(ELISA). Furthermore, no antigens that cross-react with antiserum to WGA were detected in oat embryos prepared for immunocytochemistry (Fig. 3, A and C). Similarly, pearl millet also lacked reaction product for WGA (data not shown). Thus, WGA-like lectins are not universally found in the grass family.

Tsuda (*5*) reported that a GlcNAc-binding lectin similar to WGA in amino acid composition and molecular weight can be purified from rice bran. Since rice, a member of the Oryzoideae subfamily of the Gramineae, is only distantly related to wheat (*6*), we questioned whether antibodies to WGA would cross-react with a component of rice bran. Although a precipitin reaction between a rice bran extract and antiserum to WGA was not observed on Ouchterlony double diffusion (data not shown), we found that antiserum to WGA does bind immunospecifically to sections of rice embyros (Fig. 3, B and D). The surface and a number of internal layers of the radicle display reaction product. In contrast to wheat and rye, however, reaction product in rice is located throughout all cell layers of the coleoptile (Fig. 3B). A few cells in the embryonic leaves also display reaction product (arrowhead in Fig. 3B). Rice embryo sections treated with nonimmune serum were not stained. Moreover, our results with rice, as with those from all other species, were consistent in several different experiments. The apparent discrepancy between the immunodiffusion and immunocytochemical assays may be a function of the distant relation between rice and wheat. Whereas single or weakly recognized antigenic determinants are sufficient for the detection of cross-reactivity by immunocytochemistry, multiple determinants are necessary for the formation of immunoprecipitates during double diffusion (*7*). Since wheat and rice are only distantly related, the lectins from these grasses perhaps share few cross-reactive antigenic sites. In agreement with this interpretation, we found that antiserum to WGA binds to a component of rice bran extracts in an ELISA, a test that detects nonprecipitating antibodies.

A general localization pattern of the WGA-like lectins emerges. Organs that become externalized during germination of the embryo tend to contain lectin, at least at the periphery. The radicle, root cap, and coleorhiza were found without exception to contain lectin. The extent to which the coleoptile contains lectin, however, varies; barley lacks lectin in the coleoptile, whereas rice displays lectin-containing cells throughout this organ. Thus, despite the evolutionary conservatism of these proteins, their localization patterns have diverged.

Hemagglutinating activity and immunological cross-reactivity indicative of a WGA-like lectin were detected in wheat, barley, and rye. These grasses are grouped in the Triticeae tribe of the Pooideae subfamily of the Gramineae. In addition to these cereal grains, the Triticeae tribe includes forage and range grasses. WGA-like lectins were not detected in the Aveneae tribe of the Pooideae (oats) nor in pearl millet, a member of the Panicoideae subfamily. Our data demonstrate, however, that, in addition to the characteristics noted by Tsuda (*5*), the rice lectin is similar to WGA by immunological and histological criteria. Since rice is classified in the Oryzeae tribe of the Oryzoideae, a subfamily considered to be more primitive than the Pooideae by morphological criteria (*8*), our results suggest that WGA-like lectins may be a primitive character lost in certain lineages during the adaptive radiation of the grass family but retained in species of the Triticeae and Oryzeae tribes.

A close evolutionary relation exists among the lectins of various plant families including the Leguminosae (*9*) and the Solanaceae (*10*), as well as the Gramineae (*1*). In the latter, the capacity to bind chitin has been conserved despite the variations in patterns of localization that we observed. Thus, chitin-binding may be integrally associated with lectin function. Whether the differences in patterns of localization reflect the optimization of this function in the particular environments to which these species have adapted merits further investigation.

MICHAEL L. MISHKIND
BARRY A. PALEVITZ
NATASHA V. RAIKHEL
Botany Department,
University of Georgia, Athens 30602
KENNETH KEEGSTRA
Botany Department,
University of Wisconsin,
Madison 53706

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Light-Enhanced Free Radical Formation and Trypanocidal Action of Gentian Violet (Crystal Violet)

Abstract. *Transmission of Chagas' disease by transfusion of blood containing* Trypanosoma cruzi *has often been reported, and gentian violet, a triarylmethane dye, is widely used by blood banks in attempts to eliminate such transmission. In a study of intact trypanosomes, gentian violet was found to undergo a one-electron reduction to produce a carbon-centered free radical as demonstrated by electron spin resonance spectroscopy. Either reduced nicotinamide adenine dinucleotide or the reduced dinucleotide phosphate could serve as a source of reducing equivalents for the production of this free radical by homogenates of* Trypanosoma cruzi. *The formation of this free radical, and the trypanocidal action of gentian violet, were enhanced by light. The enhanced free radical formation may be the basic cause of the selective toxicity of gentian violet to* Trypanosoma cruzi.

Transmission of Chagas' disease by blood transfusion in endemic and nonendemic areas has often been reported (*1*). The epidemiological importance of this mechanism of transmission is increasing in endemic areas, and it may be taken into consideration as a possible cause of transmission of the disease in cases encountered in nonendemic countries such as Canada (*2*).

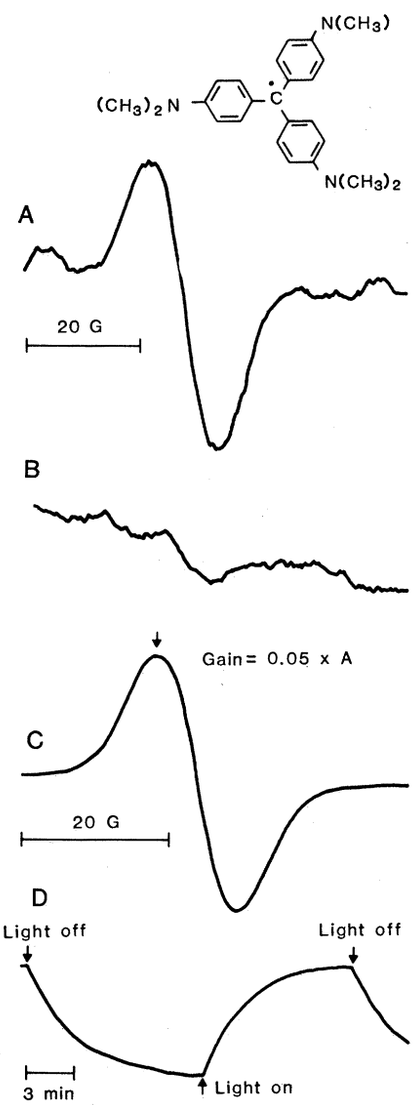
Ever since Nussensweig *et al.* (*3*) demonstrated the activity of gentian violet against the trypomastigote forms of *Trypanosoma cruzi* in vitro, this compound

has been widely used in blood banks in attempts to eliminate such transmission (*1, 4*). Gentian violet has also been used for the control of fungal and intestinal parasites in humans (*5*), since it has long been known to inhibit the growth of certain fungi, parasites, and bacteria (*3, 6*). The irreversible fixation of this dye by Gram-positive bacteria is the basis of the Gram stain (*7*). Gentian violet has also been used as an antiseptic to treat the umbilical cords of infants soon after birth (*8*).

Free radicals produced metabolically are important in the toxicity of a number of trypanocidal compounds (*9, 10*). Either the free radical metabolites of the trypanocidal agents themselves, or the superoxide anion, which results from the reduction of oxygen by the radicals, can initiate processes that lead to cell damage (*9*). The enzymatic reduction of gentian violet to a free radical metabolite by *Trypanosoma cruzi* must be considered because gentian violet can be reduced photochemically, electrochemically (*11*), or by rat liver microsomes to the tri(*p*-dimethylaminophenyl)methyl free radical (*12*). In this report we present evidence that both *T. cruzi* cells and homogenates can enzymatically reduce gentian violet to a carbon-centered free radical and that the formation of this free radical, as well as the trypanocidal action of gentian violet, are enhanced by light.

Epimastigote and trypomastigote forms of *T. cruzi*, as well as the epimastigote homogenates, were prepared as described previously (*9*) and kept on ice until use. Electron spin resonance (ESR) spectra were recorded as before (*10*) with a Varian E-9 spectrometer equipped with an E-238 TM_{110} cavity. To evaluate the growth inhibition by the drug, we cultivated the epimastigotes at 30°C for 5 days in a liquid medium (*9*), either in the dark or under incandescent light, using a General Electric tungsten lamp at a distance of 30 cm (500 W, more than 400 nm of emission). The fluence rate at this distance was 400 $\mu W/cm^2$. The final concentration of the cells was estimated as described (*9*).

When gentian violet was metabolized



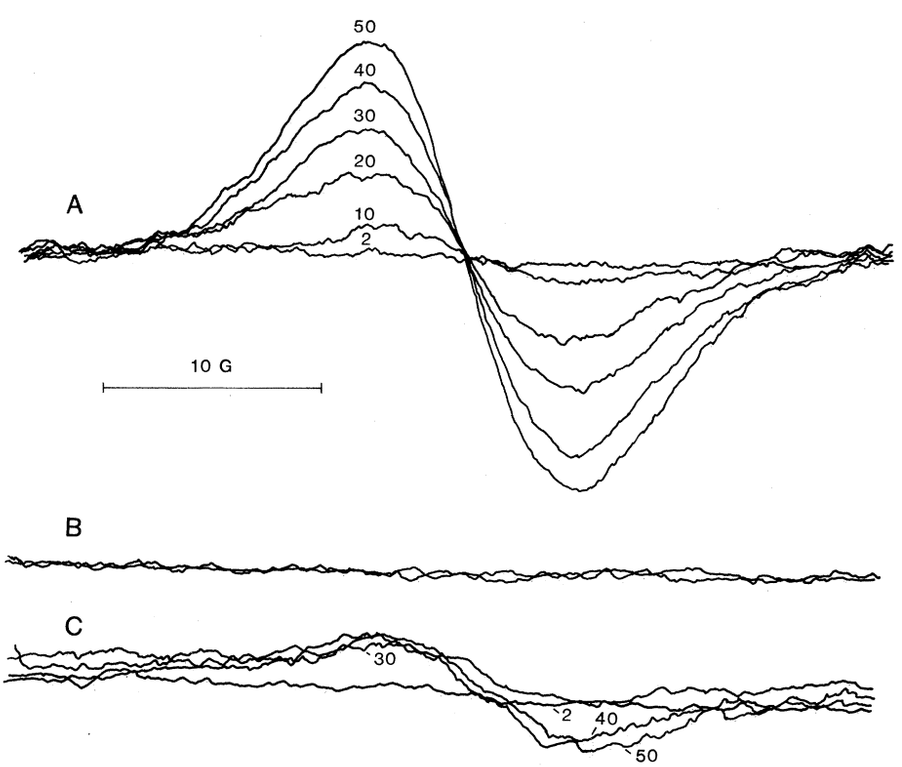


Fig. 1 (left). Electron spin resonance spectrum obtained from incubations of *T. cruzi* epimastigotes. (A) Spectrum from an incubation of epimastigotes (25 mg of protein per milliliter) prepared as described (*9*) and containing 5 m*M* gentian violet in 100 m*M* potassium phosphate buffer (*p*H 7.4). The oxygen was displaced by purging with nitrogen, and the reaction was initiated with gentian violet. The ESR spectra in (A) to (C) were recorded with a Varian E-9 spectrometer equipped with an E-238 TM_{110} cavity. The nominal microwave power was 20 mW, and the modulation amplitude was 3.5 G. The scan time was 16 minutes and the time constant was 10 seconds. (B) The same as in (A) but without gentian violet. (C) Spectrum obtained as in (A) after illuminating the flat cell with incandescent light from a slide projector (ELMO-CV-II) with a tungsten lamp of 150 W and greater than 400 nm for 10 minutes at a distance of 30 cm. The fluence rate at this distance was 1300 $\mu W/cm^2$. The nominal microwave power was 20 mW, the modulation amplitude was 3.5 G, the scan time was 16 minutes, and the time constant was 1.0 second. (D) Continuous record of the changes in the ESR signal amplitudes of the gentian violet radical during illumination, at the field position indicated by the arrow in (C). Fig. 2 (right). Electron spin resonance spectra obtained from incubations of *T. cruzi* homogenates. (A) Spectrum from a homogenate containing 5 m*M* gentian violet, an NADH generating system (1 m*M* NADH, 5.5 m*M* glucose-6-phosphate, and 1.0 U of glucose-6-phosphate dehydrogenase per milliliter) and *T. cruzi* homogenate protein (25 mg/ml) prepared as described (*9*). The oxygen was displaced by purging with nitrogen, and the reaction was initiated with NADH. The nominal microwave power was 20 mW, the modulation amplitude was 4 G, the scan time was 2 minutes, and the time constant was 3 seconds. Scans were started at six time points (2, 10, 20, 30, 40 and 50 minutes after the addition of NADH). (B) The same as in (A) but after 2 and 25 minutes with heat-denatured homogenate protein. (C) The same as in (A) but with an NADPH generating system consisting of 1 m*M* NADPH, 5.5 m*M* glucose-6-phosphate, and glucose-6-phosphate dehydrogenase (1.0 U/ml). Scans were started at four time points (2, 30, 40, and 50 minutes) after addition of NADPH.

under a nitrogen atmosphere by the intact epimastigote (Fig. 1A) or trypomastigote (not shown) stages of *T. cruzi*, a single-line ESR spectrum was obtained. This spectrum indicated the presence of an organic free radical. The line width (10 G) was similar to that found for the partially resolved spectrum detected in dimethylformamide-water mixtures, but was much broader than the line width of the free radical in aqueous solutions (1 G) (*11*). This may be explained by the binding of the free radical to the *T. cruzi* membranes. Omission of *T. cruzi* cells or gentian violet led to a total loss of signal (Fig. 1B). The free radical could not be detected in the presence of air. Care was taken to keep the incubations in the dark because after exposure to room light or the light of a slide projector (Fig. 1C) the intensity of the signal increased. The signal amplitude in Fig. 1C is approximately 17 times more intense than in the absence of light (Fig. 1A). Changes in the radical concentration during illumination showed the dependence of the signal intensity on the illuminating light (Fig. 1D). The kinetics of the ESR signal was characterized by repeatable exponential rise and decay curves with similar rates of decay of the radical in the presence or absence of light. The light-independent first-order decay constant was 0.36 per second ($t_{1/2}$ = 2 minutes).

The incubation of gentian violet with *T. cruzi* homogenates in the presence of a reduced nicotinamide adenine dinucleotide (NADH) generating system also resulted in a single-line ESR spectrum (Fig. 2A). Under the conditions used, the amplitude of the ESR spectrum increased for approximately 50 minutes after the addition of NADH. The radical formation depended on all three components: *T. cruzi* homogenates, NADH, and gentian violet. Omission of NADH or the heating of the homogenates in a steam bath for 10 minutes led to a total loss of activity (Fig. 2B). The NADH-generating system alone did not produce observable concentrations of the gentian violet radical in the dark. Although reduced nicotinamide adenine dinucleotide phosphate (NADPH) could replace NADH in these incubations, its activity was about 80 percent lower (Fig. 2C). We observed that either NADH or NADPH would support the photochemical reduction of gentian violet to the tri(*p*-dimethylphenyl)methyl free radical (data not shown) (*12*).

Growth of *T. cruzi* epimastigotes was substantially inhibited by 1.2 μM gentian violet under light (Fig. 3), since the growth constant k ($k = 0.693/T$, where T is generation time) was decreased by a factor of 60 (Fig. 3 inset). However, gentian violet was less active in the dark since 6 μM dye only decreased k by about 60 percent.

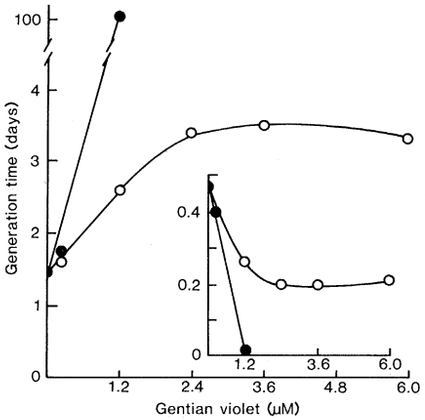


Fig. 3. Effect of gentian violet on growth of *T. cruzi* epimastigotes. The Y strain of *T. cruzi* was grown in screw-capped tubes (40 ml) in a liquid medium (*9*) at a final volume of 5 ml. Epimastigote growth was determined by cell counting with a Coulter counter. The tubes were maintained in the dark by using aluminum foil or were illuminated with an incandescent light (500 W, General Electric) at a distance of 30 cm. The temperature was maintained at 30°C. The inset shows the effect of gentian violet on the growth constant, k, in the dark (○) and in the light (●).

The formation of a carbon-centered radical metabolite of gentian violet by the action of rat liver microsomes and an NADPH-generating system has been described (*12*). Apparently, this free radical is formed in liver (at least in part) by a one-electron transfer from reduced cytochrome P-450 as indicated by its CO and metyrapone sensitivity (*12*). The information available on *T. cruzi* microsomal cytochromes is limited (*13*). The preference of NADH as the electron donor for the formation of free radicals from quinones and nitrocompounds by *T. cruzi* homogenates has been described (*9*) and may indicate the involvement of mitochondrial enzymes in the reduction of drugs to free radicals.

The biological consequences of free radical formation induced by gentian violet are unknown, but the known chemistry of the triphenylmethyl free radical suggests that the reactions of the triarylmethyl radical metabolites may be of toxicological significance. Like most carbon-centered free radicals, the triphenylmethyl radical reacts with oxygen to form a reactive peroxyl free radical (*14*), which should initiate lipid peroxidation. In addition, the triphenylmethyl free radical can be added across conjugated double bonds (*15*).

Light enhances the genetic toxicity of gentian violet (*16*). In the presence of *T. cruzi* cells, visible light photoreduces gentian violet to the same triarylmethyl free radical that is formed by enzymatic reduction, thereby enhancing its trypanocidal action. Thus, the enhanced free radical formation may be the basic cause of the selective toxicity of gentian violet to *T. cruzi*.

ROBERTO DOCAMPO*
SILVIA N. J. MORENO
Centro de Investigaciones Bioenergéticas, Facultad de Medicina, Universidad de Buenos Aires, Buenos Aires, Argentina
RAMIRO P. A. MUNIZ
FERNANDO S. CRUZ
Centro Brasileiro de Pesquisas Fisicas and *Instituto de Microbiologia, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil*
RONALD P. MASON
Laboratory of Environmental Biophysics, National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina 27709

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* Address correspondence to R.D. at Laboratory of Environmental Biophysics, National Institute of Environmental Health Sciences, P.O. Box 12233, Research Triangle Park, N.C. 27709.

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Erythrocyte and Brain Forms of Spectrin in Cerebellum: Distinct Membrane-Cytoskeletal Domains in Neurons

Abstract. *Chicken cerebellum expresses a polypeptide antigenically and biochemically related to the α subunit of spectrin, an erythrocyte membrane-cytoskeletal protein. Most of this polypeptide is associated with a brain specific spectrin subunit, γ-spectrin, and is localized in virtually all neuronal cell bodies and processes. Cerebellum also expresses polypeptides antigenically related to the β subunits of erythrocyte spectrin and these are also found in association with cerebellar α-spectrin but are confined to the plasmalemma of the neuronal cell bodies. This suggests that there is a mechanism for segregating different spectrin complexes into distinct membrane domains within a single cell.*

Red blood cells contain a protein network in close association with the cytoplasmic side of their plasma membrane. The principal component of this network is spectrin, a protein composed of two nonidentical subunits [α-spectrin, molecular weight, 240,000 (240K) and β-spectrin (220K)], which mediates linkage of actin oligomers to the plasma membrane (*1*). A number of investigators have shown that many nonerythroid cells express a polypeptide biochemically and antigenically related to erythrocyte α-spectrin (*2–5*). In avian and mammalian brain, α-spectrin specifically forms a complex with a 235K polypeptide (referred to here as γ-spectrin), which is antigenically distinct from both α- and β-spectrin and has a different peptide map (*6–8*). Brain spectrin (also termed fodrin) (*9*) has several biochemical properties similar to those of erythrocyte spectrin (*6–9*), giving rise to the suggestion that brain (αγ) spectrin is the nervous tissue analog of erythrocyte (αβ) spectrin. However, recent evidence has indicated that several nonerythroid tissues, in particular adult chicken skeletal and cardiac muscle, express polypeptides that are closely related antigenically and biochemically to the β subunit of erythrocyte spectrin and are distinct from brain γ-spectrin (*8*). In chickens, erythrocytes express two variants of β-spectrin, referred to as β and β′, in an approximate molar ratio of 5:1 (*8*). Chicken nonerythroid cells express variable amounts of polypeptides related to erythrocyte β- and β′-spectrins with the latter being the predominant polypeptide in certain cell types (*8*).

We now report that both the erythrocyte (αβ) and brain (αγ) forms of spectrin are expressed in nervous tissue. Analysis of whole extracts of chicken cerebellum by one-dimensional sodium dodecyl sulfate–polyacrylamide gel electrophoresis shows the presence of two high molecular weight polypeptides (Fig. 1). The upper component (brain α-spectrin) has the same electrophoretic mobility as chicken erythrocyte α-spectrin (Fig. 1, A and B) and has been shown previously to be antigenically related to chicken erythrocyte α-spectrin (*2–4, 6–8*). The lower component (235K, γ-spectrin) has a relative electrophoretic mobility slightly slower than the erythrocyte β- and β′-spectrins (Fig. 1, A and B) and is distinct from them by the criteria of antigenicity (*3, 7, 8*) (Fig. 1, C and D) and peptide mapping (*3, 7, 8*). Thus this polypeptide doublet appears to be identical to that previously identified as the component of axonal transport in retinal ganglion cells and termed fodrin (*9*). Immunoautoradiography of the same gel with antibodies to chicken erythrocyte β-spectrin reveals two cross-reacting polypeptides one of which comigrates with erythrocyte β′-spectrin (230K) and the other of which corresponds to erythrocyte β-spectrin; as previously shown for muscle β-spectrin (*8*), erythroid and cerebellar β-spectrins exhibit a slight difference in their relative electrophoretic mobility (Fig. 1, C and D).

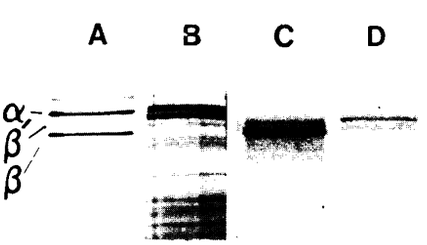

Fig. 1. Subunit composition of cerebellar spectrin. Coomassie blue stained 12.5 percent sodium dodecyl sulfate (SDS)-polyacrylamide gels (lanes A and B) and the corresponding autoradiograms after immunoautoradiography with antibodies to β-spectrin (lanes C and D) of chicken erythrocyte membranes (lanes A and C) and an SDS extract of whole chicken cerebellum (lanes B and D). Only the top portion of the gels are shown. The minor bands below β-spectrin in lanes C and D represent degradation products; SDS-polyacrylamide gel electrophoresis and immunoautoradiography were performed as described (*8, 11*). Autoradiograms were exposed without intensifying screens for 24 to 72 hours.

To examine further the presence of immunoreactive forms of β-spectrin in cerebellum, we "immunoprecipitated" spectrin from whole extracts of cerebellum with either α- or β-spectrin specific antibodies (Fig. 2). Under the conditions of immunoprecipitation used here, α-spectrin is coimmunoprecipitated with β-spectrin antiserum, and vice versa, even though each antibody is highly specific for its respective antigen. This is due to the fact that the spectrin subunits reassociate during the course of the immunoprecipitation reaction (*8, 10*). Antibodies to α-spectrin immunoprecipitate predominantly αγ-spectrin from whole cerebellar extracts (Fig. 2, lane C). Antibodies to β-spectrin do not immunoprecipitate any polypeptides detectable by Coomassie blue staining (Fig. 2, lane D). However, subsequent immunoautoradiography of the same β-spectrin immunoprecipitates with antibodies to β-spectrin establishes the presence of two polypeptides antigenically related to erythrocyte β- and β′-spectrins and that β′-spectrin is present in excess of β-spectrin in cerebellum (Fig. 2, lane I). Immunoautoradiography of the β-spectrin immunoprecipitates with α-spectrin antibodies shows also that a minor fraction of the total α-spectrin is coimmunoprecipitated as a complex with β-β′-spectrin (lane G).

To define the cell type (or types) in cerebellum that expresses the β-spectrin antigen or antigens, we used indirect immunofluorescence on frozen sections of cerebellum (*11*). Immunofluorescence with antibodies to α-spectrin reveals that, as expected (*2, 9*), the antigen is present in all three layers of the cerebellar cortex and in all discernible cell bodies and processes that populate the molecular, Purkinje cell, and granular layers (Fig. 3). In contrast, indirect immunofluorescence with antibodies to erythrocyte β-spectrin reveals that only a subpopula-