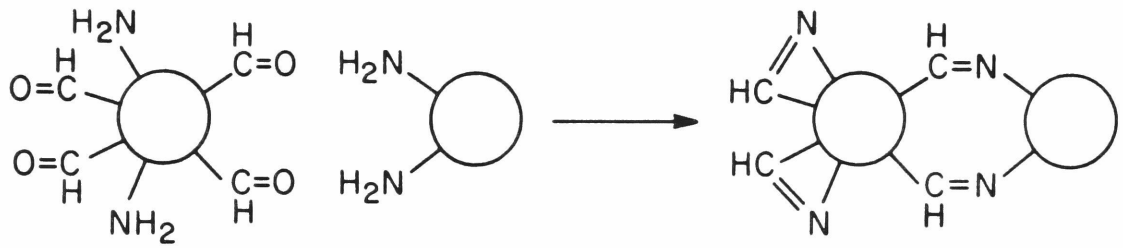



Figure 4. Postulated model for lymphocyte mitogenesis following periodate or NGO oxidation (after O'Brien and Parker, 1976). Schiff base formation may occur between cells or within the same cell surface (see text).

time-course of commitment to stimulation similar to that seen with Con A (approximately 24 hours). Interestingly, turnover experiments of periodate-oxidized and borohydride-reduced components (where the turnover was assayed as renewed responsiveness to periodate stimulation) indicated a six hour recycling time of the relevant components (Norin and Strauss, 1975). Periodate restimulation of cells from a primary periodate culture shows an acceleration of the response and an increase in magnitude reminiscent of the changes observed between primary and secondary mixed lymphocyte cultures (Beyer and Bowers, 1977a). In addition, the restimulated cells were shown to be the progeny cells of the initial proliferation (Norin and Strauss, 1975; Beyer and Bowers, 1977a). As with the other mitogens so examined, periodate induces a transient flux of calcium ions (Parker, 1974), and an increase in cyclic GMP levels (Haddox et al., 1976), as well as the longer-term increases in protein, RNA, and DNA synthesis (reviewed by O'Brien and Parker, 1976). Although oxidative mitogenesis is not antigen-specific, immune effector functions such as cytotoxicity are induced (Novogrodsky, 1975b; Beyer and Bowers, 1978). In addition, periodate oxidation of target cells has been used to facilitate non-specific killing of specifically-activated lymphocytes (Schmitt-Verhulst and Shearer, 1976), and periodate- or NGO-treated secondary cells from an initial allogeneic stimulation show reactivation and specific cytotoxicity (Kuppers and Henney, 1977; Maryanski et al., 1979).

Considerable evidence has emerged to support the concept of obligatory cell-cell interactions in mediating the mitogenesis by

periodate or NGO treatments (Kielian et al., 1977; Kondracki and Milgrom, 1977; Frost et al., 1978). In addition, it has been shown that many periodate- or NGO-oxidized cell types, including syngeneic lymphocytes (O'Brien et al., 1974; Beyer and Bowers, 1975), allogeneic lymphocytes, Hela cells, or continuous T and B lymphoid lines (O'Brien et al., 1977; Parker et al., 1977) will also mediate stimulation. Particularly interesting has been the repeated observation that macrophages (Biniaminov et al., 1974 and 1975; Greineder and Rosenthal, 1975) or at least glass-adherent cells (Novogrodsky et al., 1977; O'Brien et al., 1977; Parker et al., 1977) are absolutely required for stimulation of oxidized lymphocytes to proceed. Whether this involvement of adherent cells is through direct contact (O'Brien and Parker, 1976; Peters and Schimmelpfeng, 1979) or via the elaboration of soluble factors (Novogrodsky and Gery, 1972; Abell et al., 1974) has not been determined. In contrast to the obligatory cellular interaction seen for mouse, rat, guinea pig, and human lymphocytes stimulated by periodate or NGO, it appears that calf lymphocytes have no such interaction requirement (Presant and Parker, 1977).

Taking advantage of the unique nature of the oxidative mitogens, Bowers and Beyer (1977, 1979) have also shown the requirement for an "accessory" cell in oxidative mitogenesis. They demonstrated that "responder" lymphocytes isolated by zonal centrifugation (Bowers, 1973) or obtained as the lymphocytes from thoracic duct cannulation (Bowers and Beyer, 1977) will respond to periodate or NGO treatment only if they are cultured with

accessory cells (found in lymph node and thymus preparations, Bowers and Beyer, 1977). In such experiments, the accessory cell was shown to be a large, radio-resistant cell comprising only a very small percentage of the initial lymphocyte population (Beyer and Bowers, 1977b; Bowers and Beyer, 1979). It need not be oxidized to mediate the stimulation of oxidized responder lymphocytes, but treatment of the accessories with NGO or galactose oxidase alone (but not periodate), gave a greater than two-fold improved response of periodate-treated responder cells (Bowers and Beyer, 1979).

Characterization of cell surface glycoproteins. Since borohydride reduction or cysteine reaction with periodate- or NGO-generated aldehydes inhibits the mitogenic response (see above), it follows that the molecules labeled by radioactive borohydride or cysteine after both periodate or NGO treatment should represent the entire set of surface components which can mediate oxidative mitogenesis. Indeed, oxidation followed by reduction with high specific activity $^{3}$H-borohydride and analysis on sodium dodecyl sulfate (SDS)-polyacrylamide gels of the labeled components, has been described in the literature for a number of free and cell-bound glycosylated molecules (see Table II). To some degree, the labeling has been shown to be specific for periodate or NGO-oxidized surface molecules (Blumenfeld et al., 1972; Steck and Dawson, 1974; Gahmberg et al., 1976). However, there is some incorporation of radioactivity in the lipid fraction of erythrocytes (Blumenfeld et al., 1972; Liao et al., 1973; Steck and Dawson, 1974), and into a 57,000 molecular weight

(m.w.) component that is oxidized and reducible in the native state of mouse and human lymphocytes (Gahmberg et al., 1976; Andersson et al., 1977). The periodate/NGO, $^{3}$H-borohydride labeling technique is particularly suited to the identification and characterization of most glycoproteins, and is preferable to metabolic labeling by virtue of its ability to incorporate label at a higher specific activity (reviewed by Juliano, 1978). The $^{125}$I-lactoperoxidase labeling method (Marchalonis et al., 1971; Hubbard and Cohn, 1975) has also been used by (among others) Ladoulis et al. (1974) and Misra et al. (1978) to examine rat lymphocyte plasma membranes, and by Trowbridge et al. (1977) to look at mouse lymphocyte surface components. However, in these cases, glycosylated components were not identified; rather only total protein patterns were obtained. Obviously, the identification of glycoproteins by the periodate/NGO, $^{3}$H-borohydride reduction technique is more desirable since the oxidizable components are also the molecules mediating the mitogenesis. The $^{3}$H-borohydride reduction technique has no apparent effect on cell viability (Baumann and Doyle, 1978), and allows the identification of glycosylated components without resorting to the relatively insensitive periodic acid-Schiff stain (Zacharius et al., 1969). The principal drawback of $^{3}$H-borohydride labeling is the low emitter strength of tritium, and the consequent need for very high specific activities and/or long exposure times to identify labeled molecules by autoradiography. This has been largely overcome by the use of fluors (2,5 diphenyl oxazole: Bonner and Laskey, 1974; Laskey and Mills, 1975; or salicylate: Chamberlain,

TABLE II

A Partial Reference List of Molecules or Cell Types Labeled by Oxidation with Periodate or NGO, Followed by Reduction with $^{3}$H-Borohydride

| Reference | Molecule or Cell | Comments |
|---|---|---|
| Morell et al., 1966 | Soluble Glycoproteins, Ceruloplasmin | -Labeled following NGO oxidation. |
| Radin et al., 1969 | Isolated Glycolipid | -Labeled following NGO oxidation. |
| Van Lenten and Ashwell, 1971 | Ceruloplasmin, Orosomucoid | -Labeled after periodate oxidation; found label on sialic acid with modified C7 carbon. |
| Blumenfeld et al., 1972 | Human Erythrocytes and Membranes | -Specific labeling for periodate-oxidized sialoglycoproteins; some non-specific lipid labeling. |
| Gahmberg and Hakomori, 1973 | Human Erythrocytes and Ghosts | -Labeled after galactose oxidase treatment; saw effect of proteases and neuraminidase. |
| Liao et al., 1973 | Isolated or Membrane-Bound Erythrocyte Glycoproteins | -Specific labeling for periodate-oxidized sialoglycoproteins and gangliosides; 1% non-specific lipid incorporation. |
| Critchley, 1974 | Hamster Fibroblasts | -Labeled after periodate oxidation. |
| Steck and Dawson, 1974 | Human Erythrocytes | -Labeled after NGO oxidation. Glycosylated components shown to face on external surface of erythrocytes. Careful characterization. |
| Andersson et al., 1976 | Human T-cells, B-cells and Leukemic B-cell Lines | -Labeled after NGO oxidation; found possible high m.w. tetrad, specific T and B components, non-specific 57,000 m.w. band. |
| Gahmberg et al., 1976 | Mouse T-cells, B-cells, and Blast Cells | -Labeled after periodate and NGO; found unique T and B components, non-specific 57,000 m.w. component, extra blast bands. |
| Andersson et al., 1977 | Human T-cells, T-blasts, and Leukemic T-cell Lines | -Labeled after periodate and NGO; found that blasts have additional bands, leukemic lines extremely variable. |
| Gahmberg and Andersson, 1977 | Human Erythrocytes, Mouse Lymphocytes | -Specificity of external labeling shown for periodate oxidation systems. |
| Nilsson et al., 1977 | Human B-cells, B-blasts, and EBV-positive Lines | -Labeled after periodate and NGO; found specific bands for neoplastic B-lines, high m.w. B-cell component, possible Ia molecules. |
| Andersson et al., 1978 | MLC- or Lectin-activated Human Lymphocytes | -New band occurs at 130,000 m.w. with loss of 120,000 m.w. component. |
| Baumann and Doyle, 1978 | Hepatoma Cell Lines | -Variable rates of surface molecule turn-over. |
| Kimura and Wigzell, 1978 | MLC-induced Killer Lymphocytes | -New band occurs at 145,000 m.w. and correlates with killer cell activity. |

1979) incorporated directly into the analytical gels, which greatly increases the sensitivity of the autoradiography. A high specific activity $^{35}S$-label has also been introduced onto oxidized components by the use of [$^{35}S$]-methionine sulfone hydrazide (Itaya et al., 1975).

Lymphocyte surface molecules and antigens. Relative to the erythrocyte plasma membrane, in which the various constituents have been fairly well characterized (reviewed by Marchesi et al., 1976), little is known of most of the components in the lymphocyte plasma membrane. For rat lymphocytes (to which I will confine my remarks in this section), Goldschneider and co-workers have used antisera prepared in rabbits against various rat lymphoid populations to look at antigenic and anatomical relationships in a number of tissues (Goldschneider and Cogen, 1973; Goldschneider and McGregor, 1973; Goldschneider, 1975 and 1976). Without biochemically identifying the antigens, they developed and tested antisera specific for thymic and peripheral T-cells, B-cells, and "null" cells (from bone marrow). Their primary interest was to enumerate (by cytotoxicity or immunofluorescence) the relative percentages of each cell type in thymus, spleen, bone marrow, blood, lymph node, and thoracic duct, and to correlate those data with tissue structures (e.g., spleen red pulp) and a developmental-differentiation scheme (bone marrow precursors, to immature cortical thymocytes, to mature medullary thymocytes, to peripheral lymphocytes, which can recirculate through bone marrow and other lymphoid organs). Similar studies using xenogeneic antisera with similar goals and results have also been

undertaken by a number of other workers (Colley et al., 1970; Zeiller and Dolan, 1972; Ishii et al., 1976; Lubaroff, 1977).

More specific allogeneic antisera have also been prepared to investigate the rat histocompatibility locus (Ia and Ag-B). The rat Ia was found to be a polymorphic (Howard and Scott, 1974; Radka et al., 1977; Shinohara et al., 1978; Sporer et al., 1979) two sub-unit glycoprotein (34,000 and 27,000 m.w.; Binz et al., 1979; 33,000 and 28,000 m.w.; Sporer et al., 1979), which cross-reacted with a number of murine Ia specificities (Shinohara et al., 1978). Its tissue and cellular distribution essentially followed that of immunoglobulin-positive B-cells (Radka et al., 1977; Mason and Gallico, 1978; Shinohara et al., 1978), and the anti-Ia antisera blocked the stimulator cell in a subsequent mixed lymphocyte response (Radka et al., 1977). The Ag-B antigen was also found to be a polymorphic glycoprotein (35,000 m.w.; Callahan and DeWitt, 1975a; 44,000 m.w.; Binz et al., 1979; 45,000 m.w.; Sporer et al., 1979), which occurred on all rat cell types (including erythrocytes; Sporer et al., 1979) and was genetically-linked to the Ia antigens (Radka et al., 1977). Allogeneic antisera have also detected polymorphic 10-20,000 m.w. Ag-X (Callahan and DeWitt, 1975b) and polymorphic 35-40,000 m.w. Ag-$F_1$ (Williams and DeWitt, 1976) minor histocompatibility antigens not apparently linked to the Ag-B locus. Several monoclonal antisera, detecting both polymorphic and common determinants of the rat Ia, have also been prepared, and these antisera verify the polymorphism, species cross-reactivity, and biochemical results obtained with the allogeneic antibodies

(McMaster and Williams, 1979). In addition, it was possible to estimate the presence of 150,000 molecules of Ia on Ia-positive B-cells, as well as 45,000 molecules of Ia on a sub-population (18%) of thymocytes that was also Ia-positive (McMaster and Williams, 1979).

Immunization of $F_1$ animals with parental lymphocytes has also allowed the preparation of antisera directed against T-cell receptor (Binz and Wigzell, 1975). This has permitted the subsequent characterization of shared T and B receptor idiotypes (Binz and Wigzell, 1975), and rat histocompatibility locus antigens (Ag-B and Ia heavy chain; Binz et al., 1979).

The rat Thy-1 antigen has been very thoroughly characterized and found to be an approximately 27,000 m.w. sialoglycoprotein (30% carbohydrate with two sialic acids per molecule), cross-reactive with mouse Thy 1.1, and occurring with a frequency of 600,000 copies per thymocyte (reviewed by Williams et al., 1976). A variation in size observed in brain and two species of thymus Thy-1 have been shown to be due to differences in glycosylation (Williams et al., 1976). It has also been demonstrated that 2/3 of the immunoglobulin (Ig)-positive cells in the bone marrow are also Thy-1-positive (as part of a sequence of maturation; Thy+/Ig-, to Thy+/Ig+, to Thy-/Ig+), and that B-precursor activity in transplants was found in the Thy-1-positive cells (Hunt et al., 1977).

A number of interesting mouse monoclonal antisera, specific for rat lymphocyte membrane antigens, have also been prepared by

Williams and his co-workers. The determination of the tissue distribution of the various antigens is primarily accomplished by use of the fluorescence-activated cell sorter and fluoresceinated second antibody; enumeration of molecules on cells is by quantitation of binding of radioactively-labeled second antibody; and characterization of the antigens is by SDS-polyacrylamide gel analysis of immunoprecipitates or affinity column eluates (Williams, 1976). A monoclonal antibody denoted W3/13 is specific for a 95,000 m.w. sialoglycoprotein (not bound to lentil lectin columns (Standring et al., 1978)) that occurs with a frequency of approximately 40,000 copies on all T-cells (Williams et al., 1977). A monoclonal antibody denoted W3/25 is specific for an antigen that occurs on a subset of T-lymphocytes (80% of thymocytes and 40-50% of thoracic duct lymphocytes) which have helper activity (White et al., 1978a). Moreover, W3/25 at 3-fold site excess (the antigen occurs with a frequency of approximately 15,000 copies per positive cell) will inhibit all strains of responder lymphocytes in a mixed lymphocyte response, without affecting mitogenic responsiveness; the W3/25-negative lymphocytes were found to be the stimulators in the mixed lymphocyte response (Webb et al., 1979). Finally, a leucocyte-common antigen (LC-antigen), found exclusively on lymphoid populations and occurring at 50,000-100,000 copies per cell, has been characterized by a monoclonal antiserum called MRC OX1 (Standring et al., 1978; Sunderland et al., 1979). Originally prepared by an immunization with a 100,000 m.w. protease fragment of the LC-antigen (Sunderland et al., 1979), the low affinity MRC OX1

detects a sialoglycoprotein of 150,000 m.w. on thymocytes, 170,000 m.w. on mature T-lymphocytes, and 210,000 m.w. on B-lymphocytes (Standring et al., 1978). The LC-antigen also turns out to be the predominant antigenic specificity commonly detected by xenogeneic anti-lymphocyte sera (Fabre and Williams, 1977; Morris and Williams, 1977). At the beginning of the work described in this thesis, no functional activity had been assigned to the LC-antigen (or the Thy-1 or W3/13 antigens).

Rationale and overview. In view of the fact that borohydride inhibits oxidative mitogenesis, radioactive aldehyde-reactive reagents (such as $^{3}$H-borohydride) should label all of the components which can potentially mediate the stimulation by periodate or NGO. In the work presented herein, we sought to take advantage of this likelihood to correlate the labeling of specific surface glycoproteins with the ability of lymphocytes to undergo oxidative mitogenesis. To this end, a number of different lymphoid populations and protease treatments have been examined, and four high molecular weight components ($t_1$-$t_4$) have been implicated. Use of MRC OX1 monoclonal antiserum has identified a similar antigenicity of the $t_1$-$t_4$ molecules and has promoted lymphocyte mitogenesis by direct cross-linking of the glycoproteins on the cell surface. The involvement of the high molecular weight tetrad in stimulation by agents other than periodate or NGO, and in general lymphocyte activation is discussed.

## Materials and Methods

Materials. All enzyme substrates, cortisone acetate, sodium dodecyl sulfate (SDS), d,l-dithiothreitol, sodium borohydride, dimethyl maleic anhydride, l-cysteine methyl ester HCl, deoxyribonuclease I type III from bovine pancreas, Protein A (partially purified from S. aureus), phenyl methyl sulfonyl fluoride, Lubrol-PX, iodoacetamide, Tris-HCl, and bovine pancreatic α-chymotrypsin type II (3x crystallized, at 52 units/mg; α-chymotrypsin has major proteolytic specificity for tryptophan, tyrosine, and phenylalanine residues (White et al., 1978b)) were from Sigma, St. Louis, MO. Sodium periodate, reagent grade, and (ethylene-dinitrilo)-tetraacetic acid tetrasodium salt (EDTA) were obtained from Matheson, Coleman, and Bell, Norwood, OH. Vibrio cholerae neuraminidase, wheat germ agglutinin, deoxycholic acid sodium salt, nuclease-free pronase (B grade), and thermolysin (A grade, 3x crystallized, at 8,810 P.U./mg at 35°C; proteolytic specificity for aliphatic and some hydrophobic residues (White et al., 1978b)) were purchased from Calbiochem-Behring Corp., La Jolla, CA. Con A (2x crystallized), and pig pancreatic trypsin (powder from crystalline enzyme at 4730 or 5400 NFU/mg; proteolytic specificity for arginine and lysine (White et al., 1978b)) were obtained through Miles Laboratories, Inc., Elkhart, IN. $^{3}$H-borohydride (5-10 Ci/mmol), l-[$^{35}$S]-cysteine HCl (121 mCi/mmol), and Spectrafluor PPO-POPOP liquid scintillation concentrate were purchased through Amersham Corp., Arlington Heights, IL.

'Low-Tox' guinea pig complement was obtained from Accurate Scientific and Chemical Corp., Hicksville, NY, stored at -90°C, and was used within one hour of reconstitution in ice-cold water. Purified, lyophilized antibodies and antibody fragments were from Cappel Laboratories, Inc., Cochranville, PA. These were reconstituted with water, stored frozen, and diluted with phosphate-buffered saline (PBS) as required for use. The antisera included: rabbit anti-rat immunoglobulin (Ig), Ig fraction; rabbit anti-mouse Ig, Ig and $F(ab')_2$ fractions; and goat anti-mouse Ig, $F(ab')_2$ fraction. MRC OX1 monoclonal antibody, specific for the LC-antigen, was the kind gift of Dr. A.F. Williams, MRC, Oxford, England. W3/13 HLK monoclonal antibody, specific for the 100,000 m.w. T-cell glycoprotein, was purchased through Accurate Scientific and Chemical Corp. Both of the monoclonal antisera were obtained in ascites fluid from hybridoma-injected mice, and were purified by two sequential ammonium sulfate precipitations (3 Molar), and exhaustive dialysis against PBS. SDS-polyacrylamide gel analysis of the purified MRC OX1 antibody preparation indicated the presence of only IgG class heavy chains (55,000 m.w.) and light chains (23,000 m.w.). Protein concentrations were estimated by $O.D._{280}$, and the antibodies were stored at -90°C in 1 ml aliquots at 1 mg/ml in PBS. Normal rabbit Ig was obtained from Microbiological Associates, Inc., Bethesda, MD.

Dactylium dendroides galactose oxidase from Worthington Biochemical Corp., Freehold, NJ, was purified before use by affinity chromatography on Sepharose 6B (Hatton and Regoeczi, 1976). Cell culture medium consisted of RPMI 1640 containing 100

µg of streptomycin and 100 units of penicillin/ml (Associated Biomedic Systems, Inc., Buffalo, NY) supplemented with 15% horse serum (Grand Island Biological Co., Grand Island, NY). In experiments involving mixtures of separated responder and accessory cells, the culture medium also contained 50 µg/ml gentamycin (Schering Corp., Kenilworth, NJ). Electrophoresis purity acrylamide, and Coomassie brilliant blue were from Biorad, Richmond, CA; N,N-methylene bisacrylamide, and XR-5 X-omat X-ray film were obtained from Eastman Kodak, Co., Rochester, NY. Soluene-100 and 2,5-diphenyl oxazole (PPO) were purchased from Packard Instrument Co., Inc., Downer's Grove, IL. Formula 963 aqueous scintillation fluid and [4-$^{14}$C]-acetyl neuraminic acid (55 mCi/mmol) were obtained from New England Nuclear, Boston, MA.

Other materials and their respective suppliers were: papain (2x crystallized, 21.5 units/mg; proteolytic activity at all but acidic residues (White et al., 1978b); ICN Pharmaceuticals, Cleveland, OH); sodium metrizoate (Gallard-Schlesinger Mfg. Corp., Carle Place, NY); Lymphocyte Separation Medium (sodium diatrizoate and Ficoll to a density of 1.077-1.08 g/ml; Bionetics Laboratory Products, Kensington, MD); [methyl-$^{3}$H] thymidine (6 Ci/mmole; Schwarz/Mann, Orangeburg, NY); Bacto lipopolysaccharide W E. coli 055:85 (Difco, Detroit, MI); Heparin (Lipo-Hepin; Riker Laboratories, Inc., Northridge, CA); l-cysteine HCl monohydrate (Koch-Light Laboratories, Ltd., Colnbrook-Bucks, England); cystinyl-bis-tyrosine (Chemical Dynamics Corp., S. Plainfield, NJ); Ficoll 400 (Pharmacia, Piscataway, NJ); crystallized bovine plasma albumin (BPA)(Reheis Chemical Co., Chicago, IL); and

sodium salicylate (Fisher Scientific Co., Fair Lawn, NJ). All other chemicals were of reagent grade and obtained through conventional sources.

Cell preparations. Male Lewis rats (150-300 g) (Microbiological Associates, Inc.) were used for all experiments. Except for obtaining thoracic duct lymphocytes or erythrocytes (see below), the rats were first sacrificed by ether overdose. Cell numbers were determined with a model ZBI Coulter counter (Coulter Electronics, Inc., Hialeah, FL). Cell sizes were determined with a Coulter model $H_4$ Channelyzer in conjunction with the ZBI Coulter Counter, and size distributions were plotted on a Coulter X-Y Recorder II (Coulter Electronics, Inc.). Peaks due to debris (generally less than 60 $\mu^3$ volume) were removed from the final plots by subtracting a parabola fitted to the debris curve (Basic program for a Sol-20 micro-computer by Dr. Jon LaBadie). Cell viabilities were ascertained by Trypan blue exclusion.

Lymph node cells (LNC). Cervical and mesenteric lymph nodes were removed, trimmed free of adherent material, and rinsed in three changes of sterile Hanks' balanced salt solution (HBSS). LNC were released by pressing the nodes through a sterile steel mesh (Cellector tissue sieve; Bellco Glass, Inc., Vineland, NJ) into HBSS. After filtration through cotton to remove large debris, the cells were washed three times by centrifugation (375 x g, 10 min) and resuspension in HBSS.

Thymocytes and cortisone-resistant thymocytes. To obtain cortisone-resistant thymocytes, rats were injected subcutaneously

with cortisone acetate, 100 mg per 100 g body weight at 100 mg/ml in 0.9% saline, 3-4 days prior to sacrifice. Control animals received only an equivalent volume of 0.9% saline. Thymuses of control and cortisone-treated animals were removed, adherent lymph nodes carefully dissected away, and the lobes washed in 3 changes of HBSS. These tissues were then minced apart with sterile probes in HBSS. After filtering the cell suspension through sterile cotton to remove large fragments, the cells were washed 3 times by centrifugation (375 x g, 10 min) and resuspension in HBSS. Cell preparations from the thymuses of cortisone-treated rats averaged only 6% of the cell number obtained from control animals (which normally yielded 0.5-1 x $10^9$ cells). As reported by Blomgren and Andersson for mice (1971), and as shown in Figure 5, the cortisone-resistant thymocyte population had a larger volume (mean of 125 $\mu^3$) relative to that observed for the intact population of thymocytes (mean of 95 $\mu^3$), and almost identical to that seen for lymph node cells (mean of 120 $\mu^3$)(Blomgren and Andersson reported a shift in mode size from 105 $\mu^3$ to 125 $\mu^3$ during a 2-day cortisone treatment).

Spleen cells. For some experiments, spleens were used as a source of accessory cells. The intact spleens were removed and washed 3 times in HBSS. They were then minced apart with sterile probes and pressed through a sterile steel mesh into HBSS. After filtration through cotton, the cells were washed 3 times by centrifugation (375 x g, 10 min) and resuspension in HBSS prior to counting.

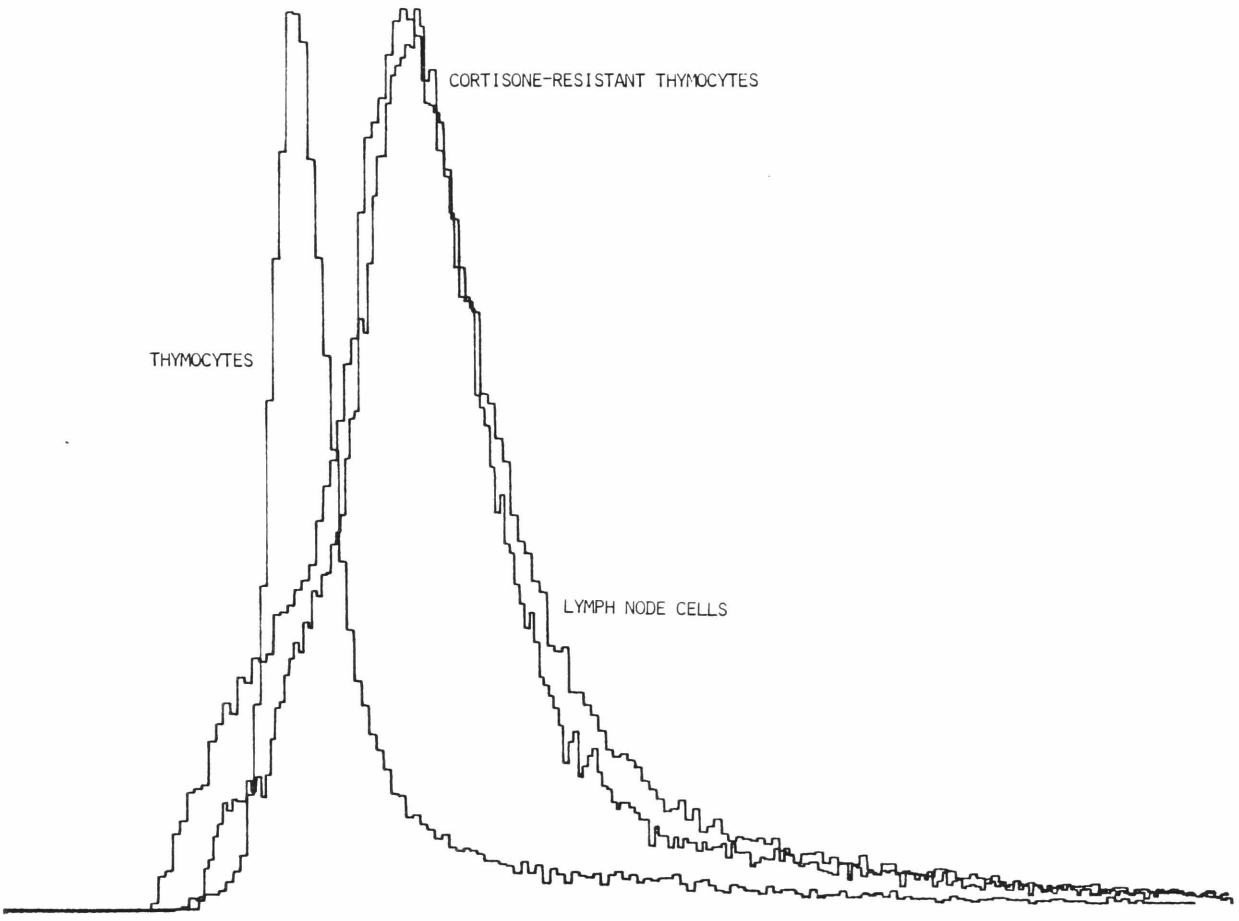



Figure 5. Volume distributions of lymph node cells, cortisone-resistant thymocytes, and total thymocyte populations, prepared as described in the Methods section. The ordinate is the relative frequency of cells in each volume channel of the Coulter Model $H_4$ Channelyzer with increasing volumes from left to right on the abscissa. The 'mean' volume is the arithmetic mean over the entire plotted population distribution. The 'mode' on these plots indicates the volume channel that contained the greatest number of particles. Lymph node cells had a mean volume of 121 $\mu^3$ and a mode at 104 $\mu^3$. Cortisone-resistant thymocytes had a mean of 125.5 $\mu^3$ with a mode at 114 $\mu^3$. Thymocytes had a mean of 97 $\mu^3$ with a mode at 75 $\mu^3$. Presumably, the slightly larger values for the cortisone-resistant thymocytes relative to LNC reflects an increased contamination of larger monocytes and other cells in the cortisone-depleted population.

Thoracic duct lymphocytes (TDL). TDL were obtained by overnight cannulation of the thoracic duct into 5 ml HBSS containing 20 U/ml heparin, 100 µg/ml streptomycin and 100 U/ml penicillin. The yield was generally 3-6 x $10^8$ cells per rat. These were then filtered through sterile cotton, washed 3 times by centrifugation with HBSS, and the erythrocytes removed by Ficoll-hypaque centrifugation (method of Parish and Hayward (1974) with slight modification). Briefly, 17 g of Ficoll 400 were added to 50 ml water, dissolved overnight, and autoclaved to clarity. Stock sodium metrizoate (hypaque, 32.8%) was diluted to a 9% solution with water and stored shielded from light. Both solutions were kept at 4°C until use. The final solution was mixed at room temperature and consisted of 10 parts Ficoll to 24 parts hypaque. Five ml of this mixture were then carefully pipetted beneath a suspension of cells at 2.5 x $10^7$/ml in RPMI 1640 containing 10% heat-inactivated horse serum (HIHS), and the two-phase system was centrifuged 20 min at 375 x g. Lymphocytes were collected from the interface and washed once with HBSS.

Erythrocytes (RBC). Rat RBC were obtained from blood drawn by cardiac puncture into tubes containing a small amount of heparin. To remove lymphocytes, the blood was diluted 3-fold with HBSS and subjected to a Ficoll-hypaque separation. As described above for TDL, the cell suspension was underlayered with a 10:24 Ficoll:hypaque solution, and the two-phase system was centrifuged 15 min at 375 x g. The resulting pellet was resuspended in 5 ml HBSS and re-centrifuged over another Ficoll-hypaque layer. The final RBC pellet was washed twice in HBSS before further use. On

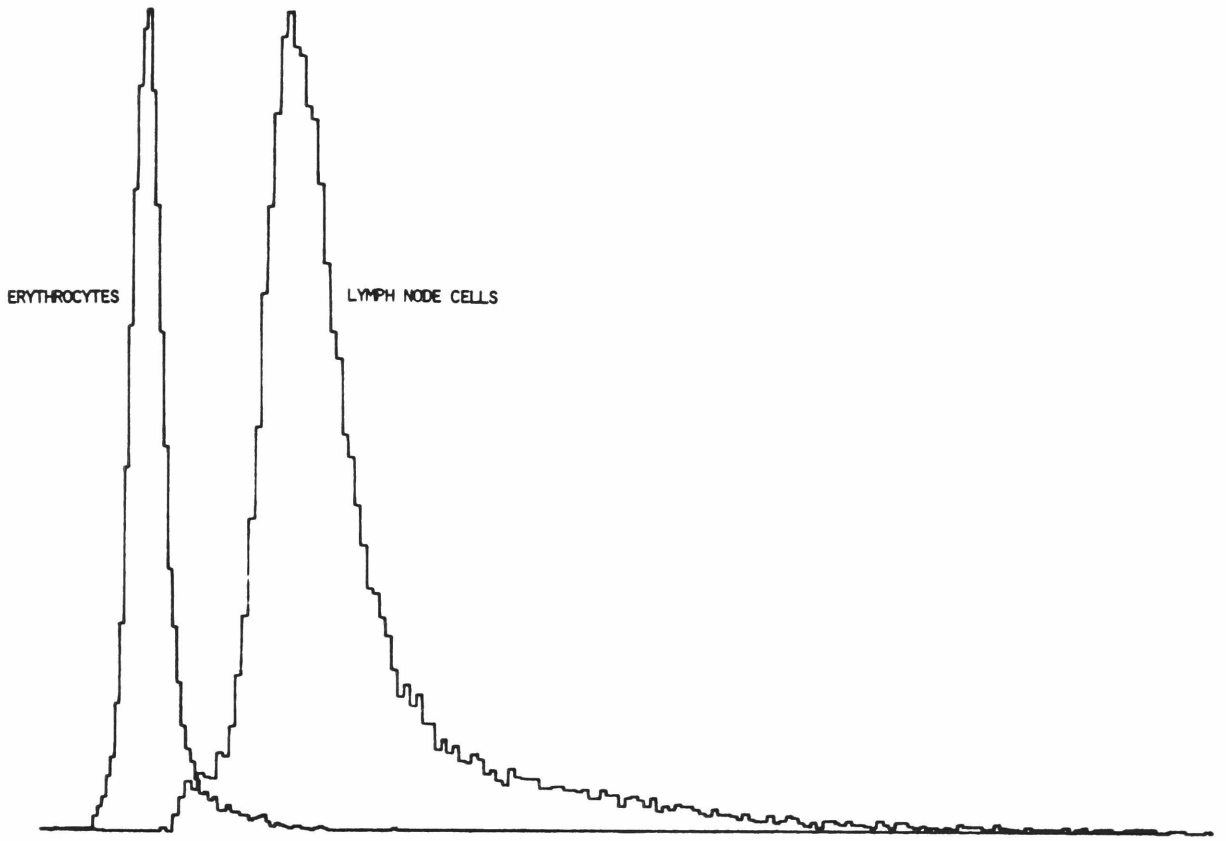



Figure 6. Volume distributions of rat erythrocytes (RBC) and lymph node cells prepared as described in the Methods section. Description of plot, ordinate, and abscissa are found in the Legend to Figure 5. RBC had a mean volume of 45 $\mu^3$ and a mode at 43 $\mu^3$. LNC had a mean volume of 121 $\mu^3$ and a mode at 104 $\mu^3$.

the average, 1 ml of blood yielded 0.3 ml of packed RBC or approximately $10^9$ erythrocytes. The volume of RBC (mean of 45 $\mu^3$) relative to LNC (mean of 120 $\mu^3$) is shown in Figure 6.

Cultured cells. LNC were periodate- or NGO-treated and plated in bulk cultures as described below. Transformed blast cells were obtained from 2-day cultures of these mitogenically-stimulated cells. Secondary cells, which had already undergone one round of division and returned to quiescence, were recovered from 6-day cultures of stimulated cells.

2-day blast cells. Mitogen-treated LNC were harvested at the peak of $^3$H-thymidine incorporation (see below). Peak incorporation equaled 21,050 cpm/$10^6$ LNC for NGO-stimulated cultures and 12,330 cpm/$10^6$ LNC for periodate-stimulated cultures. Cells were collected from bulk cultures, washed twice by centrifugation (375 x g, 10 min) with HBSS, counted, and sized (see Figs. 7 and 8). Large blast cells were isolated by flotation in bovine plasma albumin (BPA) essentially as described by Steinman et al. (1978). The cells recovered from the cultures were resuspended in 5 ml of 'dense' (1.085 g/ml) BPA and overlayered with 1 ml of 'light' (1.048 g/ml) BPA, all in cellulose nitrate centrifuge tubes (#302235 from Beckman Instruments, Inc., Palo Alto, CA). The tubes were centrifuged at 25°C for 30 min at 11,000 x g in a Sorvall HB-4 swinging bucket rotor (Sorvall, Inc., Newtown, CT), and the pelleted cells and interface 'floater' cells collected independently. The pellet and floater cells were then washed twice by centrifugation (375 x g, 10 min) in HBSS, and counted

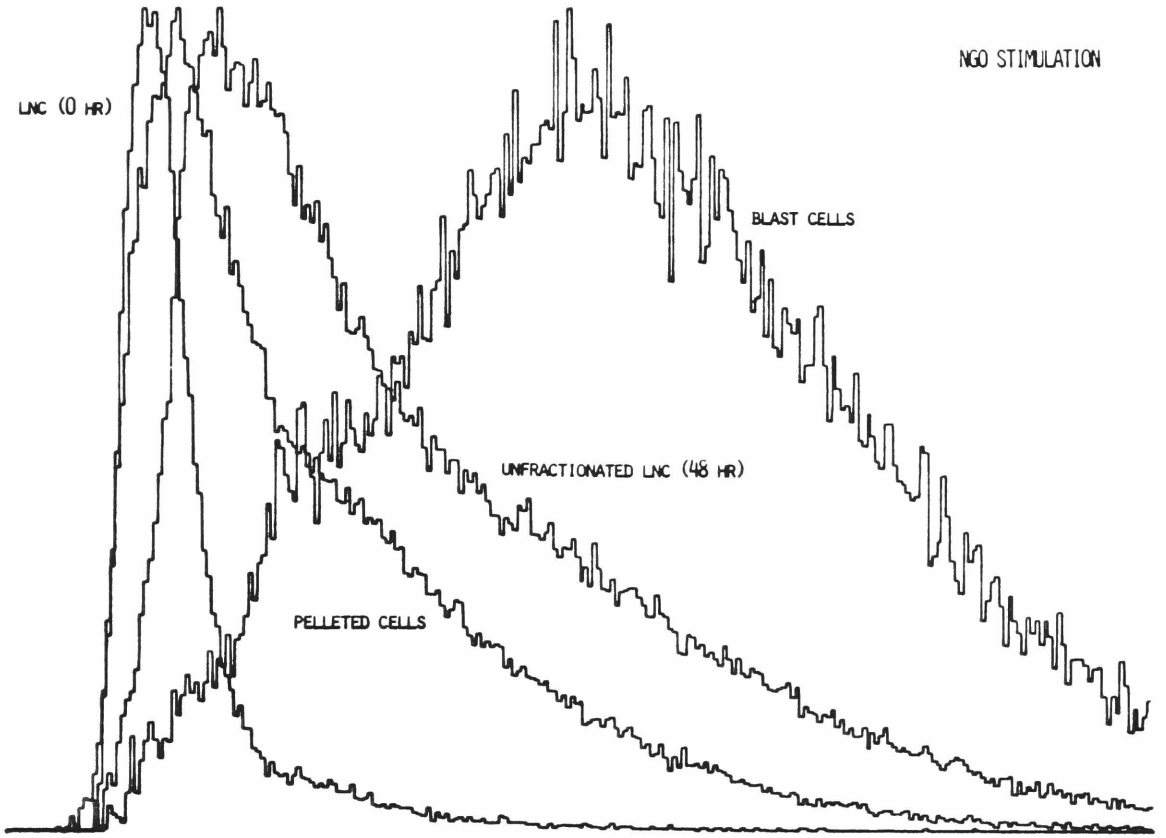



Figure 7. Volume distributions of unstimulated lymph node cells or cells from 2-day NGO cultures (unfractionated, 'floater' blast cells, and pelleted cells; see text). Description of plot, ordinate, and abscissa are found in the Legend to Figure 5. Unstimulated cells have a mean volume of 121 $\mu^3$ with a mode at 104 $\mu^3$. Unfractionated 2-day stimulated cultures had a mean of 258 $\mu^3$ and a mode at 130 $\mu^3$. Floater blast cells had a mean of 381 $\mu^3$ with a mode at 346 $\mu^3$. By comparison, pelleted 2-day cells had a mean of 196 $\mu^3$ with a mode at 105 $\mu^3$.

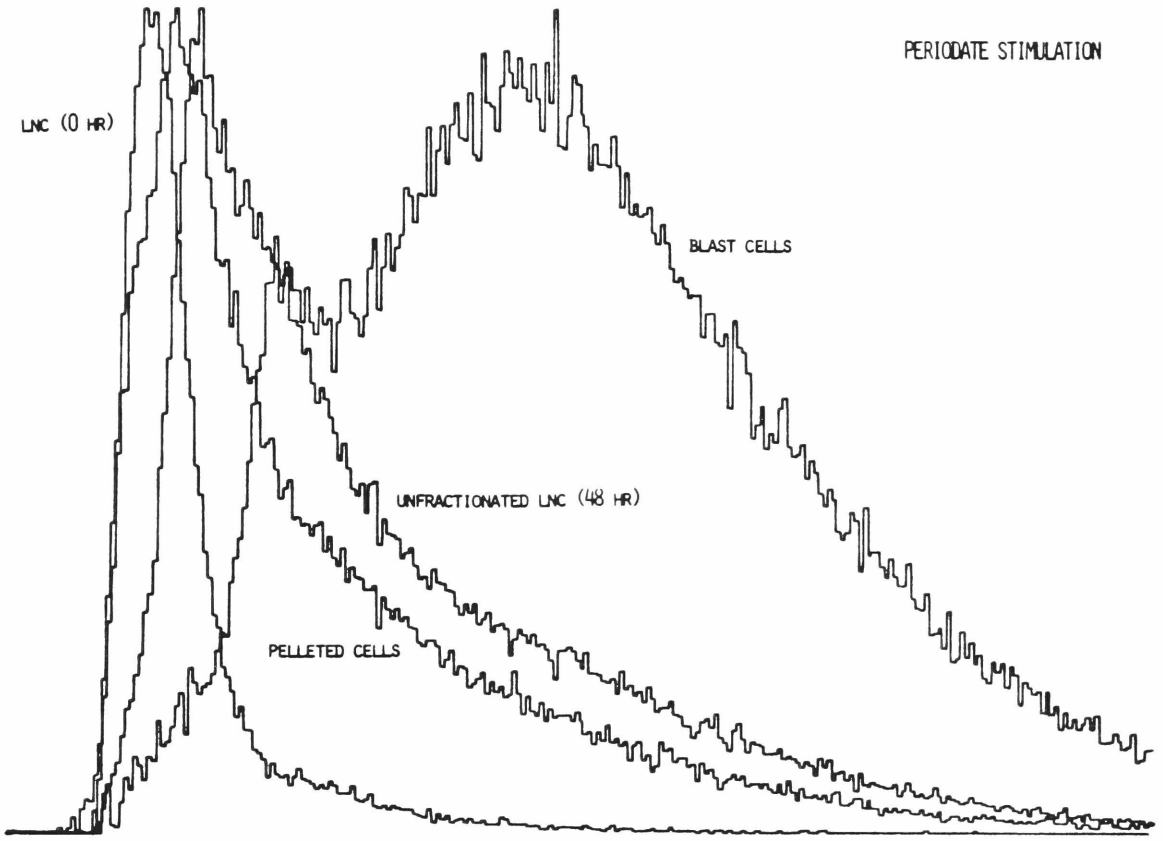



Figure 8. Same as Figure 7, except that stimulation was by periodate treatment. Unfractionated 2-day stimulated cultures have a mean volume of 234 $\mu^3$ and a mode at 119 $\mu^3$. Floater blasts had a mean of 349 $\mu^3$ with a mode at 337 $\mu^3$. Pelleted 2-day cells had a mean of 198 $\mu^3$ with a mode at 105 $\mu^3$.

and sized (see Figs. 7 and 8) before further use. The large blast cells were found predominantly in the floater population and had a mean size of 381 $\mu^3$ (NGO blasts) or 349 $\mu^3$ (periodate blasts) as compared to the pelleted cell mean size of 196 $\mu^3$ (NGO cultures) or 198 $\mu^3$ (periodate cultures). These values should be compared to the initial, unstimulated mean size of 120 $\mu^3$ and the mean size of unfractionated, stimulated 2-day cultures (NGO, 258 $\mu^3$; periodate, 234 $\mu^3$). The size values for NGO- or periodate-stimulated 2-day cultures are essentially the same as those obtained previously by LaBadie et al. (1979). Although there were clearly some large blast cells in the pelleted populations, the floater blast cells appear to be relatively free of contaminating small, unstimulated cells. Total recoveries from the BPA fractionations averaged 82.5% with approximately 84% pelleted cells and 16% floater blasts. The floater preparation had greater than 99% viability.

6-day secondary cells. As described by Beyer and Bowers (1977a), the quiescent, once-stimulated cells are capable, upon re-treatment with periodate or NGO, of mounting a more rapid stimulation (36 hr peak compared to 48 hr for a primary periodate or NGO response) with a 4-fold greater magnitude (on a per cell basis). Peak $^3$H-thymidine incorporation for these cells during intial culture was at 2 days and equaled 18,493 cpm/$10^6$ LNC for NGO-stimulated cultures and 7551 cpm/$10^6$ LNC for periodate-stimulated cultures. Cells were collected from bulk cultures on day 6, washed twice with HBSS at room temperature (375 x g, 10 min), and the cell number and viability determined. The initial

viabilities were approximately 45% and the recoveries from the initially-plated cells averaged 65%. To remove the dead cells and debris, the 6-day cells were suspended to 2 x $10^7$ in RPMI 1640 containing 10% HIHS and 100 µg/ml DNAase I. This suspension was then underlayered with Lymphocyte Separation Medium (commercial preparation of Ficoll-hypaque) and the tubes were centrifuged at 375 x g for 10 min at room temperature. The interface and pelleted cells were collected and washed once by centrifugation in HBSS (375 x g, 10 min). The cells at the interface had a viability greater than 70%, as compared to the pelleted cells with a viability less than 15%. Overall, the recovery of viable cells was approximately 35%.

Rate-zonal centrifugation. LNC were fractionated on the basis of size by velocity sedimentation in an Anderson B-XIV rotor (Bowers, 1973). Centrifugation was performed for 9 min at 1000 rpm at room temperature in a Spinco model L ultracentrifuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, CA). The cells were separated over a linear 2-4% Ficoll gradient of approximately 550 ml volume which rested on 50 ml of a 16% or 17.5% Ficoll 'cushion'; the Ficoll was dissolved in PBS, and all solutions contained 1 unit/ml heparin. The gradient was displaced with 100 ml of additional cushion followed by 32% (w/v) sucrose at a rate of 80 ml/min. Twenty 40-ml fractions were collected in tubes containing 10 ml PBS, and the cells were pelleted at 800 x g for 15 min at room temperature. The cells were washed once in HBSS and enumerated with a Coulter counter. Figure 9 shows the average distribution (n = 21) of LNC in the 20

fractions following centrifugation. The appropriate fractions were then combined (lettered Pools A-D) to give relatively discrete populations of responder and accessory LNC (Beyer and Bowers, 1977b; Bowers and Beyer, 1979). Fractions 2-6 (Pool A, having 30-35% of the total recovered cells) contained primarily small, slowly sedimenting LNC having the characteristics of responder lymphocytes (Beyer and Bowers, 1977b; Bowers and Beyer, 1979). The rapidly sedimenting cells which collected against the cushion (fractions 12-18; Pool D, containing 10-15% of the total recovered cells), were enriched for accessory cells relative to unfractionated LNC (Beyer and Bowers, 1977b; Bowers and Beyer, 1979). Overall cell recoveries from the Ficoll gradients averaged 70-85% of unfractionated controls left at room temperature in 2% Ficoll containing 1 unit/ml heparin. The viability of the fractionated cells was generally the same as unfractionated controls (80-85%).

BPA flotation of accessory cells. Jim O'Brien, and Drs. Wolfgang Klinkert and William Bowers have shown that accessory cells may also be obtained by flotation in a discontinuous BPA gradient (unpublished results), essentially as described by Steinman and Witmer for dendritic cells (1978). Spleen cells (as many as $2.5 \times 10^9$) were resuspended in 5 ml dense BPA (1.0828 g/ml) and overlayered with 1 ml light BPA (1.044 g/ml) all in cellulose nitrate tubes. After centrifugation at $4^{\circ}C$ for 30 min at 11,000 x g (Sorvall HB-4 rotor), the interface floater cells--containing the accessory cells--were removed and washed twice by centrifugation (375 x g, 10 min) and resuspension in HBSS. The

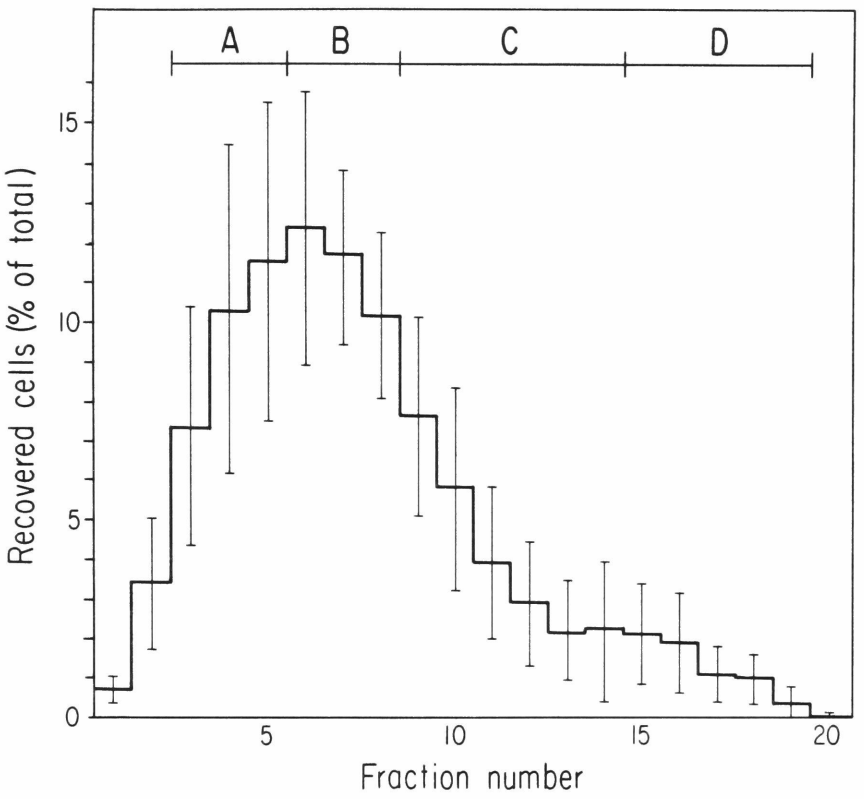



Figure 9. Averaged distribution (n = 21) of LNC after rate-zonal centrifugation (see text). The ordinate expresses the percentage of the total recovered cells found in each of the twenty fractions (fraction number expressed on the abscissa). The initial cell suspension is applied at the top of the 2-4% Ficoll gradient (left). The direction of sedimentation is from left to right, with larger, more rapidly sedimenting cells occurring most frequently in the higher numbered fractions on the right. Typically, only Pool A and Pool D populations were used for the experiments described in this thesis.

cells were then counted and suspended to 5 x $10^6$/ml in RPMI 1640 containing 15% HIHS. Typically, 5% of the starting population were recovered in the floater cell populations, with viabilities greater than 97%.

T- and B-cell preparations. Relatively purified T- and B-cell populations were obtained from initial LNC preparations by 3 separate techniques. B-cells were depleted by anti-Ig plus complement lysis, or were specifically selected for by 'panning' on rabbit anti-rat immunoglobulin-coated plates (Wysocki and Sato, 1978). T- and B-cells were also separated on nylon wool columns using the technique described by Trizio and Cudkowicz (1974).

Nylon wool columns. Nylon wool was obtained from LP-1 Leuko-Pak non-pyrogenic filters (Fenwal Laboratories, Morton Grove, IL), placed loosely in 5-ml glass syringes to the 3-ml mark, washed with water, and autoclaved. Prior to use for experiments, the syringes were each washed with 30 ml of warm (37°C) PBS followed by 30 ml of warm PBS containing 5% HIHS. They were then sealed with parafilm and incubated at 37°C for 1 hr in a vertical position. Just before use, they were flushed with 5 ml more of warm PBS containing 5% HIHS. LNC were resuspended to 5 x $10^7$/ml in PBS containing 5% HIHS, and 2 ml were added to each column followed by an additional ml of PBS + 5% HIHS. The columns were resealed and incubated 1 hr at 37°C. Effluent T-cells were collected by dropwise addition of 15 ml of PBS + 5% HIHS. Following a wash with 75 ml of PBS + 5% HIHS (again dropwise), adherent LNC (B-cells, macrophages, and adherent T-cells) were detached by

vigorous agitation of the nylon wool and eluted with 8 ml PBS + 5% HIHS. This was repeated once and the adherent cells pooled. Both adherent and non-adherent populations were washed twice by centrifugation (375 x g, 10 min) and resuspension in HBSS, and were subsequently counted. Recovery of all cells after fractionation was 60% relative to a 75% recovery of control cells not placed on the column. The viabilities of the fractionated cells were comparable to control cells and exceeded 85%. Effluent T-cells accounted for 76% of the recovered cells, while adherent cells contributed 24%. These values correspond closely to the 80% and 20% estimates for T- and B-cells in LNC obtained by immunofluorescence and cytotoxicity (Goldschneider and McGregor, 1973). Adherent cells still exhibited a stimulatory response to periodate and NGO suggesting a residual T-cell contamination in that population.

B-cell depletion. LNC were suspended to 2 x $10^7$/ml in HBSS containing 100 µg/ml rabbit anti-rat Ig and incubated for 30 min on ice. Following a centrifugation (375 x g, 10 min) and wash in HBSS, the cells were resuspended to 2.1 x $10^7$/ml in RPMI 1640 containing 10% HIHS and 100 µg/ml DNAase I. To this was added 1/20 volume of 'Low-Tox' guinea pig complement (reconstituted as recommended by the supplier) and the suspension was incubated for 30 min at 37°C. At the end of the incubation, the viabilities were assessed (averaging 70-75%) and the viable cells were recovered by centrifugation (375 x g, 10 min) over Lymphocyte Separation Medium (discontinuous Ficoll-hypaque technique). The pelleted cells had a viability in the range of 10-20% while the

interface cells commonly had viabilities between 90-95%. The total recovery of cells (interface plus pellet) generally exceeded 95% but viable cell recovery was more frequently in the range of 65-75%. The B-depleted interface cells retained normal responsiveness to concanavalin A and periodate, but did not respond to lipopolysaccharide (at 5 μg/ml optimally mitogenic dose, which normally gave 2000-3000 cpm/$10^6$ LNC). Control experiments without complement, without anti-rat Ig, or using normal rabbit Ig in place of specific antisera gave cell viabilities and recoveries comparable to untreated control cells carried through the same procedures (90-95% viability prior to discontinuous Ficoll-hypaque separation and 70% recovery of cells at the interface from the centrifugation).

B-cell panning. The B-cell panning procedure was performed by Dr. Wolfgang Klinkert. Polystyrene petri dishes (100 x 15 mm, No. 8-757-12; Fisher Scientific Co., Pittsburgh, PA) were coated with 50 μg of a mixture containing 10% rabbit anti-rat Ig and 90% non-specific rabbit Ig in 5 ml PBS, by incubating for 40 min at room temperature. The plates were then washed with 5 successive 5-ml rinses of PBS. Six ml of LNC at 6.7 x $10^6$/ml in PBS containing 5% HIHS were added to the plates, and the cells were allowed to adhere for 40 min on ice. The plates were then swirled once and adherence continued for an additional 30 min. The non-adherent cells were removed with 5 gentle 5-ml rinses of PBS, and the adherent cells were recovered by 4 vigorous 5-ml washes with a pasteur pipette. Both adherent and non-adherent cells were washed once by centrifugation (375 x g) and

resuspension in HBSS before further use. In general, the recoveries (adherent and non-adherent cells) exceeded 90%, with viabilities greater than 90%. B-cells recovered from this procedure ranged from 5-30% of the total LNC recovered.

Mitogenic treatments.

Treatment with periodate or NGO. These treatments were used both for mitogenic stimulation and to oxidize cell surface glycoproteins immediately prior to labeling with $^{3}H$-borohydride or $^{35}S$-cysteine methyl ester (CME). For periodate treatment, LNC were incubated 15 min on ice at $10^7$ cells/ml in 1.2 mM sodium periodate in PBS. The cells were subsequently centrifuged at 4°C for 5 min at 535 x g, and washed once in HBSS. For NGO treatment, LNC (2 x $10^7$/ml) were incubated 45 min at 37°C in 15 units/ml neuraminidase and 3 units/ml galactose oxidase in HBSS, and centrifuged at 375 x g for 10 min. Cells were washed once in HBSS before further treatment. For these mitogenic treatments, more than 75% of the LNC were generally recovered, and of these more than 95% were viable. Previous proteolytic digestion (see below) did not affect viabilities or recoveries.

Treatment with concanavalin A (Con A). For Con A stimulation, the final culture medium contained 25 µg/ml Con A, the concentration previously found to give optimal stimulation of rat LNC.

Monoclonal antibody (MRC OX1). LNC or B-depleted LNC were incubated at 5 x $10^6$/ml in RPMI 1640 or HBSS containing 10-25 µg/ml (15 µg/ml optimum) MRC OX1 for 30 min on ice. The cells were

then either cultured directly or were washed by centrifugation (375 x g, 10 min) and resuspension in HBSS. The MRC OX1-treated cells were cultured in medium alone (controls) or in medium containing a 1- to 20-fold excess by weight of intact (rabbit antibody) or $F(ab')_2$ fragments (goat or rabbit antibody) of anti-mouse Ig (10-fold excess was optimum). Neither MRC OX1 nor anti-mouse Ig alone were stimulatory for B-depleted LNC.

Protease treatments. Approximately 30 min prior to use, papain was activated by dissolving it in PBS containing 1 mM dithiothreitol and 1 mM EDTA. In control experiments, papain was inactivated by heating the enzyme solution at 100°C for 3.5 min. Trypsin and chymotrypsin were dissolved in PBS alone, while thermolysin solutions were prepared in HBSS. For all the protease treatments, LNC ($10^8$/ml) were incubated 45 min at 37°C with the desired concentration of enzyme and maintained in suspension by periodic shaking. The suspensions were then diluted 2-5 fold with HBSS, mixed thoroughly, centrifuged at room temperature for 15 min at 1600 x g, and the cells washed once with HBSS. The viabilities and recoveries of protease-treated cells were not significantly different from those found for control cells incubated in the absence of enzymes. Typically, 70% of the LNC were recovered, and of these, greater than 90% were viable.

Irradiation of LNC. For some experiments, the proliferation of the accessory cell preparation was prevented by irradiation with 1000 rads (8.4 min at approximately 116 rads/min) from a Gammacell 40 Small Animal Irradiator (Atomic Energy of Canada,

Ltd., Ottawa, Canada). The cells were then washed once in HBSS, counted, and resuspended in complete medium.

Cell culture conditions. After the various treatments, LNC were counted in a hemocytometer and the final concentrations adjusted to approximately 2.5 x $10^6$ cells/ml in culture medium. For experiments involving mixtures of responder lymphocytes and accessory cells, 1:1 ratios were always used. For assaying mitogenic responses, suspensions were dispensed in triplicate or quadruplicate to Microtest II plates (No. 3040, Falcon Plastics, Oxnard, CA) at 0.2 ml (approximately 5 x $10^5$ cells) per well. For determination of the viability of cultured cells after various treatments, 2 ml of LNC at a concentration of 4 x $10^6$/ml in culture medium were plated in 5 replicate 35 x 10 mm tissue culture dishes (No. 3001, Falcon Plastics). To obtain 2-day blast cells and 6-day secondary cells, bulk cultures containing 15 ml of mitogen-treated LNC at 5 x $10^6$/ml were established in 100 x 20 mm tissue culture dishes (No. 3003, Falcon Plastics). All cultures were incubated at 37°C in a humidified atmosphere of 7% $CO_2$ in air. Cell densities were re-determined after suspension in the culture medium; $^3$H-thymidine incorporation and viability counts were expressed relative to a constant $10^6$ cells as established from this count. Initial viabilities for all the cell cultures exceeded 90%.

Measurement of mitogenic stimulation. Incorporation of $^3$H-thymidine ($^3$H-dT) was used to assess lymphocyte proliferation. Cells were labeled during the final 4 hr of incubation with 5

µl/well of RPMI-1640 containing $^{3}$H-dT and unlabeled thymidine at concentrations to produce "saturating" conditions in the culture (final concentration of thymidine in each well was 1 x $10^{-5}$ M at a specific activity of 300 mCi/mmol) (Beyer and Bowers, 1977a). At the end of the 4-hr period, the cells were collected on glass fiber filter paper using a Titertek semi-automatic cell harvester (Flow Laboratories, Inc., Rockville, MD). Two ml of toluene-based scintillation fluid were added to each filter and the radioactivity was counted in a liquid scintillation spectrometer (Packard Instrument Co., Inc.). Incorporated radioactivity per $10^{6}$ cells is expressed as the average cpm ± one standard deviation of replicates.

Viability of cultured LNC. At 24-hr intervals during a 120-hr culture period, untreated LNC or LNC treated with levels of the proteases that inhibited mitogenesis (see Results section) were collected by pasteur pipette from the 35 mm tissue culture dishes. No special attempt was made to remove adherent cells, and the recovered LNC suspensions were used directly, without washing, to avoid dilution or preferential loss of dead lymphocytes. The number of viable cells in each culture was then determined, in duplicate, by trypan blue exclusion and by the pronase-Isoton-Coulter technique described by LaBadie et al. (1979). Briefly, the pronase method involves the comparison of cell numbers obtained with the Coulter counter from cell suspensions incubated either alone or in the presence of 2.5 mg/ml pronase for 15 min at 37°C. Since the pronase treatment will digest only dead cells (Stewart et al., 1975), the percentage of

viable cells in a given culture may be determined by the ratio of numbers with and without the enzyme. This procedure has the advantages over Trypan blue exclusion of objectivity and statistically large samplings.

Treatment with unlabeled borohydride or cysteine methyl ester (CME). Lymphocytes (2 x $10^7$/ml in PBS) were mixed with an equal volume of the desired concentration of borohydride or CME-hydrochloride, freshly prepared in PBS and sterilized by filtration (Millex .22 µm disposable filters, Millipore, Bedford, MA). The borohydride reductions were carried out on ice, while the CME mixtures were incubated at 37°C. At the end of 30 min, the reactions were stopped by adding 2-3 x volume of HBSS and centrifuging at 375 x g for 15 min at room temperature. The cells were washed once with HBSS before further use.

Labeling conditions.

Reduction with $^3$H-borohydride One hundred mCi batches of $^3$H-borohydride were dissolved in 0.2 ml of 0.01 N NaOH, split into two equal volumes, and stored in liquid nitrogen. For use, aliquots were thawed, diluted with 0.01 N NaOH and neutralized with equal volumes of 0.01 N HCl in 2X PBS. Oxidized LNC ($10^8$/ml in PBS) were incubated for 30 min on ice with equal volumes of the $^3$H-borohydride solutions (final conditions: 5 x $10^7$ cells/ml, and 0.625 mM to 1.26 mM $^3$H-borohydride), followed by centrifugation for 15 min, 375 x g, at room temperature. The cells were subsequently washed twice with HBSS and once with PBS before further use.

Treatment with $^{35}$S-CME. Crystallized $^{35}$S-CME hydrochloride, prepared as described below, was stored dessicated at -20°C, and fresh solutions were prepared in PBS containing 2 mM dithiothreitol as needed just prior to use. Oxidized LNC (2 x $10^7$/ml in PBS) were incubated for 30 min at 37°C with equal volumes of 0.8 mM $^{35}$S-CME solutions (final conditions: $10^7$ cells/ml,0.4 mM $^{35}$S-CME, 1 mM dithiothreitol). The reaction was stopped by adding 2-3 x volumes cold HBSS and centrifuging 15 min, 375 x g, at room temperature. The LNC were washed twice with HBSS and once with PBS before further use.

Immunoprecipitation of labeled cells. The method used was essentially that described by Standring et al. (1978) and Sunderland et al. (1979), and is outlined in Figure 10. To inhibit endogenous proteases released during solubilization in Lubrol-PX and deoxycholate, 5 mM phenyl methyl sulfonyl fluoride (PMSF) was used in place of the 5-10 mM diisopropyl fluorophosphate used by Sunderland et al. (1979). Solubilization of the PMSF was facilitated by first dissolving it in a small volume of isopropanol (usually 0.4 ml per 8.7 mg). Sample preparation of the immunoprecipitate and pellet are described below.

SDS-polyacrylamide gel electrophoresis.

Preparation of samples for SDS gel electrophoresis. Labeled cells (2 x $10^8$/ml in PBS), supernatants from pelleting procedures (e.g., immunoprecipitation), or fractions from differential or isopycnic centrifugations (see below) were solubilized by boiling for 5 min in 1% SDS. Pellets (e.g., immunoprecipitates) were

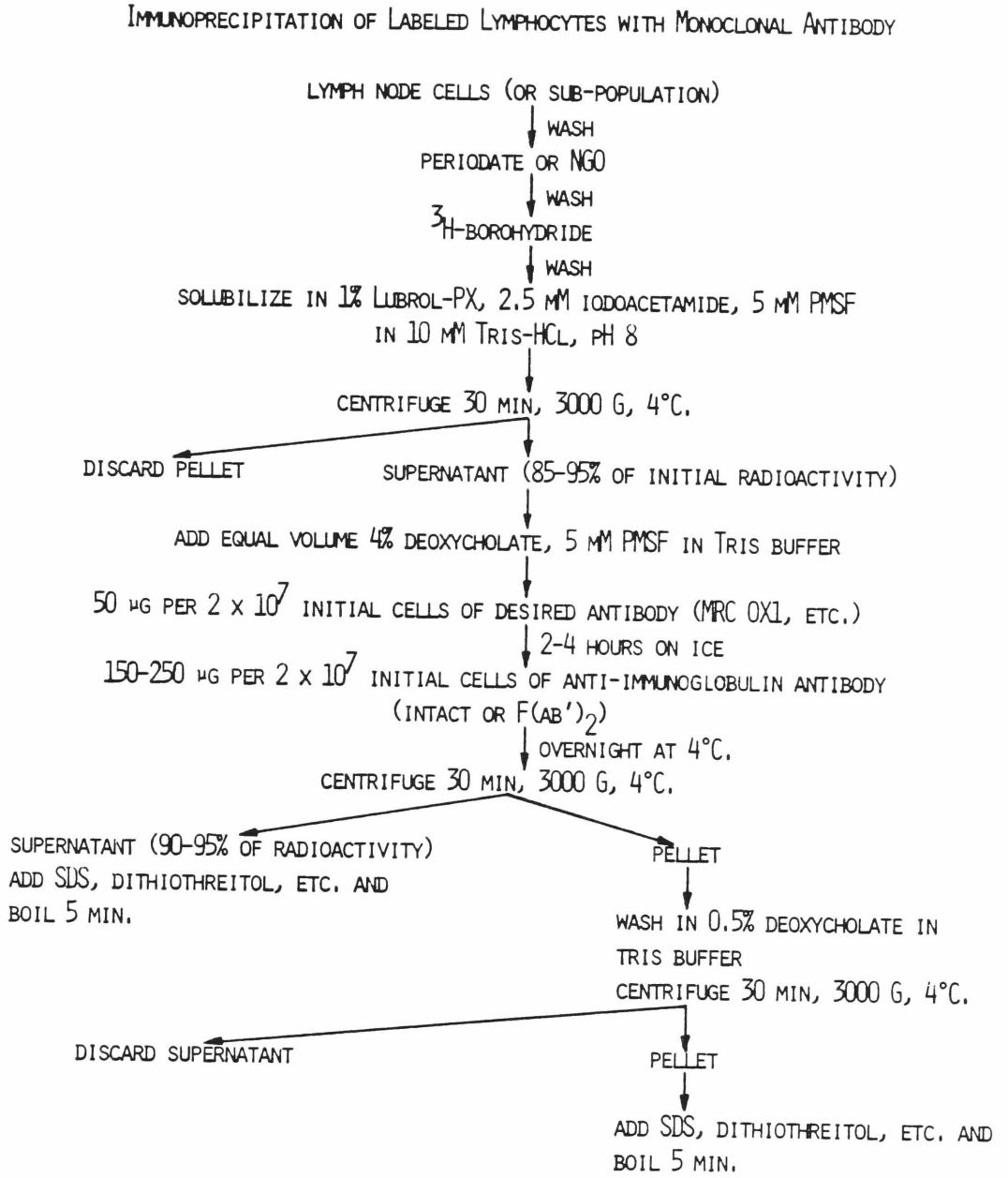



Figure 10. Scheme for immunoprecipitating labeled lymphocytes with monoclonal antiserum.

suspended in PBS to a volume which would yield the equivalent of $2 \times 10^9$ cells/ml in the starting preparations, and then solubilized in 1% SDS by boiling. The resulting solutions were diluted 1.7-fold by adjusting to final concentrations of 10% sucrose, 1.6% SDS, 10 mM Tris-HCl, 1 mM EDTA, 40 mM dithiothreitol, and 10 µg/ml bromphenol blue, and were boiled again 5 min. Incorporation of radioactivity was estimated by sampling a portion of the final preparation in scintillation fluid containing 10% Soluene-100 (see also below). For labeled cells, prepared without any subsequent fractionation, 25 µl of sample contained the equivalent of $3 \times 10^6$ lymphocytes (approximately 2.6 mg protein/ml), and averaged 100,000 cpm of incorporated tritium or 10,000 dpm of TCA-precipitable $^{35}S$ label.

SDS gel electrophoresis. The method used was that described by Scheele (1975) for SDS polyacrylamide slab gel electrophoresis, with the exception that the gel gradient extended linearly from 7% to 15% (or in one instance, from 6% to 10%; see Figure 24), with a 5% stacking gel; the total separating gel length averaged 19.5 cm with a width of 1.65 mm. Generally, between 10-100 µl of sample were layered directly under the electrode buffer into wells molded in the stacking gel. Electrophoresis was carried out at room temperature for 1700 volt-hours, at 100 volts constant voltage; under these conditions the dye front did not migrate off the gel. A set of 7 standard proteins with molecular weights ranging from 210,000 to 17,200 was used to calibrate each gel (thyroglobulin, 210,000; β-galactosidase, 117,000; catalase, 58,000; human IgG, H-chain, 50,000 and L-chain, 23,500;

ovalbumin, 43,000; carbonic anhydrase, 29,000; myoglobin, 17,200).

SDS gel analysis. Sample columns to be analyzed quantitatively were cut out from the gel slab, frozen with dry ice, and sectioned into approximately 1.5 mm slices. These slices were then placed in scintillation vials, 10 ml of scintillation fluid containing 10% Soluene-100 were added, and the capped vials incubated at 60°C for at least 48 hr. When the gel slices appeared uniformly swollen and clear, the vials were cooled to room temperature and the radioactivity was determined by scintillation counting. After subtracting blank gel background counts of 11 cpm per slice, recoveries of applied radioactivity averaged 91% ± 19% (n=12). Control experiments (not shown) indicated that all the applied material migrated into the gel slab, and that under the conditions used for 7% to 15% polyacrylamide slabs, no radioactivity was lost from the bottom of the gel.

For qualitative analysis, the appropriate gel columns were fixed 3 hr in 10% trichloroacetic acid-30% methanol, stained 5 hr with Coomassie brilliant blue (0.5 mg/ml) in 10% acetic acid-30% methanol, and destained with 2-3 washes of 10% acetic acid-30% methanol. The gels were then photographed to record the pattern of staining of the major protein bands and prepared for autoradiography. To shorten the exposure time needed to observe radioactive bands on X-ray film, fluorography was performed according to the method of Bonner and Laskey (1974) using PPO as a fluor, or by using gels which had been washed for a total of 1

hr in two changes of 0.5 M salicylate in 40% methanol (modification of the procedure described by Chamberlain, 1979). The gels were vacuum-dried and exposed to XR-5 X-omat X-ray film for the desired length of time (generally, three weeks or less sufficed) at -90°C. For some experiments, the density of the film image was made linearly proportional to the radioactivity in the samples by pre-exposing the x-ray film to light as described by Laskey and Mills (1975).

Subcellular fractionation of LNC. Differential and isopycnic centrifugation of labeled LNC were performed to identify the subcellular localization of the radioactive incorporation. The centrifugation and marker enzyme assays were performed by Dr. Earl Harrison.

Assays for marker enzymes and biochemical constituents. Protein was determined by the method of Lowry et al. (1951) or by the automated Lowry assay described by Leighton et al. (1968). In both cases bovine plasma albumin was used as a standard. DNA was assayed by the fluorometric procedure of Kissane and Robins (1958) using calf thymus DNA as a standard.

Cytochrome oxidase and N-acetyl-β-glucosaminidase were used as marker enzymes for mitochondria and lysosomes, respectively, and were assayed as described by Bowers et al. (1967). The three plasma membrane-associated enzymes, 5'-nucleotidase (Wattiaux-deConinck and Wattiaux, 1969), γ-glutamyltranspeptidase (Novogrodsky et al., 1976), and alkaline phosphodiesterase I (Beaufay et al., 1974a) were determined according to the indicated

published procedures. Under the conditions of the enzyme assays, all the activities displayed linearity with respect to both incubation time and enzyme concentration.

Subcellular fractionation of LNC. All procedures were performed at 4°C. For fractionation studies, cells were resuspended in hypotonic 42.5 mM KCl-5 mM $MgCl_2$ for 4 min, after which they were homogenized in a Dounce homogenizer with 30 up-and-down strokes of the tight-fitting pestle. Isotonicity was restored by adding an equal volume of hypertonic 242.5 mM KCl-5 mM $MgCl_2$. The resulting homogenate was then centrifuged at 650 x g for 10 min, the supernatant removed, and the pellet rehomogenized according to the above procedure. After the second centrifugation, the supernatants were combined to give the postnuclear extract (E fraction), and the remaining nuclear pellet was resuspended in isotonic 142.5 mM KCl-5mM to yield an N fraction. In some experiments the postnuclear extract was further fractionated into a high speed pellet (P fraction) and supernatant (S fraction) by centrifugation at 100,000 x g for 30 min in a type 50 TI rotor (Beckman Instruments, Inc.).

Fractionation of the postnuclear extract (E) by isopycnic density gradient centrifugation was performed with the automatic zonal rotor designed by Beaufay (1966). 10 ml of extract (E fraction) were layered over 28 ml of a sucrose gradient extending linearly with respect to volume between density limits of 1.05 and 1.20. The gradient rested on a 6 ml cushion formed by a sucrose solution with a density of 1.25. Loading and unloading

were carried out at low speed; density equilibration of the subcellular particle was achieved by centrifuging at 35,000 rpm for 40 min. Fractions of approximately 2.5 ml were collected, and the weight and mean density of each fraction was determined. The distributions of the various constituents in the gradient are presented in the form of histograms which were constructed and averaged as previously described (Leighton et al., 1968).

Analysis of incorporated radioactivity. Fractions from differential or isopycnic centrifugation of $^{35}$S-CME-labeled cells were analyzed for incorporated radioactivity by trichloroacetic acid (TCA) precipitation. Accordingly, 25 µl of samples were mixed with 225 µl (1:10) of 10 mg/ml BPA or BPA plus 0.4 M dithiothreitol, and diluted to 2 ml with water. 0.5 ml of 25% ice-cold TCA was added, the tubes were agitated, and the solutions were allowed to precipitate on ice for 2-3 hr. The precipitate was collected by centrifugation at 1500 x g, 10 min, at 4°C, and washed three times in 1-ml volumes of ice-cold 5% TCA. The supernatants were collected and 1 ml was sampled for radioactivity in 10 ml Formula 963 (containing 2 ml glacial acetic acid per 4 l of solution to quench auto-fluorescence). The TCA pellets were dissolved in 1 ml of 1 N NaOH and the solutions were transferred to scintillation vials. The tubes used for the precipitation were further washed with 2, 0.5-ml volumes of water which were then combined with the samples in the vials. Fifty µl of glacial acetic acid were added and the samples were counted in 20 ml Formula 963. Counting was carried out using a model #3255 Packard Tricarb liquid scintillation counter set at 15% gain with

a 25-1000 window. Background unlabeled CME values were 30-50 dpm. Free $^{35}$S-CME carried through the same precipitation procedure showed 0.4% and 0.04% precipitation of counts in the absence or presence of dithiothreitol, respectively.

Ten µl of $^{35}$S-CME or $^{3}$H-borohydride were also counted directly in 10 ml of a toluene-based scintillation fluid containing 10% Soluene-100. Based on direct counts of samples, recoveries of precipitates plus supernatants averaged 105%.

Chloroform-methanol extraction of glycolipid. An NGO-oxidized-, $^{3}$H-borohydride-labeled LNC preparation was extracted in 1:1 chloroform-methanol to obtain separate glycolipid and glycoprotein fractions. The method used was essentially that described by Ledeen et al. (1973). Pelleted, labeled cells were vigorously shaken for 2.5 hr in 20 x volume (2 ml) of 1:1 chloroform to methanol and the insoluble material pelleted by centrifugation at 375 x g for 15 min. The supernatant was removed and the extraction procedure was repeated twice more for 2.5 and 12 hr. The combined supernatants containing the material extracted into the organic solvent, and the remaining residue were air-dried and prepared for SDS-gel electrophoresis as described above. The total recovery of radioactivity was 92.2%, with 35.9% of that radioactivity in the organic-soluble glycolipid fraction and 64.1% in the glycoprotein residue.

Dimethyl maleic anhydride (DMMA) extraction. An NGO-oxidized-, $^{3}$H-borohydride-labeled LNC preparation was fractionated by differential centrifugation to obtain a P fraction.

This was subsequently extracted with DMMA (after the method of Shanahan and Czech, 1977) to obtain fractions containing intrinsic and extrinsic membrane proteins (Shanahan and Czech, 1977). This was accomplished by stirring 1 volume of membrane (at approximately 4 mg/ml) in 15 volumes of water, and adding 2 mg/ml DMMA while maintaining the pH at 8 by the addition of 0.1 N NaOH. When the evolution of acid ceased (approximately 40 min), the sample was centrifuged 2 hr at 100,000 x g in a type 50 TI rotor at 4°C. The supernatant (extrinsic proteins) and pellet (intrinsic proteins) were sampled for radioactivity and protein content (by the Lowry assay) and prepared for SDS-gel electrophoresis (see above). The recovery of protein was 80%, with 81% of that being in the supernatant, extrinsic fraction, and 19% being in the pelleted, intrinsic fraction. These results are comparable to those obtained by Shanahan and Czech (1977) with rat adipocyte plasma membrane preparations, where they found that 75-80% of the protein was released by the DMMA extraction procedure. The recovery of radioactivity was 120% with 19% being in the supernatant, extrinsic fraction and 81% occurring in the pelleted, intrinsic fraction. The overall enrichment of radioactivity relative to protein in the pelleted fraction was over 4-fold.

Synthesis and characterization of $^{35}$S-CME hydrochloride. The synthesis of unlabeled and radioactive CME hydrochloride primarily followed the procedure described by Sullivan et al. (1942). 0.0072 g (5.5 mCi) of l-[$^{35}$S]-cysteine hydrochloride in 1.33 ml water, and 0.1 g cysteine hydrochloride monohydrate were dissolved in 25 ml anhydrous methanol. The solution was bubbled

with dry hydrogen chloride with a reflux condenser in place. The temperature was initially 65°C and decreased gradually to room temperature within 30 min. The reaction was stopped after 1 hr and the methanol solution was roto-evaporated at 40°C almost to dryness. Cold methanol (2-3 ml) was used to dissolve and transfer the resultant oil to a small flask. Twenty-five to thirty ml of ice-cold ether and a seed crystal of CME hydrochloride were then added and the crystallization was allowed to proceed for 48 hr at 4°C. At that time only 0.5-1% of the original radioactivity remained in the ether mother liquor, and the crystals were obtained by decanting this solution. Crystals were dried in a dessicator under vacuum, and the product was stored dessicated at -20°C. The yield was approximately 50% (probably due to the use of small amounts since the yield of unlabeled product in gram-quantity syntheses was greater than 80%). Melting point of synthesized unlabeled product was 138-141°C (literature values of 140-141°C; cysteine hydrochloride has a melting point of 175°C). Amino acid analysis of the unlabeled cysteine methyl ester product (performed by Mark Riemen), showed only 0.15% cysteine contamination. Nuclear magnetic resonance (characteristic ester methyl protons at $\delta$ = 3.75 (see Fig. 11) and infrared spectra of unlabeled product matched those obtained from commercial samples of CME hydrochloride. Ascending paper chromatography was performed on Whatman #1 paper using a 30:20:6:24 butanol:pyridine:acetic acid:water solvent system. Spots were detected by autoradiography and ninhydrin spray. Greater than 95% of the radioactivity from the $^{35}$S-labeled product co-migrated

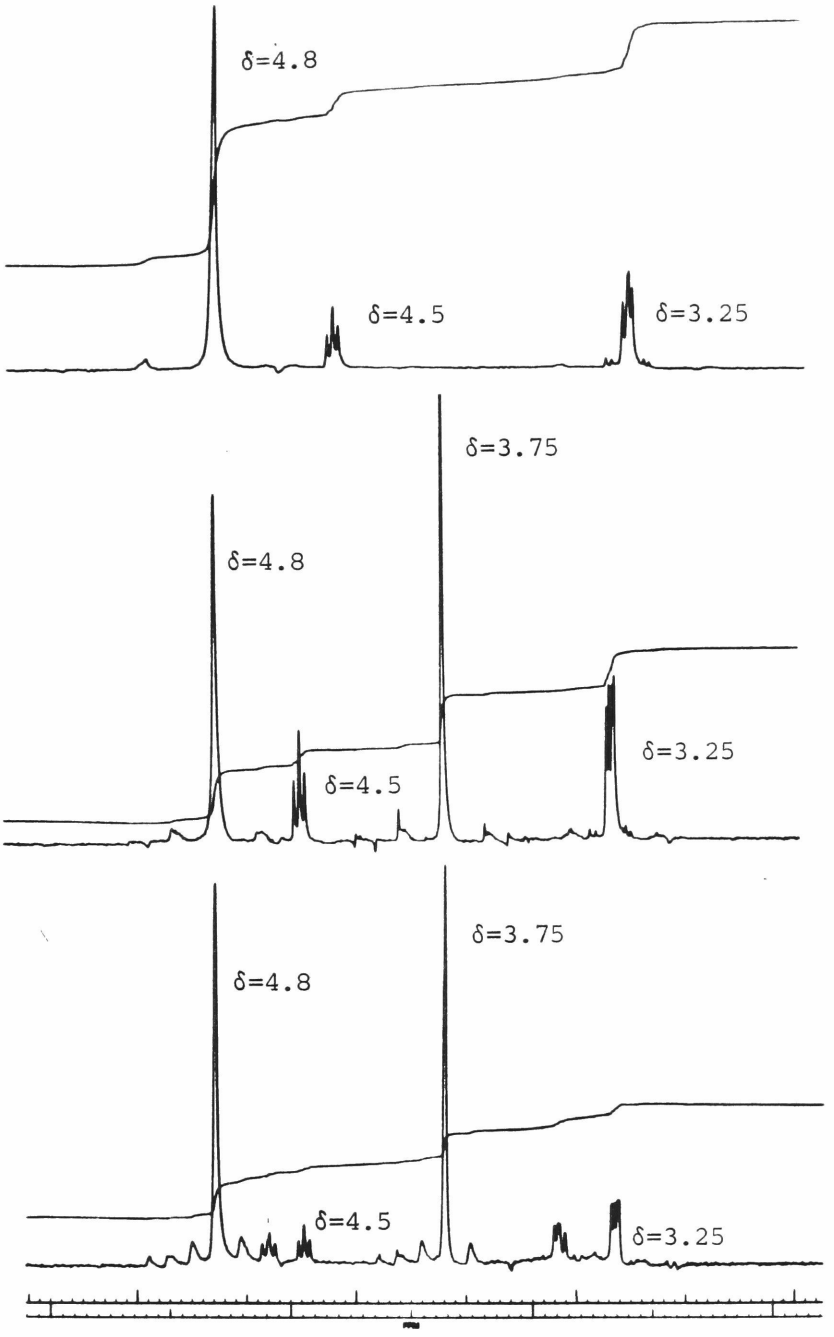



Figure 11. Proton nuclear magnetic resonance spectra (from the Varian HR-220) of cysteine and derivatives in $D_2O$. The top panel shows the spectrum for commercial l-cysteine HCl monohydrate, with a large peak at $\delta$ = 4.8 from the water molecule present. The triplet at $\delta$ = 4.5 and multiplet centered at $\delta$ = 3.25 are from the $\alpha$- and $\beta$-carbon protons, respectively. The middle panel shows the spectrum for commercial l-cysteine methyl ester HCl. The large singlet at $\delta$ = 3.75 is from the ester methyl protons. The bottom panel shows the spectrum from the synthesized cysteine methyl ester HCl. The small triplets at $\delta$ = 4.65 and $\delta$ = 3.4 are probably due to residual ether in the sample. The amino and sulfhydryl protons exchange too rapidly in $D_2O$ to be observed in these spectra. They were observed at $\delta$ = 7.4 and $\delta$ = 1.55, respectively in trifluoroacetic acid solvent.

with unlabeled product and commercial samples of CME hydrochloride ($R_f$ = 0.52); less than 5% of the radioactivity co-migrated with cysteine hydrochloride ($R_f$ = 0.25).

## Results

Inhibition of oxidative mitogenesis by reaction with borohydride or CME. Figure 12 schematically presents the reaction of a cell surface aldehyde with CME to form a thiazolidine ring (Greenstein and Winitz, 1961), or reduction of the aldehyde with borohydride to yield an alcohol. A functional assay for the interaction of such reagents with lymphocyte surface aldehydes involves the inhibition of the proliferative response to periodate or NGO treatment. As shown in Figures 13 and 14, treatment with borohydride or CME-hydrochloride after periodate or NGO oxidation caused a concentration-dependent inhibition of the mitogenesis. Without changing the kinetics of blast transformation, 10 mM borohydride caused almost complete inhibition while 1 mM inhibited approximately 50% of the response and 0.1 mM had virtually no effect. In comparison, CME was about 25 times more potent on a molar basis in inhibiting the oxidative response. Again, without change in the kinetics of proliferation, 0.4 mM CME almost completely abrogated stimulation and 0.1 mM was still 50% effective. The inhibition by either reagent was not caused by toxicity or by a non-specific 'inactivation' of the LNC, since the mitogenic responses to Con A were not affected, even at the highest concentrations used in Figures 13 and 14 (results not shown). Moreover, reversing the order of treatment (i.e., borohydride followed by periodate or NGO) showed no diminution of the stimulation (results not shown). These results suggest that the inhibition of the oxidative mitogenesis was caused by specific reaction with aldehydes generated by the

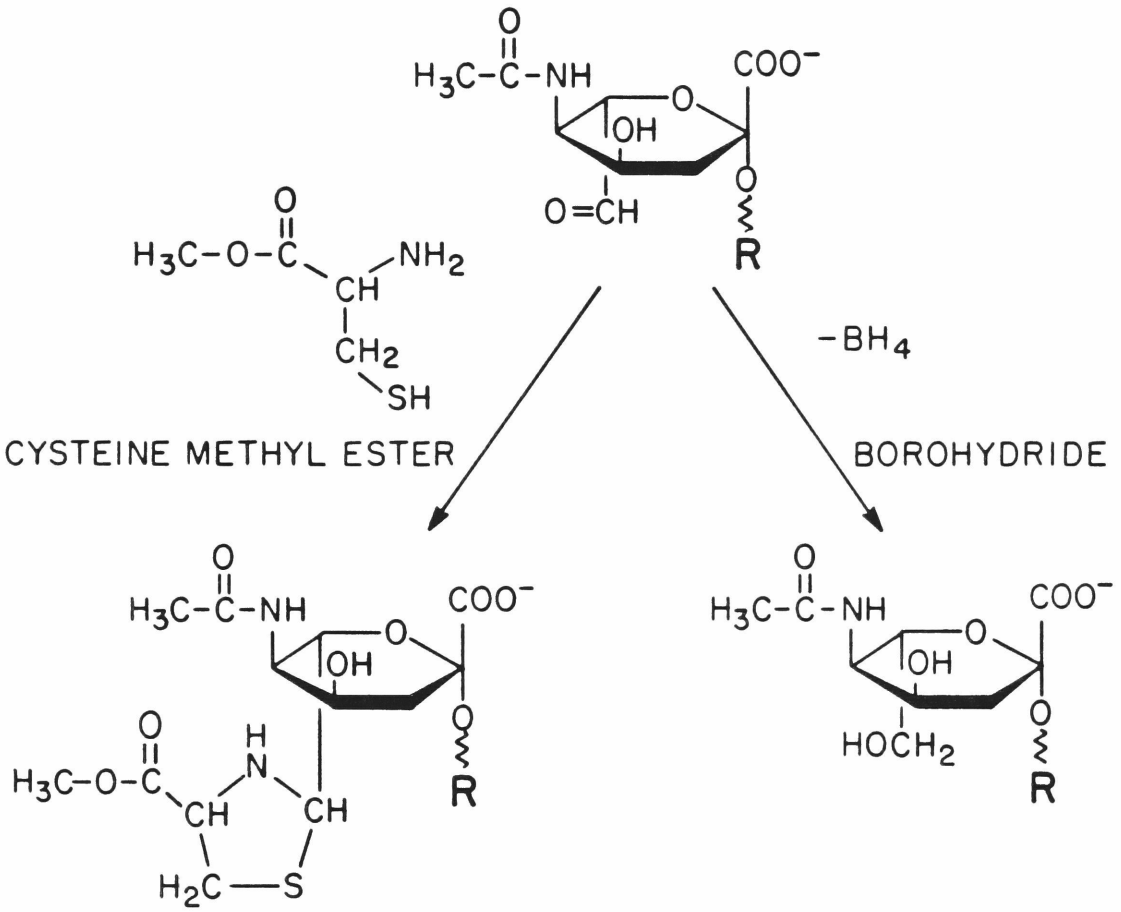



Figure 12. Reaction of C-7 aldehyde on periodate-oxidized terminal sialic acid with cysteine methyl ester to form a thiazolidine ring, or reduction with borohydride to form an alcohol.

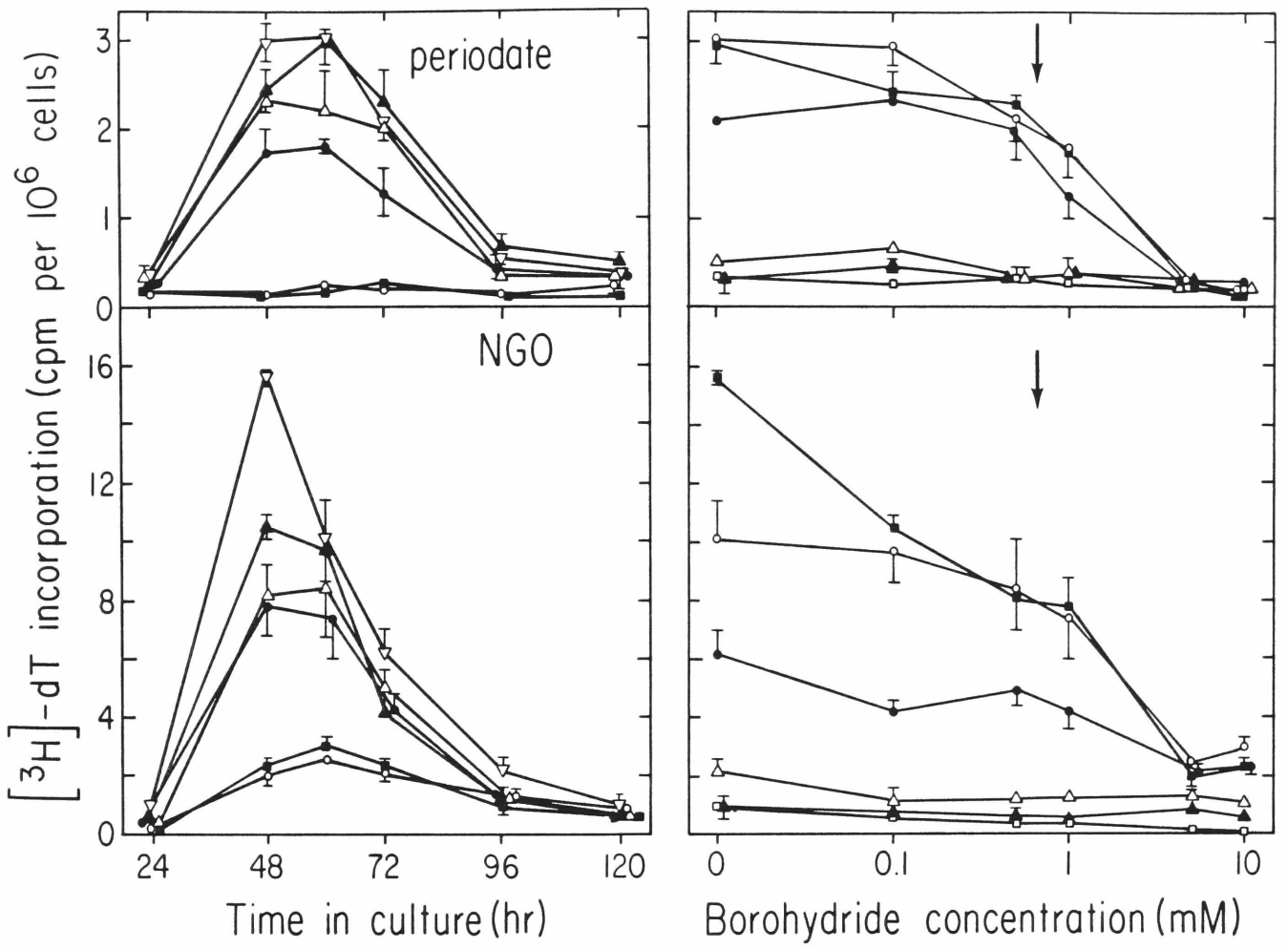



Figure 13. Inhibition of periodate- or NGO-induced stimulation by treatment with borohydride. LNC were oxidized with either periodate (upper panels) or NGO (lower panels), and were then reduced with various concentrations of borohydride before culturing. The panels on the left show the response kinetics for different concentrations of borohydride: Control, no borohydride, ▽ ; 0.1 mM, ▲ ; 0.5 mM, △ ; 1 mM, ● ; 5 mM, ○ ; 10 mM, ■ . The panels on the right show the same data plotted as a function of borohydride concentration for different time points: 24 hr, □ ; 48 hr, ■ ; 60 hr, ○ ; 72 hr, ● ; 96 hr, △ ; 120 hr, ▲ . The arrows indicate the concentration of $^{3}$H-borohydride typically used for labeling cells.

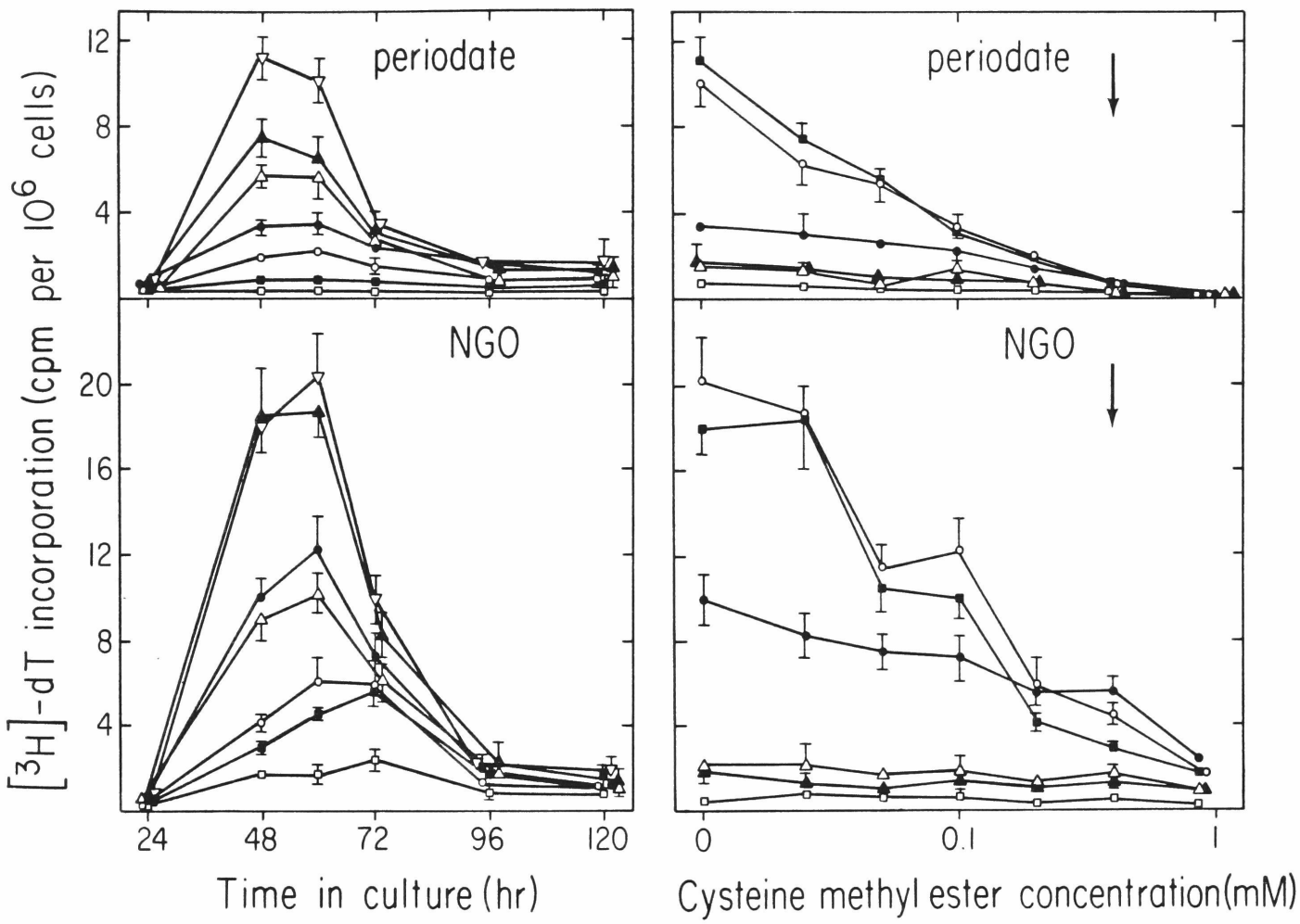



Figure 14. Inhibition of periodate- or NGO-induced stimulation by treatment with cysteine methyl ester (CME). LNC were oxidized with either periodate (upper panels) or NGO (lower panels), and were then reacted with various concentrations of CME before culturing. The panels on the left show the response kinetics for different concentrations of CME: Control, no CME, ▽ ; 0.025 mM, ▲ ; 0.05 mM, △ ; 0.1 mM, ● ; 0.2 mM, ○ ; 0.4 mM, ■ ; 0.8 mM, □ . The panels on the right show the same data plotted as a function of CME concentration for different time points: 24 hr, □ ; 48 hr, ■ ; 60 hr, ○ ; 72 hr, ● ; 96 hr, △ ; 120 hr, ▲ . The arrows indicate the concentration of $^{35}$S-CME typically used for labeling cells.

oxidizing reagents. As indicated by the positions of the arrows denoting the reagent concentration used in the labeling procedures (see Methods section), $^{3}$H-borohydride was used at sub-inhibitory levels while $^{35}$S-CME was used at a concentration well within the inhibitory range.

Cysteine ethyl ester was also found to inhibit oxidative mitogenesis at concentrations comparable to CME. In comparison, treatment with cysteine, which had been previously reported to abrogate oxidative mitogenesis (Ravid and Novogrodsky, 1976), or with cysteinyl-tyrosine (prepared by dithiothreitol reduction of cystinyl-bis-tyrosine), required concentrations in the range of 5-10 mM for maximum inhibition. Levels in excess of 10 mM of any of these reagents also tended to inhibit the Con A response.

Labeling patterns of oxidized and $^{3}$H-borohydride-reduced LNC. Viable LNC were oxidized with mitogenic concentrations of periodate or NGO, washed, and then reacted with either $^{3}$H-borohydride or $^{35}$S-CME. Analysis of the resulting pattern of labeled cell-surface components was accomplished by slicing and counting each gel column or more commonly by using autoradiography; the techniques gave complementary results. Figure 15 shows the results obtained following $^{3}$H-borohydride reduction, although comparable labeling patterns were observed after reaction with $^{35}$S-CME (see below).

Figure 15A shows a typical Coomassie blue protein staining pattern for LNC. A reproducible pattern of 35 Coomassie blue-stained bands was observed from whole LNC. A number of stained

components from crude plasma membrane fractions migrated similarly to Coomassie blue-stained bands from intact LNC; in addition, there were 10 proteins which were detectable only with partially purified and concentrated P fractions. With the exception of rat erythrocytes and a mouse macrophage line that were examined (#P388D1, gift of Dr. Jay Unkeless), all the lymphoid populations had the same protein staining pattern. Based on a comparison with the reported molecular weights of purified material (Elgin and Weintraub, 1975), the four low molecular weight, heavily-stained bands (16,000, 15,400, 14,5000, and 12,400 m.w. on these gels), and the intermediate molecular weight doublet (33,600 and 30,600 m.w.) are probably histones (H3, H2a, H2b, H4, and H1, respectively). In support of this contention, these Coomassie-stained proteins were found in nuclear (N) fractions obtained by differential centrifugation, but were absent in the post-nuclear extracts. Figures 15B, 15C, and 15F show respectively the autoradiographic patterns of cells labeled with $^{3}H$-borohydride after treatment with periodate, NGO, or galactose oxidase alone. There was no apparent correlation between stained protein bands from whole LNC and labeled cell-surface components. Since membrane proteins constitute only approximately 1% of the total cell protein in lymphocytes (Ladoulis et al., 1974), and because glycoproteins generally stain poorly with Coomassie blue (Fairbanks et al., 1974), this result was not surprising. Even in crude plasma membrane preparations which contained only approximately 20% of the total cellular protein and had a 4- to 5-fold enrichment of membrane enzyme markers (see below), the

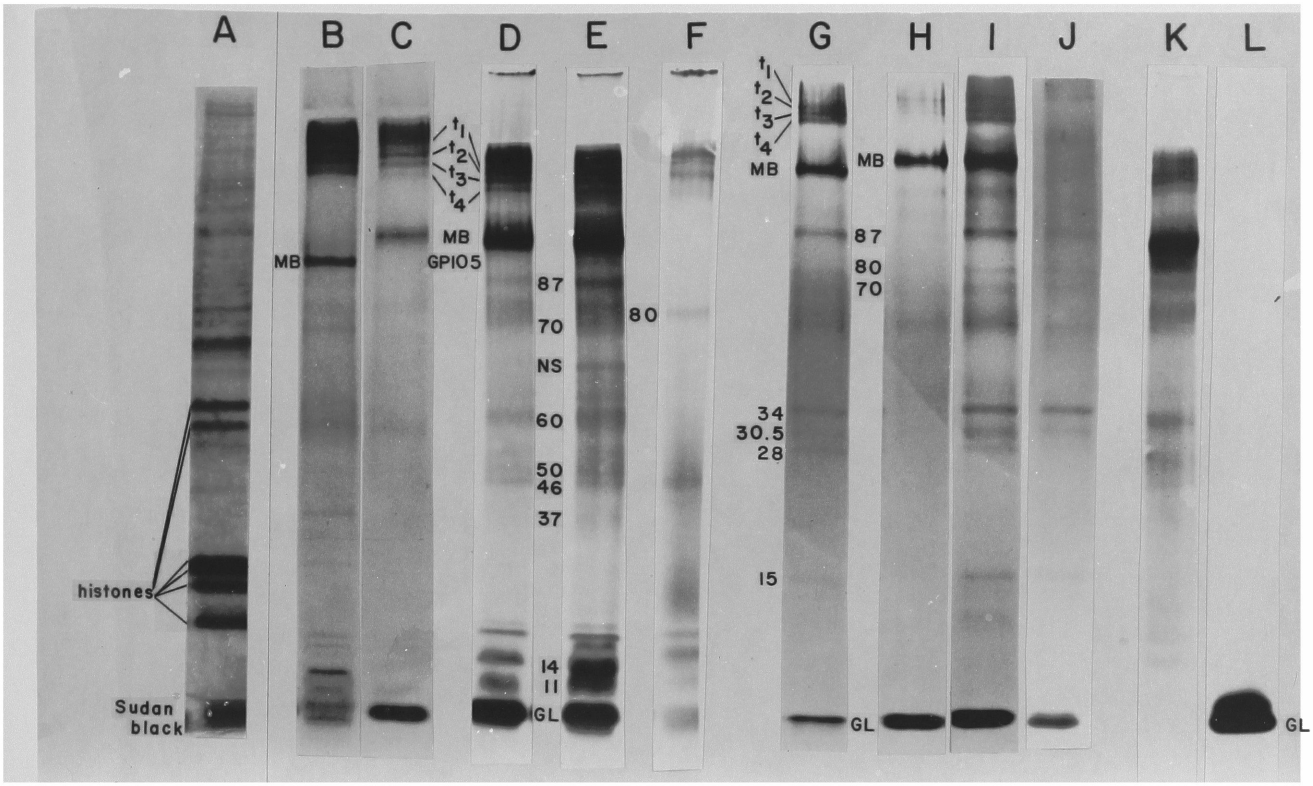



**Figure 15.** SDS-polyacrylamide slab gel banding patterns for rat LNC. A: Coomassie blue and Sudan black staining pattern. Sudan black was added directly to samples before electrophoresis. B-L: autoradiographic patterns of $^{3}$H-borohydride-labeled components involving different methods of oxidation and/or various cell treatments (listed sequentially). B: 10 mM unlabeled borohydride; periodate; $^{3}$H-borohydride. C: 10 mM unlabeled borohydride; NGO; $^{3}$H-borohydride. D: same as C except that the sample was run on a different gel. E: NGO; $^{3}$H-borohydride. F: 10 mM unlabeled borohydride; galactose oxidase; $^{3}$H-borohydride. G: periodate; $^{3}$H-borohydride. H: NGO; $^{3}$H-borohydride. I: NGO; periodate; $^{3}$H-borohydride. J: 60 units/ml neuraminidase; periodate; $^{3}$H-borohydride. K-L: NGO; $^{3}$H-borohydride; lipid and glycolipid extraction (as described in the Methods section). K: residue. L: material extracted into organic phase. GP105: glycoprotein with an apparent molecular weight of 105,000, etc. MB, $t_1$, $t_2$, $t_3$, $t_4$, etc.: arbitrary nomenclature for commonly referred to components (see Table V for a complete list). NS: protein labeled by $^{3}$H-borohydride in the absence of oxidation by NGO or periodate. GL: glycolipids. To facilitate comparison of the autoradiographic bands, sample columns taken from the same gels are grouped together. Thus, B-C, D-F, G-J, and K-L display the same absolute migration of comparable bands. Column A is from the same gel slab as samples G-J. Molecular weights were obtained from calibration curves calculated from the relative migrations of standard proteins for each gel (see Methods section).

Coomassie staining pattern did not correspond to the pattern seen by autoradiography. The observations suggested that $^{3}H$-borohydride was not reacting with the major cell proteins in a non-specific manner.

Most of the labeled bands from NGO-treated and periodate-treated LNC had similar migrations (e.g., $t_1$-$t_4$, GP87, GP70, etc.; compare Figs. 15B and 15C or Figs. 15G and 15H). However, there were three notable exceptions:

1) NGO-oxidized LNC (Figs. 15C and 15H) showed a main band of label at an apparent molecular weight of 120,000 (MB), whereas the major band in periodate-oxidized LNC (Figs. 15B and 15G) had an apparent molecular weight of 110,000. Treating LNC with NGO followed by periodate, and then $^{3}H$-borohydride, led to incorporation of radioactivity only into the 120,000 m.w. band without any label appearing at the 110,000 m.w. position (Fig. 15I). This result suggested that the two oxidizing agents were not generating aldehydes on unique cell surface molecules. Neither position showed any label if the LNC were reduced with $^{3}H$-borohydride after treatment with 60 units/ml neuraminidase prior to periodate oxidation (Fig. 15J), which indicated that the periodate was not cleaving and oxidizing the MB component at some mid-chain point and thereby inducing the labeling of an artifactually lower m.w. fragment. Finally, no radioactivity was incorporated into either MB position by treatment with galactose oxidase alone (Fig. 15F), which demonstrated that the labeling by NGO was dependent on the activity of the neuraminidase and that the MB component must be terminated by sialic acid.

2) Four low molecular weight bands (GP34, GP30.5, GP28, and GP15) were labeled with $^{3}$H-borohydride after periodate treatment, but not after oxidation by NGO (compare Figs. 15G and 15H). These bands were periodate-oxidized and labeled even if LNC were first treated with neuraminidase (60 units/ml) (Fig. 15J).

3) NGO-oxidized LNC showed at most two heavily-labeled low molecular weight components (GL) migrating to approximately 10,000 m.w. By comparison, following periodate oxidation these low molecular weight components generally appeared as an array of low molecular weight, lightly-labeled bands, and in some preparations could not be visualized at all. Extraction into 1:1 chloroform:methanol (Ledeen et al., 1973) (Fig. 15L), staining with Sudan black (Ladoulis et al., 1974)(Fig. 15A), and some variability in molecular weights indicative of auto-oxidation (Fogarty, 1971) imply that these bands were most likely glycolipid. The array of low molecular weight components from periodate-oxidized LNC may also be partially attributable to gangliosides containing multiple sialic acids attached to one another (Ledeen, 1978). Periodate treatment would oxidize the sialic acids but would otherwise leave the poly-sialylated molecules relatively intact. The resultant labeling pattern would therefore appear as a cluster of molecules which varied in molecular weight depending on the degree of sialylation (Fig. 15B). NGO oxidation, on the other hand, would remove the sialic acids and leave only a lower molecular weight core glycolipid (Fig. 15C).

Only one glycoprotein band was labeled if LNC were reduced with $^{3}$H-borohydride without prior oxidation (see also below, Fig. 20). This band (NS; 62,000 m.w.; Fig. 15E) was not labeled if cells were reduced with non-radioactive borohydride prior to either NGO or periodate oxidation (Figs. 15B and 15D); all the other radiolabeled bands were unaffected. It therefore follows that the specifically-labeled radioactive components on the gels were not oxidized in their natural state and reacted with $^{3}$H-borohydride only after oxidation by the mitogenic agents.

Reduction with $^{3}$H-borohydride after galactose oxidase treatment alone (Fig. 15F) showed only light, if any, labeling of the predominant radioactive bands (MB, $t_1$-$t_4$ and GL) relative to that seen after NGO treatment. In general, only the radioactive band at 80,000 m.w. (GP80) showed any significant labeling under these conditions.

Controls for radioactive labeling procedures. In order to validate the radiolabeling procedures, a number of criteria had to be satisfied. The labeling should be restricted to plasma membrane sites without labeling internal components, and should be specific with regard to the oxidized molecules. In addition, the labeling should be either quantitative, or at least uniform in randomly tagging all oxidized surface components. Finally, it was important to show for the $^{3}$H-borohydride reduction that the tritium was incorporated into radioactive alcohols and not into radioactive Schiff bases, such as could form between aldehydes and surface amino groups.

Distributions of labeled components and of marker constituents in subcellular fractions of LNC homogenates. In order to determine if the $^{3}H$-borohydride and $^{35}S$-CME labeling procedures used here were specifically labeling plasma membrane components, analytical subcellular fractionation experiments were carried out. Table III shows the distributions of marker constituents and radiolabel after fractionation of rat LNC homogenates by differential centrifugation.

The data indicate that the low-speed nuclear (N) fraction contained about 25% of the total homogenate protein, and 95% of the DNA, but only small amounts of constituents known to be localized in other organelles. Thus, the homogenization conditions led to good cell breakage, with no evidence of nuclear damage or agglutination of organelles.

The high-speed pellet (P fraction) contained approximately 20% of the homogenate protein, almost all of the mitochondrial enzyme, cytochrome oxidase, and about one-third of the lysosomal marker, N-acetyl-β-glucosaminidase. The remaining two-thirds of the N-acetyl-β-glucosaminidase activity was recovered in the high-speed supernatant (S fraction). Apparently, some of the lysosomes of LNC were ruptured by homogenization in the hypotonic medium needed for adequate cell breakage. Most important in the present regard, however, was the fact that 80% of the plasma membrane markers was recovered in the P fraction. Since only 20% of the total cell protein was present in the P fraction, this fraction was therefore enriched 4-fold over the homogenate in plasma

TABLE III

Distributions of Marker Constituents and Radiolabels after Fractionation of Rat LNC Homogenates by Differential Centrifugation

| Constituent | | Percent of Recovered Constituent | | | Recovery |
|---|---|---|---|---|---|
| | | N | P | S | |
| Protein | (8)[a] | 24.7 ± 10.3 | 19.0 ± 5.1 | 55.9 ± 6.9 | 100.2 ± 5.9 |
| DNA | (1) | 94.7 | 3.9 | 1.4 | 100.2 |
| Cytochrome oxidase (mitochonria) | (1) | 9.3 | 90.7 | 0.0 | 62.8 |
| N-acetyl-β-glucosaminidase (lysosomes) | (5) | 7.6 ± 2.1 | 26.6 ± 9.6 | 65.8 ± 10.6 | 86.5 ± 8.6 |
| Alkaline phosphodiesterase (plasma membrane) | (6) | 9.1 ± 5.3 | 85.9 ± 6.4 | 4.9 ± 2.2 | 94.0 ± 10.5 |
| γ-glutamyl transpeptidase (plasma membrane) | (2) | 16.0 ± 4.7 | 76.8 ± 7.7 | 7.2 ± 3.0 | 104.9 ± 30.4 |
| 5'-nucleotidase (plasma membrane) | (2) | 10.5 ± 2.1 | 76.0 ± 4.2 | 13.5 ± 2.2 | 81.8 ± 1.7 |
| NGO/$^{3}$H-borohydride | (3) | 8.9 ± 6.7 | 79.8 ± 1.6 | 11.3 ± 6.4 | 95.8 ± 7.6 |
| Periodate/$^{35}$S-CME (percent acid-precipitable, without dithithreitol) | (2) | 1.5 ± 1.3 | 5.8 ± 0.0 | 92.3 ± 1.3 | 93.9 ± 1.6 |
| | | (57.6 ± 54.0) | (63.2 ± 1.4) | (4.8 ± 1.9) | |

a = number of determinations

membrane.

Oxidation of LNC with NGO or periodate, followed by reaction with $^{3}$H-borohydride or $^{35}$S-CME, had no effect on the subcellular distribution of marker constituents. As shown in Table III, LNC labeled by NGO-oxidation and $^{3}$H-borohydride reduction, and fractionated by differential centrifugation, had a distribution of tritium which was identical with that of the plasma membrane marker enzymes. A similar result was obtained by $^{3}$H-borohydride labeling following periodate oxidation. These results were consistent with the localization of the $^{3}$H-borohydride label on the plasma membrane components.

Table III also shows the subcellular distribution of radioactivity from LNC which were oxidized with periodate and reacted with $^{35}$S-CME. Similar distributions were obtained by labeling after an NGO oxidation. Most of the cell-associated radiolabel (>90%) was recovered in the high-speed supernatant (S) fraction, and only about 5% was recovered in the plasma membrane-enriched P fraction. The high levels of radioactivity were found in the S fraction regardless of whether the labeling was performed at 37°C, 25°C, or on ice, suggesting that much of the reagent entered the LNC by diffusion as opposed to active transport. However, the distribution of total radioactivity did not reflect the subcellular sites which were labeled by CME since little, if any, of the radioactivity in the S fraction was covalently incorporated into proteins. This statement is supported by the following observations. First, as shown in Table

III, only 5% of the radiolabel in the S fraction was precipitable by trichloroacetic acid (TCA) while about 2/3 of that in the P fraction was TCA-precipitable. Second, gel electrophoresis of the P fraction revealed a number of specifically-labeled glycoproteins while the S fraction showed almost no specific labeling of macromolecular components (see Fig. 16). Finally, paper chromatography of the S fraction showed that greater than 90% of the radioactivity was associated with a component which migrated like authentic, free cysteine. Of the remaining radioactivity, approximately half had a mobility identical to that obtained with CME. There was an amount of label that persisted at the origin but this was comparable to that observed following chromatography of $^{35}$S-CME alone (data not shown). Presumably, the radioactivity present in the S fraction represented free $^{35}$S-cysteine that originated from the uptake and hydrolysis of $^{35}$S-CME. Significantly, this material was not apparently incorporated into protein over the course of the labeling period.

Gel electrophoresis of the various subcellular fractions obtained from labeled LNC provided further confirmation of the localization of the radiolabels. In Figure 16 is shown the gel patterns of labeled molecules from solubilized, intact LNC labeled by $^{3}$H-borohydride reduction (Fig. 16; whole cells), or from homogenates and subcellular fractions prepared from both NGO-oxidized, $^{3}$H-borohydride-labeled LNC (Fig. 16; $^{3}$H-borohydride, H, N, S, and P) and periodate-oxidized, $^{35}$S-CME-labeled LNC (Fig. 16; $^{35}$S-CME, H, N, S, and P). It was apparent

that $^{35}$S-CME labeled the same specifically-oxidized molecules on LNC as did $^{3}$H-borohydride (see also Figs. 20C and 20D).

By comparing the labeling patterns of solubilized whole cells and homogenates (Fig. 16; $^{3}$H-borohydride, whole cells and H), it was clear that the banding was essentially identical. Degradation due to proteases did not appear to be a problem using the homogenization and fractionation procedures described in the Methods section. Indeed, almost all of the specific bands seen in whole homogenates were also present in the crude plasma membrane P fractions (Fig. 16; $^{3}$H-borohydride, P). No labeled bands were detected in the N fraction (Fig. 16; $^{3}$H-borohydride, N). The absence of label in the nuclear fraction indicated that the homogenization of the cells was complete and that the cell membrane remained intact during the labeling procedures. This contention was also supported by the lack of labeling observed in the S fraction (Fig. 16; $^{3}$H-borohydride, S). Interestingly, two of the bands seen in whole homogenates were present in the S fraction but were not present in the high-speed pellet P fraction. These two components (GP50 and GP12) may be extrinsic glycoproteins which were only loosely associated with the plasma membrane and which were removed from the membrane by washing and fractionation in KCl solutions.

The patterns observed for each of the fractions from LNC treated with periodate and $^{35}$S-CME were almost identical to those observed following $^{3}$H-borohydride labeling. Especially important was the fact that the P fraction (Fig. 16; $^{35}$S-CME, P), which

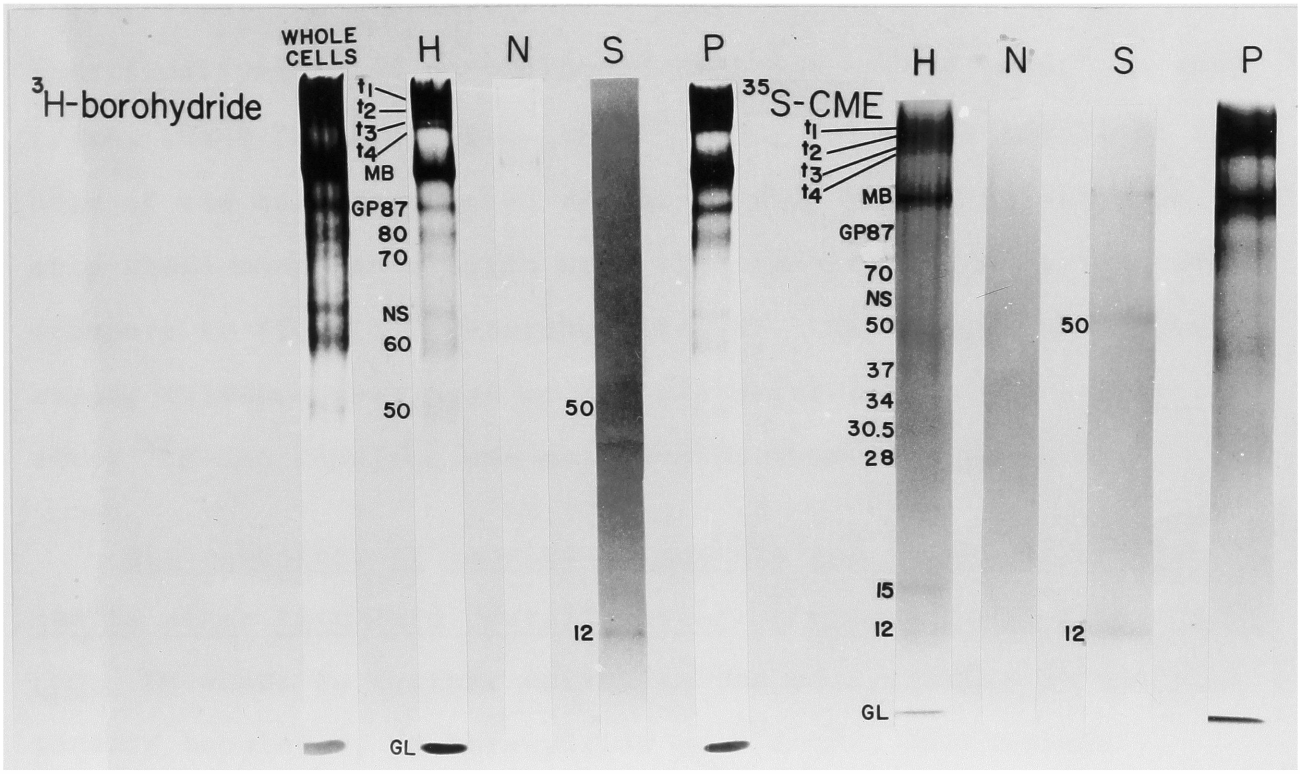



Figure 16. Labeling patterns of fractions obtained by differential centrifugation of NGO-oxidized, $^{3}$H-borohydride-reduced or periodate-oxidized, $^{35}$S-CME-reacted LNC. Whole cells: labeled cells, directly solubilized in 1% SDS; see Methods section. H: total cell homogenate. N: nuclear fraction. S: supernatant from high-speed centrifugation of post-nuclear extract. P: pellet from high-speed centrifugation of post-nuclear extract. Samples prepared as described in Methods section. For NGO/$^{3}$H-borohydride-labeled LNC, the radioactivity and protein applied were: H: 22,000 cpm, 24 µg protein; N: 9,000 cpm, 14 µg protein; S: 6000 cpm, 32 µg protein; P: 26,000 cpm, 8 µg protein. For periodate/$^{35}$S-CME-labeled LNC, the radioactivity and protein applied were: H: 58,000 dpm, 72 µg protein; N: 500 dpm, 4 µg protein; S: 27,000 dpm, 23 µg protein; P: 36,000 dpm, 192 µg protein. The apparent band at approximately 40,000 m.w. in the S fraction of the $^{3}$H-borohydride-labeled cells is an artifact from the development of the X-ray film.

contained only about 5% of the total cell-associated radioactivity after $^{35}$S-CME labeling, contained almost all of the specifically-labeled components detectable on the gels. In contrast, the S fraction (Fig. 16; $^{35}$S-CME, S), which contained the bulk of the cell-associated radioactivity, showed labeling of only those components which were also labeled by $^{3}$H-borohydride (compare to Fig. 16; $^{3}$H-borohydride, S). This provided further, strong evidence that most of the radioactivity in the S fraction after $^{35}$S-CME labeling was not incorporated into protein.

Distributions of labeled components and of marker constituents after isopycnic centrifugation of post-nuclear extracts of LNC. In order to further establish the localization of the components labeled by $^{3}$H-borohydride or $^{35}$S-CME, postnuclear extracts (E fractions) of LNC homogenates were fractionated by isopycnic density gradient centrifugation. Figure 17 shows the distributions of radiolabels and marker enzymes from such gradients.

Most of the protein in the E fraction was soluble and was recovered from the position occupied originally by the sample at the top of the density gradient. That portion of the protein distribution which was sedimentable was more or less evenly apportioned among the various fractions with a small peak at a density of about 1.16. The distribution of cytochrome oxidase, indicating the position where the mitochondria equilibrated in the gradient, showed a sharp, symmetrical peak at a density of about 1.19. N-acetyl-β-glucosaminidase activity was recovered

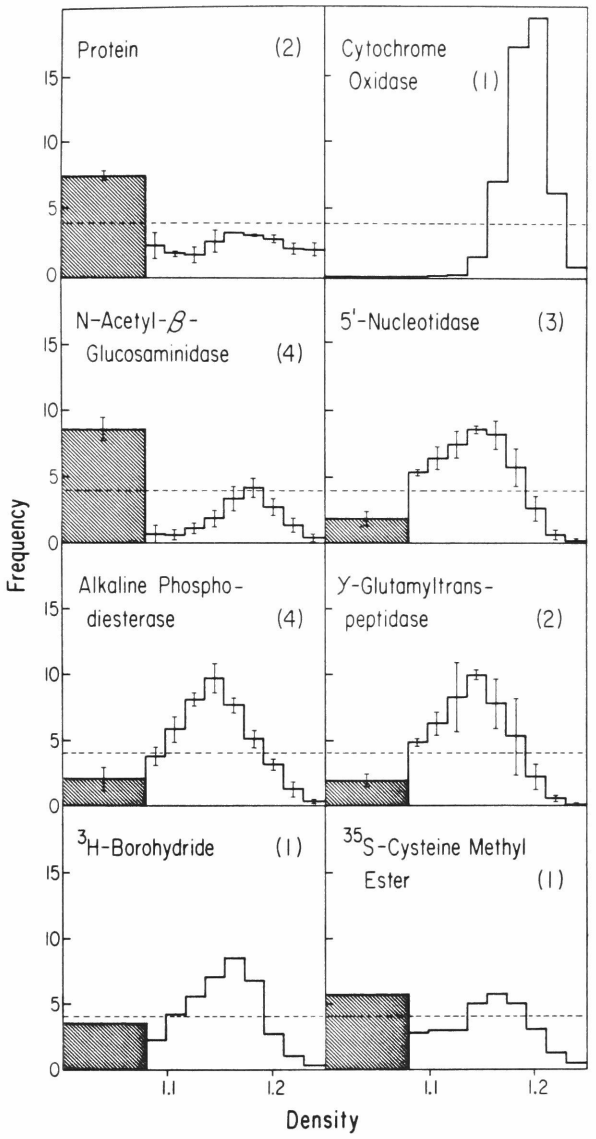



Figure 17. Histograms showing the distributions of marker enzymes and radioactivity from $^{3}H$-borohydride- or $^{35}S$-CME-labeled LNC following isopycnic centrifugation. The direction of sedimentation and increasing density of the gradient is from left to right. Hatched boxes at the left of each distribution indicate the averaged activity found in the soluble region of the gradients where the samples were applied. The numbers in parentheses indicate the number of experiments. The dashed horizontal line represents the value for the mean activity of each enzyme or measurement, assuming it is distributed equally across the gradient. Values above this line indicate enrichment in a particular fraction. Frequency (ordinate) specifies the percent of total activity in each fraction as a function of the interval of density spanned by the fraction. Enzyme assays are described in the text.

primarily in the soluble fractions with the remainder being sedimentable. The presence of large amounts of this enzyme in the soluble fractions was expected on the basis of the results of differential centrifugation studies (Table III), and presumably resulted from the breakage of lysosomes. The characteristics of lysosomes in lymphoid cells have been previously studied and discussed in some detail (Bowers, 1972). In general, it was found that lysosomes in lymphoid cells were more fragile than those found, for example, in liver (Leighton et al., 1968); the lysosomes in LNC were found to be even more susceptible to breakage than those in thoracic duct lymphocytes or spleen cells. That portion of the N-acetyl-β-glucosaminidase activity which was sedimentable showed a broad symmetrical peak in the gradient with a mode at a density of about 1.18, and presumably reflected the distribution of intact lysosomes.

Figure 17 also shows the distributions of three plasma membrane markers, 5-nucleotidase, γ-glutamyltranspeptidase, and alkaline phosphodiesterase I. All three enzymes were largely sedimentable, and distributed throughout the gradient with peaks in the region of density 1.14-1.16. The broad distribution of these enzymes reflected the heterogeneity in equilibrium densities of plasma membrane vesicles. This heterogeneity in density most likely resulted from variations in the relative proportions of lipid, protein, carbohydrate and entrapped water in individual vesicles (Beaufay et al., 1974b).

The distribution of radioactivity in the density gradients

after fractionation of labeled cells further supported the argument that the labeling techniques were specifically introducing the radioactivity onto cell surface components. Thus, after labeling with $^{3}$H-borohydride, most of the radioactivity was sedimentable and had a distribution similar to the plasma membrane markers. It is apparent, however, that there was slightly more radioactivity in the soluble region of the gradient than might be expected from the activity of the plasma membrane marker enzymes. This may be due to the release of labeled cell surface proteins during homogenization and fractionation of the cells as mentioned above, or may be attributable to un-incorporated $^{3}$H-borohydride label that was contained in the homogenate despite extensive washing of the LNC. The distribution of acid-precipitable radioactivity from $^{35}$S-CME-labeled LNC was also similar to that of the plasma membrane marker enzymes. In the case of the $^{35}$S-CME labeling, however, there was substantially more acid-precipitable radioactivity in the soluble region of the gradient than could be accounted for on the basis of the activity of the plasma membrane marker enzymes, or of released, labeled cell surface components. However, as demonstrated below, most of the acid-precipitable radioactivity represented non-specific trapping of radiolabel in the TCA precipitates rather than incorporation of radiolabel into proteins.

The top half of Figure 18 shows the distributions of total and acid-precipitable radioactivity after isopycnic centrifugation of extracts prepared from periodate-oxidized, $^{35}$S-CME-labeled LNC. In agreement with the results of fractionation

studies employing differential centrifugation (Table III), almost all of the total cell-associated radioactivity was in the soluble region of the gradient. However, only 5-10% of the radioactivity in this region was precipitable by TCA while that associated with sedimentable components was about 80% acid precipitable. Moreover, when TCA precipitation was carried out in the presence of 40 mM dithiothreitol to reduce potential $^{35}$S-CME-protein disulfide links, the acid-precipitable radioactivity in the soluble fraction was decreased by approximately 50%. There was no effect of dithiothreitol observed on the acid-precipitable radioactivity in fractions representing sedimentable material.

The gel patterns shown in the bottom portion of Figure 18 also supported the contention that the small amount of TCA-precipitable radioactivity in soluble fractions was not incorporated into protein. Thus, labeled molecules were observed primarily only in those fractions containing sedimentable radioactivity. Although roughly the same amount of acid-precipitable radioactivity from the soluble region of the gradient was applied to the gels, little radioactive labeling was observed (first 5 slots on the left). Indeed, the only specific labeling seen in the soluble region were the 2 bands which also appeared there after $^{3}$H-borohydride labeling (Fig. 19). Figure 19 further indicates that the specifically-labeled $^{3}$H-borohydride-tagged components sediment with the total radioactivity. All of these results were consistent with a localization of the $^{3}$H-borohydride- or $^{35}$S-CME-labeled molecules on the lymphocyte plasma membrane.

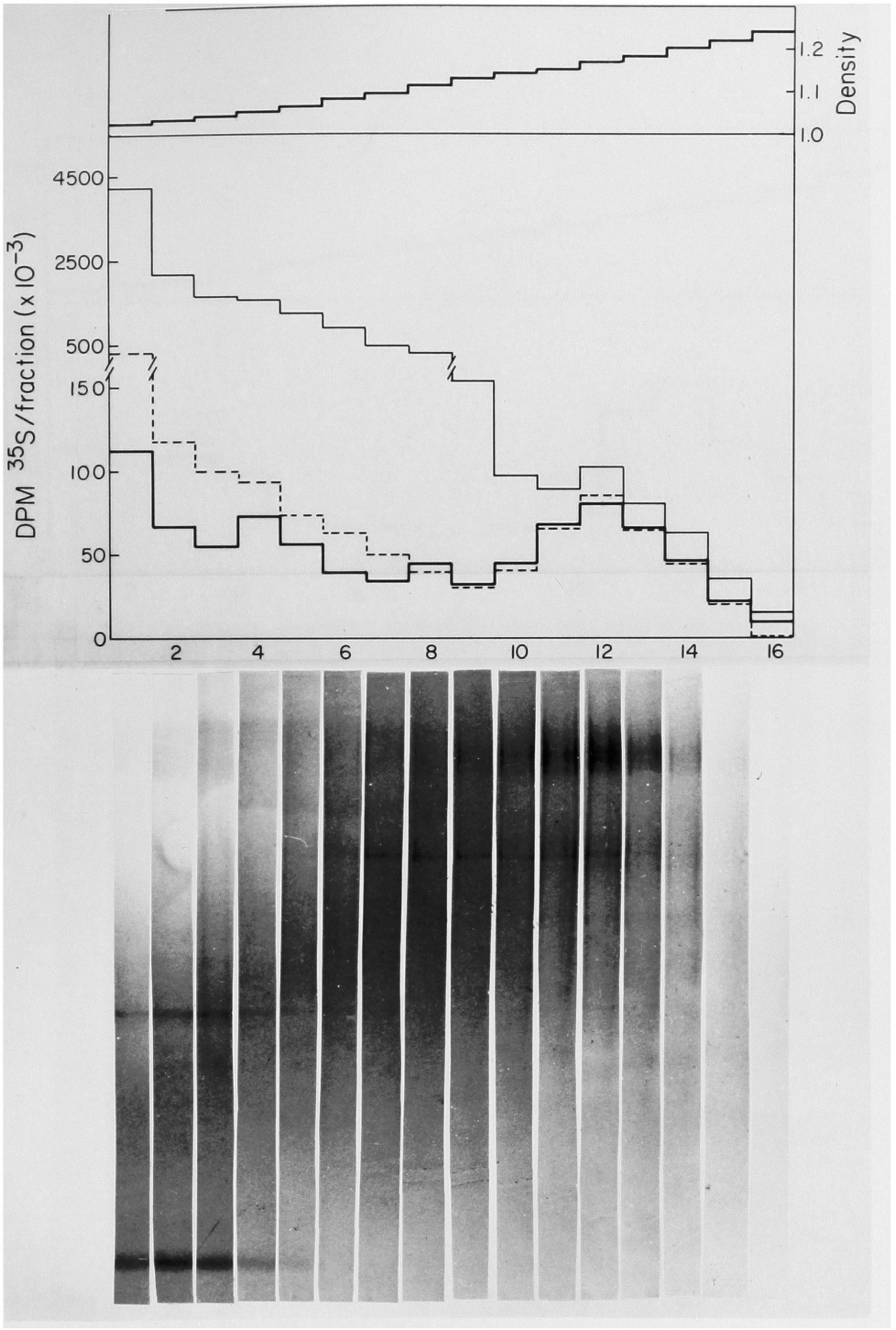



Figure 18. Distribution of radioactivity from an isopycnic centrifugation of periodate-oxidized, $^{35}$S-CME-reacted LNC. The density of each fraction (fraction number on the abscissa) is displayed in the uppermost panel. The middle panel shows the distribution of: total dpm (——), TCA-precipitable dpm (— — —), and TCA-precipitable dpm in the presence of 40 mM dithiothreitol (▬▬▬). The gel patterns in the bottom panel derive from samples of the same fractionation. 100 µl each of samples prepared from the gradient fractions (not TCA-precipitates) were used for each lane. Radioactivity varied from 52,000 dpm in Fraction 1, to 2200 dpm in Fraction 9, 2100 dpm in Fraction 12, and 350 dpm in Fraction 16.

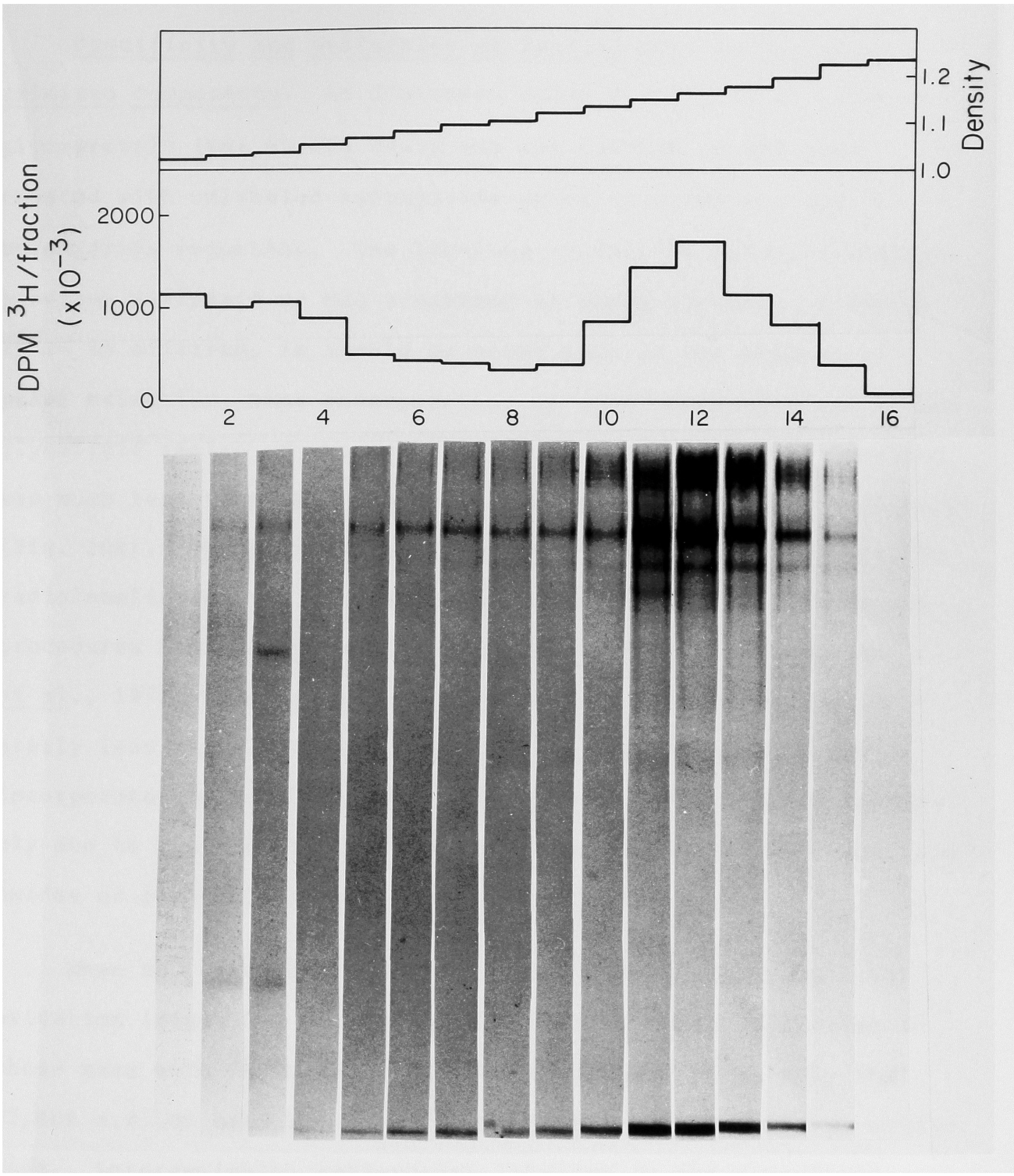



Figure 19. Distribution of radioactivity from an isopycnic centrifugation of NGO-oxidized, $^3$H-borohydride-reduced LNC. The density of each gradient fraction (fraction number on the abscissa) is displayed in the top panel. The gel patterns in the bottom panel derive from 100 µl equal volumes of samples from the same fractionation. The applied radioactivity was 4800 cpm in Fraction 1, 7400 cpm in Fraction 3, 3200 cpm in Fraction 8, 12,200 cpm in Fraction 12, and 1500 cpm in Fraction 16.

Specificity and uniformity of labeled NGO- or periodate-oxidized components. As discussed above for Figure 15, only one glycoprotein (NS; 62,000 m.w.) was not labeled if LNC were treated with unlabeled borohydride prior to oxidation and $^{3}$H-borohydride reduction. The labeling of this NS molecule without previous periodate or NGO treatment is shown directly in Figure 20A. In addition, it should be noted that in the absence of prior oxidation, some incorporation of label also occurred in the glycolipid region of the gel (Fig. 20A). However, this labeling was much less than that observed after periodate or NGO oxidation (Fig. 20B). The phenomenon of apparent non-specific glycolipid radiolabeling during otherwise specific $^{3}$H-borohydride labeling procedures has also been described for erythrocytes (Blumenfeld et al., 1972; Liao et al., 1973; Steck and Dawson, 1974). Generally less than 1% of the specific glycolipid labeling was incorporated non-specifically (Liao et al., 1973), and is possibly due to the reduction of double bonds conjugated to glycolipid amides or phospholipid esters (Kadin, 1966).

When LNC were reacted with $^{35}$S-CME with and without prior oxidation (Figs. 20D and 20E, respectively), results similar to those seen with $^{3}$H-borohydride were obtained. Thus, only the 62,000 m.w. NS band was labeled in the absence of previous oxidation. Interestingly, radioactive labeling in the glycolipid region was extremely faint unless LNC were oxidized with either NGO or periodate (Fig. 20E).

Because high specific activity $^{3}$H-borohydride had to be used

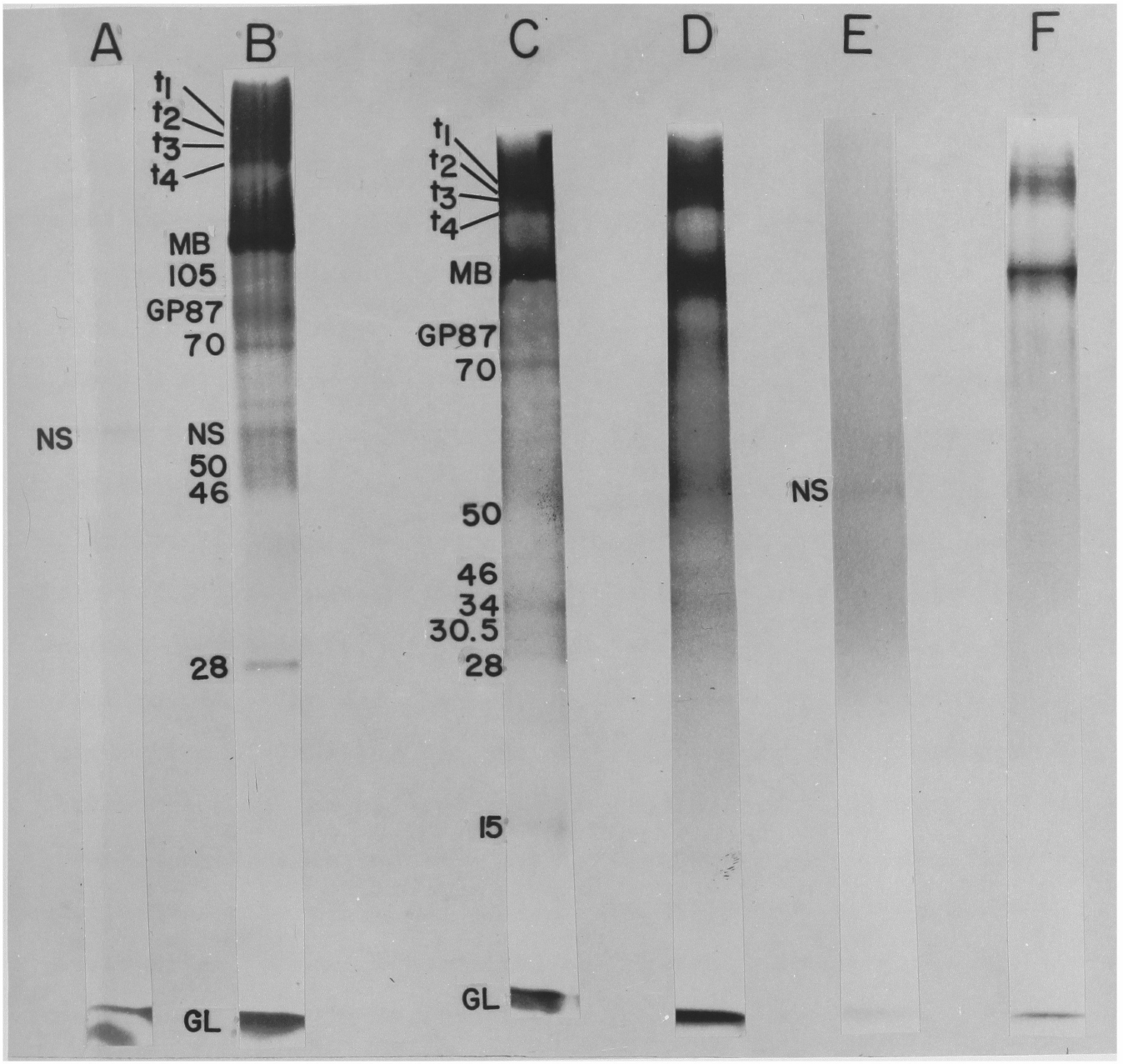



Figure 20. Controls for the specificity and uniformity of labeling by $^{3}H$-borohydride and $^{35}S$-CME. A: no oxidation; $^{3}H$-borohydride. B: periodate; $^{3}H$-borohydride. C: periodate; $^{3}H$-borohydride; P fraction. D: periodate; $^{35}S$-CME; P fraction. E: no oxidation; $^{35}S$-CME; P fraction. F: periodate; 0.4 mM unlabeled CME; $^{35}S$-CME; P fraction. Samples A and B are from one slab gel; samples C-F are from a different gel.

to readily observe labeled glycoproteins from whole cell preparations applied to gels, and because large quantities of such $^{3}H$-borohydride preparations were neither safe nor economical, LNC were labeled at concentrations of $^{3}H$-borohydride which were not completely inhibitory for the NGO or periodate stimulation. Thus, it was conceivable that at the levels of $^{3}H$-borohydride used, a highly susceptible subset of glycoproteins was reduced, rather than a random sample of all the oxidized cell-surface molecules. To address this possibility, LNC were first oxidized with NGO and then pre-reduced with increasing concentrations of non-radioactive borohydride, before a final reduction with the normal concentration of $^{3}H$-borohydride. If the reduction was in fact random, then all the radioactive bands would show a uniform diminution of labeling for successively higher concentrations of borohydride in the pre-reduction. Figure 21 demonstrates that pre-reduction with non-radioactive borohydride produced a qualitatively similar labeling pattern for all concentrations of borohydride, and thus supported the contention that under the conditions used, $^{3}H$-borohydride reduced a random sample of all the oxidized cell-surface molecules.

$^{35}S$-CME labeling was performed at concentrations of $^{35}S$-CME which were completely inhibitory for oxidative mitogenesis (Fig. 12). Thus, the $^{35}S$-CME reacted with at least all of the oxidized molecules which were involved in the stimulatory response. Moreover, in a fashion similar to $^{3}H$-borohydride, oxidized LNC incubated with 0.4 mM unlabeled CME prior to treatment with $^{35}S$-CME, showed a labeling pattern which was substantially reduced in

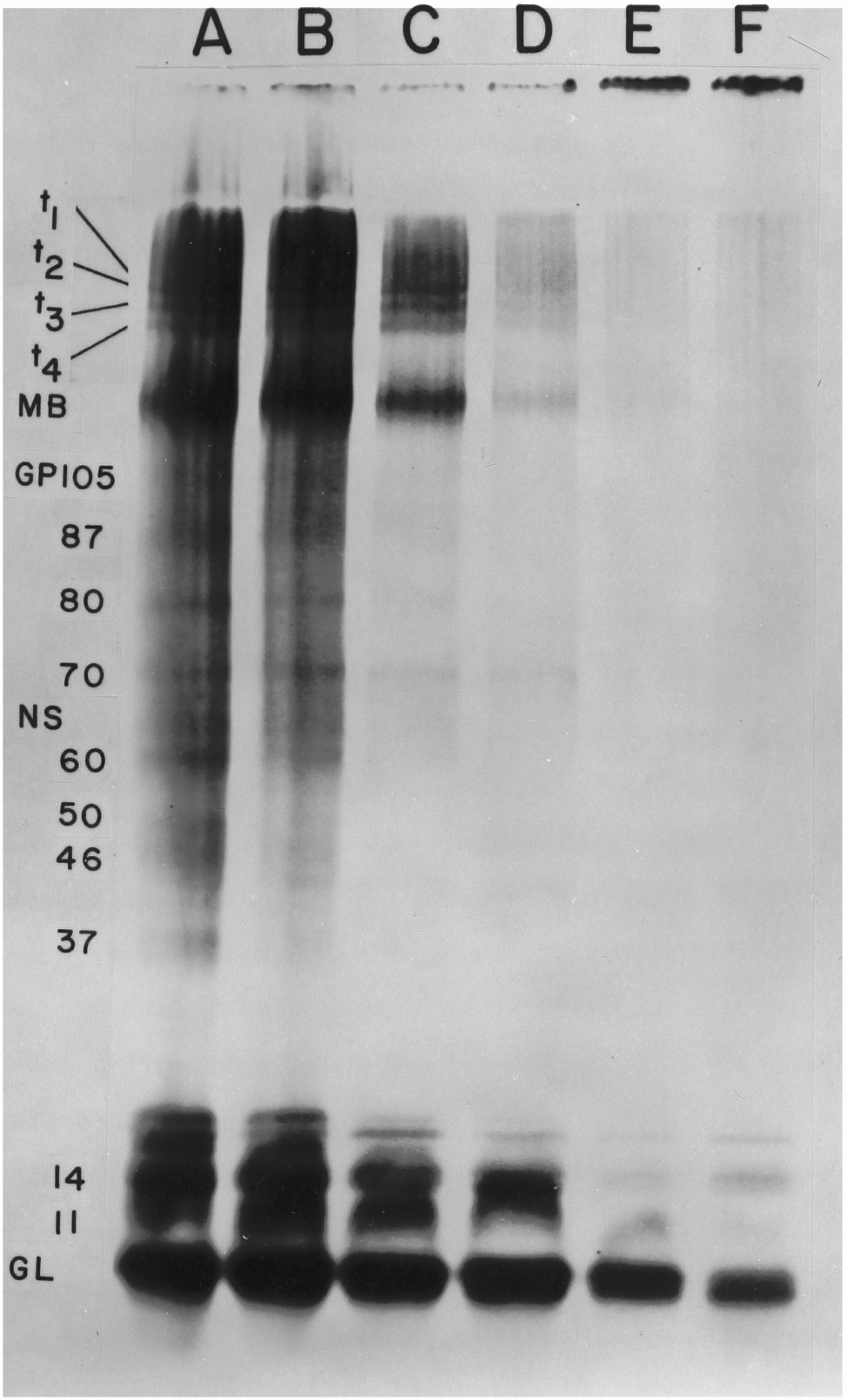



Figure 21. Control for uniform labeling of oxidized surface components. LNC were oxidized with NGO and reduced with increasing concentrations of unlabeled borohydride. The cells were then reduced with 0.93 mM $^{3}H$-borohydride and subsequently prepared for SDS gel electrophoresis. Concentrations of borohydride in pre-reduction step: A: control, no borohydride. B: 0.625 mM. C: 1.25 mM. D: 2.5 mM. E: 5 mM. F: 10 mM.

intensity but was otherwise unchanged (Fig. 20F). Thus, the set of results shown in Figure 20 indicate that $^{35}$S-CME specifically and uniformly labels oxidized cell surface components. In addition, the $^{35}$S-CME labeling reagent yielded a radioactive banding pattern indistinguishable from that obtained by $^{3}$H-borohydride reduction (compare Figs. 20C and 20D).

Control for Schiff base formation. CME can react with surface aldehydes only via formation of a thiazolidine ring. Thus, although there is a possibility of $^{35}$S-CME labeling by disulfide formation, the reducing SDS-polyacrylamide gels that were used obviate this difficulty, and the $^{35}$S-CME incorporation was therefore limited to the specifically-generated cell surface aldehydes. However, in addition to the reduction of aldehydes to form radioactive alcohols, $^{3}$H-borohydride can reduce Schiff bases that form between aldehydes and primary amino groups on other proteins. If Schiff base reduction occurred to a significant extent, the radioactive bands seen would not represent specific cell-surface molecules, but rather cross-linked adducts of unknown composition. This possibility, however, has been ruled out by acid hydrolysis (0.01 N HCl for 15 min at 100$^{o}$C) of periodate-oxidized, $^{3}$H-borohydride-reduced LNC prior to solubilization in SDS. Such treatment breaks the glycosidic linkage of terminal sialic acids without affecting the integrity of the sialic acid itself, or of other glycosidic or peptide bonds (Aminoff, 1961). If $^{3}$H-borohydride incorporation was due to reduction of Schiff bases, then radioactivity would remain associated with high molecular weight proteins after acid hydrolysis.

Conversely, if $^{3}$H-borohydride only reduced aldehyde moieties, then no radioactivity would be found in higher molecular weight regions after acid hydrolysis. In fact, such acid treatment should cause a shift of radioactivity to lower molecular weights nearly coincident with authentic sialic acid. The results (Fig. 22) supported the latter of these possibilities, showing a nearly quantitative loss of label in the high and intermediate molecular weight peaks. It was noteworthy that the low molecular weight peaks representing glycolipid (just above the tracking dye) showed a lesser decrease in radioactivity upon hydrolysis than did the glycoproteins. Since the overall pattern of incorporation was not changed by treatment with acid, the results probably reflected incomplete hydrolysis of the glycolipid sialic acids rather than the presence of Schiff bases. Thus, the results clearly indicated that the $^{3}$H-borohydride did indeed label the great majority of glycoproteins by specifically reducing aldehydes.

Although it is apparent that $^{3}$H-borohydride and $^{35}$S-CME were entirely comparable as specific labeling reagents (each with certain advantages; see Discussion section), the $^{35}$S-CME procedure was developed relatively late in the course of the thesis. Thus, for all the rest of the work presented below, the $^{3}$H-borohydride labeling method was used exclusively. Presumably, similar results would be obtained with the radioactive $^{35}$S-CME procedure.

Mitogenic responsiveness and labeling patterns of different lymphoid populations. Various populations of lymphoid cells were

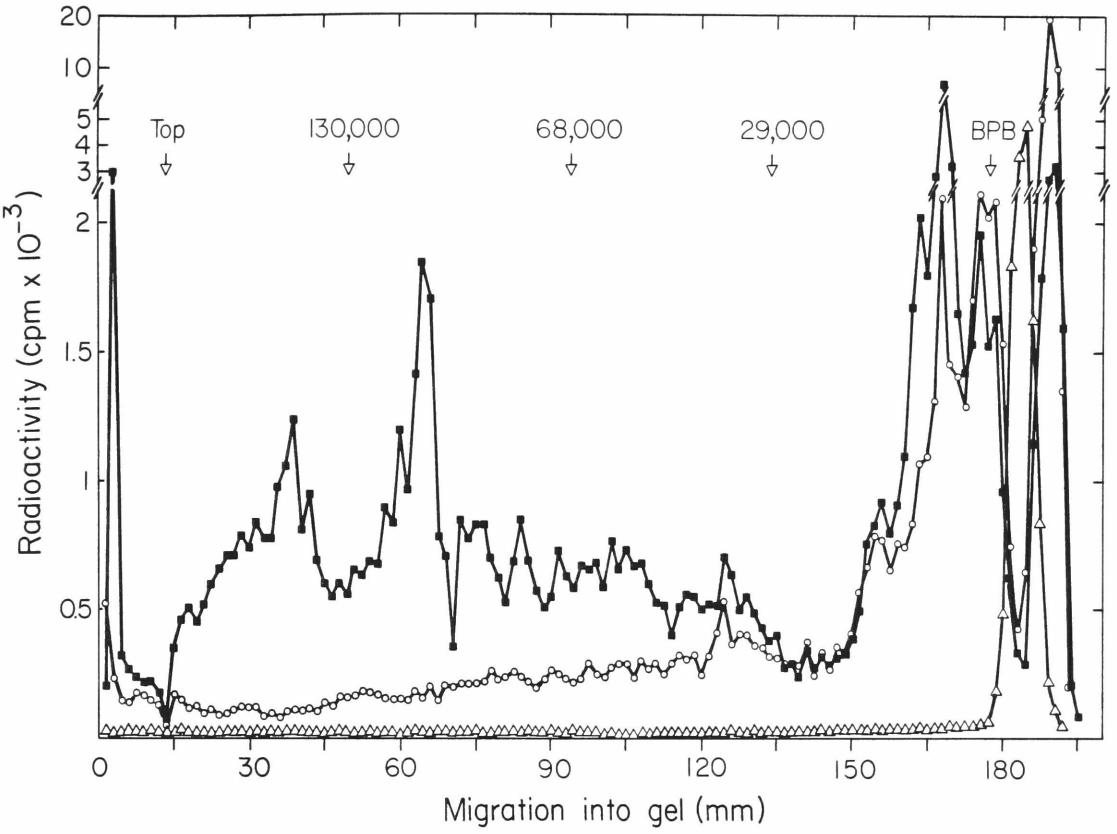



Figure 22. Quantitative analysis of radioactive banding patterns for rat LNC: effect of acid hydrolysis. Cells were reduced with 10 mM unlabeled borohydride and then oxidized by periodate and treated with $^{3}$H-borohydride. The sample was split and then either prepared for gel electrophoresis by the standard procedure (see Methods section) or acid-hydrolyzed to release terminal sialic acids (0.01 N HCl for 15 min at 100°C, followed by neutralization), and then prepared for polyacrylamide gel analysis. Non-hydrolyzed sample, ■ ; acid-hydrolyzed sample, O ; [4-$^{14}$C]-acetyl neuraminic acid, added to the acid-hydrolyzed sample before application to the gel, Δ . Recovery from the non-hydrolyzed sample was 88.1% of 129,000 cpm applied to the gel. Recovery from the acid-hydrolyzed sample was 75.5% of 128,000 cpm applied. Top: interface of stacking and separating gels. BPB: bromphenol blue tracking dye. 130,000: E. coli β-galactosidase standard. 68,000: bovine serum albumin standard. 29,000: horse erythrocyte carbonic anhydrase standard.

examined for their responsiveness to periodate or NGO stimulation, and a correlation was sought between ability to respond and the $^{3}$H-borohydride labeling of specific surface molecules after oxidation. It was known that rat LNC are induced to undergo blastogenesis by periodate or NGO (Novogrodsky and Katchalski, 1971b; Kielian et al., 1977; vida infra), as are TDL (in the presence of non-oxidized accessory cells; Bowers and Beyer, 1977). Periodate and NGO are also specific T-lymphocyte mitogens, and do not stimulate B-lymphocytes to proliferate to any significant degree (Novogrodsky, 1974). Since cortisone-resistant thymocytes have been reported to represent the subpopulation of 'mature', immuno-competent lymphocytes in the thymus (Blomgren and Andersson, 1971), it was also of interest to examine the periodate- or NGO-responsiveness of those cells relative to the predominantly 'immature' total thymocyte populations. Thus, thymocytes and cortisone-resistant thymocytes were prepared as described in the Methods section. As shown by the recoveries and sizing data (Fig. 5), the cortisone-depletion procedure gave a relatively pure population of large cells (125 $\mu^3$ mean volume) relative to the control thymocyte population (97 $\mu^3$ mean volume). When similar preparations of cortisone-resistant thymocytes and thymocytes were treated with periodate or NGO, the responses presented in Table IV were obtained. The responses of untreated, or periodate- or NGO-treated LNC are shown for comparison. Untreated cell preparations showed no significant incorporation of $^{3}$H-thymidine other than the 24 hr time-point for thymocytes. The early elevated DNA synthesis in these cells probably

TABLE IV

Incorporation of $^{3}$H-thymidine in untreated, periodate-treated, or NGO-treated LNC, thymocytes, and cortisone-resistant thymocytes

| Untreated | Time of Harvest (hr) 24 | 48 | 60 | 72 | 96 | 120 |
|---|---|---|---|---|---|---|
| LNC | 71 ± 17[a] | 98 ± 39 | 67 ± 19 | 161 ± 66 | 90 ± 35 | 246 ± 50 |
| thymocytes | 509 ± 32 | 88 ± 25 | 80 ± 45 | 150 ± 55 | 127 ± 34 | 60 ± 15 |
| cortisone-resistant thymocytes | 274 ± 67 | 147 ± 41 | 166 ± 91 | 227 ± 61 | 164 ± 24 | 267 ± 87 |
| Periodate-treated | | | | | | |
| LNC | 425 ± 14 | 5397 ± 442 | 3459 ± 547 | 1983 ± 355 | 627 ± 61 | 686 ± 201 |
| thymocytes | 440 ± 56 | 500 ± 60 | 300 ± 69 | 201 ± 77 | 157 ± 51 | 177 ± 81 |
| cortisone-resistant thymocytes | 485 ± 85 | 6144 ± 324 | 3142 ± 121 | 1467 ± 49 | 518 ± 294 | 353 ± 29 |
| NGO-treated | | | | | | |
| LNC | 753 ± 85 | 11291 ±1066 | 6715 ± 654 | 3486 ± 359 | 662 ± 96 | 391 ± 58 |
| thymocytes | 444 ± 30 | 1333 ± 375 | 1434 ± 218 | 929 ± 190 | 261 ± 29 | 167 ± 27 |
| cortisone-resistant thymocytes | 734 ± 124 | 7581 ± 931 | 6592 ±2032 | 3672 ± 938 | 785 ± 201 | 343 ± 144 |

[a] Incorporation is the average cpm from quadruplicate cultures ± one standard deviation. Cultures were pulsed with tritiated thymidine under 'saturating' conditions for 4 hr prior to harvest.

reflected the high innate metabolic activity and rapid division of the thymocytes freshly isolated from the animal (Metcalf and Wiadrowski, 1966). The peak $^{3}$H-thymidine incorporation for all three mitogenically-stimulated cell populations occurred at approximately 48 hr. Clearly, the cortisone-resistant thymocytes were enriched for responsive cells relative to non-depleted thymocyte populations, and showed $^{3}$H-thymidine incorporation much like that seen for comparable numbers of periodate- or NGO-treated LNC. The lack of responsiveness of thymocyte populations to periodate or NGO, despite the presence of accessory cells in these populations, has been described previously (Bowers and Beyer, 1977).

The $^{3}$H-borohydride labeling patterns of LNC, TDL, thymocytes, and cortisone-resistant thymocytes are shown in Figure 23 (A-G). In Figure 24 are also shown nylon wool column-separated adherent ('B-cells') and non-adherent ('T-cells') lymphocytes (Figs. 24B and E, and 24C and F, respectively). T-lymphocytes following B-depletion (Fig. 24H) and B-lymphocytes selected by anti-Ig panning (Fig. 24I) are also shown. Control, unfractionated LNC (Figs. 24A and 24D) from the same gels as the various T- and B-cell preparations are also shown as a basis for comparison. LNC (Figs. 23A and 23B) and TDL (Figs. 23C and 23D) had essentially identical labeling patterns. Thymocytes (Fig. 23F), however, differed from LNC in that the $t_1$-$t_3$ components were poorly labeled, if at all, while the $t_4$ component often had an intensity greater than that seen for LNC. The GP87 and GP70 glycoproteins, and GL were also weakly labeled relative to LNC.

Thymocytes, however, did show high levels of radioactivity in a band at approximately 27,000 m.w., which would correspond to the rat Thy-1 sialoglycoprotein (Williams et al., 1976). Thy-1 was relatively weakly labeled in LNC and was presumably present only in greatly reduced numbers. The MB and other components showed comparable radioactive incorporation in both LNC and thymocyte populations. Cortisone-resistant thymocytes, in comparison to thymocytes, showed labeling patterns more similar to those seen for LNC. Although it was somewhat difficult to discern on this gel, the $t_1$-$t_4$ glycoproteins showed increased labeling (but still much less intense than that seen for LNC), with some faint incorporation as well in the GP87 and GP70 bands. The glycolipid radioactivity was comparable to that seen for LNC, and the incorporation into the 27,000 m.w. Thy-1 band was intermediate between LNC and thymocytes. It is noteworthy that the band at 46,000 m.w. on cortisone-resistant thymocytes had much more intense labeling than the same band from LNC or thymocytes. Whether the heavily-labeled GP46 reflected a contaminating cell type (or molecule) in these preparations, or was a specific component which occurred with increased density on cortisone-resistant thymocytes is not known.

Non-adherent T-lymphocytes from the nylon wool columns (Figs. 24B and 24E) showed the same overall labeling pattern as unfractionated LNC (Figs. 24A and 24D). The expanded 6%-10% gradient gels (Figs. 24D-F) very clearly resolved the $t_1$-$t_4$ tetrad. The only obvious difference detected between these cell preparations was diffuse radioactivity above the $t_1$-$t_4$ tetrad in LNC

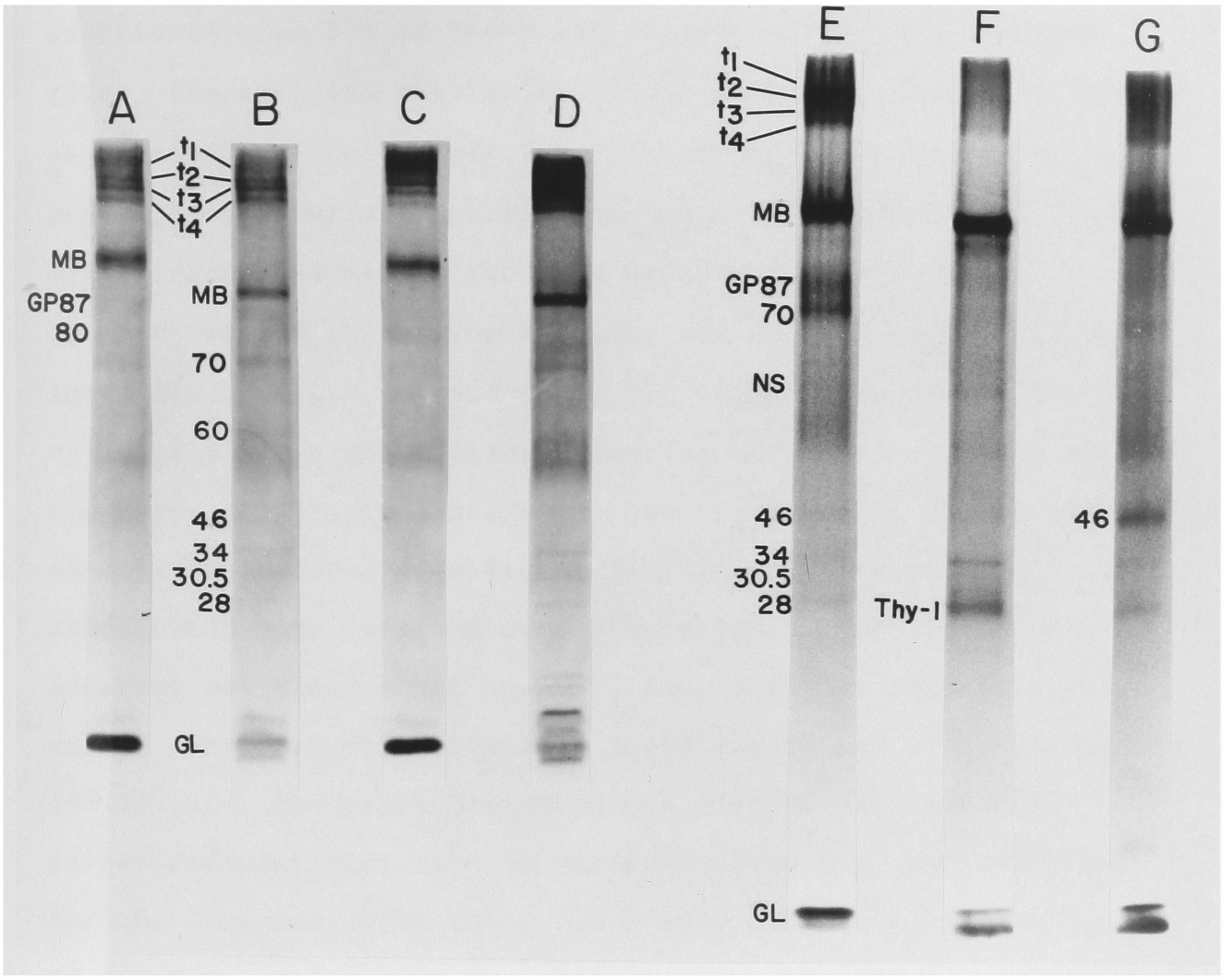



Figure 23. $^{3}$H-borohydride-labeling patterns of various rat lymphoid populations. A: LNC; 10 mM unlabeled borohydride; NGO; $^{3}$H-borohydride. B: LNC; 10 mM unlabeled borohydride; periodate; $^{3}$H-borohydride. C: TDL; 10 mM unlabeled borohydride; NGO; $^{3}$H-borohydride. D: TDL; 10 mM unlabeled borohydride; periodate; $^{3}$H-borohydride. E: LNC; periodate; $^{3}$H-borohydride. F: thymocytes; periodate; $^{3}$H-borohydride. G: cortisone-resistant thymocytes; periodate; $^{3}$H-borohydride. Samples A-D are from the same gel; samples E-G are from a different electrophoresis.

which was not observed with the T-lymphocytes. Since T-cells constitute some 80% of total LNC (Goldschneider and McGregor, 1973), the striking similarity of the banding patterns of the two populations was not surprising. Interestingly, the adherent, presumptive B-cells prepared from the nylon wool columns (Figs. 24B-F) showed generally the same radioactive bands as the T-lymphocytes and unfractionated LNC, albeit at a much diminished intensity (e.g., $t_1$-$t_4$ and MB). Since the nylon wool-adherent cells retained a significant oxidative mitogenic response (Bowers and Beyer, 1977), it was likely that the faint $t_1$-$t_4$ and MB components reflected a significant T-cell contamination rather than bands which also occurred on B-lymphocytes. The gel of the adherent cells did show, however, a unique very high molecular weight component which migrated above the tetrad at approximately 190,000 m.w. and corresponded to the diffuse high molecular weight radioactivity seen on unfractionated LNC. In addition, for the adherent cells there was a very broad intense band at 86,000 m.w., and a band at 54,000 m.w. which probably represented glycosylated heavy chain from surface Ig. Figures 24H and 24I show B-depleted LNC and B-lymphocytes selected by anti-Ig panning, respectively, compared to unfractionated LNC from the same gel electrophoresis (Fig. 24G). The patterns for the B-depleted presumptive T-cells and unfractionated LNC were essentially identical, in agreement with the results seen above with the non-adherent cells from nylon wool columns and LNC. The very faint bands obtained from the panned B-lymphocytes (the faint labeling was due to small numbers of cells) corresponded to the

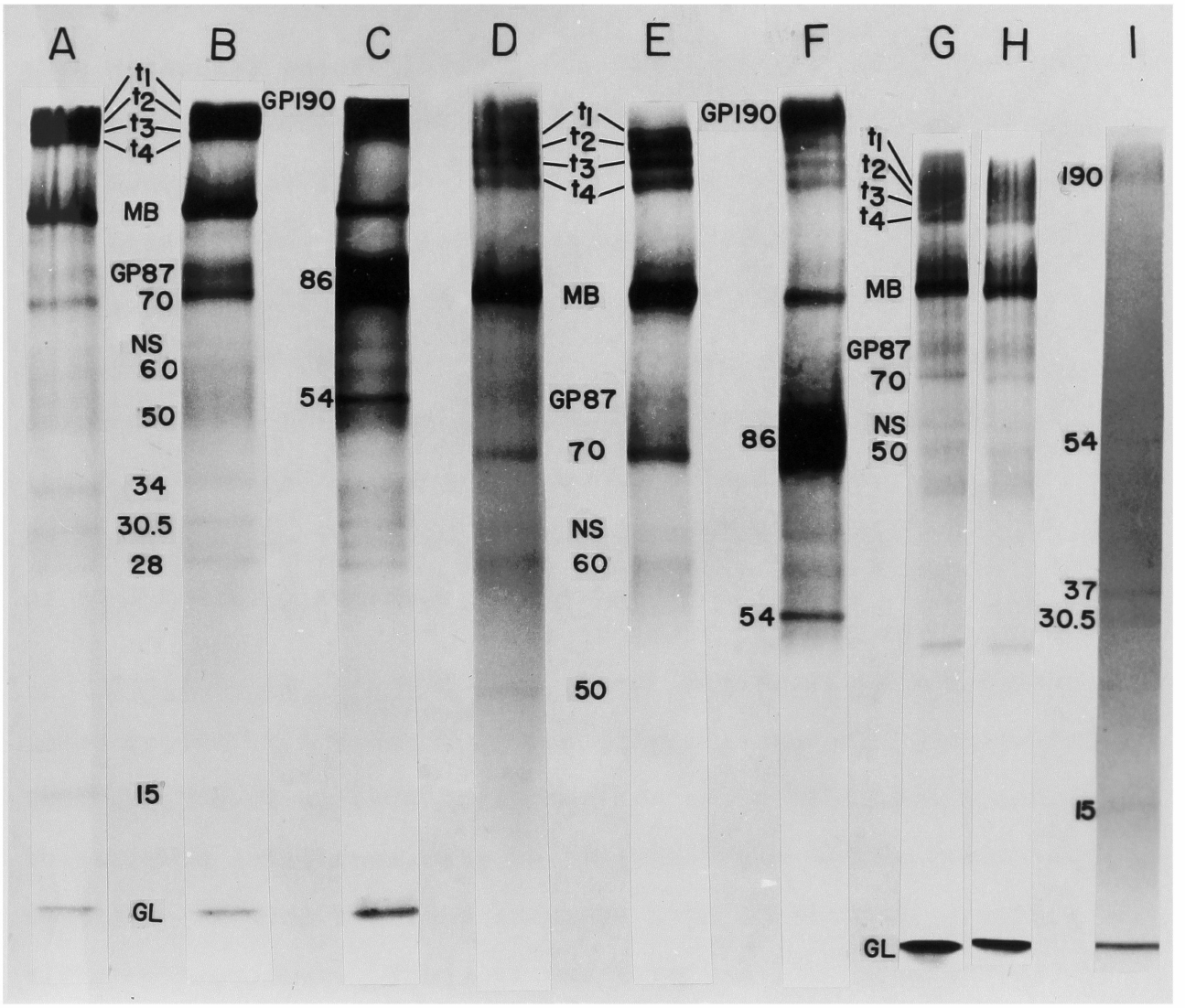



Figure 24. $^{3}$H-borohyaride-labeling patterns of LNC sub-populations. LNC were fractionated on nylon wool columns (columns B,C and E,F) into adherent and non-adherent populations, or were prepared by anti-Ig-plus-complement B-cell depletion (column H) or anti-Ig B-cell panning (column I) as described in the Methods section). A-C are from the same 7-15% polyacrylamide gradient gel; D-F are samples identical to those electrophoresced in A-C, but were separated on a 6-10% polyacrylamide gradient gel. G-I are from a different 7-15% polyacrylamide gradient gel. A and D: LNC; periodate; $^{3}$H-borohydride. B and E: nylon wool non-adherent ('T') cells; periodate; $^{3}$H-borohydride. C and F: nylon wool adherent ('B') cells; periodate; $^{3}$H-borohydride. G: LNC; periodate; $^{3}$H-borohydride. H: B-depleted LNC; periodate; $^{3}$H-borohydride. I: panned B-lymphocytes; periodate; $^{3}$H-borohydride.

high molecular weight 190,000 and the intermediate molecular weight 54,000 bands which were observed to intensify in nylon wool-adherent cells. The broad, intense 86,000 m.w. component seen from labeled adherent cells was not observed in this panned B-cell preparation, which suggested that this molecule occurred on a macrophage or other nylon wool-adherent non-lymphocyte. In agreement with the contention that the residual radioactivity in the $t_1$-$t_4$ and MB molecules on nylon wool-adherent cells was due to contaminating T-cells, panned B-cells showed no incorporation of radioactivity at those positions.

Labeling of cultured LNC. Beyer and Bowers (1977a) have demonstrated that 6-day cultures of periodate- or NGO-stimulated LNC, which have already undergone one round of division and returned to quiescence, may be re-stimulated by the oxidizing agents to undergo a second response with accelerated kinetics and elevated magnitude. Thus, it was expected that once-stimulated 6-day cells would display the molecule(s) relevant to oxidative mitogenesis. It was also of some theoretical interest to examine the labeling pattern of 2-day blast cells obtained at the peak of $^3$H-thymidine incorporation. In addition to the radioactive bands observed on unstimulated lymphocytes, others have also found a number of new components on T-blasts obtained from phytohemagglutinin- or MLR-stimulated lymphocyte cultures (Andersson et al., 1977; Andersson et al., 1978; Kimura and Wigzell, 1978). One of these surface molecules has been implicated as a specific marker of cytotoxic T-lymphocytes (Kimura and Wigzell, 1978). Furthermore, since Norin and Strauss (1975) had

reported a 6-hr turn-over of the relevant oxidizable surface components, it was anticipated that new molecules would have been synthesized which would be oxidizable and $^{3}$H-borohydride-reducible after 2 days in culture.

These points were examined by the SDS-polyacrylamide gels presented in Figure 25. In general, 2-day blast and 6-day secondary LNC (prepared as described in the Methods section) had all the same radiolabeled components as fresh, unstimulated LNC, including the $t_1$-$t_4$ and MB glycoproteins (although the labeling intensity of those molecules was somewhat diminished; Figs. 25A-C). Although the patterns shown in Figures 25B and 25C are from periodate-stimulated cultures of LNC, identical results were obtained with NGO-stimulated cultures. In addition to the bands which were the same, 2-day blasts (Fig. 25B) showed intense labeling of a glycoprotein at 62,000 m.w. which co-migrated with the NS band. The intensity of labeling of this molecule had diminished almost to normal by day 6 (Fig. 25C). However, secondary cells displayed a number of other new, predominantly low molecular weight components (less than 30,000 m.w.) which were not found on fresh LNC or 2-day blasts. The additional bands were probably not from glycoproteins adsorbed from the culture medium, since the cells were purified and washed extensively following harvest (see Methods section). Furthermore, no similar bands were apparent from 2-day blasts, which had been cultured in the same medium.

Labeling of RBC and analysis of intrinsic vs. extrinsic mem-

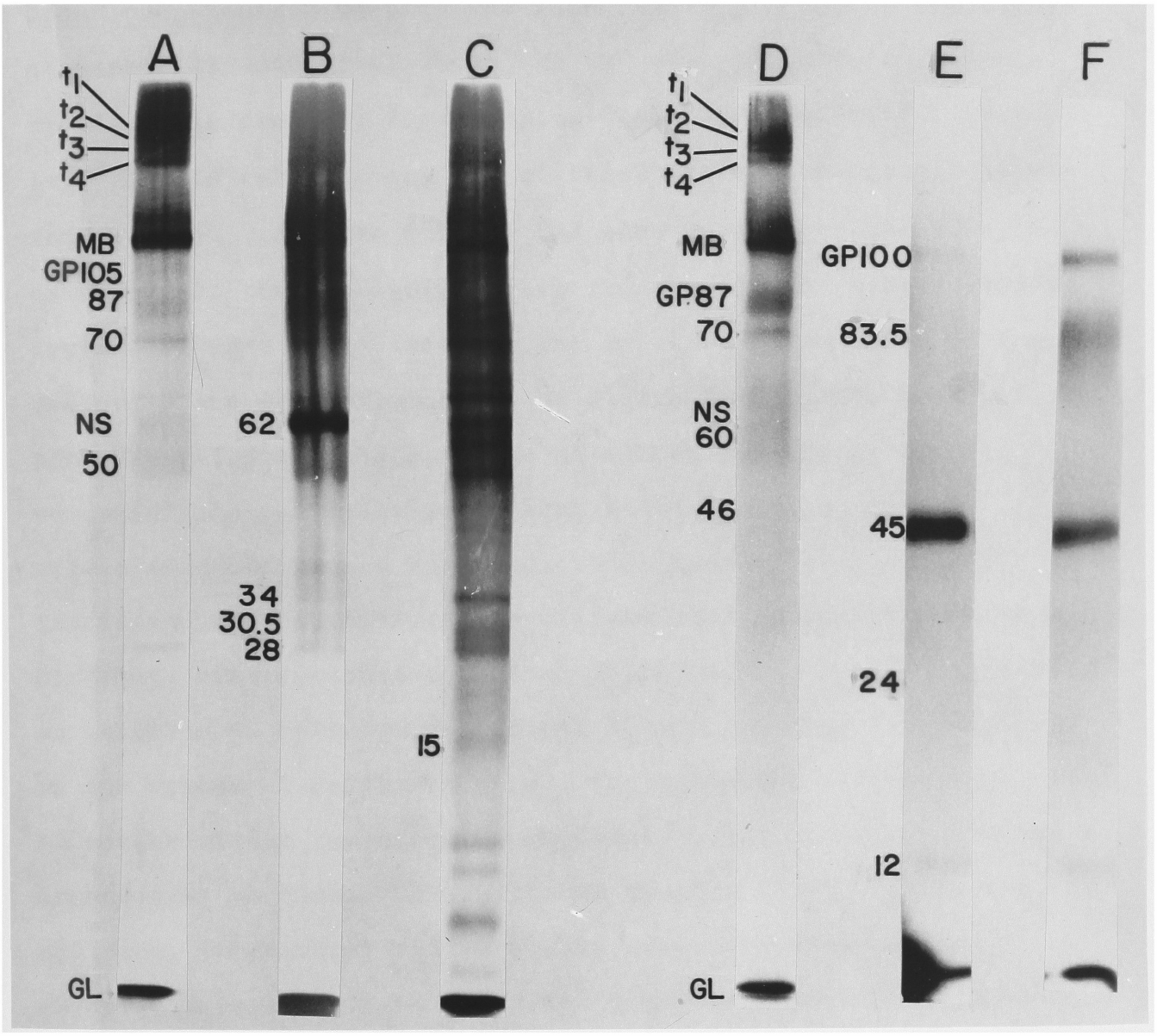



Figure 25. $^{3}$H-borohydride-labeling patterns of 2-day blast cells, 6-day secondary cells, and rat red blood cells (RBC). Preparation of cells is described in the Methods section. A: fresh, unstimulated LNC; periodate; $^{3}$H-borohydride. B: 2-day blast cells from a periodate-stimulated culture; periodate; $^{3}$H-borohydride. C: 6-day secondary cells from a periodate-stimulated culture; periodate; $^{3}$H-borohydride. D: LNC; periodate; $^{3}$H-borohydride. E: RBC; periodate; $^{3}$H-borohydride. F: RBC; NGO; $^{3}$H-borohydride. Samples A-C are from one gel; samples D-F derive from a different electrophoresis.

brane components. Despite the fact that erythrocytes constituted at most a 1% contamination in the LNC preparations (and were specifically removed, for example, from TDL populations) it was important to rule out any RBC contribution to the overall labeling patterns. Figures 25E and 25F show periodate- and NGO-oxidized RBC, respectively, after reduction with $^{3}$H-borohydride. There were some differences in the relative intensities of the various bands depending on the oxidizing agent used, and this probably reflected the presence of either periodate- or neuraminidase-resistant acetylated sialic acids on these molecules (Schauer and Faillard, 1968; Buscher et al., 1974; Sarris and Palade, 1979). The diffuse band at 83,500 m.w., the distinct, heavily-labeled band at 45,000 m.w., and the faint band at 24,000 m.w. corresponded to PAS I, II, and III, respectively, in the scheme of Fairbanks et al. for human RBC (1971). The high molecular weight radioactive component at 100,000 m.w. also had a counterpart on human RBC (Fairbanks et al., 1971). There was, in addition, substantial radioactivity incorporated into the glycolipid region of the gel. The periodic acid-Schiff (PAS) staining technique of Sarris and Palade (1979) was less sensitive than the $^{3}$H-borohydride labeling method used here, and could identify only PAS I and II on rat RBC, in addition to glycolipid. Significantly, although longer exposure of the labeled samples in the gels showed a plethora of other less intensely labeled molecules, the overall labeling patterns bore no resemblance to those obtained from LNC (Fig. 25D).

To determine which labeled cell surface molecules were

firmly embedded in the plasma membrane matrix (intrinsic), and which had comparatively loose surface attachment (extrinsic), a P fraction of NGO-oxidized, $^{3}$H-borohydride-reduced LNC was subjected to the dimethyl maleic anhydride (DMMA) extraction procedure described by Shanahan and Czech (1977)(see Methods section). The results of this extraction are shown in Figure 26. With the exception of two very weakly labeled components at 125,000 and 135,000 m.w., no other radioactive bands were found in the DMMA-extracted 'extrinsic' fraction (Fig. 26C), despite the fact that the extracted material contained over 80% of the total P fraction protein. The vast majority of the radioactive components (including the glycolipids) were found in the pelleted, 'intrinsic' fraction (Fig. 26B), suggesting that the bulk of the oxidizable surface glycoproteins were integral (and likely to be trans-membrane) components of the lymphocyte plasma membrane (Steck and Yu, 1973; Yu et al., 1973; Shanahan and Czech, 1977). The DMMA does not interact with the non-proteinaceous glycolipid, and therefore does not extract it from the plasma membrane matrix (Steck and Yu, 1973). That the $t_1$-$t_4$ tetrad is absent and the MB band appears augmented in the intrinsic fraction (Fig. 26B) is suggestive of substantial endogenous proteolytic activity during the extraction procedure (Standring et al., 1978).

Table V presents a cumulative list of the predominant, oxidizable rat lymphocyte surface components, their molecular weight, periodate- or NGO-sensitivity, and occurrence on the dif-

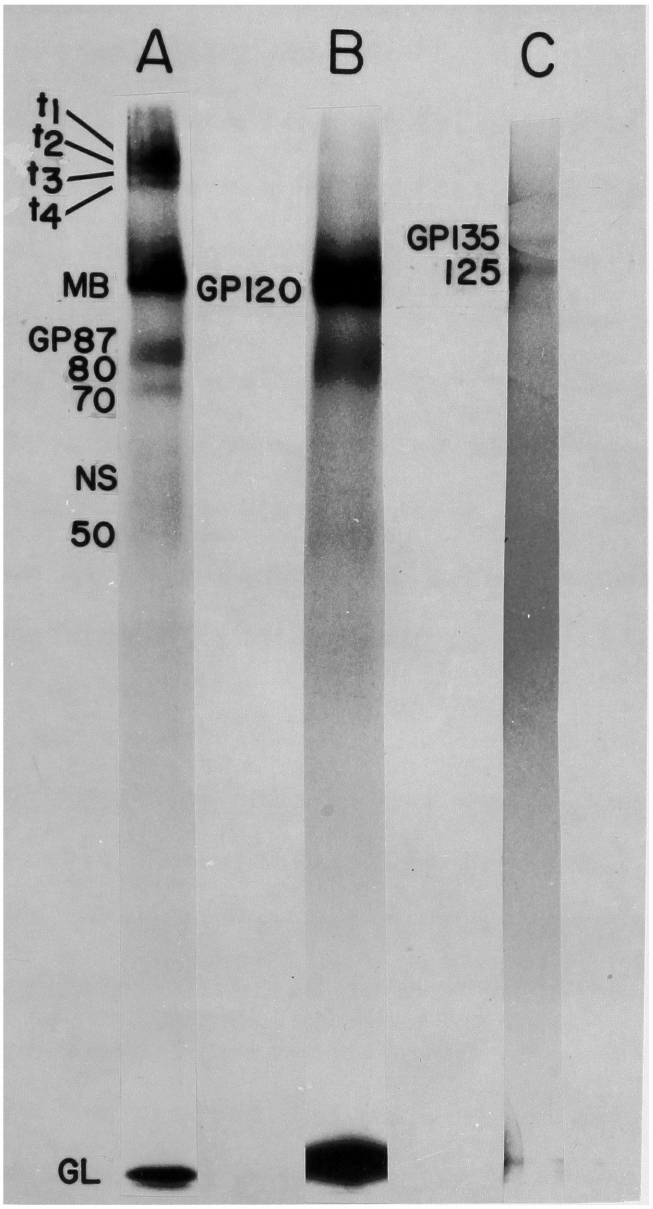



Figure 26. $^{3}$H-borohydride-labeled LNC and DMMA-extraction fractions. A: NGO; $^{3}$H-borohydride. B: NGO; $^{3}$H-borohydride; P fraction; pelleted 'intrinsic' components from DMMA extraction. C: NGO; $^{3}$H-borohydride; P fraction; extracted 'extrinsic' components from DMMA extraction.

ferent cell types examined. More than 20 reproducible bands were observed after the oxidation and $^{3}$H-borohydride reduction of LNC. Those components which were labeled following both periodate or NGO treatment, which were not appreciably labeled by galactose oxidase alone, and which occurred on periodate- or NGO-responsive cell types (e.g., LNC, TDL, cortisone-resistant thymocytes, T-lymphocytes, etc.) were candidates for involvement in oxidative mitogenesis. Obviously, the molecules meeting those criteria represented a rather large list; however, the number of viable possibilities was greatly reduced by labeling analysis following proteolytic dissection of the lymphocyte cell surface (see below).

Protease treatment of LNC: effect on mitogenesis. Figure 27 shows the proteolytic dose-inhibition curves for periodate (Panel B), NGO (Panel C), or Con A (Panel D) stimulations following mild digestion with trypsin. Similar dose-inhibition curves for the same mitogens are shown for chymotrypsin (Fig. 28), or thermolysin (Fig. 29). The results indicate that inhibition of the oxidative mitogenesis was not attributable to changes in the kinetics of proliferation. For Con A, although the peak responses were delayed approximately 12-24 hr, the magnitudes of the responses were not affected by the enzyme digestions. Thus, the inhibition of the periodate and NGO proliferations was not caused simply by toxic effects or a non-specific 'inactivation' of the cells. At no concentrations were any of the proteases found to be stimulatory for LNC by themselves (Panel A in Figs. 27-29).

TABLE V

Summary of the nomenclature, apparent m.w., periodate- and NGO-sensitivity, and cell-type association of the predominant oxidizable rat lymphocyte surface molecules

| Labeled Component | | Apparent m.w. ($\times 10^{-3}$)[a] | NGO[b] | Periodate[b] | Notes; Cell Type Association |
|---|---|---|---|---|---|
| systematic name | trivial name | | | | |
| GP190 | | 190 | + | + | B-cell component |
| GP175 | $t_1$ | 175 | + | + | T-cell component; not found on thymocytes |
| GP170 | $t_2$ | 170 | + | + | T-cell component; not found on thymocytes |
| GP160 | $t_3$ | 160 | + | + | T-cell component; not found on thymocytes |
| GP155 | $t_4$ | 155 | + | + | T-cell component; found on all T-cells |
| GP120 | $MB_{NGO}$ | 120 | + | - | T-cell component; found on all T-cells |
| GP110 | $MB_{periodate}$ | 110 | - | + | Probably most accurate m.w. for MB |
| GP105 | | 105 | + | + | Found on all lymphocyte preparations |
| GP87 | | 87 | + | + | Found on LNC, TDL, cortisone-resisant thymocytes |
| GP80 | | 80 | + | - | Found on all lymphoid cells; oxidized by GO alone |
| GP70 | | 70 | + | + | Found on LNC, TDL, cortisone-resistant thymocytes |
| | NS | 62 | - | - | Oxidized in natural state; elevated on 2-day blasts |
| GP60 | | 60 | + | + | Found on all lymphocyte preparations |
| GP55 | | 55 | + | + | Found on all lymphocyte preparations |
| GP50 | | 50 | + | + | Found on all lymphocyte preparations; 'extrinsic'[c] |
| GP46 | | 46 | + | + | Found on all lymphoid cells; high for cortisone-resistant thymocytes; possibly Ag-B |
| GP37 | | 37 | + | + | Found on LNC, B-cell preparations; possibly Ia |
| GP34 | | 34 | - | + | Oxidized by periodate only |
| GP30.5 | | 30.5 | - | + | Oxidized by periodate only; found on LNC, B-cell preparations; possibly Ia |
| GP28 | | 28 | - | + | Oxidized by periodate only |
| GP27 | Thy-1 | 27 | + | + | Probably rat Thy-1; elevated on thymocytes |
| GP15 | | 15 | - | + | Oxidized by periodate only |
| GP14 | | 14 | + | + | Found on all lymphocyte preparations |
| GP12 | | 12 | + | + | Found on all lymphocyte preparations; 'extrinsic' |
| | GL | 10 | + | +/- | Glycolipid |

[a] The molecular weight of glycoproteins as determined by SDS-polyacrylamide gel electrophoresis is often inaccurate (Segrest et al., 1971; Segrest and Jackson, 1972). Thus, the molecular weight of these components are technically only apparent. The values shown represent the averages of at least 15 independent determinations, and in most cases, greater than 25. The standard deviations are less than 10% in all cases, and generally less than 5%.

[b] + = labeled after treatment with th indicated agent; - = not labeled; +/- = variable labeling.

[c] 'extrinsic' refers to the observation that these components were found in soluble fractions of differential and isopycnic centrifugations.

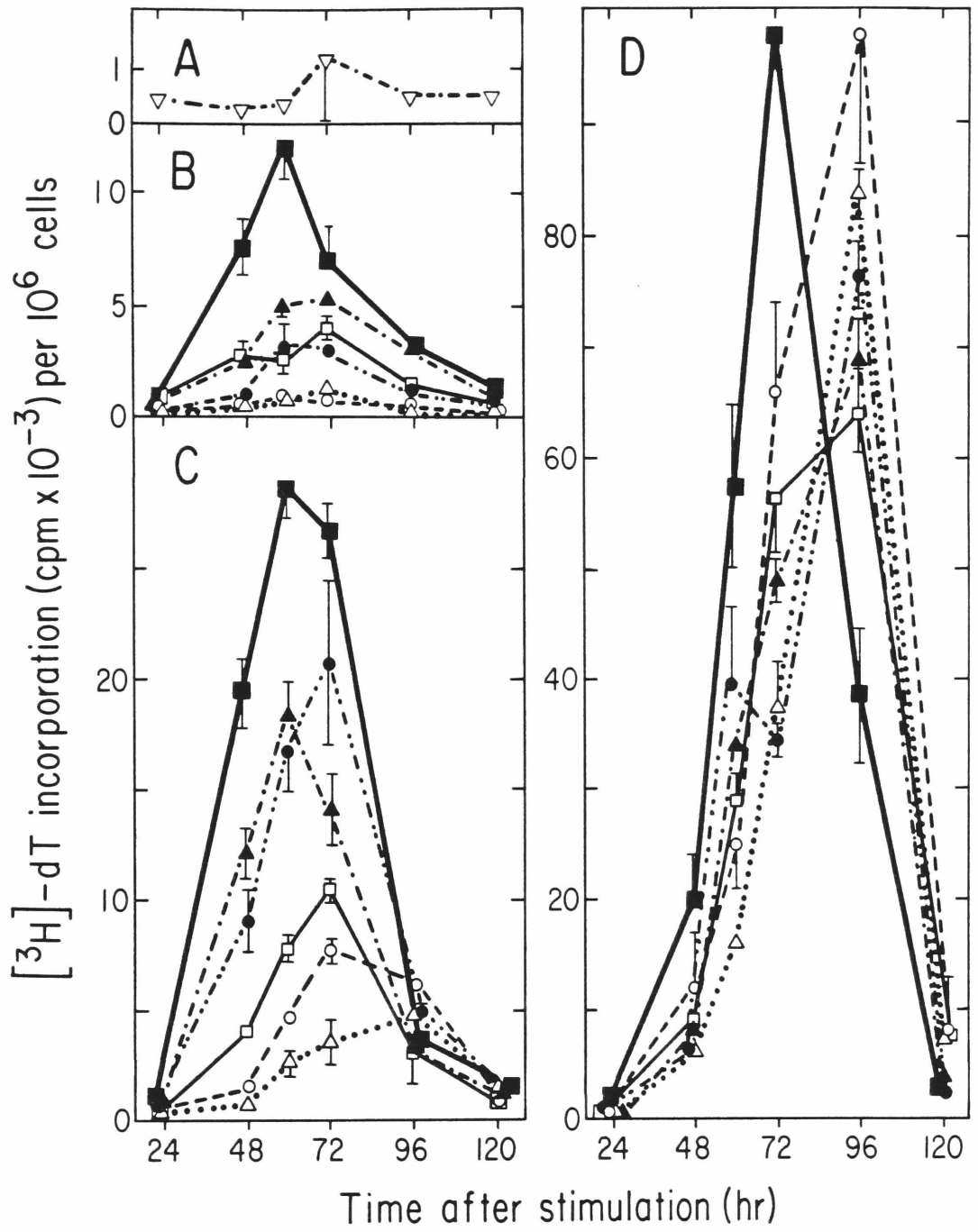



Figure 27. Dose-response curves for the effect of trypsin on mitogenic stimulation. LNC were incubated in various concentrations of trypsin as described in the Methods section. They were then either cultured without further treatment (Panel A), or after treatment with mitogenic levels of periodate (Panel B), NGO (Panel C), or Con A (Panel D). Control, no trypsin, ■ and heavy line; 33.7 NFU/ml, ● ; 67.5 NFU/ml, ▲ ; 135 NFU/ml, □ ; 270 NFU/ml, ○ ; 540 NFU/ml, △ . The response in the absence of any mitogens (Panel A, ▽ ) represents the greatest incorporation among all the trypsin concentrations used.

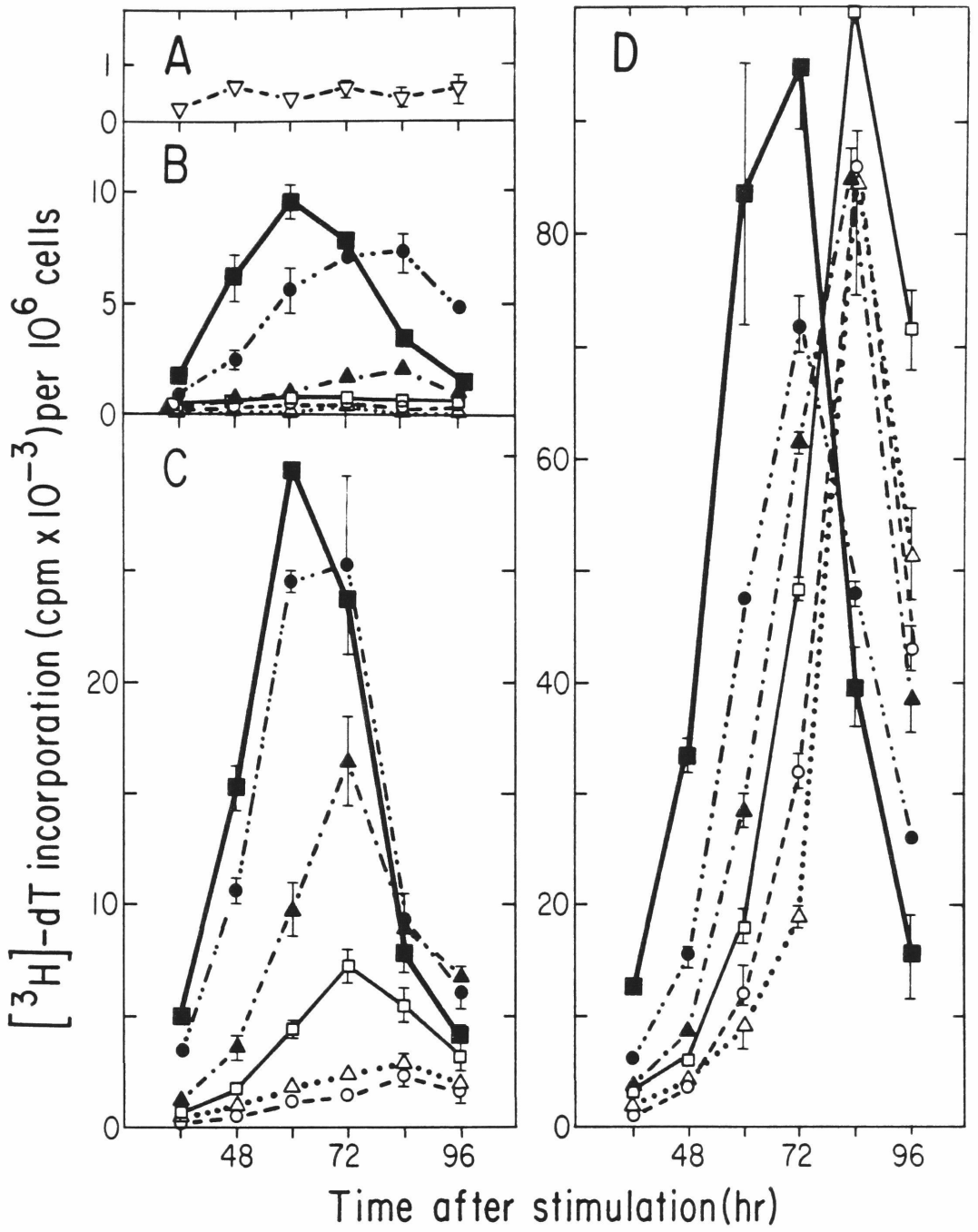



Figure 28. Dose-response curves for the effect of chymotrypsin on mitogenic stimulation. Panels A-D represent the same treatments as described in Figure 27. Control, no chymotrypsin, ■ and heavy line; 13 units/ml, ● ; 26 units/ml, ▲ ; 52 units/ml, □ ; 104 units/ml, ○ ; 208 units/ml, △ . The response in the absence of any mitogens (Panel A, ▽ ) represents the greatest incorporation among all the chymotrypsin concentrations used.

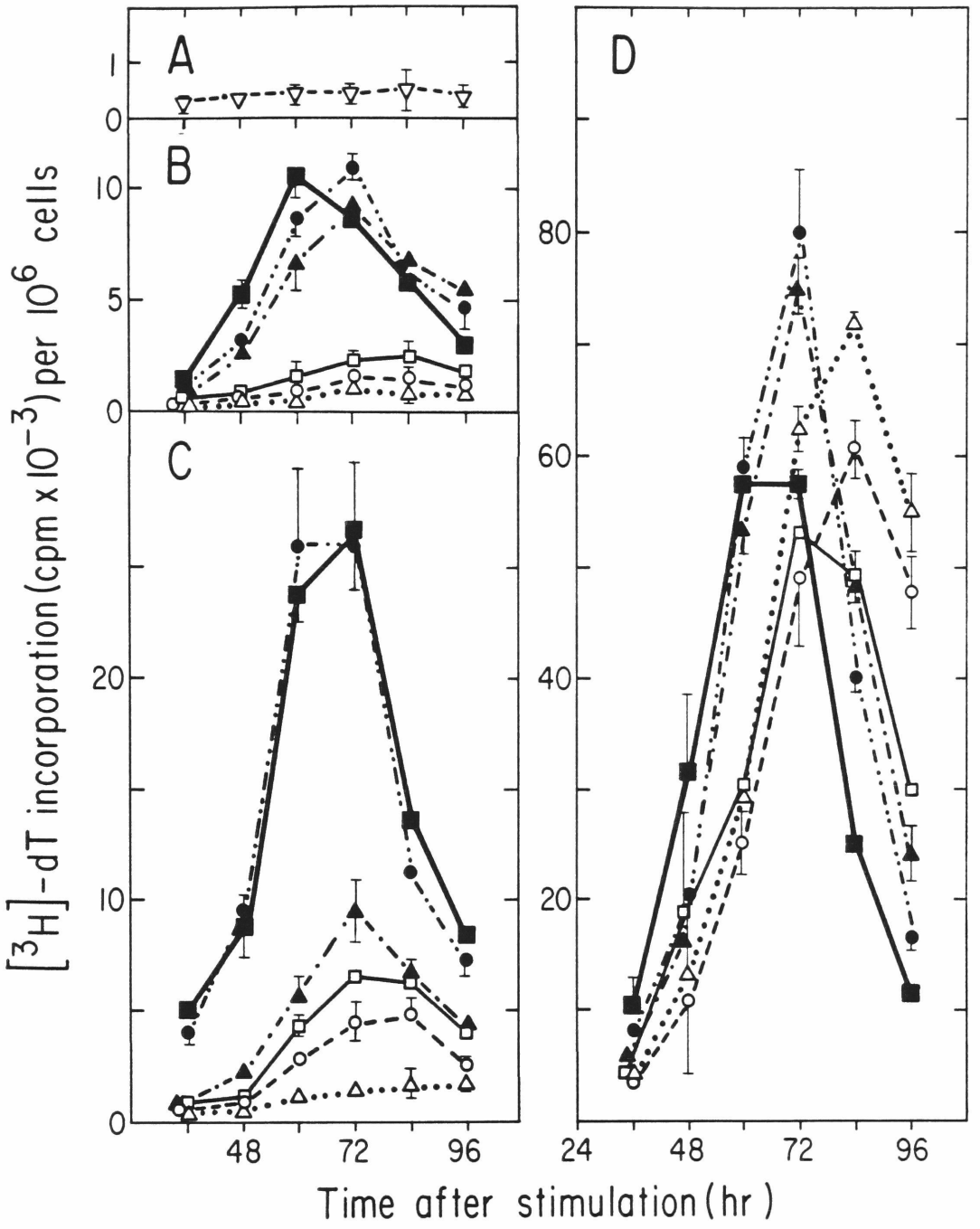



Figure 29. Dose-response curves for the effect of thermolysin on mitogenic stimulation. Panels A-D represent the same treatments as described in Figure 27. Control, no thermolysin, ■ and heavy line; 550 P.U./ml, ● ; 1100 P.U./ml, ▲ ; 2200 P.U./ml, □ ; 4405 P.U./ml, ○ ; 8810 P.U./ml, △ . The response in the absence of any mitogens (Panel A, ▽ ) represents the greatest incorporation among all the thermolysin concentrations used.

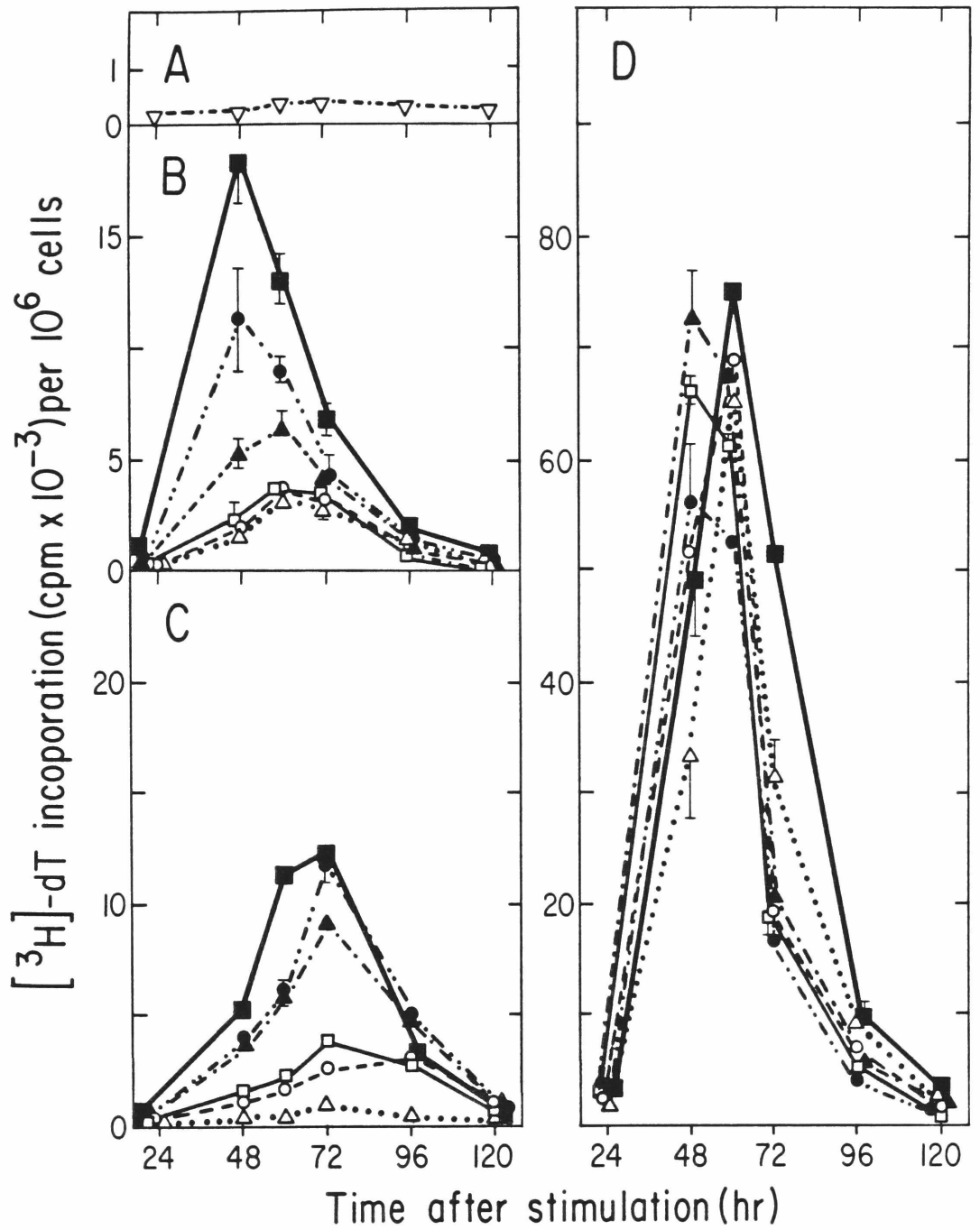



Figure 30. Dose-response curves for the effect of papain on mitogenic stimulation. Panels A-D represent the same treatments as described in Figure 27. Control, no papain, ■ and heavy line; 1.25 units/ml, ● ; 2.5 units/ml, ▲ ; 5 units/ml, □ ; 10 units/ml, ○ ; 20 units/ml, △ . The response in the absence of any mitogens (Panel A, ▽ ) represents the greatest incorporation among all the papain concentrations used.

The curves shown in Figures 27-29 should be compared to those for LNC treated with various concentrations of papain (Fig. 30). Responses to periodate and NGO were inhibited by 75-95% at enzyme concentrations (up to 10 units/ml) that did not significantly alter any aspect of the Con A stimulation. If anything, low doses of papain tended to slightly accelerate the Con A blastogenesis. Only at higher concentrations of papain (20 units/ml and more), was the Con A response somewhat delayed; again, there was no reduction in the magnitude of the mitogenesis. No concentration of papain was found to be stimulatory for LNC by itself.

To exclude the possibility that papain was non-specifically sticking to the cell surface and was thereby interfering with oxidation by periodate or NGO, LNC were subjected to the reverse order of treatments (i.e., periodate or NGO followed by papain) (results not shown). The periodate and NGO responses were inhibited to an equal extent regardless of whether the papain digestion preceded or followed the oxidation treatments. Further, incubation in the presence of a non-proteolytic protein like bovine plasma albumin, either before or after oxidation, had no effect on the oxidative mitogenesis (results not shown). Finally, incubation of LNC with heat-inactivated papain before NGO or periodate oxidation was not inhibitory (Fig. 31). Taken together, these results suggest that the inhibition of oxidative mitogenesis by papain was due to the proteolytic activity of the enzyme.

Unless noted otherwise, for the remainder of the experiments described here, the following concentrations of the respective enzymes were used: papain, 5 units/ml; trypsin, 270-540 NFU/ml; chymotrypsin, 78-156 units/ml; thermolysin, 4405-8810 P.U./ml. These levels consistently led to 90% or greater inhibition of peak oxidative mitogenesis without diminishing the Con A response (Figs. 27-30).

Viability of protease-treated LNC. The initial viabilities of cultured LNC always exceeded 90% and were unaffected by previous protease treatment, oxidation, or both (see Methods section). The following experiment was performed to examine the long-term viability of protease-treated cells. After treatment with the various proteases, unstimulated LNC were cultured for a 5-day period. At 24-hr intervals, viabilities of the cells were determined by trypan blue exclusion, and by Coulter counter enumeration. The results of this experiment (Fig. 32) demonstrated that protease concentrations which were inhibitory for periodate- or NGO-induced mitogenesis did not affect the LNC viability during culturing, and indicated therefore that the protease inhibition was not attributable simply to cell death. In addition, incubation of LNC with papain followed by periodate or NGO did not affect the magnitude or delay the kinetics of a subsequent Con A stimulation (results not shown), indicating that the combined protease and oxidative treatments also did not affect viability or responsiveness.

Protease treatment of responder LNC: effect on mitogenesis.

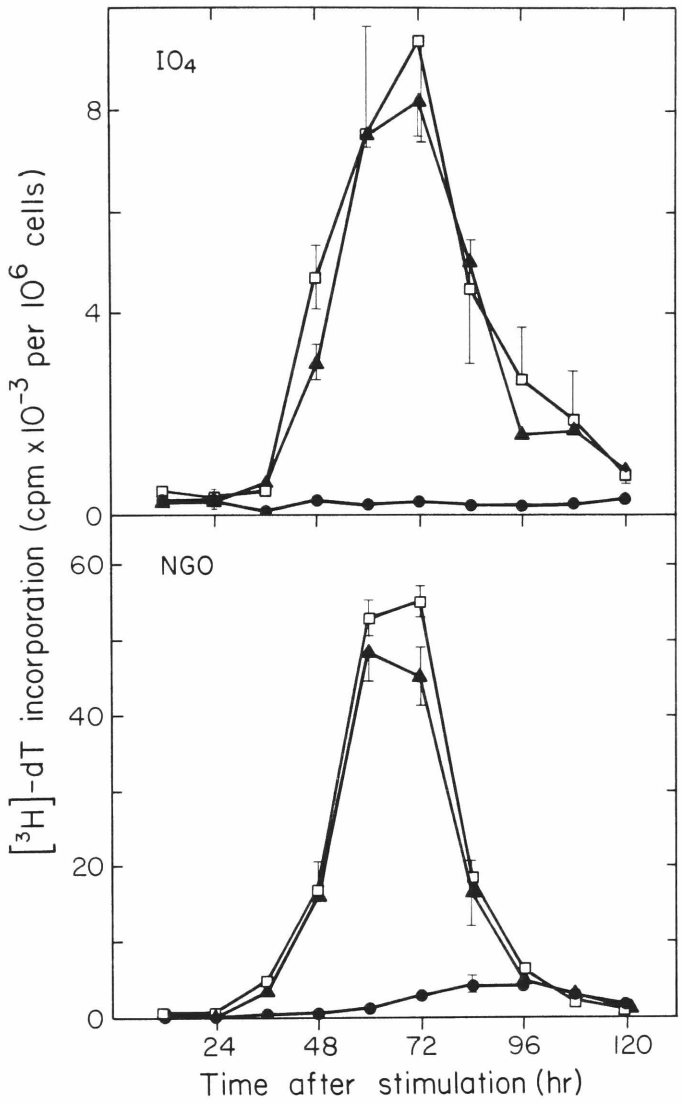



Figure 31. Inhibition of periodate and NGO mitogenesis by papain: heat-inactivated papain control. LNC were incubated for 30 min at 37°C with 5 units/ml activated papain ( ● ), an equivalent amount of heat-inactivated papain ( □ ), or with the dithiothreitol-EDTA buffer used to activate the enzyme ( ▲ ). After washing, the cells were treated with periodate ($IO_4$, upper panel), or NGO (lower panel) and subsequently cultured.

As discussed above, it has been demonstrated that periodate- or NGO-treated lymphocytes ('responders') undergo blast transformation only in the presence of another type of cell ('accessory') that need not be oxidized, and that occurs as a very small percentage of LNC populations (Beyer and Bowers, 1977b; Bowers and Beyer, 1979). Thus, proteolytic digestion of whole LNC populations could conceivably inhibit oxidative mitogenesis by removing essential molecules from either the responding lymphocytes or from the non-responding accessory cells.

To determine whether there was an effect on the responding lymphocyte or on the accessory cell in causing the protease inhibition of oxidative mitogenesis, the following experiment was performed. LNC were fractionated by rate-zonal centrifugation (Bowers, 1973), and, as described in the Methods section, appropriate fractions were combined to yield populations of responder and accessory cells. The responder populations were then treated with papain, trypsin, chymotrypsin, thermolysin, or in HBSS alone (control), oxidized with periodate, and mixed with an equal number of non-oxidized accessory cells (either $\gamma$-irradiated or untreated). For comparison, unfractionated LNC were also treated with the various proteases, oxidized with periodate, and cultured. As shown in Figure 33, the inhibition of oxidative mitogenesis by any of the four proteases could be attributed almost entirely to an effect on the responder cell population. The Con A response for mixtures of protease-treated responders and $\gamma$-irradiated accessories was the same as for protease-treated unfractionated cells (results not shown),

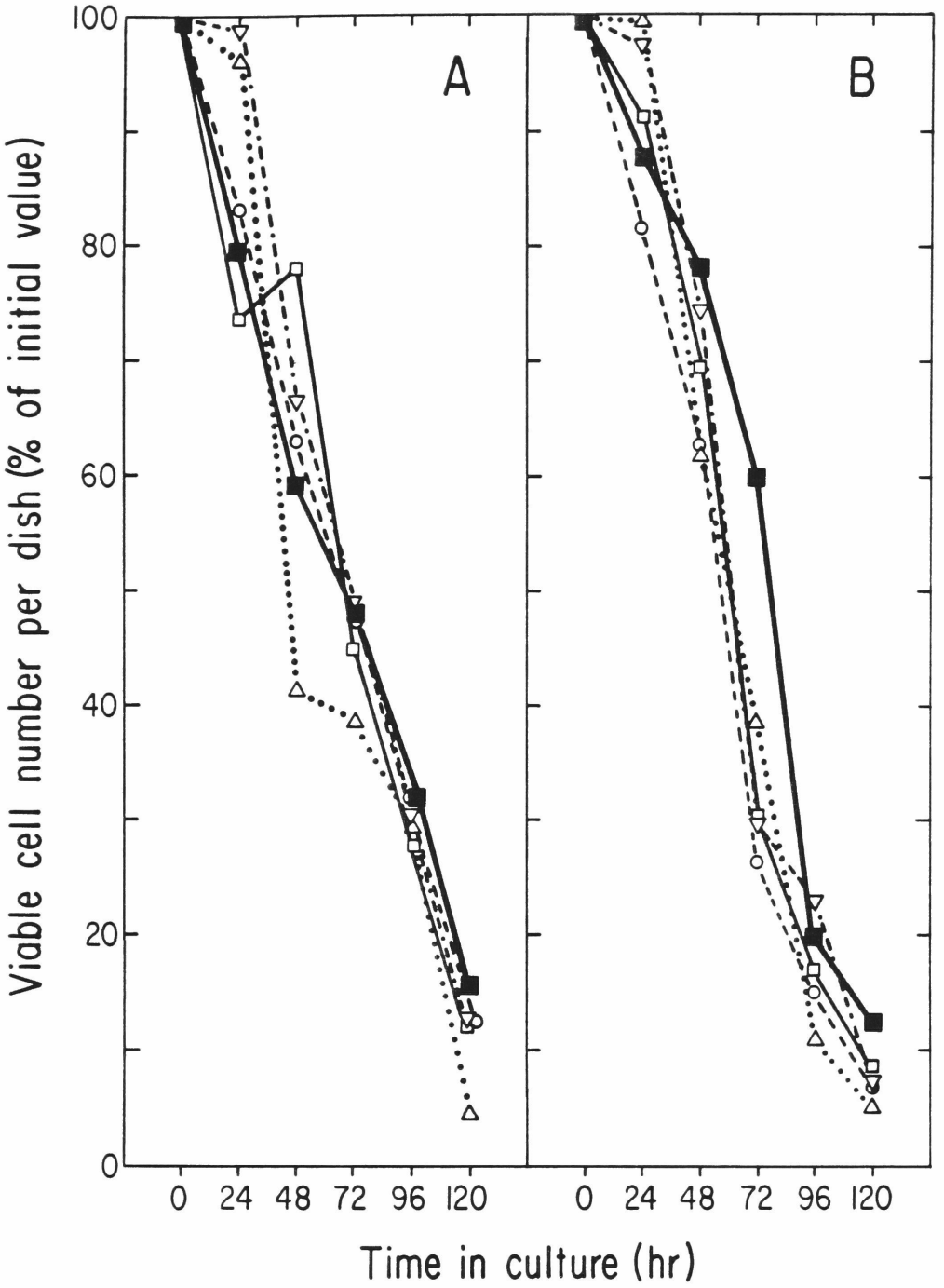



Figure 32. Viability of protease-digested LNC in culture. LNC were treated with 540 NFU/ml trypsin ( □ ), 156 units/ml chymotrypsin ( ▽ ), 8810 P.U./ml thermolysin ( ○ ), 5 units/ml papain ( △ ) or HBSS alone (control, ■ , and heavy line). At various intervals after culturing, the viabilities of the cells were determined by trypan blue exclusion (Panel A), and by Coulter Counter analysis (Panel B) (see Methods section).

indicating that the fractionation and digestion of responder lymphocytes did not kill or 'inactivate' these cells. Finally, mixtures of equal numbers of protease-treated accessory cells and periodate-oxidized responder lymphocytes showed proliferative responses that averaged 60% of the stimulations in combinations where neither cell type was protease-treated (measured at the peak of the responses; range from 30%-96%, n=6). In all cases, mixing protease-treated, oxidized responders with the protease-treated accessory cells showed a further substantial reduction of the proliferation (peak responses averaging 14% of the responses seen with undigested mixtures; range from 6.5%-30%, n=6). Overall, the results indicated that most of the inhibition resulting from protease treatment was due to the specific cleavage of molecules from responder lymphocytes.

Protease treatment of LNC: effect on $^{3}$H-borohydride labeling. Viable, unfractionated LNC were reduced with $^{3}$H-borohydride after oxidation with periodate (Fig. 34A) or NGO (Fig. 34H), and the distributions of the labeled molecules in SDS-polyacrylamide gel slabs were analyzed by autoradiography. As described above, the labeling patterns from NGO- or periodate-treated LNC were similar (e.g., $t_1$-$t_4$, GP87, GP70, etc.).

Treatment of unfractionated LNC with inhibitory concentrations of any of the four proteases, followed by periodate or NGO oxidation, and reduction with $^{3}$H-borohydride, led to the generation of radioactive banding patterns different from undigested controls, and unique for each of the four enzymes (compare Figs.

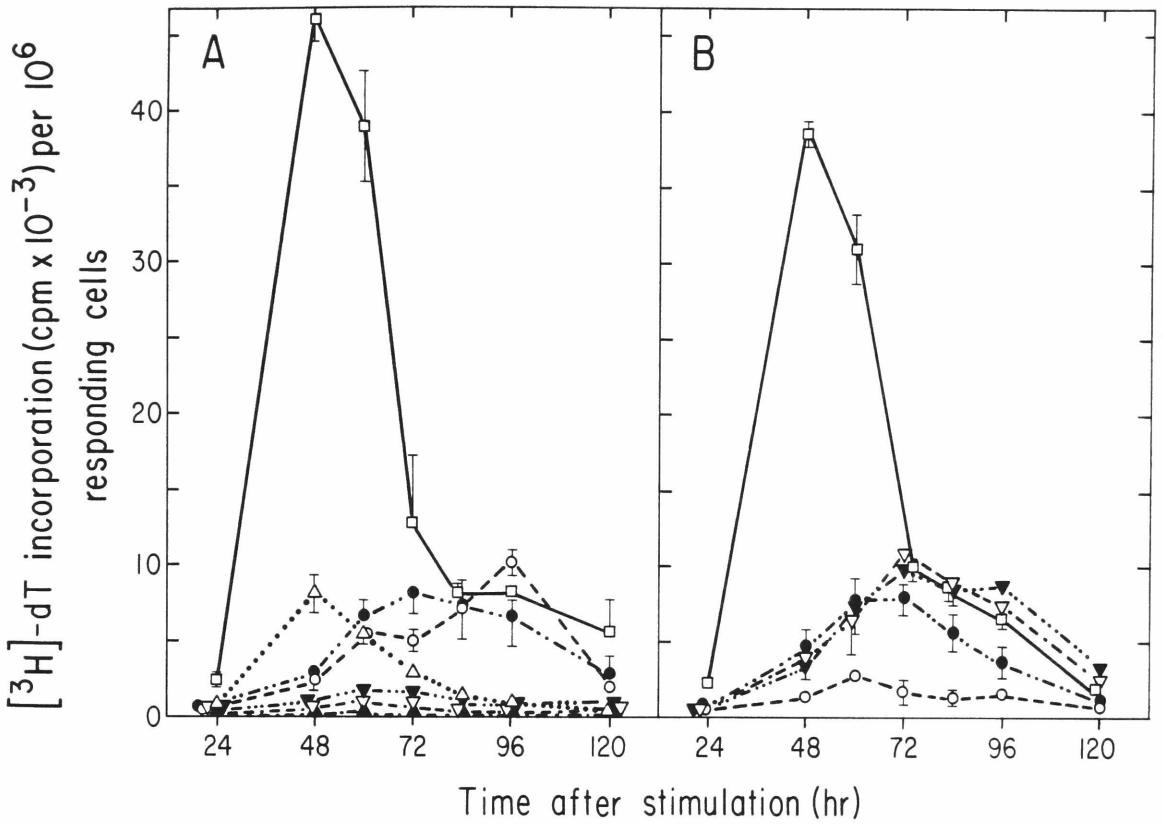



Figure 33. Inhibition of periodate mitogenesis by protease treatment of responder LNC (Panel A). LNC were separated by size using rate-zonal centrifugation, and the appropriate fractions were combined to give responder and accessory populations (see Methods section). Responder lymphocytes were treated with 540 NFU/ml trypsin ( ● ), 156 units/ml chymotrypsin ( ▼ ), 8810 P.U./ml thermolysin ( ▽ ), or 5 units/ml papain (○). Control responders were incubated in HBSS alone ( □ , △ ). The variously-treated responder cells were then washed and oxidized with periodate. Fractions enriched in accessory cells were irradiated (1000 rads) and mixed 1:1 with responders to a final cell density of 2.5 x $10^6$ cells/ml. Accessory cells plus oxidized, undigested responders, □ ; oxidized, undigested responders alone, △ ; accessory cells alone, ▲ .

For comparison, unfractionated LNC (Panel B) were subjected to treatment with the same concentrations of proteases, oxidized with periodate, and cultured. Trypsin, ● ; chymotrypsin, ▼ ; thermolysin, ▽ ; papain, ○ ; HBSS control, □ . It is important to note that the $^3$H-dT incorporation (ordinate) has been normalized relative to $10^6$ responding lymphocytes. This is to take into account that half the cells in the mixtures were irradiated and unable to respond.

34A-34E or Fig. 34F and Fig. 34H). Despite the differences in the amino acid specificities of the four proteases (see Materials section; White et al., 1978b), a number of common features of the resulting radioactive banding patterns did emerge. Consistently, the MB glycoprotein was either absent (papain and chymotrypsin) or present at reduced labeling intensity (trypsin and thermolysin). The $t_1$-$t_4$ bands disappeared and most likely were digested to smaller membrane-bound fragments. No other labeled glycoproteins were digested by every one of the enzymes. As expected, the glycolipid (GL) band was unaffected by proteolysis. The 'new' bands that appeared after proteolytic digestion of LNC probably originated from larger glycoproteins in undigested cells. Specifically, the radioactive bands of 150,000 m.w. from papain-treated cells, and of 140,000 m.w. from cells treated with trypsin, chymotrypsin, and thermolysin most likely represented the protease-resistant parts of one or all of the $t_1$-$t_4$ glycoproteins. It is important to note that concentrations of papain that were not inhibitory for oxidative mitogenesis (1 unit/ml) removed the MB band and GP70 completely (as did inhibitory levels of papain), but tended to leave the $t_1$-$t_4$ bands relatively intact (compare Figs. 34G and 37H).

Reversing the procedure, i.e., oxidizing and reducing with $^3$H-borohydride before protease treatment, gave rise to the same pattern of radioactive banding (results not shown). In addition, supernatants recovered from papain digests of labeled cells contained low molecular weight glycopeptides (approximately 10,000 m.w.) consistent with a derivation from the papain-sensitive

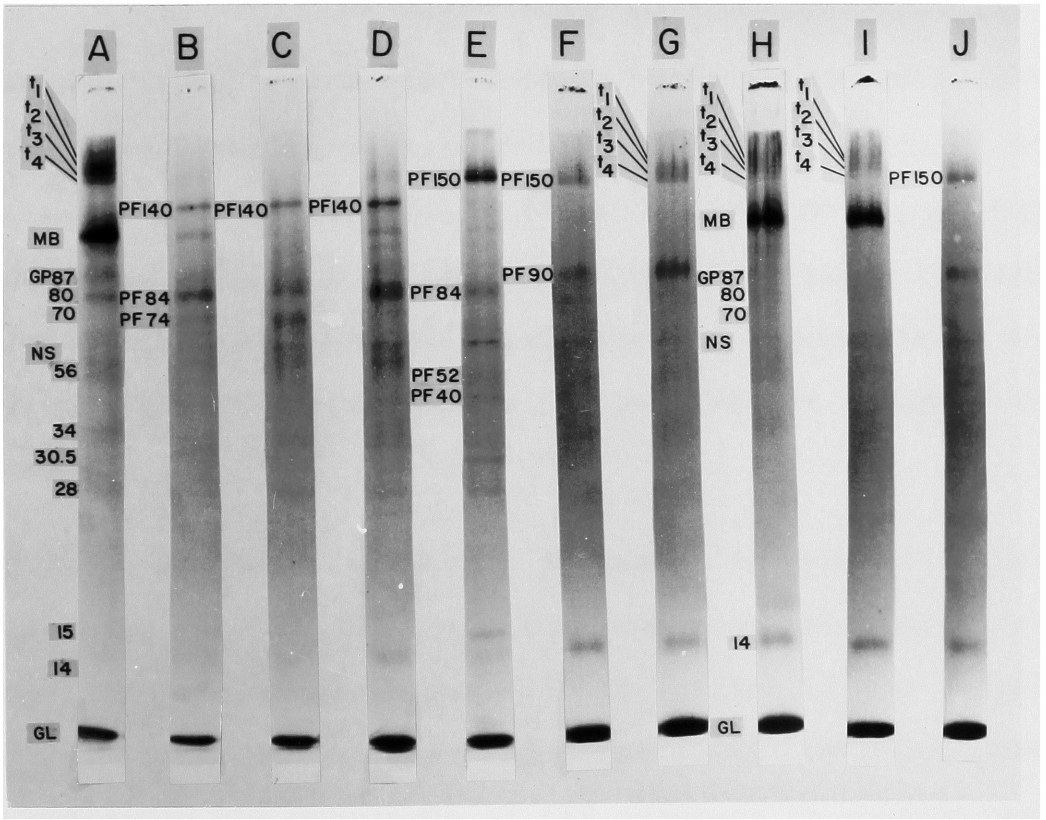



Figure 34. SDS-polyacrylamide slab gel autoradiographic patterns of $^{3}$H-borohydride-labeled LNC. Cell treatments are listed sequentially and in all cases were terminated by $^{3}$H-borohydride reduction and solubilization in SDS (see Methods section). A: periodate. B: 260 NFU/ml trypsin; periodate. C: 78 units/ml chymotrypsin; periodate. D: 4405 P.U./ml thermolysin; periodate. E: 5 units/ml papain; periodate. F: 5 units/ml papain; NGO. G: 1 unit/ml papain; NGO. H: NGO. I: Ficoll-gradient fractionated responder lymphocytes; NGO. J: Ficoll-gradient fractionated responder lymphocytes; 5 units/ml papain; NGO. PF150: glycoprotein of 150,000 apparent molecular weight which occurs after protease treatment, etc. (see Table VI for a complete list).

bands (results not shown). Heat-inactivated papain had no effect on the labeling patterns (results not shown).

Responder lymphocytes fractionated by velocity sedimentation showed the same labeling pattern following NGO oxidation as did unfractionated LNC (compare Figs. 34H and 34I). Moreover, papain treatment (5 units/ml) of these responder cells caused the loss and/or fragmentation of the same cell surface glycoproteins visualized from papain-digested, whole LNC populations (compare Figs. 34F and 34J).

A summary of these results is presented in Table VI. Taken as a whole, the results indicated that the proteases specifically cleaved pertinent glycoproteins from the surface of the responder lymphocytes. Since the enzymes abrogated oxidative mitogenesis without affecting the magnitude of the response to another mitogenic stimulus (Con A), the implication was that the protease-sensitive sialoglycoproteins mediated NGO- and periodate-induced blast transformation. Specifically implicated from the results were the $t_1$-$t_4$ sialoglycoproteins.

The lymphocyte distribution and molecular weight characteristics of the $t_1$-$t_4$ tetrad were very similar to those observed for the diffuse 170,000 m.w. T-cell LC (leucocyte common)-antigen described by Standring *et al.* (1978) and Sunderland *et al.* (1979), and suggested that some of the $t_1$-$t_4$ glycoproteins might represent the same surface molecules. In addition, the work of Wynne *et al.* (1976) and Ravid *et al.* (1978) had indicated that cross-linking the specifically-oxidizable surface molecules was

stimulatory for lymphocytes. Thus, it was conceivable that an antisera directed against any, or all of the $t_1$-$t_4$ components would be directly mitogenic for LNC. To examine this possibility, the monoclonal antiserum MRC OX1, specific for the LC-antigen, was obtained from Dr. A.F. Williams.

Monoclonal antibody (MRC OX1); immunoprecipitation of components from $^3$H-borohydride-labeled LNC. Periodate-oxidized, $^3$H-borohydride-reduced, B-depleted LNC were immunoprecipitated as described in the Methods section, and the precipitates and supernatants were examined by SDS-polyacrylamide gel electrophoresis. The autoradiographic banding patterns are shown in Figures 35B and 35C, respectively. B-depleted LNC, electrophoresced on the same gel are shown for comparison in Figure 35A. Also shown is the banding pattern of precipitates obtained when MRC OX1 was left out of the immunoprecipitation procedure. MRC OX1 alone resulted in no precipitable material. It was clear that MRC OX1 plus anti-mouse Ig (in this case, $F(ab')_2$ fragments, although intact antibody was also satisfactory) specifically immunoprecipitated all four components of the $t_1$-$t_4$ tetrad (Fig. 35B); the residual $t_1$-$t_4$ left in the supernatants (Fig. 35C) was probably due to sub-optimal levels of the MRC OX1 first antibody. Similar results were obtained if non-depleted total LNC were immunoprecipitated by the same procedure, although additional diffuse high m.w. radioactivity above $t_1$-$t_4$ was also recovered in the precipitate (results not shown). Preliminary results with W3/13 HLK antibody, which is specific for a 100,000 m.w. T-lymphocyte component (Williams et al., 1977; White et al., 1978a), have thus

TABLE VI

Summary of the Protease Sensitivities and Fragments following Periodate- or NGO-oxidation $^{3}$H-borohydride-labeled Components from LNC following Treatment with Inhibitory Doses Trypsin, Chymotrypsin, Thermolysin, or Papain

| Labeled Component | | Apparent | | | | | |
|---|---|---|---|---|---|---|---|
| systematic name | trivial name | m.w. (x $10^{-3}$) | Trypsin[a] (270 units/ml) | Chymotrypsin[a] (78 units/ml) | Thermolysin[a] (4405 units/ml) | Papain[a] (5 units/ml) | Notes |
| GP190 | | 190 | - | - | - | - | |
| GP175 | $t_1$ | 175 | - | - | - | - | |
| GP170 | $t_2$ | 170 | - | - | - | - | |
| GP160 | $t_3$ | 160 | - | - | - | - | |
| GP155 | $t_4$ | 155 | - | - | - | - | |
| PF150[b] | | 150 | - | - | - | + | |
| PF140 | | 140 | + | + | + | - | |
| GP120 | $MB_{NGO}$ | 120 | - | - | - | - | Absent at 1 unit/ml papain |
| GP110 | $MB_{periodate}$ | 110 | - | - | - | - | |
| GP105 | | 105 | - | + | + | + | |
| PF100 | | 100 | - | - | + | - | |
| PF90 | | 90 | + | + | + | - | |
| GP87 | | 87 | - | - | + | + | |
| PF84 | | 84 | - | - | - | + | |
| PF80 | | 80 | + | - | + | + | |
| PF75 | | 75 | + | + | + | - | |
| GP70 | | 70 | + | + | - | - | Absent at 1 unit/ml papain |
| | NS | 62 | + | + | + | + | |
| GP60 | | 60 | + | + | + | + | |
| GP55 | | 55 | + | - | + | + | |
| PF52 | | 52 | - | - | - | + | |
| GP50 | | 50 | + | - | - | + | |
| PF49 | | 49 | - | - | + | + | |
| GP46 | | 46 | - | - | + | + | |
| PF40 | | 40 | + | - | + | - | |
| GP37 | | 37 | - | - | - | + | |
| PF36 | | 36 | + | + | + | + | |
| GP34 | | 34 | + | + | + | - | |
| GP30.5 | | 30.5 | + | - | - | - | |
| GP28 | | 28 | + | + | + | + | |
| GP15 | | 15 | + | + | + | - | |
| PF14 | | 14 | - | - | - | + | |
| GP12 | | 12 | + | + | + | + | |
| | GL | 10 | + | + | + | + | |

[a] + = present after protease treatment; - = absent after protease treatment.

[b] PF designates "protease fragment"; '+' indicates that treatment with that enzyme generates that size labeled fragment; '-' indicates that no fragment appeared after protease digestion.

Molecular weights of fragments in this Table are averaged from at least 5 different determinations and more commonly 8-10. The standard deviations were generally less than 5%.

far been equivocal; the MB glycoprotein was immunoprecipitated, but the $t_1$-$t_4$ tetrad and some other lower molecular weight components were also brought down to a small extent.

Monoclonal antibody (MRC OX1); induction of mitogenesis. B-depleted LNC coated with MRC OX1 (15 μg/ml was optimal) were stimulated to undergo division if cultured in the presence of anti-mouse Ig (intact Ig or $F(ab')_2$ fragments)(Fig. 36); generally, a 10-fold excess by weight of second antibody was found to be optimal. MRC OX1 treatment alone, or anti-mouse Ig treatment alone was not mitogenic for B-depleted LNC (bottom 2 curves of MRC OX1 panel in Fig. 36). However, in non-depleted total LNC populations, the response of cells in the presence of anti-mouse Ig alone was as much as one-half the stimulation seen with both antibodies. This response was probably due to an anti-Ig-induced B-cell proliferation (Weiner et al., 1978) since mouse and rat Ig are known to be antigenically cross-reactive. In the non-depleted LNC population, MRC OX1 alone was still without effect (results not shown). The response to MRC OX1 plus anti-Ig had kinetics identical to those obtained for a periodate response (first panel in Fig. 36); however, the magnitude of the stimulation was approximately 10-fold less than that observed for periodate-treated B-depleted LNC (Fig. 36). Various approaches including: 1) neuraminidase treatment of the cells (15 units/ml for 30 min, 37°C), 2) addition of non-mitogenic doses of a lympho-agglutinating lectin (10 μg/ml of wheat germ agglutinin in the final culture medium), 3) use of anti-Ig-aggregated or heat-aggregated MRC OX1 (heat aggregation after the method of Gamble,

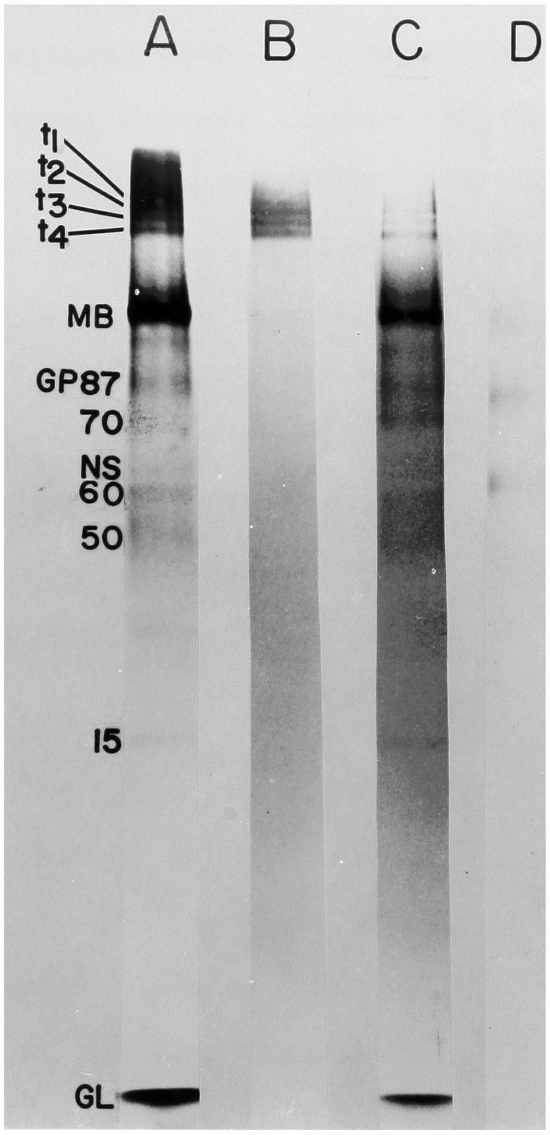



Figure 35. Immunoprecipitation with MRC OX1 and goat anti-mouse $F(ab')_2$ fragments of periodate-oxidized and $^3H$-borohydride-reduced B-depleted LNC. The immunoprecipitation was carried out as described in the Methods section and in Figure 10. 50 µg/ml MRC OX1 and 175 µg/ml anti-Ig $F(ab')_2$ fragments were used. A: B-depleted LNC; periodate; $^3H$-borohydride. B: immunoprecipitate. C: supernatant from immunoprecipitate. D: immunoprecipitate obtained when MRC OX1 was excluded from the immunoprecipitation procedure.

1966; 30 min at 65°C, followed by chilling), or 4) supplementation of the final cultures with 3% accessory cells (purified from spleen by BPA flotation) did not improve the MRC OX1 response. No concentration of Protein A was found to be stimulatory for MRC OX1-coated LNC. MRC OX1-coated cells were not killed or subsequently inactivated in culture since the response of these cells to Con A was normal in magnitude and even slightly accelerated relative to cells treated with Con A alone (middle panel, Fig. 36). Moreover, periodate-oxidized, MRC OX1-coated LNC showed a 1.5-fold enhanced response compared to LNC which were treated with periodate alone (results not shown).

Preliminary results obtained with W3/13 HLK antibody (range of 10-100 µg/ml) indicated a small response (less than 20% of that seen with MRC OX1) at optimal concentrations of first and second antibodies (25 µg/ml and 250 µg/ml, respectively). It is not as yet clear whether this was due to the apparent cross-reactivity of the antiserum with $t_1$-$t_4$, or was attributable to a non-specific stimulation of the LNC (e.g., by immune complexes or by general surface cross-linking).

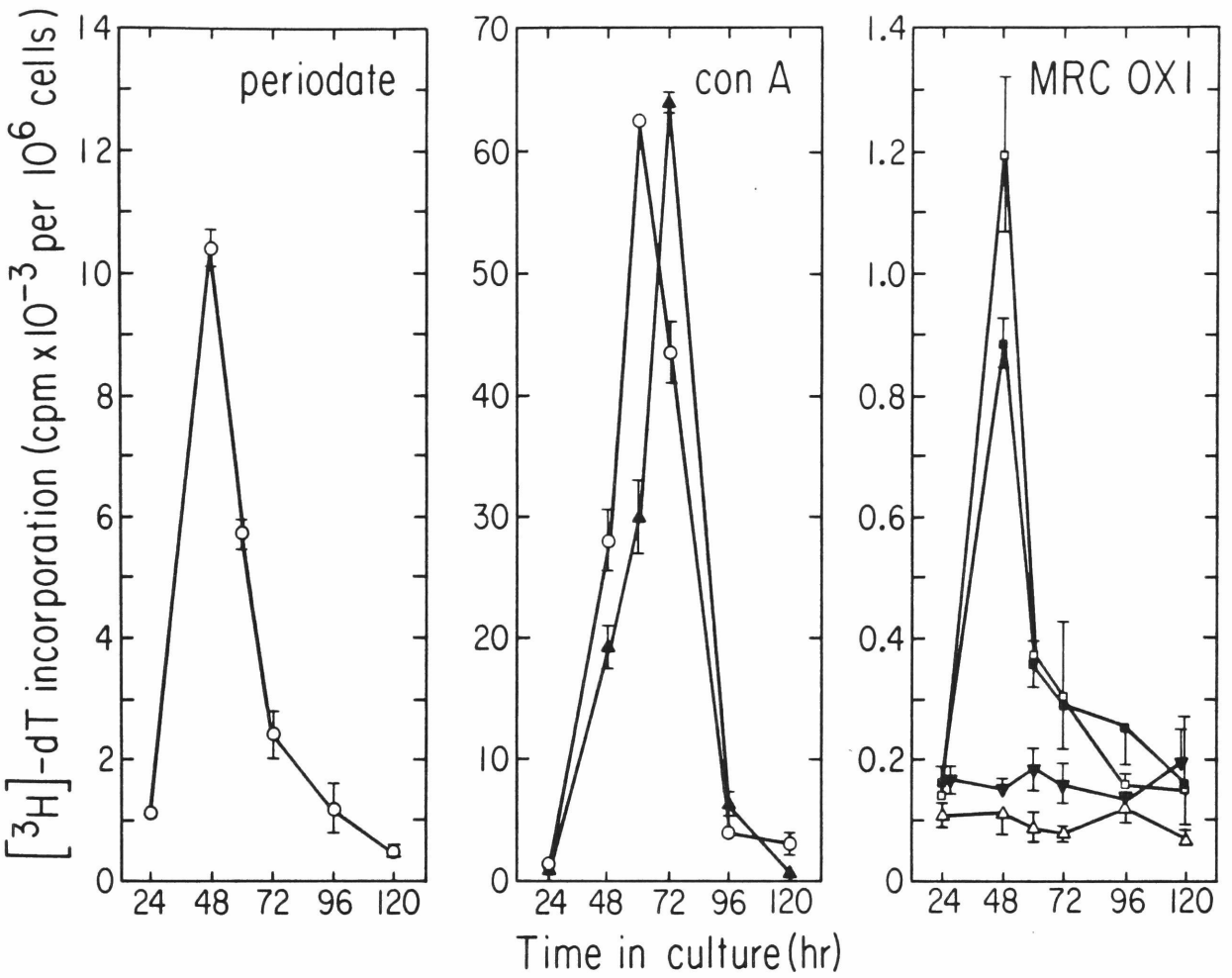



Figure 36. Stimulation of B-depleted LNC by periodate, Con A, or MRC OX1 plus goat anti-mouse Ig $F(ab')_2$ fragments. In the Con A panel (middle): O , cells coated with 15 µg/ml MRC OX1 for 30 min at room temperature, and washed before culturing in the presence of Con A. ▲ , cells subjected to the same incubation without MRC OX1 present. In the MRC OX1 panel (right):

△ , cells coated with 15 µg/ml MRC OX1 alone, and washed.

▼ , cells incubated in the presence of 125 µg/ml goat anti-mouse Ig $F(ab')_2$ fragments alone. □ and ■ , cells coated with 15 µg/ml MRC OX1 and cultured in the presence of 125 µg/ml and 150 µg/ml goat anti-mouse Ig $F(ab')_2$ fragments, respectively.

## General Discussion

The work presented here has sought to correlate the presence on lymphocytes of specific periodate- or NGO-oxidizable surface components with the ability of the lymphocytes to undergo blastogenesis in response to periodate or NGO treatments. The chemically- or enzymatically-modified lymphocyte surface molecules have been identified by SDS-polyacrylamide gradient slab gel electrophoresis of oxidized cells tagged by reduction with $^{3}H$-borohydride or by reaction with a new aldehyde-specific labeling reagent, $^{35}S$-CME. Since borohydride and CME both inhibit oxidative mitogenesis (Figs. 13 and 14), the components labeled by reaction with the radioactive derivatives represent the entire set of molecules which can mediate the mitogenic response. On the assumption that periodate and NGO promote mitogenesis via modification of the same surface components, it was expected that relevant molecule(s) would be radiolabeled after oxidation with either agent. Similarly, it was anticipated that the important oxidizable stimulatory components would also occur on those lymphocytes which were capable of undergoing oxidative mitogenesis (LNC, TDL, T-lymphocytes, etc.). Proteolytic inhibition of the mitogenic proliferation and the protease effect on labeling patterns were further used to identify specific sets of pertinent oxidizable molecules. The cumulative results implicated the importance of a high molecular weight (175,000 m.w. to 155,000 m.w.) $t_1$-$t_4$ tetrad of sialoglycoproteins in mediating the periodate or NGO stimulation. Subsequent work with a monoclonal antiserum that cross-reacted with all four molecules demonstrated

that direct cross-linking of the components induced lymphocyte proliferation.

Labeling of oxidized surface molecules. Mild oxidation of rat LNC with NGO or periodate, followed by reduction with $^{3}$H-borohydride or reaction with $^{35}$S-CME, specifically and uniformly label a set of glycosylated cell-surface molecules. Analysis of the distribution of radioactivity from labeled LNC by differential (Table III, Fig. 16) or equilibrium density centrifugation (Figs. 17-19) indicates that the incorporated label from either of the $^{3}$H-borohydride or $^{35}$S-CME procedures has a distribution consistent with a localization on the plasma membrane. That two labeled components (GP50 and GP12) consistently appear in high-speed centrifugation supernatants, or isopycnic gradient fractions containing primarily soluble components, is most likely due to extraction of these molecules by changing the ionic strength of the medium (Steck and Yu, 1973), rather than to labeling of truly soluble molecules, or proteolytic fragmentation of surface components. This contention is supported by the following points: 1) Neither component is labeled without prior treatment by periodate or NGO (Fig. 20A), and both NGO and periodate treatments cause the subsequent $^{3}$H-borohydride or $^{35}$S-CME labeling of the components (Figs. 16, 18, and 19). Thus, the molecules must be specifically oxidized and will not spontaneously react with radioactive molecules that might diffuse across the membrane. Moreover, that they are oxidized by both periodate and NGO indicates that they must be on the external membrane surface since the neuraminidase and galactose oxidase are large (approximately

70,000 m.w.), membrane-impermeant proteins. 2) GP50 and GP12 appear on labeled whole cells which have been directly solubilized by boiling in 1% SDS, and are therefore not subject to any substantial proteolytic digestion (diagnostic of which would be the collapse of $t_1$-$t_4$ to approximately 100,000 m.w.; Sunderland et al., 1979). Therefore, it is likely that they are not radiolabeled fragments of glycoproteins cleaved by endogenous proteases released during homogenization and centrifugation.

The considerable amount of radiolabel that appears in the high-speed supernatants and isopycnic gradient soluble fractions following $^{35}$S-CME labeling, has been shown to be largely unincorporated, non-acid-precipitable cysteine (or possibly cystine). While the presence of this unincorporated radioactivity is a potential draw-back of this labeling procedure for examining membrane events in long-term cultures (where incorporation of the $^{35}$S-cysteine precursor into protein could be significant), it is apparently not a problem using the conditions and duration of the labeling protocol described here (30 min, 37°C in PBS). In the absence of periodate or NGO oxidation, $^{35}$S-CME labels only the 62,000 m.w. NS protein which is also labeled by $^{3}$H-borohydride without prior oxidation (Fig. 20E).

Generally, unless the lymphocytes were first either chemically or enzymatically oxidized, the only labeled protein observed was this 62,000 m.w. NS component (Figs. 20A and 20E). If the cells were reduced with unlabeled borohydride prior to the normal oxidation, $^{3}$H-borohydride reduction procedure, the NS protein was

not labeled (Figs. 15D and 15E), suggesting that it is oxidized in its native state. Gahmberg et al. (1976) and Andersson et al. (1977) have described a similar molecule on murine and human lymphocytes, respectively (57,000 m.w. on both species), and it has been speculated that it may be a pyridoxal-containing enzyme (Gahmberg et al., 1976). It is also important to note that $^{3}$H-borohydride reduction in the absence of prior oxidation gave rise to some radioactivity that migrated with the glycolipid material. The label was not free $^{3}$H-borohydride since that was shown to diffuse out of gels during the fixing, staining, and destaining procedures. Interestingly, very little, if any radioactivity was found in this region after $^{35}$S-CME labeling of non-oxidized lymphocytes, suggesting that the incorporation is unique to the $^{3}$H-borohydride reagent. Under the physiologic conditions of the labeling procedure, borohydride does not normally reduce double bonds, or ester or amide carbonyls. However, borohydride will reduce double bonds conjugated to ester carbonyls (Kadin, 1966), such as could occur on some species of phospholipids, and presumably would also reduce double bonds conjugated to amide carbonyls on certain sphingolipids. CME, on the other hand, would not react with double bonds in this fashion (see below). The phenomenon of non-specific glycolipid labeling by $^{3}$H-borohydride has been widely noted by a number of other workers using erythrocytes (Liao et al., 1973; Steck and Dawson, 1974), lymphocytes (Andersson et al., 1977), and cultured hepatoma cells (Baumann and Doyle, 1978), and is generally less than 1% of the specific, NGO- or periodate-induced incorporation (Liao et al., 1973).

From Figures 21, 20D, and 20F, it can be concluded that the autoradiographic patterns represent the labeling of a random sample of all the oxidized cell-surface molecules. Therefore, even though $^{3}$H-borohydride is used at concentrations which are not completely inhibitory for oxidative mitogenesis, the resultant set of labeled molecules must include the cell-surface components which mediate the stimulatory response. In addition, since mild acid hydrolysis releases radioactivity from the high and intermediate molecular weight labeled glycoprotein bands (Fig. 22), it can be concluded that under the conditions used, $^{3}$H-borohydride specifically reduces glycoprotein aldehydes to radioactive alcohols and does not reduce any significant number of Schiff base complexes. The incorporation of $^{3}$H-borohydride non-specifically into the glycolipid contributes relatively little to the specific labeling seen following NGO or periodate oxidation (see above). Thus, the persistence of radioactivity in the glycolipid region of the gels after acid hydrolysis (Fig. 22) is only partially attributable to the non-specific label. Rather, the linked sialic acids of poly-sialylated glycosides may be comparatively acid-resistant and therefore retain more of the tritium label than sialic acids on glycoproteins.

The important point remains that the labeling pattern obtained with $^{35}$S-CME, which will not react with Schiff bases (see below), is identical to that obtained with $^{3}$H-borohydride, suggesting that they are both reacting with the same aldehyde moieties. Consequently, each band on the slab gel represents a unique cell-surface molecule rather than a cross-linked adduct of

unknown composition. It should be emphasized that this by no means disproves the notion of Schiff base formation as a mechanism for the mitogenesis induced by periodate and NGO (Novogrodsky and Katchalski, 1973; O'Brien and Parker, 1976); only that Schiff bases could not be detected under the labeling conditions used (30 min, on ice in PBS). It is entirely possible that Schiff base formation could occur later in culture at 37°C as the lymphocyte plasma membrane becomes more fluid and has a greater chance for interaction with serum or cell-surface proteins.

That CME inhibits oxidative mitogenesis much more efficiently (at lower concentrations) than borohydride (compare Figs. 13 and 14) or cysteine, has led to the development of $^{35}$S-CME as a useful labeling reagent. The effectiveness of the CME reagent can be explained as follows. In cysteine, the amino and sulfhydryl groups are electrostatically and inductively coupled, and have similar pK's. The result is that the ionization of one functionality affects the pK of the remaining undissociated group; consequently, both the amino and sulfhydryl moieties have 'mixed pK's'. In the particular case of cysteine, the amino functionality has a pK of 8.86 if it is the initial group to dissociate; however, the pK shifts to 10.36 if the sulfhydryl loses its proton first (Benesch and Benesch, 1955). Since the first sulfhydryl pK is 8.53, cysteine exists at neutrality primarily as the $NH_3$+, S- species (Benesch and Benesch, 1955). Cysteine and its derivatives react with aldehydes first via rate-limiting formation of a Schiff base with the amino group, followed by nucleophilic attack by the sulfur and consequent ring closure to

generate the thiazolidine ring (Schauenstein et al., 1977)(see Fig. 12). Since the initial Schiff base will form only between aldehydes and uncharged amino groups (March, 1968), modifications of cysteine which decrease the amino pK (8.86 and 10.36) relative to the sulfhydryl pK (8.53 and 10.03)(Benesch and Benesch, 1955), will enhance the rate of reaction. By virtue of the reduced electrostatic effect of the ester in CME, as compared to the carboxylic acid in cysteine, cysteine methyl ester (mixed amino pK of 6.56 and 8.99; Greenstein and Winitz, 1961) and ethyl ester (mixed amino pK of 6.77 and 8.41; Greenstein and Winitz, 1961) exist as predominantly uncharged species at neutral pH. In addition to explaining the efficient inhibition of oxidative mitogenesis by these compounds relative to cysteine, this may also provide a rationalization for the unhindered low temperature diffusion of the labeled molecules into the cells. According to Greenstein and Winitz (1961, p.486), and Benesch and Benesch (1955), cysteinyl-peptides should also have low amino group pK's (6.75-7.25). In addition, those peptides would have the advantage of a carboxyl terminal charge which would diminish membrane diffusion. However, when cysteinyl-tyrosine was examined (it was selected for its potential as a reagent that could be radiolabeled with iodine), the inhibitory concentrations for oxidative mitogenesis were in the same range as cysteine (5-10 mM). Conceivably, the effective concentration of the cysteinyl-tyrosine is substantially decreased by either a hyperactive dipeptide transport or dipeptidase activity.

Despite its high rate of diffusion across plasma membranes,

the specificity of the radioactive CME reagent is the same as that found for $^{3}H$-borohydride, and CME has some clear advantages over borohydride as a glycoprotein/glycolipid aldehyde labeling compound. Obviously, radiolabeled CME may be made up as the $^{35}S$, $^{14}C$, or $^{3}H$ (and possibly $^{125}I$) derivatives, where the high-activity strong β- or γ-emitters could shorten autoradiographic exposure times, increase the sensitivity of the autoradiographic method, and/or eliminate the the need for incorporating fluors into the slab gels (Bonner and Laskey, 1974; Laskey and Mills, 1975). The variety of isotopes which may be used also allows the design of a number of double-label experiments which are not possible with $^{3}H$-borohydride. Moreover, CME is fairly stable (Greenstein and Winitz, 1961), and does not suffer the problem of spontaneous hydrolysis in the presence of water (and the associated practical problem of tritiated hydrogen gas) which occurs with $^{3}H$-borohydride. Finally, despite the fact that even at 0.625 mM concentration, where the $^{3}H$-borohydride concentration is in a 100-fold molar excess over sialic acids (assuming $7.5 \times 10^{7}$ sialic acids per lymphocyte; Lichtman and Weed, 1970), the $^{3}H$-borohydride reacts with less than 0.5% of the total surface aldehydes (assuming all sialic acids are oxidized; calculations made from high-speed pellet P fraction). By comparison, at 0.4 mM, $^{35}S$-CME reacts with greater than 70% of the surface sialic acids after periodate oxidation, and greater than 35% after NGO treatment (the diminished incorporation after NGO oxidation possibly reflecting incomplete NGO reaction with glycosylated surface components. These calculations were made from TCA-

precipitable counts in the presence of 40 mM dithiothreitol of high-speed pellet P fractions). Thus, the reason that the CME reagent is more efficient in inhibiting oxidative mitogenesis is probably due to its more quantitative reaction.

The major current stumbling block to the use of radioactive CME as a glycoprotein/glycolipid labeling reagent is its non-availability as a commercial preparation. Consequently, the radiolabeled CME product has a low specific activity which results from the addition of unlabeled carrier cysteine for the reagent synthesis (see Methods section). In addition, the permeability of the CME and potential incorporation of the radioactivity into newly-synthesized proteins, makes the use of the reagent in long-term cultures undesirable. However, suitable modification of the ester group, or possibly use of cysteinyl-d-amino acid dipeptides could obviate this difficulty. It is worth mentioning that $^{125}$I-Con A labeling of glycosylated molecules in SDS-polyacrylamide slab gels (after the method of Burridge, 1978) was also attempted. Because problems were encountered with high background labeling, and since Con A does not necessarily bind to the specific molecules which are oxidized by periodate or NGO, the technique was not extensively pursued.

$^{3}$H-borohydride-labeled molecules on lymphocytes. Reduction with $^{3}$H-borohydride after either NGO or periodate oxidation labels similar sets of molecules (see complete list of bands in Table V). However, some variability in the relative migration or intensity of some labeled components is apparent when correspond-

ing bands from NGO- or periodate-oxidized cells are compared (Fig. 15, Table V). In addition, periodate-oxidized cells, exclusively, show four low molecular weight bands (GP34, GP30.5, GP28, GP15) upon subsequent reduction with $^{3}$H-borohydride (Fig. 15, Table V).

It is noteworthy that the heavily-labeled main band glycoprotein (MB) migrates to different apparent molecular weights depending on whether the LNC are oxidized by NGO (120,000 m.w.) or periodate (110,000 m.w.). Gahmberg et al. (1976), and Gahmberg and Andersson (1977) have observed a similar phenomenon for a single glycoprotein (GP9; 125,000 m.w. after NGO oxidation) on murine lymphocytes; labeling with $^{3}$H-borohydride after treatment with NGO shows a slightly higher molecular weight than labeling after treatment with periodate.

In the absence of more detailed structural analyses of the MB glycoproteins, it is impossible to conclude that they represent the same molecule. However, indirect evidence has accumulated which suggests that these two radioactive bands do indeed derive from the same glycoprotein. Since no radioactivity is incorporated into either position following treatment with galactose oxidase alone (Fig. 15F), the possibility of a single parent molecule with a variable amount of sialylation (and thus with a variable amount of terminal galactose or N-acetyl galactosamine residues) can be excluded. The possibility that periodate acts on some especially sensitive site to cause a bond breakage with a net loss in apparent molecular weight, is ruled out by

treating LNC with neuraminidase and galactose oxidase prior to periodate oxidation. Radioactivity is incorporated only into the 120,000 m.w. MB band during a subsequent $^{3}$H-borohydride reduction (Fig. 15I), indicating that mitogenic levels of periodate do not cleave bonds other than those on terminal sialic acids (see also Presant and Parker, 1976). In addition, the acid hydrolysis results (Fig. 22) and the absence of labeling with periodate following neuraminidase treatment (Fig. 15J) demonstrate that the radioactivity associated with the MB band following periodate oxidation is on an acid-labile linkage (Aminoff, 1961), in this case on sialic acids.

A likely explanation for the variation in the molecular weights is suggested by the work of Segrest et al. (1971) and Segrest and Jackson (1972), which showed that the removal of sialic acid can slow the migration of some glycoproteins in polyacrylamide gels by as much as 20-100%. Because of the anomalous binding of SDS to the oligosaccharide chains of glycoproteins (Segrest et al., 1971), the charge from sialic acids can contribute substantially to the charge/mass ratio of highly glycosylated molecules. When sialic acids are removed by treatment with neuraminidase (or with NGO), there is a significant drop in charge for only a small decrease in actual mass, with the net result being an overall apparent increase in molecular weight. Thus, the molecular weight calculated for the MB protein after periodate oxidation (with the charge from the sialic acids intact) is probably the more accurate value. Because the other labeled glycoprotein bands have the same

mobilities regardless of the method of oxidation, they probably represent glycoproteins which are not as heavily glycosylated as the MB glycoprotein, and which bind sufficient SDS to overcome the inherent molecular charge. Williams and his co-workers (Williams et al., 1977; Standring et al., 1978) have partially purified the MB glycoprotein with a monoclonal antiserum (W3/13 HLK) and have found it to be composed of approximately 50% carbohydrate by weight (A.F. Williams, personal communication).

As mentioned above, reduction with $^{3}$H-borohydride after periodate oxidation labels four low molecular weight components (GP34, GP30.5, GP28, and GP15) which are not labeled if LNC are instead oxidized by NGO (Figs. 15G, 15H). Even though these bands are visualized if the cells are treated with neuraminidase first (Fig. 15J), it seems likely that the label is on terminal sialic acids because mild acid hydrolysis releases all of the radioactivity (Fig. 22). A possible explanation for this apparent paradox has been described in the literature. 4-O-acylated, neuraminidase-resistant sialic acids have been isolated from a variety of biological sources, and in some cases constitute up to 50% of the total sialic acid (Schauer and Faillard, 1968; Buscher et al., 1974). These derivatives, however, can still be readily oxidized by periodate at the exocyclic carbons (Schauer and Faillard, 1968; Buscher et al., 1974). Thus, although glycoproteins terminated by such sialic acids are not modified by treatment with NGO, they can be labeled with $^{3}$H-borohydride after oxidation with periodate.

In contrast to the situation described above, the very low molecular weight GL component(s) are heavily labeled as a single (or occasionally double) band following NGO oxidation, but are only lightly labeled at an array of molecular weights after periodate treatment (Figs. 15B and 15C). These bands probably represent glycolipids because they are extracted into 1:1 chloroform:methanol (Fig. 15L) (Ledeen et al., 1973), and because they show a variable and time-dependent decrease in apparent moleclular weight which may reflect a significant amount of auto-oxidation (Fogarty, 1971). In addition, the GL bands are heavily stained by Sudan black (Fig. 15A), but are not stained by Coomassie blue. As suggested previously, the array of molecular weights observed for the glycolipids after periodate oxidation may be accounted for by poly-sialylated gangliosides which will display unique migrations as a function of the number of sialic acids (Ledeen, 1978). Removing the sialic acids (as with NGO treatment) will result in labeled core molecules with generally homogeneous molecular weight (about 10,000 m.w.). Low molecular weight (8000 m.w.) components with similar properties have been described by Ladoulis et al. (1974) and by Misra et al. (1978) in rat spleen and thymus membrane preparations. Because of the anomalous binding of SDS to glycosylated molecules (Segrest et al., 1971; Segrest and Jackson, 1972), the calculated molecular weights (approximately 10,000 m.w.) probably represent an upper estimate.

Since the glycolipid bands are not labeled to any significant extent after galactose oxidase treatment alone (Fig. 15F),

the difference in labeling intensity after NGO or periodate oxidations is not attributable to a terminal galactose or N-acetyl galactosamine residue on these molecules. Instead, it is possible that the terminal sialic acids on the glycolipids are acylated in the 0-8 or 0-9 positions and are therefore relatively insensitive to periodate oxidation (Schauer and Faillard, 1968). Modified sialic acids of this sort have been previously characterized from various sources and have been found to be susceptible to neuraminidase cleavage (Schauer and Faillard, 1968; Buscher et al., 1974), or to periodate oxidation following deacetylation with 0.1 N NaOH (Sarris and Palade, 1979).

In contrast to the many labeled bands obtained following oxidation with NGO, treatment with galactose oxidase alone shows virtually no labeling except for the GP80 component (Fig. 15F). The results are in agreement with the findings of Gahmberg et al. (1976), describing only one high molecular weight component of murine lymphocytes which is preferentially labeled after treatment with galactose oxidase alone. The galactose oxidase-modified bands probably represent molecules with a predominance of terminal galactosyl or N-acetyl galactosamine residues, and therefore are not labeled after treatment with periodate. The light labeling observed for bands other than GP80 after galactose oxidase oxidation can most likely be attributed to minor amounts of glycoprotein microheterogeneity (Spiro, 1970) or positional isomerism (e.g., sialic acids linked 2-4 to penultimate galactoses, rather than the more common 2-6 linkage; Dixon et al., 1976). It is interesting, in view of the generally poor labeling

after exposure to galactose oxidase alone, that treatment with this enzyme in the absence of neuraminidase is weakly mitogenic for rat (Kielian et al., 1977) and murine LNC (Novogrodsky and Katchalski, 1973). In contrast, human lymphocytes are mitogenically stimulated by galactose oxidase alone (Dixon et al., 1976). In addition, subsequent $^{3}$H-borohydride reduction of galactose oxidase-treated human lymphocytes shows labeling of several components, including a predominant incorporation into a high m.w. tetrad of glycoproteins (GP1-4, 200,000-160,000 m.w.; Andersson et al., 1976).

Labeling of different cell populations. As expected from the predominance of T-lymphocytes in LNC (80%; Goldschneider and McGregor, 1973), the labeling patterns of nylon wool-purified (Figs. 24B and 24E) or antibody and complement, B-depleted T-cells (Fig. 24H), closely resemble the patterns observed for unfractionated LNC populations. The major exception was the absence of high molecular weight (190,000 m.w.) radioactivity in the T-cell preparations. In comparison, the nylon wool-adherent (Figs. 24C and 24F) and anti-Ig-panned B-lymphocytes (Fig. 24I), showed diminution (Figs. 24C and 24F) or complete absence (Fig. 24I) of the labeling of the $t_1$-$t_4$ and MB bands and increased intensity of four components: a high 190,000 m.w. and 3 intermediate molecular weight (54,000, 37,000, and 30,500 m.w.) glycoproteins. Similar differences have been observed in the labeling patterns of human T- and B-lymphocytes (prepared by nylon wool column filtration and T-rosetting, respectively; Andersson et al., 1976), murine T- and B-lymphocytes (prepared by

preparative cell electrophoresis; Gahmberg et al., 1976), and with rat T- and B-lymphocytes (obtained after separation by T-rosetting; Standring et al., 1978); however, Gahmberg et al. (1976) could not detect a band corresponding to glycosylated surface Ig (54,000 m.w.) or the lower molecular weight (37,000 and 30,500 m.w.) components possibly corresponding to Ia molecules (Standring et al., 1978). The distribution of the various components; that is, $t_1$-$t_4$ and MB on T-lymphocytes, and the high 190,000 m.w. glycoprotein on B-lymphocytes, is consistent with the characteristics and localization of the LC-antigen (diffuse, 170,000 m.w. on T-cells, 200,000 m.w. on B-cells; Standring et al., 1978), and the T-cell-specific 95,000 m.w. glycoprotein recognized by the monoclonal W3/13 HLK antiserum (MB) (Standring et al., 1978). Furthermore, the numbers of each of these components as determined by antibody binding, is slightly less than 100,000 per cell (Sunderland et al., 1979); this matches the numbers ($1\text{-}5 \times 10^5$) obtained from specific activity values by calculating the $^3$H-borohydride incorporation of each of the various bands and assuming one reactive aldehyde per glycoprotein (obviously, fewer molecules would obtain for greater numbers of sialic acids per molecule).

In a similar fashion, Ficoll gradient-purified responder lymphocytes (Fig. 34I) representing the bulk of LNC preparations (Beyer and Bowers, 1977b; Bowers and Beyer, 1979), had labeling patterns essentially the same as unfractionated LNC. That result, coupled to the similar patterns obtained from purified TDL (Figs. 23C and 23D) which lack accessory cells (Bowers and

Beyer, 1977), and the obviously different labeling seen from RBC (Figs. 25E and 25F), strongly suggests that there is a negligible contribution from non-lymphocytes in the overall labeling patterns obtained from responsive lymphocyte populations.

With the exception of Con A, the majority of thymocytes respond only poorly to mitogens (Greaves and Janossy, 1972) or other stimuli (Blomgren and Andersson, 1971). Even in cases where thymocytes were stimulated by other lectin mitogens (Keller, 1975), allogeneic cells (Han et al., 1976), or antibody against grafted sites (Ravid et al., 1978), T-lymphocytes (Decker et al., 1977), or gangliosides (Sela et al., 1978), it is not clear that the responding cells were not from the 'mature', cortisone-resistant sub-population. Thus, even though thymocyte preparations have been shown to contain accessory cells (Bowers and Beyer, 1977), it is not too surprising that the predominantly 'immature' thymocytes did not exhibit a blast response following periodate or NGO treatment (Table IV; Bowers and Beyer, 1977). In comparison, cortisone-resistant thymocytes, which also have most of the same attributes as peripheral lymphocytes (Blomgren and Andersson, 1971), were capable of undergoing oxidative mitogenesis. At the same time, they also displayed new $^{3}H$-borohydride-labeled components corresponding to the $t_1$-$t_3$, and GP87 and GP70 glycoproteins found on LNC (Fig. 23). The correlation is interesting, and is suggestive of a surface 'maturation' process in the course of functional thymocyte differentiation (Goldschneider, 1975; Williams, 1976), but may only be coincidental. The increased intensity of the 46,000 m.w. glycoprotein on

cortisone-resistant thymocytes may reflect an elevated density of the rat Ag-B histocompatibility antigen (45,000 m.w.; Sporer et al., 1979) on these cells. It is not likely to be due to an RBC contamination (which carry a predominant, labeled 46,000 m.w. glycoprotein component; see Figs. 25E and 25F), since the cortisone-resistant thymocyte preparation had few cells of the appropriate size class (Fig. 5). The generally intense 155,000 m.w. $t_4$ glycoprotein and the 27,000 m.w. band on thymocytes (Fig. 23F) correspond to the thymocyte LC-antigen (150,000 m.w.) and Thy-1 glycoprotein, respectively (Standring et al., 1978).

The labeling patterns observed from 2-day mitogen-stimulated blast cells (Fig. 25B) were very similar to those obtained from murine Con A and phytohemagglutinin blasts (Gahmberg et al., 1976), and from human phytohemagglutinin blasts (Andersson et al., 1977). However, only in the murine blasts was there also an increased intensity of labeling of the band co-migrating with the NS component. As with the periodate- or NGO-induced rat blasts, the high molecular weight (greater than 150,000 m.w.) glycoproteins in human and murine blasts were diminished in intensity relative to resting cells. No molecules were observed on either 2-day blasts or 6-day secondary cells (Fig. 25C) from mitogen-treated rat cultures which would correspond to the GP130 (mouse lymphocytes; Andersson et al., 1978) or T145 (human lymphocytes; Kimura and Wigzell, 1978) components which develop on MLR-blasts and appear to correlate with cytotoxic T-cells.

The results obtained from the labeling of various cell

populations helped to delineate several consistent features of oxidizable surface components (Table V), but did not substantially reduce the numbers of candidate molecules to be considered for a central role in oxidative mitogenesis. The experiments with thymocytes and cortisone-resistant thymocytes were very suggestive ($t_1$-$t_3$, GP87, and GP70 seemed pertinent; $t_4$, MB, Thy-1, and other components seemed uninvolved). However, the results could be easily attributed to a native unresponsiveness of the thymocytes rather than to an absence of relevant molecules, and a coincidental intensification of certain bands on the responsive cortisone-resistant thymocytes. In the final analysis, use of proteases as tools for dissecting out specific lymphocyte surface molecules turned out to be much more informative for identifying the central, relevant sialoglycoproteins.

Proteolytic dissection of LNC. Mild digestion of whole LNC populations with trypsin, chymotrypsin, thermolysin, or papain is inhibitory for periodate- or NGO-induced mitogenesis (Figs. 27-30). That the Con A stimulation under the same conditions is not inhibited (Figs. 27-30) indicates the LNC are not rendered unresponsive by protease treatment. It is clear, however, that for the first three of these enzymes (trypsin, chymotrypsin, and thermolysin), the peak Con A response is delayed 12-24 hr (Figs. 27-29). Because the initial viabilities and the viabilities of cultured LNC are not affected by treatment with inhibitory levels of the proteases (Fig. 32), it can be concluded that the enzymes do not inhibit oxidative mitogenesis or delay the Con A response by simply killing cells. Further, controls involving proteolysis

of LNC following oxidation, incubation with bovine plasma albumin, or treatment with heat-inactivated papain (Fig. 31) demonstrate that the inhibition due to papain treatment is not caused by non-specific absorption to cell surfaces. Rather, trypsin, chymotrypsin, and thermolysin, as well as papain, almost certainly exert their effect by cleaving essential peptide pieces from membrane glycoproteins. In contrast to periodate or NGO treatments which occur shortly after protease digestions, Con A remains in the culture medium at all times. Thus, digested receptors which might be regenerated over a 12-24 hr period could mediate a delayed, but otherwise normal Con A blastogenesis. Similar conclusions were reached by Lindahl-Kiessling and Peterson (1966a and b). They showed that trypsin caused a transient inhibition of the phytohemagglutinin response of human peripheral blood lymphocytes without affecting stimulation by allogeneic cells in an MLR.

It should be emphasized that these results do not necessarily require that the surface molecules involved in oxidative mitogenesis and Con A stimulation be the same (see also below). In fact, the differential sensitivities of Con A, and periodate or NGO blastogenesis to papain suggest that unique sites are involved (Fig. 30; Novogrodsky and Katchalski, 1972 and 1973). It is possible, however, that those different sites reside on the same glycoprotein. In such a case, papain could digest portions of the molecule relevant to oxidative mitogenesis without affecting the Con A binding sites. Accordingly, trypsin, chymotrypsin, and thermolysin could cleave at positions on the glycoprotein

such that both Con A and oxidizable sites are lost. In light of this proposal, it is interesting that papain leaves a 150,000 m.w. fragment from the digestion of the $t_1$-$t_4$ molecules, while the other three proteases yield a significantly smaller 140,000 m.w. piece (Figs. 7B-7E).

The inhibitory effect of the proteases on oxidative mitogenesis is also interesting in view of current work suggesting a central importance of glycolipids in mitogenic stimulation (Spiegel et al., 1979). In those experiments, DNP was coupled to periodate-oxidized brain gangliosides via the appropriate hydrazide, and the DNP-glycolipid was incubated with cortisone-resistant thymocytes until some $10^7$ molecules per cell were incorporated (although less than half of those had an exterior orientation). Subsequent culturing in the presence of divalent anti-DNP antibody or fragments induced lymphocyte aggregation and surface capping, and was mitogenic. Similar effects have also been observed with divalent antibodies with presumptive specificity for $G_{M1}$ gangliosides (Sela et al., 1978). The implication from this work is that mitogenic stimulation may proceed primarily via a cross-linking of glycolipid moieties.

There is, however, considerable evidence that argues against a significant role for glycolipids. Allan et al. (1972) found that the Con A response was not affected by incubating the lectin with a lymphocyte surface fraction enriched in glycolipid, while the glycoprotein fraction removed all mitogenic activity. Their conclusion was that Con A interacted with intact lymphocytes pri-

marily via glycoproteins. Further, the inhibitory or delaying effects of proteases on oxidative mitogenesis and Con A responsiveness (Novogrodsky and Katchalski, 1972 and 1973; vida infra), argue that the mitogens periodate, NGO, and Con A all act through glycoprotein moieties, especially since glycolipids are not affected by proteolytic digestion. It is also difficult to envisage how glycolipid cross-linking could mediate capping and other surface modulation, since the glycolipids do not span the membrane and presumably have no direct means of interaction with the sub-membranous modulating assembly (Edelman, 1976). It seems likely, therefore, that the glycolipid stimulation described by Sela et al. (1978) and Spiegel et al. (1979) is attributable to tight hydrophobic interaction of the glycolipid with pertinent surface proteins (Yu et al., 1973). Subsequent antibody cross-linking of the glycolipid then leads to functional cross-linking of the relevant membrane proteins and lymphocyte triggering. Since biological membranes do not exist without glycolipids, it is difficult to perform the appropriate experiment to rule out an involvement of gangliosides. It is also arguable (however unlikely), that agents that stimulate via cross-linking of membrane proteins are mitogenic only by virtue of the subsequent interaction of tightly-associated glycolipids. The overall evidence, however, seems more to support a role for glycoprotein rather than glycolipid in mitogenic stimulation.

Enzyme treatment by itself did not induce mitogenesis of LNC (Panel A in Figs. 27-30). These results are in agreement with previous work (Kaplan and Bona, 1974; Vischer, 1974). Although

long-term incubation in the presence of trypsin, chymotrypsin, or papain caused a stimulation of mouse B-cell preparations, it was found that short pre-treatments with trypsin (the only enzyme so tested) gave variable and generally poor responses (Kaplan and Bona, 1974; Vischer, 1974). Moreover, T-cells were not stimulated by protease treatment (Kaplan and Bona, 1974; Vischer, 1974; Hart and Streilen, 1974). Thus, it is reasonable that short-term protease incubations of rat LNC (composed of some 80% T-cells; Goldschneider and McGregor, 1973) did not lead to a blastogenic response.

Protease effect on responder lymphocytes. The results in Figure 33, with mixtures of protease-treated, oxidized responder lymphocytes and untreated accessory cells, indicate that the inhibition of oxidative mitogenesis by the protease treatments in unfractionated LNC can be attributed primarily to an effect on the major population of responders. In mixture experiments involving protease-treated accessory cells and oxidized responder lymphocytes, a moderate but extremely variable inhibition was also observed; however, this is not entirely unexpected. If aldehydes on the responding lymphocytes interact via Schiff bases with amino groups on surface molecules of the accessory cells (Novogrodsky and Katchalski, 1973; O'Brien and Parker, 1976), it is possible that the proteases cause a degree of inhibition by digesting some of these accessory cell proteins. However, a number of trivial effects could also account for this inhibition, e.g., accessory cells may be specifically lost by adherence during the 45 min protease incubation period. Since accessory cells

cannot currently be quantitated by other than their ability to enable oxidized lymphocytes to respond, these possibilities cannot be distinguished. Because the protease treatment of accessory cells alone only partially inhibits oxidative mitogenesis, and because addition of protease-treated responders shows a substantial further inhibition of the response, it is reasonable to conclude that most of the relevant accessory cell proteins are comparatively protease-resistant. Nonetheless, the observation of some effect on the accessory cells does merit further investigation to delineate whether specific surface modifications or non-specific phenomena (i.e., loss of accessory cells) is involved.

In contrast to the lack of a major effect on accessory cells, protease treatment of the responder lymphocytes alone resulted in inhibition of oxidative mitogenesis which was the same as for protease treatment of the whole LNC population. The simplest interpretation of this result is that the the various enzymes exert their inhibitory action by digesting important periodate- or NGO-oxidizable surface molecules on the responder cells. These results are particularly significant since responder lymphocytes constitute the greatest proportion of the total LNC populations (Beyer and Bowers, 1977b; Bowers and Beyer, 1979) and undoubtedly contribute the most substantial amount of radioactive incorporation to the overall labeling pattern. In support of this, responder lymphocytes have essentially the same labeling pattern as whole LNC (Figs. 34H-34I). Moreover, the labeling patterns of responder lymphocytes and unfractionated LNC

are affected in the same way by papain treatment (Figs. 34F and 34J). Thus, the inhibition of oxidative mitogenesis following papain digestion is clearly associated with a loss of molecules from the surface of responding cells.

Inhibitory levels of the four proteases indicate that a subset of five oxidizable cell surface sialoglycoproteins is involved in stimulation by periodate and NGO (specifically $t_1$-$t_4$ and MB). However, using sub-inhibitory doses of papain (1 unit/ml), the MB band at approximately 110,000 m.w. (and GP70) is completely digested while $t_1$-$t_4$ remain relatively intact (Fig. 34F). Thus, the integrity of only $t_1$-$t_4$ (or some subset thereof) seems critical for stimulation by periodate or NGO. Interestingly, that conclusion agrees with the conclusion arrived at from the labeling pattern results obtained with various periodate- or NGO-responsive lymphocyte populations; specifically, the thymocyte and cortisone-resistant thymocytes results had indicated that the $t_1$-$t_3$ molecules were relevant to oxidative mitogenesis while the MB band was not.

Significance of surface aldehyde density. An alternate suggestion that should be considered at this point is that general surface aldehyde density, rather than the presence of specific oxidizable sialoglycoproteins, is the critical parameter for oxidative mitogenesis. According to such a model, virtually any aldehyde would suffice to interact with another surface moiety in facilitating the stimulation by periodate or NGO. Thus, there could be some threshold density of aldehydes required

to mediate the response. By virtue of their large molecular weight, the $t_1$-$t_4$ molecules would be most susceptible to proteolytic cleavage; conceivably, the same inhibitory effect would be observed if the lower molecular weight proteins could be cleaved to achieve a similar overall reduced density of aldehydes. This possibility can be argued against on two points: 1) Based on the cpm in various surface molecule classes (as determined by slicing and counting SDS-polyacrylamide slab gels which contain electrophoresced samples from oxidized and $^3$H-borohydride-labeled LNC), greater than 65% of surface aldehydes are present on glycolipids (Fig. 22). These molecules would not be affected by protease treatment. 2) Again, based on the analysis of sliced and counted slab gels, inhibitory levels of papain (which remove MB and GP70 as well as $t_1$-$t_4$) caused the loss of only 15% of the total radioactivity, and presumably only 15% of the total surface aldehydes. Thus, it seems unlikely that oxidative mitogenesis is dependent only on the density of surface aldehydes available for surface cross-linking. Rather, the simplest interpretation of the results presented here is that the presence specifically of the $t_1$-$t_4$ sialoglycoproteins on responding lymphocytes is necessary to effect oxidative mitogenesis.

Stimulation by specific monoclonal antibodies. The interpretation presented above is in some measure confirmed by the results obtained with the monoclonal anti-LC antiserum, MRC OX1 (Sunderland et al., 1979). Since MRC OX1 plus anti-mouse Ig specifically immunoprecipitated all four bands of the $t_1$-$t_4$ tetrad (Fig. 35B), the $t_1$-$t_4$ sialoglycoproteins must have at

least one common antigenic site, and possibly represent the same core protein with differing degrees of glycosylation (see below). Certainly the uniform sensitivity of the four bands to protease digestion, and the apparent consolidation of the tetrad to a single heavily-labeled protease fragment (PF140 or PF150), are consistent with a similar basic protein structure (Fig. 34). The observation by Standring et al. (1978) of a single diffuse 170,000 m.w. T-lymphocyte LC-antigen, as opposed to the tetrad that was observed in this work, is most likely attributable to the uniform 10% polyacrylamide gels used by those workers (Standring et al., 1978). The 7-15% gradient gels used here and by Trowbridge et al. (1977) for mouse lymphocytes, give better high molecular weight resolution and clearly identify discrete heterogeneous molecular weight species of LC-antigen. Even a uniform 8% gel was successful in resolving a high molecular weight tetrad of sialoglycoproteins on human lymphocytes (GP1-GP4, 200,000-160,000 m.w.; Andersson et al., 1976 and 1977), although it is not clear whether those components truly represent human analogues of the rat (Sunderland et al., 1979) and murine (Trowbridge et al., 1977; Trowbridge, 1978) LC-antigens.

A plausible explanation for the variation in molecular weight of the $t_1$-$t_4$ components would be an increase in glycosylation of a core glycoprotein (e.g., $t_4$), possibly as discrete blocks of 5,000 to 7,000 m.w. A structure such as that depicted in Figure 1 would contribute directly about 3000 m.w.; however, the anomalous SDS binding and migration in polyacrylamide gels brought about by the extra carbohydrate could conceivably cause a

shift in apparent molecular weight of 2-3 times that (Segrest et al., 1971; Segrest and Jackson, 1972). A variation in molecular weight of greater than 3,000 (12%) was observed for rat brain and thymus Thy-1 antigens, where the only molecular difference was additional terminal glycosylation on the 3 existing core saccharide structures (6% increase in real mass due to the carbohydrate; Williams et al., 1976). Further, Standring et al. (1978) and Trowbridge et al. (1978) working with monoclonal antisera, have described unique molecular weight species of antigenically-cross-reactive LC-antigens on different cell types (B-cells, T-cells, and thymocytes; range from 150,000 to 210,000 m.w. for the same species). These results demonstrate that extreme molecular weight variation can occur for a common antigenic structure. Similar results have been obtained with polyclonal anti-LC antisera (Sunderland et al., 1979), and the LC-antigenicity was lost upon heating (A.F. Williams, personal communication), indicating that the common antigenicity is not due to recognition of very small peptide sequences, or to specifity for carbohydrate moieties.

As predicted from the work of Wynne et al. (1976) and Ravid et al. (1978), and based on the evidence delineating an important role for the $t_1$-$t_4$ tetrad in oxidative mitogenesis, cross-linking the LC-antigens directly with MRC OX1 and anti-Ig is mitogenic for rat LNC (Fig. 36). That MRC OX1 alone was not stimulatory for the lymphocytes is most likely due to the monoclonal nature of the antibody. A monoclonal antiserum directed against some component can, by definition, recognize only a single antigenic

configuration on that molecule. Since the relevant site probably occurs only once on each molecule, the divalent monoclonal antibody can cross-link at most 2 antigens. Cross-linked doublets are probably not sufficient to induce the surface modulation necessary to facilitate a subsequent mitogenic response. However, further cross-linking of the MRC OX1 antibody with polyclonal anti-Ig will give sufficient surface aggregation to trigger blast transformation. Based on such arguments, it is expected that polyclonal anti-LC antisera (Sunderland et al., 1979) might be directly stimulatory for rat lymphocytes. Indeed, Decker et al. (1977) have described a horse anti-murine lymphocyte antiserum which immunoprecipitated $^{125}$I-labeled components of 200,000-150,000 m.w. and was also mitogenic for murine thymocytes. Coincidentally, the anti-lymphocyte serum at low levels also blocked Con A stimulation, suggesting that the antiserum and lectin were stimulatory by interacting with and cross-linking the same surface glycoprotein (Decker et al., 1977). That most xenogeneic anti-lymphocyte antisera may be preferentially elicited against LC-antigens (Fabre and Williams, 1977), suggests that the mitogenicity of these preparations (which have not been generally examined for antigenic specificity) may be attributable to reactivity with and cross-linking of similar LC-antigens in other species (Holt et al., 1966; Woodruff et al., 1967).

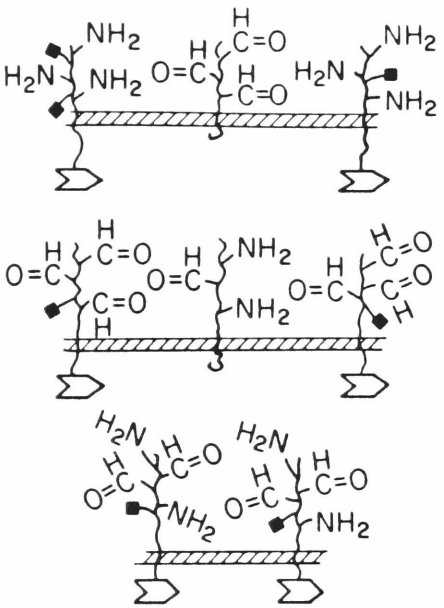
The 10-fold weaker response by MRC OX1 and anti-Ig is not due to a loss of accessory cells during B-depletion, since addition of 3% accessory cells (obtained from BPA flotation of spleen cells) had no effect, and the periodate and Con A responses of

the B-depleted preparations were normal. Rather it seems most likely that the relatively weak stimulation is attributable to the low affinity of the MRC OX1 monoclonal antisera (Sunderland et al., 1979). Attempts to improve the MRC OX1 avidity by heat- or anti-Ig-aggregation met with no success, and may be due to insufficient aggregation or again may be caused by the innate low affinity of the monoclonal antibody. The failure of protein A to mediate stimulation is possibly because the MRC OX1 may be of a mouse Ig sub-class which does not associate with protein A under physiologic conditions (Kessler, 1976). Whether the MRC OX1-responsive cells represent a lymphocyte sub-population with high levels of LC-antigen (all lymphoid cells carry some form of LC-antigen, but the surface density may vary; Standring et al., 1978; Sunderland et al., 1979), or whether the response reflects a general low level responsiveness of the entire population is unknown. It is also currently unknown whether MRC OX1 will stimulate B-lymphocytes, although the presence of a high molecular weight LC-antigen on these cells suggests that blast transformation may occur (Standring et al., 1978). Conversely, that periodate and NGO are exclusively T-cell mitogens (Novogrodsky, 1974), despite their ability to oxidize the 'relevant' 190,000 m.w. LC-antigen on B-lymphocytes, indicates the requirements for B-cell stimulation may involve more than simple surface aggregation. Such a conclusion is also derived from the requirement for lectins to be insolubilized before they become mitogenic for B-cells (Andersson et al., 1972b; Greaves and Bauminger, 1972). The response to MRC OX1 is probably specific, since prel-

iminary results indicate that mouse antibody directed against another rat surface molecule (W3/13 HLK anti-T-cell specific antigen, which is probably the same as MB) gives only a very weak stimulation. However, further characterization of the specificity of the stimulation, and the use of polyclonal anti-LC antisera for mitogenic stimulation will help elucidate these points.

Another approach that has been used to examine the relevant molecules involved in mitogenic stimulation is that of Axelsson et al. (1979). They sought to correlate the mitogenicity of various lectins with $^{125}$I-labeled surface glycoproteins which are recovered from the appropriate lectin affinity columns. Their results, which indicate a potential role for glycoproteins of 105,000, 125,000, and 175,000 m.w., are also of some interest as they pertain to lymphocyte stimulation in general. For example, are the molecules which mediate stimulation by the lectins the same as those which have been implicated here as relevant for oxidative mitogenesis? The results of Decker et al. (1977) indicate that at least Con A and a mitogenic anti-lymphocyte serum do interact with the same surface component. Further, do any of these molecules have anything to do with antigenic stimulation?

Models examining the roles of surface glycoproteins in oxidative mitogenesis. What has been shown is that the $t_1$-$t_4$ tetrad of glycoproteins (LC-antigens) is important for stimulation by periodate and NGO, and that direct cross-linking of those molecules by a specific monoclonal antibody is also mitogenic.



Arguments for: indirect stimulation
differential protease sensitivity

Against: biotin/avidin and
DNP/anti-DNP experiments

Arguments for: biotin/avidin and DNP/anti-DNP experiments

Against: indirect stimulation
[differential protease sensitivity]

Arguments for: biotin/avidin and DNP/anti-DNP experiments; indirect stimulation

Against: [differential protease sensitivity]

■ Con A binding sites

subunits of "activating complex"

Figure 37. Possible models for the role of the cell surface glycoproteins involved in mitogenic stimulation by periodate or NGO.

These, and other observations have led to the formulation of the three models shown in Figure 37. These models attempt to explain how LNC may be stimulated by the oxidative mitogens and/or lectins (such as Con A), and how the same surface molecule could mediate transformation by both types of agents.

An arbitrary assumption in these models is that the lymphocyte triggering is facilitated by the formation of some sort of internal sub-membranous 'activating complex' (Edelman, 1976). In the diagrams in Figure 37, this activating complex is formed by the aggregation of transmembrane molecules (based on the results with the monoclonal antiserum, more than the two that are drawn are probably required). In contrast to these models, it is equally conceivable that the components of the activating complex could also be entirely isolated on the internal face of the membrane, or attached to unrelated molecules. In that instance, the formation of the activating complex could occur indirectly as a consequence, for example, of the activation of transglutaminase (Novogrodsky et al., 1978). In the diagrams in Figure 37, it has also been assumed that the primary aldehyde interaction is via Schiff base formation with an amino group, although other possibilities are equally possible (Novogrodsky, 1975b). For the sake of simplicity, and due to a lack of any evidence, the role of the accessory cell has been completely ignored.

In the first model (top), the relevant oxidizable molecules (shown with aldehydes) are depicted as anchor sites which then promote the aggregation of the molecules which carry the activat-

ing complex sub-units; lectins would directly cross-link the molecules carrying the activating complex sites. In favor of this model are the experiments demonstrating that inactivated, periodate-oxidized cells can indirectly stimulate non-oxidized lymphocytes (Beyer and Bowers, 1975; O'Brien et al., 1977; Parker et al., 1977). Hypothetically, the oxidized molecules on the stimulator cells would act like exogenous lectins to cause the aggregation of the pertinent proteins on the responder cells. This model also explains how papain could inhibit oxidative mitogenesis but not affect the Con A response; the protease would specifically cleave only the central anchor such that insufficient numbers of aldehydes would be left for cross-linking to the relevant proteins. At the same time the lectin receptors would be relatively unaffected. The arguments against this model, however, are too strong to overlook. Indirect stimulation is not mediated by any oxidized cell (such as RBC), but rather generally requires oxidized lymphocytes (O'Brien et al., 1977; Parker et al., 1977). Further, the stimulation obtained by directly cross-linking oxidizable molecules (see Fig.3; Wynne et al., 1976; Ravid et al., 1978; vida infra) does not make sense in terms of an oxidized passive anchor with no direct activity.

The second model (middle) addresses the issues raised by the antibody and biotin/avidin experiments (see Fig. 3). In this scheme, the oxidizable glycoproteins also carry the relevant lectin-binding sites and make up the sub-units of the activating complex. The oxidized glycoproteins are subsequently aggregated by interaction with a central anchor protein which carries

several convenient free amino groups. Lectin stimulation, and the response induced by aggregating oxidizable sites, occurs by direct cross-linking of these relevant glycoproteins. The protease inhibition could be explained by either of two possibilities; in one, the inhibition of oxidative mitogenesis results from cleavage of the central anchor protein. This would obviously not affect the Con A response, and should not affect the response obtained by direct antibody cross-linking. The other possibility is that the papain acts to cleave primarily only the 'oxidizable portion' of the relevant glycoproteins, and leaves the putative Con A receptor relatively intact. Depending on where on the LC-antigen that the MRC OX1 binds, the MRC OX1 response could also be unaffected by proteolysis. In the former case, where the protein anchor is digested, the protease effect on the subsequent labeling patterns would only be coincidental. The major draw-back of this model is that it fails to explain how indirect stimulation could occur; the responder cell in this particular scheme must carry the pertinent oxidized components.

The final model (bottom) takes into consideration all of the various experimental observations, and is a likely explanation for the triggering induced by periodate or NGO. In this scheme, the relevant, oxidizable glycoproteins carry the lectin binding sites and the amino functionalities. The direct cross-linking of the oxidizable components by either antibody, biotin/avidin, or lectin leads to direct formation of the activating complex. Indirect stimulation is mediated by interaction of the aldehydes on the inactivated stimulators with the amino groups on the

relevant components of the responders. The only possible problem associated with this model is in explaining the differential effects of papain on the Con A and oxidative mitogen responses. What must be concluded (if the same molecule carries both lectin and oxidizable sites) is that papain cleaves at a point on the peptide chain which removes most of the saccharide groups, but leaves enough sites to facilitate a Con A response. Interestingly, it may be speculated that trypsin, chymotrypsin, and thermolysin (which leave a smaller 140,000 m.w. protease fragment, compared to the 150,000 m.w. papain fragment; see Fig. 34) may all cleave at a point which causes the coincidental loss of the putative Con A receptor. Again, depending on the position of the antigenic site recognized by the MRC OX1, the stimulation by the monoclonal antibody may not be affected by the protease digestions. In fact, that MRC OX1 was initially raised against a 100,000 m.w. membrane-bound proteolytic fragment (Sunderland et al., 1979), suggests that the MRC OX1 response will be unaffected by the proteases. Of course, another explanation for the differential inhibitory effects of papain is that the Con A could act on and aggregate a completely different molecule, which would then induce cross-linking of the relevant glycoprotein/activating complex perhaps via a transglutaminase effect (Novogrodsky et al., 1978).

Since the LC-antigen is ubiquitous on lymphoid cells (Standring et al., 1978), it is tempting to assign it a role in general lymphocyte activation. However, whether it is itself an antigen receptor, a sub-unit of an activating complex which is

somehow aggregated by antigen interaction, or simply an interesting molecule in oxidative mitogenesis, remains to be established.

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