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Monoclonal Antibodies to Rhodopsin: Characterization, Cross-Reactivity, and Application as Structural Probes†

R. S. Molday* and D. MacKenzie

ABSTRACT: Two monoclonal antibodies designated as rhodopsin (rho) 1D4 and rho 4A2 were obtained from hybridoma cells cloned after the fusion of mouse myeloma cells with spleen cells of a mouse immunized with bleached bovine rod outer segment disk membranes. These antibodies were specific for rhodopsin as determined by radioimmune labeling of bovine rod outer segment disk membrane proteins electrophoretically transferred from sodium dodecyl sulfate gels to CNBr-activated paper. Limited proteolytic digestion of rhodopsin in sealed disk membranes in conjunction with radioimmune assays indicated that the rho 1D4 antibody bound to the carboxyl-terminal segment of rhodopsin on the cytoplasmic side of disk membranes, whereas the rho 4A2 antibody bound to a determinant along the amino-terminal third of the rhodopsin polypeptide chain. Binding of the rho 4A2 antibody was sensitive to solubilization and photobleaching of rhodopsin. The rho 4A2 antibody did not bind to rhodopsin in sealed membrane disks but did bind to detergent-solubilized rhodopsin. Detergent-solubilized bleached rhodopsin was 13 times more antigenic than unbleached rhodopsin. Rhodopsin solubilized in Triton X-100 was more antigenic than rhodopsin solubilized in cholate. These results indicate that the 4A2 antibody serves as a sensitive immunological probe for structural changes of rhodopsin caused by solubilization and photobleaching. Both the rho 1D4 and 4A2 antibodies were also found to cross-react with frog rhodopsin but not *Halobacterium halobium* bacteriorhodopsin. The rho 4A2 antibody bound to the three forms of frog rhodopsin resolved by sodium dodecyl sulfate gel electrophoresis whereas rho 1D4 bound to only the two higher molecular weight frog rhodopsins. Finally, lectin inhibition studies using ^{125}I-labeled succinyl-Con A and antibody inhibition studies confirmed previous results indicating freshly prepared bovine disks were sealed with the lectin binding sites oriented toward the inside of the disk, whereas frozen–thawed disks were predominantly unsealed with both membrane surfaces exposed. Frog disk membrane vesicles were shown to have the same orientation.

Rhodopsin is the major membrane glycoprotein in rod outer segment (ROS)[1] disk membranes of vertebrate retinal rod photoreceptor cells. Biochemical and electron microscopic studies indicate that rhodopsin is a transmembrane protein (Jan & Revel, 1974; Fung & Hubbell, 1978; Molday & Molday, 1979) with the amino-terminal segment containing two Con A specific and WGA-specific carbohydrate chains (Steineman & Stryer, 1973; Hargrave, 1977; Fukuda et al., 1979) oriented toward the interior of the disks (Röhlich, 1976; Clark & Molday, 1979) and a protease-sensitive carboxyl-terminal segment and internal segments of the polypeptide chain exposed on the interdisk or cytoplasmic side (Pober & Stryer, 1975; Sale et al., 1978; Molday & Molday, 1979). Although the detailed organization of rhodopsin in the disk membrane is not yet known, it has been proposed that the rhodopsin polypeptide traverses the membrane an odd number of times, possibly as many as 7 times, in α-helical conformations (Fung & Hubbell, 1978; Albert & Litman, 1978; Michel-Villaz et al., 1979). Rhodopsin, therefore, would have structural features similar to those of bacteriorhodopsin (Henderson & Unwin, 1975; Engelman & Zaccai, 1980).

In order to obtain further insight into the structure and function of ROS disk membrane proteins, we have recently prepared and characterized several monoclonal antibodies against rhodopsin and the M_r 220 000 glycoprotein (MacKenzie & Molday, 1982). These antibodies were used in conjunction with radioimmune assays and immunoferritin electron microscopic techniques as probes to study the organization of these proteins in ROS disk membranes. We have extended these initial studies, and in this paper, we report on the binding properties and cross-reactivity of two other mo-

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[1] Abbreviations: ROS, rod outer segment(s); Con A, concanavalin A; FCS, fetal calf serum; BSA, bovine serum albumin; RIA, radioimmune assay; PBS, phosphate-buffered saline; Ig, immunoglobulin; $NaDodSO_4$, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; DEAE, diethylaminoethyl; rho, rhodopsin; WGA, wheat germ agglutinin.

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noclonal antibodies directed against different antigenic sites on bovine rhodopsin.

Experimental Procedures

ROS Disk Membranes and Proteolysis. Sealed ROS disks were isolated from frozen bovine retinas (Hormel) as previously described (Molday & Molday, 1979) by using the method of Smith et al. (1975). Disks were unsealed by repeated freezing and thawing (3–4 times) from liquid N_2. Frog ROS disk membranes were prepared from retina tissue of dark-adapted *Rana pipiens* by differential gradient centrifugation (Molday & Molday, 1979).

In proteolytic digestion studies, 300 μL of unbleached sealed disks (4–6 mg of protein/mL) was treated with an equal volume of 200–300 μg/mL TPCK-trypsin (Worthington Biochemical), 250 μg/mL *Staphylococcus aureus* protease (strain V-8, Pierce Chemical Co.), 400 μg/mL *Streptomyces griseus* protease (Sigma Chemical Co.), or 20 μg/mL thermolysin (Sigma) containing 1 mM $CaCl_2$ for 2 h at 23 °C. The enzyme digestion was stopped by the addition of 20 μL of soybean trypsin inhibitor (8 mg/mL) for trypsin, 50 μL of 0.1 M EDTA for thermolysin, and 2 mL of 0.4 mM phenylmethanesulfonyl fluoride for *S. aureus* protease and *S. griseus* protease. The disks were washed twice with 2 mL of 0.01 M Tris buffer, pH 7.4, by centrifugation at 27000*g* for 30 min, and the final pellet was resuspended in Tris buffer at a concentration of 4–6 mg/mL as determined by the Lowry protein assay with BSA as a standard. In some experiments, trypsin- and thermolysin-digested disks were treated with inhibitor as described above and used directly in competition assays along with the supernatant fraction from these samples after the disks had been centrifuged at 27000*g* for 30 min.

Immunizations and Hybridoma Cell Production. Female Balb/c mice were immunized 3 weeks apart with two intraperitoneal injections of 100 μg of bleached bovine ROS disks emulsified in 0.2 mL of Freund's complete adjuvant as recently described (MacKenzie & Molday, 1982). Mice were given a final intraperitoneal injection of the disks on day 30. Four days later, 1×10^8 spleen cells were fused with 1×10^7 NS-1 mouse myeloma cells in 1 mL of 50% poly(ethylene glycol) 1500 (BDH Chemicals). The cells were suspended in 50 mL of Dulbecco's modified Eagle's minimum essential medium containing 100 μM hypoxanthine, 0.4 μM aminopterin, 16 μM thymidine, and 20% FCS from Gibco and 5×10^6 Balb/c feeder thymocytes per mL. Aliquots (100 μL) of the cell suspension were seeded into 96-well culture plates and grown in a 37 °C humidified incubator in a 10% CO_2–90% air atmosphere. Antibody-secreting hybridomas were cloned by limiting dilution. Culture supernatants from cloned hybridoma cells were used in the experiments.

Standard Solid-Phase Radioimmune Assay. Antibody-secreting hybridomas were detected by an indirect solid-phase radioimmune assay (MacKenzie & Molday, 1982). Bleached ROS disk membranes (2.5 mg/mL protein) were solubilized with 1% (w/v) Triton X-100 and diluted to 0.25 mg/mL with distilled water. Aliquots (25 μL) were dried on flex vinyl microtiter wells at 60 °C for 2 h. The wells were then rinsed with distilled water and treated with RIA buffer consisting of 1% BSA, 1% FCS, and 0.1% NaN_3, in phosphate-buffered saline. After the RIA buffer was removed, the wells were incubated with 25 μL of hybridoma culture fluid for 60 min at 23 °C, washed in PBS, and then incubated with 25 μL of ^{125}I-labeled goat anti-mouse Ig [15–40 μg/mL; $(1–2) \times 10^6$ dpm/μg] in RIA buffer for 30–60 min at 23 °C. The plates were then rinsed extensively in PBS, cut into individual wells, and counted in a Beckman 8000 γ counter.

Solid-Phase Radioimmune Competition Assay. Competition assays were used to study the antigenic properties of disks subjected to various treatments. Briefly, 25 μL of varying concentrations of pretreated ROS disks in RIA buffer was incubated at 23 °C with 25 μL of hybridoma culture fluid diluted to a concentration which gives 80–90% saturation of binding by the standard solid-phase RIA. After 1 h, 25 μL of the mixture was removed and screened for remaining antibody activity by the standard solid-phase radioimmune assay described above.

The binding of ^{125}I-labeled succinyl-Con A (Clark & Molday, 1979) to ROS disks was measured by a similar assay. ROS disks (25 μL) of various concentrations in Tris-buffered saline containing 2 mM $CaCl_2$, 2 mM $MnCl_2$, 10 mM NaN_3, and 1% BSA were added to 25 μL of 30 μg/mL ^{125}I-labeled succinyl-Con A (4.6×10^5 dpm/μg). After 1 h at 23 °C, 25 μL of the mixture was added to Triton X-100 solubilized, immobilized disks. The wells were extensively washed in Tris-buffered saline after 60 min, and the extent of ^{125}I-labeled succinyl-Con A bound to the wells was determined. In these lectin competition assays and the radioimmune competition assays, background binding, i.e., binding of ^{125}I-labeled antibody or ^{125}I-labeled succinyl-Con A to immobilized disks in the presence of a large excess of competing disks, was less than 20% of the maximum binding and usually 5–10%. This value representing nonspecific binding was not subtracted from the data presented.

Disks used in these studies were either unbleached, completely bleached in room light, frozen and thawed 3–4 times from liquid N_2, or solubilized with 1% Triton X-100 or 1% sodium cholate as indicated. Unbleached ROS disks in cholate and Triton X-100 had A_{400}/A_{500} ratios of 0.24 and 0.25, respectively.

$NaDodSO_4$ Gel Electrophoresis and Gel Transfer. $NaDodSO_4$ solubilization of disks in the presence of excess 2-mercaptoethanol and discontinuous polyacrylamide gradient slab gel electrophoresis were carried out as previously described (Molday & Molday, 1979; MacKenzie & Molday, 1982). Routinely, 5–30 μg of disk proteins was applied to each well. Gel slices were either stained with Coomassie Blue or subjected to electrophoretic transfer as adapted from the blotting transfer procedure of Bhullar et al. (1981). Briefly, $NaDodSO_4$ gels were washed with three 100-mL changes of 0.1 M sodium phosphate, pH 7.4, containing 0.1% $NaDodSO_4$ over 20 min and two 100-mL changes of 0.02 M sodium phosphate, pH 7.4, over a 10-min period. The washed gel was sandwiched against a wet sheet of CNBr-activated paper, prepared by the method of Clarke et al. (1979), and placed in a Bio-Rad Transblot apparatus (Bio-Rad, Richmond, CA). Electrophoretic transfer was carried out at 35 V and 1.5 A at 4 °C in 0.02 M sodium phosphate, pH 7.4, for 2–4 h. Following transfers, the remaining reactive groups on the CNBr-activated paper were quenched by incubation in Tris buffer, pH 9.0, containing 0.06 M glycine and 1% BSA overnight at 23 °C. The paper containing the transferred proteins was rinsed in distilled water, air-dried, and stored in a desiccator at 4 °C until used.

Detection of Polypeptides Which Bind Monoclonal Antibodies and Con A. Transfer papers were rinsed in RIA buffer and incubated with 10 mL of hybridoma culture fluid containing the mouse monoclonal antibody for 1 h at 23 °C. The papers were then washed with several changes of PBS containing 0.4% *N*-lauroylsarcosine (Sigma) over a period of 1–2 h and subsequently incubated with 10 mL of ^{125}I-labeled goat anti-mouse Ig [$(1–2) \times 10^6$ dpm/mL] for 1 h at 23 °C.

Table I: Solid-Phase Radioimmune Assay for Disk-Specific Monoclonal Antibodies[a]

hybridoma cell supernatant	^{125}I-labeled goat anti-mouse Ig bound (dpm)	
	Triton X-100 solubilized disks	$NaDodSO_4$-solubilized disks
rho 1D4	68 290	54 547
rho 4A2	69 939	50 492
control	2 435	3 100

[a] ROS disks (2.5 mg/mL) were solubilized in either 1% Triton X-100 or 1% $NaDodSO_4$ diluted 10-fold with water, and 25 μL was dried in microtiter wells. After being washed in RIA buffer, the wells were treated with 25 μL of hybridoma cell supernatant for 30 min, washed in RIA buffer, and treated with 25 μL of 40 μg/mL ^{125}I-labeled goat anti-mouse Ig antibody (sp act. 4.42 × 10^5 dpm/mg) for 30 min. The wells were then washed, cut, and counted for ^{125}I. Hybridoma supernatant was omitted in the control although similar results were obtained when nonspecific hybridoma supernatant was used.

Finally, the papers were washed extensively with PBS containing 0.4% *N*-lauroylsacrosine over 1–2 h, air-dried, and subjected to autoradiography on preflashed Kodak Royal X-Omat film with an X-ray intensifying screen for 6–24 h at −70 °C.

Con A binding glycopeptides were detected in a single step. Transfer papers were treated with 10 mL of ^{125}I-labeled succinyl-Con A [(3–5) × 10^6 dpm/mL] in Tris-buffered saline (0.02 M Tris-HCl, pH 7.4, 0.15 M NaCl, 2 mM $CaCl_2$, 2 mM $MnCl_2$) containing 1% BSA for 1 h at 23 °C. The paper was then washed with several changes of the same buffer over 1–2 h, dried, and subjected to autoradiography.

Immunoaffinity Chromatography and Amino Acid Analysis. The 1D4 antibody was purified from ascites fluid by precipitation with 50% ammonium sulfate followed by DEAE-cellulose chromatography (Levy & Sober, 1960). Analysis by $NaDodSO_4$–polyacrylamide gel electrophoresis indicated that the antibody was pure except for a trace amount of transferrin.

The 1D4 monoclonal antibody was convalently coupled to Sepharose 2BCl by the CNBr activation method described by Cuatrecasas (1970). Approximately 5 mg of antibody was coupled to 5 mL of a packed Sepharose column. Immunoaffinity chromatography was carried out as follows: 0.5 mL of the supernatant from trypsin-treated sedimented disks was applied to the column. The column was then washed with 20 mL of 0.05 M ammonium acetate, and the bound peptide was eluted with 1 M formic acid. The fractions were lyophilized and subsequently assayed for their antigenicity by the solid-phase inhibition assay. Fractions with 1D4 antibody binding activity were pooled and subjected to analysis on a Dionex amino acid analyzer after acid hydrolysis.

Results

Analysis of Monoclonal Antibody Activity. When mouse myeloma cells were fused with spleen lymphocytes from a mouse immunized with purified bleached bovine disk membranes, several hybridoma cell lines were obtained which secreted antibodies reactive toward Triton X-100 solubilized and $NaDodSO_4$-solubilized disk membranes. Two of these monoclonal antibodies designated as rho 1D4 and rho 4A2 were characterized in detail. Table I gives typical results for the solid-phase binding assay in which detergent-solubilized disk membranes immobilized in microtiter wells were sequentially treated with hybridoma cell supernatant and ^{125}I-labeled goat anti-mouse Ig antibody. Both ρ 1D4 and 4A2 antibodies were found to bind to Triton X-100 solubilized and $NaDodSO_4$-solubilized disk membranes. Controls in which specific hybridoma cell supernatant was omitted or substituted with control hybridoma supernatant (i.e., culture supernatant from hybridoma cells which secrete nonspecific antibody) routinely gave 2–6% of the counts obtained when rho 1D4 or 4A2 hybridoma supernatants were used.

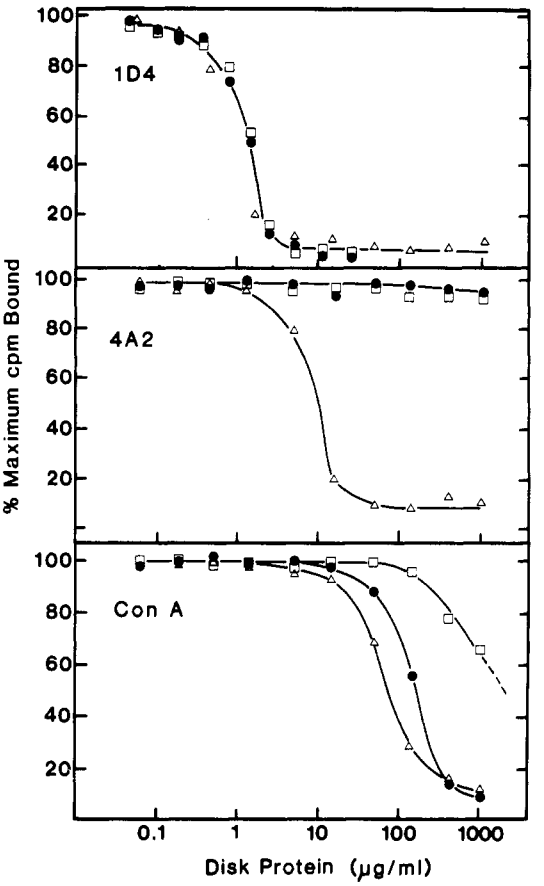

FIGURE 1: Competitive inhibition of monoclonal antibodies and Con A binding to bleached Triton X-100 solubilized, immobilized disk membranes by bleached bovine ROS disk membranes. Supernatants from hybridoma cell cultures rho 1D4 (top) or rho 4A2 (middle) or ^{125}I-labeled succinyl-Con A (bottom) were preincubated with serially diluted sealed disks (□), unsealed frozen–thawed disks (●), or Triton X-100 solubilized disks (△) and subsequently tested for their capacity to bind to Triton X-100 solubilized immobilized bovine disks by the indirect radioimmune assay using ^{125}I-labeled goat anti-mouse Ig or the direct lectin assay.

Competitive Inhibition of Monoclonal Antibody and Con A Binding to Bovine Disk Membranes. The effectiveness of bleached sealed disks, frozen–thawed disks, and Triton X-100 solubilized disks to inhibit antibody binding to Triton X-100 treated immobilized disks was studied in order to determine the relative accessibility of the antigenic sites on disk membranes. The inhibition profiles for the 1D4 and 4A2 monoclonal antibodies are shown in Figure 1.

Sealed disks, frozen–thawed disks, and Triton X-100 treated disks were all equally effective in inhibiting the 1D4 antibody binding to immobilized Triton X-100 solubilized disks.

In contrast, only Triton X-100 treated disks were found to inhibit the 4A2 antibody binding to immobilized and Triton X-100 solubilized disks at disk concentrations below 0.1 mg/mL. Sealed disks showed no inhibition even at high disk concentrations. Inhibition by frozen–thawed disks, however, was variable. For some disk preparations, freezing and thawing did not induce inhibition of the 4A2 antibody binding even at the highest disk concentrations used (1 mg/mL) as

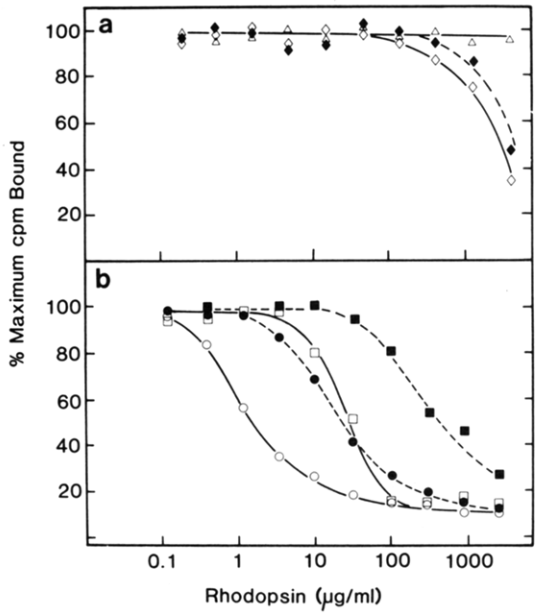

FIGURE 2: Effect of bleaching and detergent solubilization of bovine disks on rho 4A2 antibody binding as measured by solid-phase competition assays. (a) Supernatants from rho 4A2 hybridoma cell cultures were preincubated with serially diluted sealed, bleached disks (△), frozen–thawed bleached disks (◇), or frozen–thawed unbleached (◆) disks as competing antigens and subsequently tested for the capacity of the rho 4A2 antibody to bind to bleached, Triton X-100 solubilized, immobilized bovine disks by using the indirect radioimmune assay. (b) Competing antigens used in the same solid-phase radioimmune assay were unbleached (■) or bleached (□) disks solubilized in 1% cholate or unbleached (●) or bleached (○) disks solubilized in 1% Triton X-100.

shown in Figure 1. In other preparations, however, frozen–thawed disks showed some inhibition at high antigen concentrations (Figure 2). The cause of this variation is currently under investigation.

The sidedness of the disk preparations used in these assays was determined in Con A inhibition studies. As illustrated in Figure 1, a significantly higher concentration of sealed disks was required to inhibit ^{125}I-labeled succinyl-Con A binding to immobilized and detergent-treated disks compared to frozen–thawed or detergent-treated disks. Extrapolation of the inhibition curve for sealed disks to 50% inhibition (dashed line in Figure 1) indicates that the concentration of sealed disks required for 50% inhibition of ^{125}I-labeled succinyl-Con A binding is 30 times higher than for Triton X-100 treated disks. This is in agreement with quantitative ^{125}I-labeled succinyl-Con A binding studies using centrifugation assays (Clark & Molday, 1979). In these studies, 30 times more ^{125}I-labeled succinyl-Con A bound to Triton X-100 treated disks compared to sealed disks. Competitive inhibition studies also indicated that frozen–thawed disks were 10 times more effective in inhibiting ^{125}I-labeled succinyl-Con A binding than sealed disks. This is also in general agreement with previous reports (Adams et al., 1978; Clark & Molday, 1979) showing that most Con A binding sites become accessible after freezing and thawing of sealed disks.

Effect of Bleaching and Detergent Solubilization of Disks on Antibody Binding. Competitive inhibition assays were also used to study the effect of bleaching and detergent solubilization on the antigenicity of bovine disk membranes. As shown in Figure 2a, freshly prepared bleached or unbleached sealed disks did not inhibit rho 4A2 antibody binding to bleached, Triton X-100 solubilized disks immobilized on microtiter plates. At high rhodopsin concentrations, bleached and unbleached frozen–thawed disks were found to show some competitive inhibition. Fifty percent inhibition, however, required over 1000 times higher rhodopsin concentration compared with bleached Triton X-100 solubilized disks (Figure 2a,b).

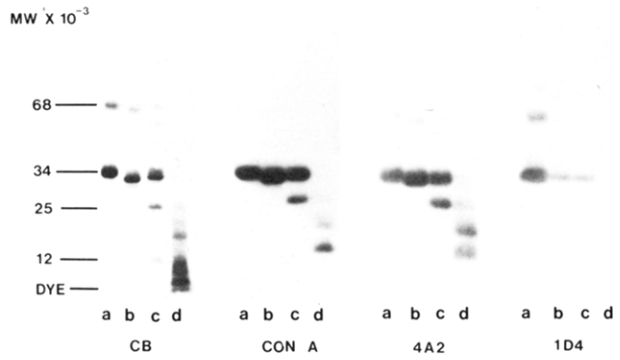

FIGURE 3: Analysis of polypeptides from untreated and protease-digested ROS disk membranes which bind Con A and monoclonal antibodies. Untreated disk membranes (gel a) and sealed disk membranes treated with 100 μg/mL trypsin (gel b), 125 μg/mL *S. aureus* protease (gel c), and 200 μg/mL *S. griesus* protease (gel d) for 2 h were washed and solubilized in $NaDodSO_4$ in the presence of 2-mercaptoethanol and subjected to electrophoresis on polyacrylamide gradient slab gels. Gel slices were either stained with Coomassie Blue (CB) or electrophoretically transferred to CNBr-activated paper and treated with ^{125}I-labeled succinyl-Con A or indirectly labeled with rho 4A2 or rho 1D4 monoclonal antibody by using ^{125}I-labeled goat anti-mouse Ig as a second antibody.

The antigenicity of detergent-solubilized disks was strongly dependent on the state of bleaching of the disks. As illustrated in Figure 2b for both cholate-solubilized and Triton X-100 solubilized disks, a 13 times higher concentration of unbleached rhodopsin was required to obtain half-maximum inhibition of rho 4A2 antibody compared to bleached rhodopsin. The type of detergent used in the solubilization of the disks also affected the antigenicity of the disks. In general, Triton X-100 solubilized disks were over 15 times more effective in inhibiting rho 4A2 antibody binding to immobilized, solubilized disks than where cholate-solubilized disks (Figure 2b).

In contrast, photobleaching and detergent solubilization had no measurable effect on the antigenicity of disks toward rho 1D4 antibodies as revealed in similar solid-phase competitive inhibition studies (not shown).

Identification of rho 1D4 and 4A2 Antigens of Bovine Disk Membranes. In order to identify and localize the antigenic sites for rho 1D4 and 4A2 antibodies, we subjected sealed ROS disks and disks treated with various proteolytic enzymes to $NaDodSO_4$ gel electrophoresis, and the separated polypeptides were electrophoretically transferred to CNBr-activated paper. The paper strips were then either directly treated with ^{125}I-labeled succinyl-Con A or indirectly labeled with rho 1D4 and 4A2 antibodies by using ^{125}I-labeled goat anti-mouse Ig as a second antibody. Results are shown in Figure 3 along with the Coomassie Blue staining patterns.

Con A and the monoclonal antibodies rho 1D4 and 4A2 were found to bind to rhodopsin, the major Coomassie Blue staining glycoprotein of apparent M_r = 34 000 and its oligomeric forms often seen on $NaDodSO_4$ gels. When sealed disks were treated with trypsin prior to $NaDodSO_4$ gel electrophoresis, rhodopsin migrated with an apparent M_r of 32 000, just ahead of undigested rhodopsin as previously reported (Molday & Molday, 1979). Both Con A and rho 4A2 antibodies bound to this large membrane-bound fragment of rhodopsin. The rho 1D4 antibody, however, did not bind to this fragment. The low degree of labeling observed for rho 1D4 corresponded to residual undigested rhodopsin of M_r 34 000.

When sealed disks were subjected to limited proteolysis with *S. aureus* protease, which cleaves peptide bonds on the carbonyl side of Glu and Asp residues (Drapeau et al., 1972), several fragments were observed, in agreement with proteolytic studies of rhodopsin from ovine disks reported by Findlay et al. (1981). As shown in Figure 3, one fragment migrated just ahead of the leading edge of undigested rhodopsin and appears to be rhodopsin minus a seven amino acid fragment from the carboxyl terminus (Findlay et al., 1981). This large rhodopsin membrane fragment (apparent $M_r \simeq 33000$) bound Con A and rho 4A2 antibody, but not rho 1D4 antibody. As in the case of trypsin digestion, the 1D4 antibody did bind to residual undigested rhodopsin. The smaller fragment of apparent M_r 25000 [designated as V8-L by Findlay et al. (1981)] was found also to bind Con A and rho 4A2 antibody, but not rho 1D4 antibody. A fragment at M_r 12000, on the other hand, did not bind Con A or rho 4A2 antibody. A low degree of 1D4 antibody binding to a band in this molecular weight region was observed when autoradiography was carried out for a relative long time, i.e., 24 h. This labeling most likely results from rho 1D4 antibody binding to the carboxyl-terminal third of rhodopsin in which the seven amino acid carboxyl terminus had not been cleaved. This, however, represents a minor proteolytic fragment.

More extensive digestion of sealed disks with *S. griseus* protease revealed that two additional Con A specific glycopeptides of rhodopsin having molecular weights less than 26000 were generated. Monoclonal antibody rho 4A2 was also observed to label these fragments.

Competitive Inhibition Studies of Trypsin- and Thermolysin-Treated Disks. In order to gain more insight into the location of the binding site for the rho 1D4 antibody along the carboxyl-terminal region of rhodopsin, we treated sealed bovine disks with either trypsin or thermolysin. The reaction was stopped by the addition of soybean inhibitor for trypsin and EDTA for thermolysin. The total mixture and the supernatant obtained after the disk membranes were centrifuged into a pellet were compared for their ability to inhibit the binding of rho 1D4 antibody to solubilized immobilized disks. Results are shown in Figure 4.

Unwashed, trypsin-treated disks and the supernatant obtained from these disks both inhibited rho 1D4 antibody binding to the same degree. This indicates that the soluble tryptic peptide from the C-terminal region of rhodopsin still serves as an antigen. However, the effective concentration required to obtain half-maximum inhibition was 16 times higher than that for undigested rhodopsin. Inhibition by unwashed thermolysin-treated disks required a 100-fold higher disk protein concentration compared to untreated disks. Inhibition by thermolysin-treated disks could be accounted for by a small amount of undigested rhodopsin observed by NaDodSO$_4$ gels. No significant inhibition was observed for the supernatant fraction from thermolysin-digested disks. Thus, the antigenic determinant for the 1D4 antibody was destroyed by the action of thermolysin.

Analysis of the 1D4 Antibody Binding Peptide. The supernatant fraction from trypsin-treated disks was applied to a rho 1D4 antibody–Sepharose column. The bound peptide which was eluted with formic acid was found to exhibit 1D4 antibody binding activity as measured by the solid-phase radioimmune competition assay. The amino acid analysis of this peptide is given in Table II.

Cross-Reactivity of rho 1D4 and 4A2 Antibodies with Frog Rhodopsin. The cross-reactivity and binding characteristics of rho 4A2 and 1D4 antibodies to frog ROS disk membranes

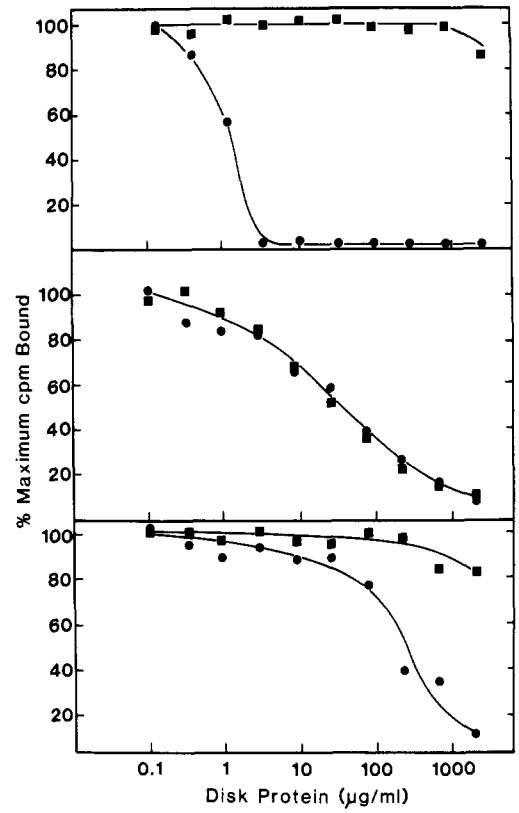

FIGURE 4: Competitive inhibition of rho 1D4 antibody binding to bleached, Triton X-100 solubilized, immobilized bovine disk membranes by untreated, trypsin-treated, and thermolysin-treated bovine disks. Supernatants from rho 1D4 hybridoma cell cultures were pretreated with the following competing antigens: (top) untreated disks (●) and supernatant (■) after the disks were centrifuged into a pellet; (middle) reaction mixture of disks digested with 150 μg/mL trypsin for 2 h and subsequently inhibited with soybean trypsin inhibitor (●) and the supernatant (■) after trypsin-treated disks were centrifuged into a pellet; (bottom) reaction mixture of disks digested with 10 μg/mL thermolysin for 2 h and inhibited with EDTA (●) and the supernatant (■) after thermolysin-treated disks were centrifuged into a pellet.

Table II: Amino Acid Composition of Immunoaffinity-Purified C-Terminal Tryptic Peptide of Bovine Rhodopsin

amino acids detected	amount present[a] (nmol of amino acid/nmol of peptide)	integral values
Asp	0.26	
Thr	2.04	2
Ser	1.19	1
Glu	2.27	2
Pro	1.22	1
Ala	2.00	2
Val	1.13	1
Phe	0.14	
Lys	0.19	

[a] Values were normalized with respect to Ala.

were also studied by using competitive inhibition assays. As shown in Figure 5, rho 1D4 antibody binding to solubilized and immobilized bovine disk membranes was competitively inhibited by bleached frog disk membranes. rho 4A2 antibody binding, however, was not inhibited by bleached or unbleached frog disk membranes. Unbleached Triton X-100 solubilized frog disks also did not inhibit rho 4A2 antibody binding up to a disk protein concentration of 1 mg/mL, whereas bleached Triton X-100 solubilized disks were highly effective.

Competitive inhibition of ^{125}I-labeled succinyl-Con A

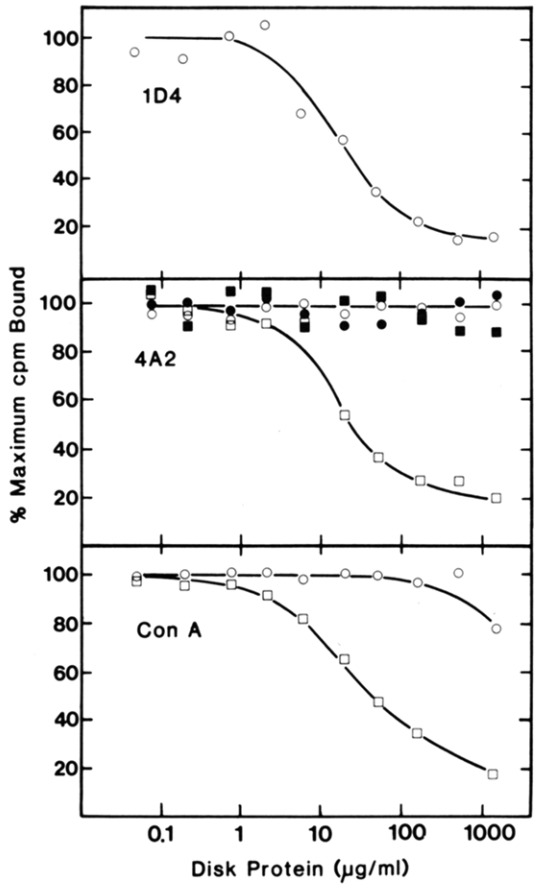

FIGURE 5: Competitive inhibition of monoclonal antibody and Con A binding to bleached, Triton X-100 solubilized, immobilized disk membranes by frog disk membranes. Supernatants from rho 1D4 (top) and rho 4A2 (middle) hybridoma cell lines or ^{125}I-labeled succinyl-Con A (bottom) were preincubated with serially diluted unbleached (●) or bleached (○) frog disk membranes or Triton X-100 solubilized unbleached (■) or bleached (□) frog disk membranes and subsequently tested for the capacity of these antibodies or succinyl-Con A to bind to Triton X-100 solubilized bovine disks by the indirect radioimmune or direct lectin binding assay.

binding to solubilized, immobilized bovine disks by frog disk membranes was also carried out to determine the accessibility of lectin receptors on frog disk vesicles. As shown in Figure 5, Triton-solubilized frog disk membranes readily inhibited ^{125}I-labeled succinyl-Con A binding, whereas intact, freshly prepared disk vesicles were less effective by several orders of magnitude. This suggests that the freshly prepared frog disk vesicles were right side out with the Con A binding site oriented toward the interior of the disk.

Analysis of rho 4A2 and rho 1D4 antibody binding to frog disk proteins separated on discontinuous $NaDodSO_4$–polyacrylamide slab gels verified that both these antibodies cross-react with frog rhodopsins (Figure 6). As previously shown (Molday & Molday, 1979; Bridges & Fong, 1980; Fatt, 1981), multiple bands of frog rhodopsin can be resolved on these gels by Coomassie Blue staining. Three forms of rhodopsin bind the rho 4A2 antibody, but only the two higher molecular weight frog rhodopsins bind the rho 1D4 antibody.

Cross-reactivity of these antibodies to bacteriorhodopsin from Triton X-100 treated *Halobacterium holobium* purple membranes was also investigated. No cross-reactivity of either rho 1D4 or rho 4A2 antibodies to bacteriorhodopsin was observed.

Discussion

Two monoclonal antibodies raised against ROS disks were found to bind specifically to rhodopsin as determined by radioimmune labeling of disk proteins electrophoretically transferred from $NaDodSO_4$ gels to CNBr-activated paper. Radioimmune competition studies and limited proteolytic digestion of rhodopsin further indicated that these antibodies bound to different antigenic sites along the polypeptide chain of rhodopsin.

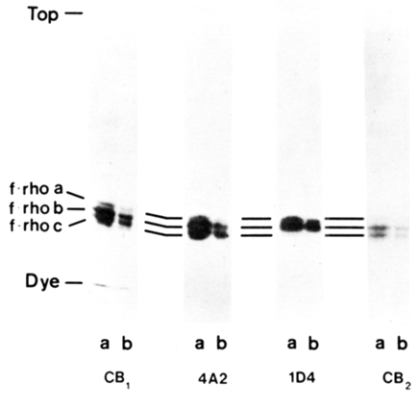

FIGURE 6: Analysis of polypeptides from frog ROS disk membranes which bind monoclonal antibodies. Purified ROS disk membranes were solubilized in $NaDodSO_4$ in the presence of 2-mercaptoethanol, and either 30 μg (gel a) or 10 μg (gel b) was subjected to electrophoresis on polyacrylamide slab gels. Gel slices were either directly stained with Coomassie Blue (CB_1) or first subjected to electrophoretic transfer to CNBr-activated paper and then stained with Coomassie Blue (CB_2). Transfer papers were treated with hybridoma supernatant 4A2 or 1D4 and subsequently with ^{125}I-labeled goat anti-mouse Ig to detect antigens.

The antigenic site for the rho 1D4 antibody is located along the carboxyl-terminal segment of rhodopsin. This conclusion is based on limited proteolytic digestion studies in which removal of a seven or nine amino acid peptide from the C terminus of rhodopsin by *S. aureus* protease or trypsin digestion results in the loss of rho 1D4 antibody binding to the large rhodopsin fragment (Figure 3). Furthermore, the C-terminal peptide released by trypsin digestion of rhodopsin in disk membranes was shown to bind to the 1D4 antibody by both immunoaffinity chromatography and solid-phase competition analysis. When the conversion of Gln to Glu during acid hydrolysis is taken into account, the amino acid composition of this peptide (Table II) corresponds to the nine amino acid C-terminal fragment of rhodopsin (Hargrave et al., 1980). The finding that thermolysin digestion of rhodopsin, which cleaves between Val-4′ and Gln-5′ as well as between Val-12′ and Thr-13′ from the C terminus (Hargrave et al., 1980), abolishes rho 1D4 antibody binding activity further suggests that the region around the 4′ and 5′ positions is required for antibody binding. On the basis of these studies and the reported C-terminal sequence of rhodopsin (Hargrave & Fong, 1977), the approximate location of the primary rho 1D4 antibody binding site is illustrated in Figure 7. The finding that undigested rhodopsin is a more effective inhibitor of rho 1D4 antibody binding relative to the trypsin peptide by over an order of magnitude, however, suggests that polypeptide conformation and/or additional amino acids along the C terminal enhances its antigenic properties.

The rho 1D4 antibody does not bind to the same site along the C-terminal region of rhodopsin as the previously reported 3D6 monoclonal antibody (MacKenzie & Molday, 1982). This is evident by comparison of the binding activities of these two antibodies to the trypsin digest of rhodopsin. Cleavage of the peptide bond between Lys-10′ and Thr-9′ has been shown to essentially abolish the 3D6 antibody binding activity to both the C-terminal peptide and the remaining rhodopsin

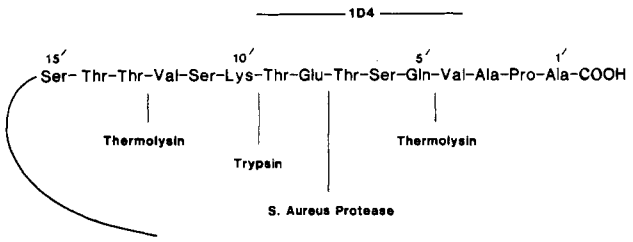

FIGURE 7: Carboxyl-terminal amino acid sequence of bovine rhodopsin based on the studies of Hargrave (1977) showing the points of peptide bond cleavage by trypsin, *S. aureus* protease, and thermolysin (Hargrave et al., 1980; Findlay et al., 1981) and the approximate location of the rho 1D4 monoclonal antibody binding to the C-terminal rhodopsin peptide based on proteolytic digestion studies and amino acid analysis reported here.

fragment, whereas the rho 1D4 antibody still binds to the C-terminal peptide. Thus, the rho 3D6 antibody is more sensitive than the rho 1D4 antibody to trypsin digestion of rhodopsin.

The rho 4A2 antibody binds to an antigenic site along the amino-terminal third of rhodopsin. Evidence for this is also based on radioimmune labeling of peptides from disks treated with proteases. In particular, Con A specific glycopeptides of molecular weight less than 26 000 obtained after *S. griseus* protease digestion of rhodopsin in disks were found to bind the rho 4A2 antibody. The carbohydrate chains which bind Con A have been shown to be conjugated to Asn residues near the amino terminus (Hargrave, 1977).

Solid-phase radioimmune competition studies yielded some insight into the accessibility of rho 4A2 antibody binding sites. The rho 4A2 antibody did not bind to bleached or unbleached rhodopsin in sealed disks and exhibited little, if any, binding to rhodopsin in frozen–thawed disks. Rhodopsin, however, was seen to be highly antigenic after it had been bleached and solubilized in detergent. The antigenicity was greatest when bleached rhodopsin was solubilized in relatively strong detergents such as Triton X-100 and $NaDodSO_4$ which cause irreversible structural changes as measured by the inability of opsin to recombine with 11-*cis*-retinal to regenerate rhodopsin (McCaslin & Tanford, 1981). On the basis of these results, it appears that the rho 4A2 antigenic determinant is inaccessible to the antibody when rhodopsin is in the disk membrane. This inaccessibility of the antigenic site may be due to the phospholipid bilayer or more likely the native rhodopsin protein conformation. When rhodopsin is bleached and solubilized in detergent, a structural change takes place such that the rho 4A2 antigenic determinant becomes available for binding by the antibody. Triton X-100 and $NaDodSO_4$ are more effective in exposing this peptide segment of rhodopsin compared to the mild detergent cholate in which regeneration of rhodopsin with 11-*cis*-retinal is possible (Henselman & Cusanovich, 1974).

It is uncertain as to whether this antigenic determinant is partially exposed in unbleached solubilized rhodopsin and promotes weak binding of the antibody or if it is totally inaccessible. In the latter case, the observed inhibition of 4A2 antibody binding by unbleached solubilized rhodopsin may be due to a small amount of bleached rhodopsin present in these preparations. This is now under investigation. In any case, the rho 4A2 antibody appears to be a sensitive probe for the conformation of rhodopsin in detergents.

Both monoclonal antibodies against bovine rhodopsin were found to cross-react with frog rhodopsin and exhibit similar binding properties. This supports the view that the structures of bovine and frog rhodopsins are similar. The rho 4A2 antibody was found to bind to the three forms of frog rhodopsin, whereas rho 1D4 antibody only bound to the two higher molecular weight forms (rho a and rho b). This supports earlier proteolytic digestion studies (Molday & Molday, 1979) suggesting that rho c does not have the characteristic trypsin-sensitive carboxyl-terminal segment which binds the rho 1D4 antibody.

Finally, these studies lead to further insight into the orientation of disk membrane vesicles. Solid-phase competitive inhibition studies using ^{125}I-labeled succinyl-Con A confirm previous quantitative lectin binding studies (Clark & Molday, 1979) indicating that freshly prepared bovine disk membranes are predominantly right side out and sealed with the lectin binding sites oriented toward the interior of the disk. Frog disk vesicles which are formed from frog disks during preparation are also sealed with the same orientation. Solid-phase radioimmune competition studies using rho 1D4 antibody in conjunction with solid-phase lectin competition studies using Con A further confirm previous studies (Adams et al., 1978; Clark & Molday, 1979) that freezing–thawing disrupts the disks. Under the conditions of freezing–thawing described here, however, the disks are predominantly unsealed rather than inverted in orientation (Shichi, 1981) since the intradisk membrane surface is accessible to Con A and the interdisk surface is accessible to rho 1D4 binding and limited proteolytic digestion of the C terminus.

These and related immunological studies (MacKenzie & Molday, 1982; Kimura et al., 1982) point to the usefulness of monoclonal antibodies in studying the structural and functional domains of energy-transducing proteins both in membranes and in the detergent-solubilized state.

Acknowledgments

We are grateful to L. Molday for her technical assistance.

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Purification and Characterization of the Mammalian β_2-Adrenergic Receptor†

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ABSTRACT: Conditions for the effective solubilization and assay of the mammalian β-adrenergic receptor from canine lung were determined. The detergent to protein ratio and the absolute detergent concentration were key factors in releasing the membrane-bound receptor in high yield and in permitting detection of the solubilized receptor by a direct binding assay. Scatchard analysis of equilibrium binding utilizing [^{3}H]dihydroalprenolol showed a single class of binding sites with an affinity (K_D = 1.3 nM) identical with that of the membrane-bound receptor. Stereospecificity and agonist affinity were comparable and characteristic of the β_2 subtype. New affinity supports consisting of alprenolol, acebutolol, or nadolol, linked to agarose via different-length spacer arms and substituted in the 1-6 mM range, were synthesized. By comparison with values obtained from direct ^{14}C-labeled ligand incorporation, radioimmunoassay indicated that only 10-20% of the resin-immobilized ligand was available for binding. Each of the three affinity supports adsorbed the receptor, but biospecific elution was consistently achieved only from the resin substituted with acebutolol, the ligand that exhibited the lowest intrinsic affinity (K_D = 170 nM) for the receptor. Purified receptor from canine lung eluted from an AcA34 column equilibrated in 0.1% digitonin, with a Stokes radius of 49 Å. The sedimentation coefficient of the receptor–digitonin complex was measured at 7.1 S by utilizing 5-20% sucrose gradients. The entire yield of purified receptor from an entire lung was then subjected to sodium dodecyl sulfate gel electrophoresis under reducing conditions. A major band of 52 000-53 000 daltons was visualized, and coincident binding activity was detected with the use of the photoaffinity label [^{3}H]acebutolol azide, indicating that this subunit contains the hormone-binding site.

The pharmacologic properties of the β-adrenergic receptor have been extensively investigated. Over the past several years, several groups have had success in solubilizing and assaying β-receptor activity in solution (Caron & Lefkowitz, 1976; Vauquelin et al., 1977; Kleinstein & Glossman, 1978; Schocken et al., 1980). Other investigators (Haga et al., 1977; Strauss et al., 1979) have attempted to label the receptor prior to solubilization because they were unable to measure specific binding in the presence of detergent. Two groups (Limbird & Lefkowitz, 1977, 1978; Vauquelin et al., 1979) have explored the molecular characteristics of the receptor solubilized from either turkey or frog erythrocyte. Shorr et al. (1981) have recently reported on several of the biophysical parameters of a purified receptor preparation from frog erythrocyte. However, less progress has been achieved in characterizing the mammalian β-adrenergic receptor. In this paper, we detail the optimal conditions that permit the solubilization and characterization of the β-adrenergic receptor from canine lung. A new affinity purification procedure is described that employs a precursor of the β antagonist, acebutolol, and several of the physical properties of the highly purified receptor are reported.

Experimental Procedures

Materials

(−)- and (+)-alprenolol tartrate were the kind gifts of Ayerst and Hassle. [^{14}C]Alprenolol was donated by Hassle, as well. (+)- and (−)-propranolol were generously provided by ICI, Ltd., England. Acebutololamine and the ^{14}C-labeled derivative were the generous gifts of Dr. Wooldridge of May and Baker, Ltd. (±)-Nadolol and the ^{14}C-labeled derivative were kindly provided by Dr. Bruce Migdalof of Squibb. (−)-Isoproterenol, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride, poly(ethylene glycol) 8000, bovine γ-globulin, bovine serum albumin (fatty acid free), Tris-HCl,[1] lysozyme, and sodium periodate were obtained from Sigma. 3-Iminobis(propylamine) was purchased from Pfaltz and Bauer. ϵ-Aminocaproic acid, succinic anhydride, *N*-acetylhomocysteine

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[1] Abbreviations: Tris, tris(hydroxymethyl)aminomethane; NaDodSO$_4$, sodium dodecyl sulfate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

0006-2960/83/0422-0660$01.50/0