J. Biosci., Vol. 5, *Supplement* 1, December 1983, pp. 83–91.

Cell binding and mitogenic properties of the lima bean lectins

J. A. MAGNUSON, E. R. PANDOLFINO, G. R. MUNSKE, A. E. NAMEN and M. S. NISSEN

Biochemistry/Biophysics Program, Department of Chemistry, Washington State University, Pullman, Washington 99164, USA

Abstract. Two lectins, a tetramer designated LBL_4 and an octamer LBL_8 designated have been purified from the lima bean *Phaseolus lunatus*. The tetramer appears to be nonmitogenic for human lymphocytes and is a weak mitogen for bovine cells. The octamer and a chemically cross-linked form of the tetramer are good mitogens. The lima bean lectin binds to only certain sub-populations of human lymphocytes. The primary class which does not bind appears to be a sub-population of *T*-lymphocytes. Comparisons of cell binding with other lectins which bind to 2-acetamido-2-deoxy-D-galactose have been carried out. Quantitative analysis of the binding to human erythrocytes is co-operative but binding to lymphocytes is non-co-operative. These results show that there may not be a direct correlation between mitogenic stimulation and co-operative binding to membrane receptors.

Keywords. Lima bean; lectin; metal ions; lymphocytes; mitogenesis.

Introduction

The lectins from lima beans *Phaseolus lunatus* have been examined since 1945. Early studies showed them to be blood group specific, preferentially agglutinating type *A* human erythrocytes (Boyd and Reguera, 1949). These lectins are one of many which show specificity for binding 2-acetamido-2-deoxy-D-galactose (or N- acetyl-D-galactosamine).

The lectins have been purified by gel (Gould and Scheinberg, 1970) and affinity (Galbraith and Goldstein, 1972) chromatogrphy. This work by Gould and Scheinberg (1970) and Galbraith and Goldstein (1972) did much to establish a basis for understanding the protein structure. Two lectins are obtained, one of 240,000 daltons, and another of 120,000 daltons. These have been designated in earlier literature, after the order in which they elute from chromatographic columns, as component II and component III (Gould and Scheinberg, 1970). Each lectin is composed of 31,000-dalton monomers and we have designated the tetramer as LBL_4 and the octamer as LBL_8. These early studies identified two cysteine residues per monomer. Dodecyl sulphate electrophoresis with and without reducing agent led Gould and Scheinberg (1970) to propose that each subunit had one of its cysteines involved in a disulphide linkage to another subunit. Thus, each subunit should have one free sulphydryl. Each lectin is composed of disulphide-linked dimers;

Abbreviations used: LBL_4, Lima bean lectin tetramer; LBL_8, lima bean lectin octamer; Mops, morpholinopropanesulphonate; EDTA, ethylenediaminotetraacetate; bicine,N,N-bis(2-hydroxyethyl)-glycine.

LBL_4 is two dimers and LBL_8 is four dimers. Each dimer appears to represent the binding site for one 2-acetamido-2-deoxy-D-galactopyranoside. Although LBL_4 and LBL_8 are composed of identical subunits, no interconversion between the two forms occurs. It is, therefore, possible to work with one form and study its characteristics without having any of the other forms present. As is found with many other lectins, metal ions are required for saccharide binding activity.

LBL_8 is a potent mitogen for a variety of lymphocytes, while LBL_4 is a relatively poor mitogen in comparable systems (Reichert *et al.,* 1973; Ruddon *et al.,* 1974; Bessler *et al.,* 1976). The set of identical proteins differing apparently only in aggregation states presents two intersting probes for the mitogenic response. Both the lectin structure and the usefulness as a mitogen are two avenues of research to be pursued. In this report, we present some of our early results on studies with this lectin. For a more complete background survey on the lima bean lectins, see the comprehensive review on lectins by Goldstein and Hayes (1978).

Materials and methods

Lectins were prepared from Thorogreen lima beans (Pandolfino and Magnuson, 1980). Beans were ground in a blender and suspended in 0.1 M phosphate buffer, pH 6.8. After stirring for 1 h, the suspension was filtered and the filtrate was centrifuged. A 45–50% $(NH_4)_2SO_4$ fraction was obtained from this clarified extract. After suspension in 0.5 M NaCl, 0.01 M morpholinopropanesulphonate (Mops), pH 7.0, and dialysis against the same buffer, the sample was applied to an Ultragel AcA 34 column. LBL_4 obtained was pure as determined by gel electrophoresis. LBL_8 was almost pure by the same criteria, but sometimes required a further chromatography step using SP-Sephadex.

For metal ion binding studies the lima bean lectins were first demetallized by dialysis against 0.1 M ethylenediaminotetraacetate (EDTA), 0.5 M NaCl, pH 7.0. EDTA was removed by dialysis against 0.5 M NaCl, 0.01 M Mops, pH 7.0, which had been demetallized by passage over Chelex 100. Atomic absorption showed that Mn^{2+}, Ni^{2+}, Co^{2+}, and Zn^{2+} were negligible (<1% with respect to 31,000-dalton subunits) and Ca^{2+} was less than 4%.

Metal ion binding experiments were carried out by dialysing 1 ml of lectin against 4 ml of buffer containing ^{45}Ca or. ^{54}Mn. Experiments were conducted in polypropylene tubes at 5°C with gentle agitation for 5 days.

Lima bean lectins were labelled with fluorescein by treating the lectin (3–4 mg/ml) in 0.5 M NaCl, 0.05 M N,N-bis (2-hydroxyetliyl) glycine (bicine), 0.4 M 2-acetamido-2-deoxy-D-galactose, pH 8.4, with saturating concentrations of fluorescein isothiocyanate. Following treatment for 16 h at 4° C, dialysis against 0.5 M NaCl, 0.01M Mops, pH 7.0, gave labelled lectin with essentially 100% of the original agglutination activity. Other fluorescein-labelled lectins were purchased from Sigma Chemical Co., St. Louis, Missouri, USA.

LBL_4 was gross-linked with dimethyl suberimidate. To 1 ml of LBL_4, 4–10 mg/ml in 0.5 M NaCl, 0.2 M triethanolamine, 0.4 M 2-acetamido-2-deoxy–D-galactose, pH 8.5, was added 5 mmol aliquots of suberimidate. After 5 additions during a 12-h period, the reaction mixture was maintained at 4°C for another 24 h and then dialysed against 0.5 M NaCl, 0.01 M Mops, pH 7.0.

Iodination of the lectins was done following protection of the free sulphydryls with, mercuric ion. Lectin (4–10 mg/ml) in 0.3 M NaCl, 0.05 M bicine, pH 8.2, was

treated with a slight excess of $HgCl_2$ (with respect to 31,000 subunits). Lectin solution was then added to a glass scintillation vial with 150 µg of Iodogen (Pierce Chemical) coating the inside. To the solution was added 0.5–2 mCi of ^{125}I–and after 10 min at 0°C, a two-fold excess of dithiothreitol was added. This solution was extensively dialysed first against 0.05 M NaCl, 0.01 M Mops, 10^{-5} M dithiothreitol, pH 7.0, and then against phosphate-buffered saline, pH 7.2. Lectin thus treated retained 100% of its haemagglutination activity.

Human peripheral blood lymphocytes were prepared by Ficoll-Hypaque density centrifugation (Boyum, 1968). Viability of cells was always greater than 95% as determined by trypan blue exclusion. Lymphocyte fractions enriched for *T* or *B* cells were obtained by E-rosette separation (Brown *et al.,* 1976). E-rosettes were subjected to three centriragations through Ficoll-Hypaque, and residual monocytes were depleted by culturing cells overnight in glass Petri dishes. Monocyte identification was carried out with fluoresceinated dextran (Berlin and Oliver, 1980). Rhodamine-labelled rabbit $(F_{ab})_2$ fragments directed against human IgM and used here to identify *B* cells were a gift from Dr. Janet Oliver of the University of Connecticut Health Centre, Farmington, Connecticut, USA. Lymphocytes were cultured in RPMI 1640 supplemented with 100 units/ml penicillin, 100 µg/ml dihydrostreptomycin sulphate, and heat-inactivated foetal calf serum. Cells were maintained at 37°C under 5% CO_2 in microtiter plates. After 72 h, 1 µCi of [^{3}H]-thymidine (6.67 Ci/mmol) was added and cells were harvested 24 h later. In some cases, when noted, cells were cultured in Iscove-Melcher media (Iscove and Melcher, 1978). Bovine lymph node lymphocytes were obtained from lymph nodes of freshly slaughtered cattle by passage over glass wool (Peters, 1975). Cells were cultured in RPMI 1640 or Iscove-Melchers media. Human erythrocytes were obtained from normal healthy volunteers. Cells were washed in phosphate-buffered saline with 10 mg/ml human serum albumin.

For lectin binding studies, cells were suspended in phosphate-buffered saline with 10 mg/ml bovine serum albumin and 1 mg/ml glucose. ^{125}I-labelled lectin was added to lymphocytes ($5–18\times10^7$/ml) or erythrocytes ($8–20\times10^8$/ml) in plastic culture tubes and after 90 min, 330 µl of cell suspension was placed above 60 µl of Dow Corning 704 silicone oil in a 40 µl microfuge tube. Tubes were centrifuged in a Beckman Microfuge B, frozen in dry ice/acetone, and cut at the oil-water interface. Cells were pelleted below the interface, and free and bound lectin were determined directly by counting water and oil phase, respectively. Experiments to determine nonspecific binding were carried out in the presence of 0.2 M 2-acetamido-2-deoxy-D-galactose or used lectin inactivated with N-ethyl maleimide. Nonsipecific binding, always less than 10% of total, has been subtracted in all experiments reported.

Results

Characteristic of the lima bean lectins

The lectins LBL_4 and LBL_8 are similar to those described earlier by Gould and Scheinberg (1970) and Galbraith and Goldstein (1972). Gel electrophoresis under non-denaturing conditions gave a single, but different, band for each of the two lectins. Electrophoresis with dodecyl sulphate and 2-mercaptoethanol gave one band apparently identical for each of the lectins. The lectins appear to be iden-

tical in Ouchterlony diffusion tests with antibodies raised to each lectin independently (Galbraith and Goldstein, 1972; Pandolfino and Magnuson, 1980) and CD spectra of both are identical (Pandolfino and Magnuson, 1980). The two lectins, although composed of identical subunits, are not interconvertible. Homogeneous LBL_4 and homogeneous LBL_8 were subjected to various concentrations of urea and reducing agent. In analysis by non-denaturing electrophoresis at pH 4.3, after extensive dialysis against pH 7.0 buffer, no LBL_4 was found in LBL_8 solutions and *vice versa.*

Tow-dimensional gel electrophoretic analysis (O'Farrell, 1975) has been carried out on LBL_4 and LBL_8. All analysis gave identical patterns for LBL_4 and LBL_8. When the original system of O'Farrell (1975) was employed, no heterogeneity was observed for molecular weight but marked heterogeneity was observed for iso-electric points of the 31,000-dalton monomers. We also carried out the 2-D-analysis and omitted 2-mercaptoethanol and dithiothreitol from all the reagents, from initial extracts of the bean to final electrophoresis steps. We expected to observe less heterogeneity as disulphide-linked dimers should have migrated as units in the first dimension and spots with molecular weights approximately corresponding to dimers should have been observed in the second dimension. This result would have been expected if all monomers were linked to second monomers through a disulphide bond. A large amount of monomer was detected even when the lectin had not been exposed to any reducing agent.

Metal ion binding studies

Like many lectins, LBL_4 and LBL_8 require metal ions for saccharide binding activity. We chose to examine the stoichiometry of Mn^{2+} and Ca^{2+} binding to the lectins. Detailed Scatchard analysis were done on LBL_4 but only saturating stoichiometry was determined for LBL_8. This was done because of relatively low amount of LBL_8 available. Figure 1 shows the results for Mn^{2+} binding to demetallized LBL_4, Ca^{2+} binding to demetallized LBL_4 and Ca^{2+} binding to LBL_4 presaturated with Mn^{2+}. This binding study shows that there are 4 metal binding sites per tetramer of LBL_4. Two sites can be occupied by Mn^{2+} and the remaining two with Ca^{2+}. Alternately, all 4 sites can be filled with Ca^{2+}. When only Ca^{2+} is binding to the lectin, binding is positively co-operative, but co-operativity is not observed with either Mn^{2+} binding or Ca^{2+} binding to LBL_4 with Mn^{2+} already present. This interpretation has assumed that all sites might bind either Ca^{2+} or Mn^{2+}. This is the simplest interpretation but other possibilties do exist.

Mitogenic characteristics of LBL_4 and LBL_8

Mitogenic stimulation of lymphocytes by the lima bean lectins had been demonstrated early (Reichert *et al.*, 1973) and more recent reports have examined the characteristics in more detail (Ruddon *et al.,* 1974; Bessler *et al.,* 1976). We have examined the stimulation of human lymphocytes by LBL_4, LBL_8, and the cross-linked LBL_4. This product of suberimidate cross-linking was actually a mixture of octamers and higher aggregates. Unreacted LBL_4 was separated by chromatography on Ultragel AcA 34. For human lymphocytes, LBL_4 was found not to be mitogenic, LBL_8 was a good mitogen, and the cross-linked LBL_4 closely resembled LBL_8. Mitogen stimulation increased with increasing levels of lectin.

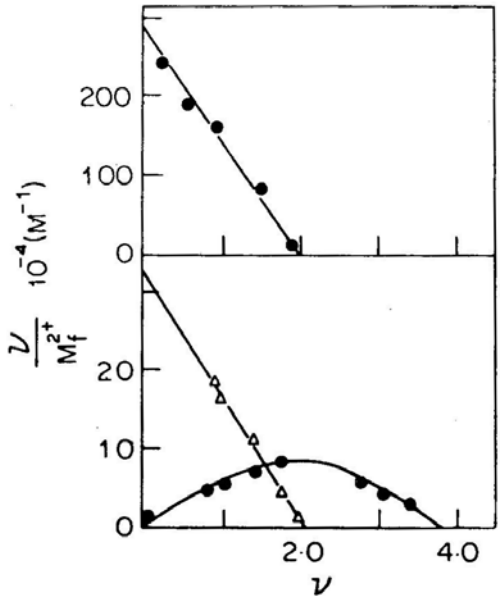

Figure 1. Scatchard plots of Mn^{2+} and Ca^{2+} binding to demetallized LBL_4. Top panel: Binding of Ca^{2+} to demetallized LBL_4 pretreated with Mn^{2+}. Panel B: Binding of Mn^{2+} to demetallized LBL_4 (Δ); binding of Ca^{2+} to demetallized LBL_4 (●). Ratio of bound metal ions to total LBL_4 molecules is represented by v.M f^{2+} is free molar concentration of either Ca^{2+} or Mn^{2+}.

For cells cultured at 2×10^6/ml, maximum stimulation was reached at concentrations of 25 μg/ml with a constant level of stimulation being observed at 25,50, and 100 μg/ml.

Fractionation of human peripheral blood lymphocytes by E-rosetting was used to give enriched populations of *T*- and *B*-lymphocytes. The *T*-lymphocyte response to LBL_8, was high, but the *B*-lymphocyte response was reduced. In addition, blasts from stimulated cultures were shown to form rosettes with sheep red blood cells showing that the blasts were stimulated *T* cells (Abauf and Daguillard, 1977). When monocytes were depleted from the E-rosette fraction by adherence to glass dishes, the mitogenic response was reduced to background but could be restored by supplementation with unfractionated cells.

A lectin with similar specificity to the lima bean lectins is soybean agglutinin. Previous studies had shown that soybean agglutinin was not mitogenic to untreated human lymphocytes but would become mitogenic if cells were treated with neuraminidase. We compared mitogenic stimulation of LBL_4 and LBL_8 with untreated and neuraminidase-treated cells. These neuraminidase-treated cells were stimulated by soybean agglutinin. No mitogenic activity was observed for LBL_4 and the activity for LBL_8 was unchanged with these treated cells.

Our work with bovine lymphocytes is not as extensive as with human cells. A dose response curve for bovine lymph node lymphocytes is shown in figure 2. LBL_8 is a good mitogen while LBL_4 is a weak mitogen.

Cell binding characteristics of the lima bean lectins

Binding to cell surfaces was first investigated by using LBL_4 and LBL_8 which had been labelled with fluorescein. Competition experiments were set up in which

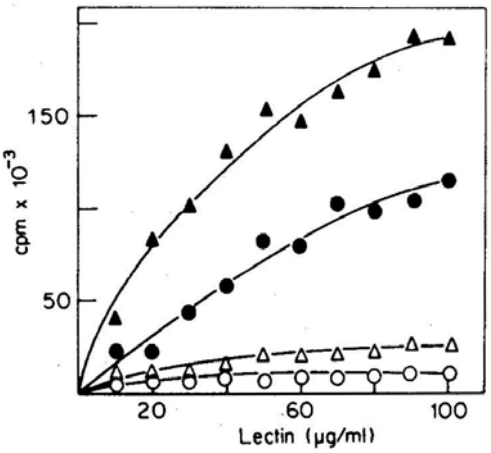

Figure 2. Mitogenic response for bovine lymph node lymphocytes stimulated with various doses of LBL. LBL_4 at various concentrations in RPMI 1640 supplemented with 50% foetal bovine serum, O;and LBL_4 in Iscove-Melchers media, Δ. LBL_8 at various concentrations in RPMI 1640 supplemented with 5% foetal bovine serum, (●); and LBL_8 in Iscove-Melchers media, (Δ).

Table 1. Binding of fluorescein-labelled lectins to lymphocytes.

Treatment[a]	Cells labelled %
F1-LBL_4	66± 5
F1-LBL_8	66±12
F1-SBA	1
F1-L-PHA	99
F1-LBL_4 *vs.* LBL_8	1
F1-LBL_8 *vs.* LBL_4	1
F1-LBL_4 *vs.* SBA	66± 1
F1-LBL_8 *vs.* L-PHA	1
F1-L-PHA *vs.* LBL_8	99

[a] In competition experiments, human lymphocytes were treated with unlabelled lectin (1.5–20 mg/ml) for 15 min at 0°C prior to addition of fluorescein-labelled lectin.

human lymphocytes were pretreated with an unlabelled lectin and then treated with a labelled lectin. Results of these experiments are shown in table 1. LBL_4 and LBL_8 only bind to approximately 70% of all human lymphocytes. They clearly bind to the same receptor as pretreatment of the cells with non-fluorescent lectin prevents binding of fluorescent lectin in subsequent treatments. Soybean agglutinin binds almost to no cells and also has no effect on subsequent binding of fluorescent LBL_8. The lectin PHA binds almost to all cells and prevents subsequent binding: of fluorescent LBL_8. LBL_8, however, does not block binding of fluorescent PHA. Of course these fluorescence experiments are not quantitative in the sense that they do not easily detect reduced levels of binding.

Neuraminidase treatment of the cells did alter some of the binding patterns. Although the mitogenic response to LBL_4 was not altered, as indicated earlier, essentially 100% of the lymphocytes bound fluorescent LBL_4 and LBL_8 after treatment. Before neuraminidase treatment, we could detect no binding by fluorescent soybean agglutinin, but after treatment essentially 100% of all lymphocytes bound the fluorescent lectin. About 40% of cells binding soybean agglutinin would not bind this agglutinin after treatment with LBL_8.

We attempted to compare the quantitative aspects of LBL_4 and LBL_8 binding to lymphocytes to establish, whether or not, the mitogenic capability of the two was reflected in binding characteristics. Bovine lymph node lymphocytes were used because large quantities of cells were needed. As shown in figure 2, LBL_8 is a potent mitogen and LBL_4 is only a weak mitogen for bovine cells. Binding analysis were done using iodinated LBL_4 and LBL_8. The results of binding assays are shown in figure 3. The binding is characterized by one type of site for both LBL_4 and LBL_8. Affinities are approximately the same for each. LBL_4 has an association constant of 4×10^5 M^{-1}, and LBL_8 has a constant of 6×10^5 M^{-1}. Approximately 50% more LBL_4 molecules can bind to the cell surface but a larger mass of LBL_8 can bind.

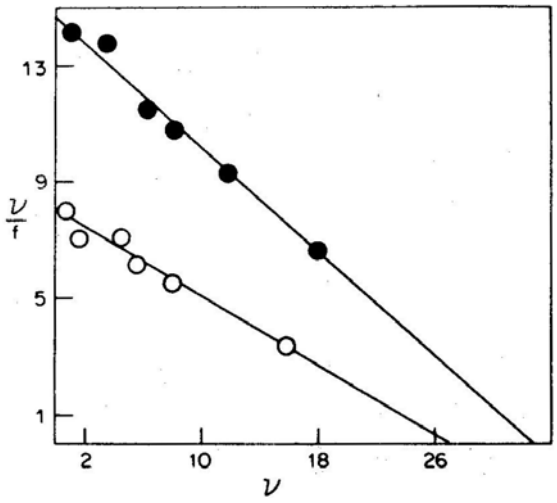

Figure 3. Representative plot for binding of LBL_4 (O) and LBL_8 (●) to bovine lymph node lymphocytes obtained from a single animal. The value of is the ratio of 60,000-dalton dimers/cell where dimer represents one saccharide binding site. Values presented are actual values multiplied by 10_{-5}. The value of f is molar concentration of dimers. The $/f$ values presented have been multiplied by 10^{-11}.

Discussion

We have described a relatively easy method for the purification of lima bean lectins to homogeneity as shown by gel electrophoresis. Mitogenic characteristics, molecular size, and sugar binding specificity are similar to those reported by earlier workers. LBL_4 and LBL_8 appear to be made of identical protomers and vary only with respect to degree of aggregation. We have subjected the lectins to two-dimensional gel electrophoresis analysis and find that a simple model in

which all monomers are cross-linked through disulphides into dimers may not hold for the lectin we have purified. We found significant amount of monomers in our preparations. Variations in lectin structure may exist when comparing different strains of lima beans. We have found that agglutination activity in extracts varies significantly with the strain of the bean. In studies using antisera to the lima bean lectin we have also observed grossly varying amounts of lectin in antibody-antigen precipitin tests. Variation with source of the lectin is not entirely surprising. The lentil lectin is a good example of previously demonstrated variations (Goldstein and Hayes, 1978).

Early experiments by Bessler and Goldstein (1974) demonstrated that each LBL_4 molecule has two saccharide binding sites and each LBL_8 had four sites. Our metal ion binding studies indicate also that stoichiometry associated with these lectins is reflected in half the number of subunits present. We find that when Mn^{2+} is present, only two Mn^{2+} and two Ca^{2+} ions bind to each LBL_4 tetramer. Although Ca^{2+} can replace the Mn^{2+}, strong interaction between the subunits is then observed.

The pairing of LBL_4 with LBL_8 poses interesting possibilities for studying cell binding and mitogenic characteristics. Our fluorescent lectin studies show that LBL_4 and LBL_8 bind to the same cell receptor. Receptor binding, in itself, is not enough to initiate the mitogenic response as LBL_4 is a weak mitogen. The work on cross-linked LBL_4 supports the concept that receptor cross-linking is very important for mitogenic stimulation of lymphcoytes. The competition studies with other lectins again indicate that cell receptor binding cannot be totally explained by specificity to a single monosaccharide.

Prujansky *et al.* (1978) have proposed a model for mitogenic stimulation which requires that binding of a mitogenic lectin to a lymphocyte surface should demonstrate positive co-operativity. We felt that comparisons of LBL_4, a very weak mitogen, and LBL_8, a potent mitogen, would be valuable in testing the above hypothesis. Interestingly we found that neither LBL_4 nor LBL_8 bound co-operatively to bovine lymphocytes, although it was easily demonstrated that binding to human type A erythrocytes is positively co-operative (unpublished observations). Based on our results, we believe that co-operativity in binding may not be an absolute requirement for mitogenesis.

References

Abauf, N. and Daguillard, F. (1977) *Cell Immunol.,* **29,** 251.
Berlin, R. D. and Oliver, J. M. (1980) *J. Cell Biol.,* **85,** .660.
Bessler, W. and Goldstein, I.J. (1974) *Arch: Biochem. Biophys.,* **165,** 444.
Bessler, W., Resch, K. and Ferber, E. (1976) *Biochem. Biophys. Res. Commun.,* **69,** 578.
Boyd, W. C. and Reguera, R. M. (1949) *J. Immunol.,* **62,** 333.
Boyum, A. (1968) *Scand. J. Clin. Lab. Invest.,* **21,** 77.
Brown, J. M., Leon, M. A. and Lightbody, J. J. (1976) *J. Immunol.,* **117,** 1976.
Galbraith, W. and Goldstein, I.J. (1972) *Biochemistry,* **11,** 3976.
Gould, N. R. and Scheinberg, S. L. (1970) *Arch. Biochem. Biophys.,* **137,** 1.
Goldstein, I. J. and Hayes, C. E. (1978) *Adv. Carbohyd. Chem., Biochem.,* **35,** 127.
Iscove, N. N. and Melcher, S. F. (1978) *J. Exp. Med.,* **147,** 923.
O'Farrell, P. H. (1975) *J. Biol. Chem.,* **250,** 4007.
Pandolfino, E. R. and Magnuson, J. A. (1980) *J. Biol. Chem.,* **255,** 870.
Peters, J. H. (1975) *Methods Cell Biol.,* **9,** 1.

Prujansky, A., David, A. and Sharon, N. (1978) *Biochim. Biophys. Acta,* **508,** 137.
Reichert, C. F., Pan, P. M., Mathews, K. P. and Goldstein, I. J. (1973) *Nature New Biol.,* **242,** 146.
Ruddon, R. W., Weisenthal, L. M., Lundeen, D. E., Bessler, W. and Goldstein, I.J. (1974) *Proa Natl. Acad. Sci. USA,* **71,** 1848.