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Immunohistochemical Localization of a Phosphoprotein (B-50) Isolated From Rat Brain Synaptosomal Plasma Membranes

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OESTREICHER, A. B., H. ZWIERS, P. SCHOTMAN AND W. H. GISPEN. *Immunohistochemical localization of a phosphoprotein (B-50) isolated from rat brain synaptosomal plasma membranes.* BRAIN RES. BULL. **6**(2) 145–153, 1981.—The endogenous phosphorylation *in vitro* of at least 5 protein bands of rat brain synaptosomal membranes (SPM) is inhibited by the behaviorally active peptide $ACTH_{1-24}$. One of these proteins, the phosphoprotein band B-50, was isolated from rat brain SPM by SDS-slab gel electrophoresis. An antiserum to B-50 was raised in rabbits. The presence of antibodies to B-50 in the antiserum was demonstrated by immunoperoxidase staining of cryostat sections of a polyacrylamide gel containing the antigen. The production of antibodies was monitored by an indirect immunofluorescence technique using cryostat sections of quick-frozen rat cerebellum. Immunofluorescent staining of the molecular and granular layers was observed, whereas the white matter and the perikarya and Purkinje cells of the cerebellum were not stained. With the use of the peroxidase-antiperoxidase (PAP) method, the immunohistochemical localization of the antigen in the molecular and granular layer of the cerebellum was confirmed. Regions rich in neuropil, like the CA_1, CA_2, CA_3, CA_4 and dentate gyrus showed an intense immunostaining. Thus, in agreement with the synaptic origin of B-50, the antiserum reacted with tissue components present in brain regions rich in synaptic contacts.

Phosphoproteins Synaptosomal proteins Immunohistochemistry ACTH

N-TERMINAL peptides of ACTH are known to influence specific behaviours in animal and man [4] and to affect brain metabolism [5,16]. Zwiers *et al.* [21] demonstrated that behaviorally active ACTH peptides cause a dose-dependent inhibition of the *in vitro* phosphorylation of at least 5 protein bands in SPM [21,22]. Recently, one of these bands, B-50, a protein with an MW of 48,000 and an ACTH-sensitive protein kinase which phosphorylates B-50, have been extracted from rat brain membranes [23] and purified [24]. A great similarity in structure-activity relationship between ACTH-induced inhibition of SPM phosphorylation and ACTH-induced excessive grooming in the rat was found [6,22].

At present, information on sites in the brain where ACTH acts to induce the various effects on behavior and brain metabolism is still scarce [4]. In an effort to obtain information on the distribution in various brain regions of ACTH-sensitive protein kinase and its substrate proteins, we started a pilot study of the immunohistochemical localization of the synaptosomal phosphoprotein (B-50). Here, we report on the distribution of immuno-B-50-like components in rat cerebellum and hippocampus, using an antiserum raised against the phosphoprotein band B-50 isolated from rat SPM.

METHOD

Isolation of B-50

The antigen B-50 was isolated from SPM of rat brain after SDS-polyacrylamide slab gel electrophoresis. SPM was prepared from the forebrains of male albino rats of an inbred Wistar strain (TNO, Zeist, The Netherlands) weighing 150–200 g, as described by Zwiers *et al.* [21].

To 1 ml, containing 2–4 mg SPM protein, 0.5 ml stopmix A was added to solubilise the proteins, resulting in a mixture containing in a final concentration of 62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol 0.001% bromophenolblue and 5% β-mercaptoethanol. The solubilized SPM proteins were separated on an 11% SDS-polyacrylamide slab gel (11×9 cm). The gel electrophoresis was performed according to Lugtenberg *et al.* [12] and as described by Zwiers *et al.* [21]. The protein band B-50 was identified by its relative mobility after staining of the gel with Fast Green.

In order to confirm the identity of band B-50, aliquots of SPM (110 μg protein) were phosphorylated by incubation for 15 sec at 30°C with 7.5 μM ATP (final volume 50 μl), containing 2–6 μCi $|\gamma\text{-}^{32}P|$ ATP (approx.: 15 Ci/mmol). The incuba-

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tion medium contained 50 mM Na-acetate and 10 mM Mg-acetate (pH 6.5), with or without 10^{-4} M $ACTH_{1-24}$. The reaction was stopped by adding 25 μl stopmix A. 30 μl aliquots of this phosphorylated SPM were added to two slots of an analytical slab gel, on which samples of unphosphorylated SPM for the separation of B-50 (see above) and/or samples of isolated B-50 had also been applied. After electrophoresis, gels were fixed and stained [21]. The wet gels were sealed in a thin polyethylene bag and placed together with a Kodak X-omat X-ray film in a holder, for exposure to autoradiography [21]. After exposure, the films were developed, dried and used for locating the exact position of the band B-50 by means of its ACTH-sensitive ^{32}P-incorporation. The band B-50 was cut out and extracted from the gelstrip overnight, with 2 ml 0.1% SDS in 0.1 M Tris pH 8.6. The extract was lyophilized and desalted on a small Sephadex G-25 column. The yield was about 6–10 μg B-50 protein/mg SPM protein, while 2.5 mg SPM protein was obtained per rat brain.

Preparations of B-50 protein band identified both by protein staining and phosphorylation procedures were used for immunization. The detection of the antibody was mainly carried out with antigen isolated and characterized by the second method.

Production of Antiserum

Two white New Zealand rabbits (weighing ca. 3 kg) were immunized with intradermal injections on multiple sites of the back.

The immunogen consisted of 0.5–1.0 ml B-50 solution (100–150 μg B-50 in 50 mM Tris, pH 7.2) mixed with an equal volume of Freund's adjuvant. Complete adjuvant was used for the first injection, while boosters were given in incomplete adjuvant.

The rabbits received at first 4 immunizations, each containing ca. 100 μg B-50, at intervals of 3 weeks. These immunizations were followed by 4 injections, each containing ca. 150 μg B-50, at intervals of 5 weeks.

Two and four weeks before starting the immunization, rabbits were prebled and then bled 7 to 10 days after each immunization. The serum samples were stored at −20°C. The most potent antiserum (the 7th bleeding) was concentrated about 4 times by precipitation in 43% saturated $(NH_4)_2SO_4$, followed by dialysis against distilled water, 0.05 M Na-acetate-0.021 M acetic acid (pH 5.0), distilled water and finally against 0.15 NaCl and 15 mM NaN_3 [3].

Detection of Antibodies

Since the availability of the B-50 antigen was limited, we used the indirect immunofluorescence technique, with cryostat sections of quick-frozen rat cerebellum to monitor semi-quantitatively the production of antibodies in the serum samples taken from the rabbits. Cryostat sections (6 μm) from frozen cerebellum tissue, post fixed in acetone, were incubated with 1:80 dilutions of the primary test serum and then stained for indirect immunofluorescence using a procedure as described by Swaab *et al.* [19]. The second antibody was anti-rabbit immunoglobulin conjugated to fluorescein isothiocyanate (FITC). To check for method specificity, sections of the same series were incubated with preimmune and normal serum. The measurement of the immunofluorescence yield of the tissue sections was performed as described by Swaab *et al.* [19] using a Leitz orthoplan microscope.

The specificity for antibodies to B-50 was investigated by the SDS-Gel-Immuno-Peroxidase method (SGIP) introduced by van Raamsdonk *et al.* [20]. 10.7% polyacrylamide gels of 3 mm thickness containing per slot 12 μg B-50, 100–200 μg SPM protein and 50–200 μg proteins of rat serum were electrophorezed as described previously [21]. After the electrophoresis, gel pieces of 40×20×3 mm each containing 3 sample lanes were cut. These pieces were frozen in liquid nitrogen. Longitudinal sections of about 30–45 μm were cut from the frozen gel pieces with a cryostat microtome at a temperature of −20 to −30°C. About 30 sections were obtained and stored in a protein fixation solution, consisting of 15% glacial acetic acid and 85% ethanol. The fixative was removed from the sections by washing in buffer [20]. The sections were incubated with various dilution (up to 1:20) of the antiserum, first for 30 min at 30°C, followed by incubation overnight at 4°C. Thereafter the antibody-antigen complex was detected by a second antiserum goat anti-rabbit immunoglobulin conjugated to peroxidase. The bound peroxidase reacted with its substrate (3,3′-diaminobenzidine and hydrogenperoxide) in 50 mM Tris-HCl, pH 7.6 to form a characteristic brown reaction product [20]. The reaction was stopped in distilled water. The gel sections were stretched out on microscope slides and dried. In order to detect which bands of the separated proteins might have reacted with the antiserum, the remaining gel piece was stained in Fast Green to visualize the total protein pattern.

The immunocytochemical localization of B-50-like material in fixed tissue was performed according to the unlabeled antibody enzyme method (peroxidase-anti-peroxidase, PAP; [17]). Three male Wistar rats (~ 150 g body weight) were perfused. We used the perfusion, fixation and staining procedures as described by Buijs *et al.* [2].

Materials

Horse anti-rabbit immunoglobulin conjugated to fluorescein isothiocyanate (FITC) was obtained from the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam; goat anti-rabbit immunoglobulin conjugated to peroxidase from Nordic Immunological Laboratories, Tilburg, The Netherlands; PAP-complex from Union Chimique Belgique (UCB), Brussels, Belgium. A preparation of PAP-complex was kindly provided by Dr. C. W. Pool, Netherlands Institute for Brain Research, Amsterdam, The Netherlands. 3,3′-diaminobenzidine tetrahydrochloride was purchased from BDH Chemicals, Ltd, Poole, England; Synthetic $ACTH_{1-24}$ from Organon International BV, Oss, The Netherlands; Freund's adjuvant from Difco Laboratories, Detroit, USA.

RESULTS

Formation of Antibodies Investigated by Immunofluorescence Microscopy

In order to follow the production of antibodies, the serum samples of the immunized rabbits were screened using immunofluorescence microscopy of frozen sections of rat cerebellum. To this end, the sections were incubated with the test samples and thereafter with FITC-conjugate of anti-rabbit immunoglobulin. The serum samples showed an increase of immunofluorescence after the 6th immunization, 4 to 6 months after the first injection with B-50 was given (see Fig. 1). Examination of the immunofluorescence of the cerebellum sections demonstrated bright yellow-green immuno-

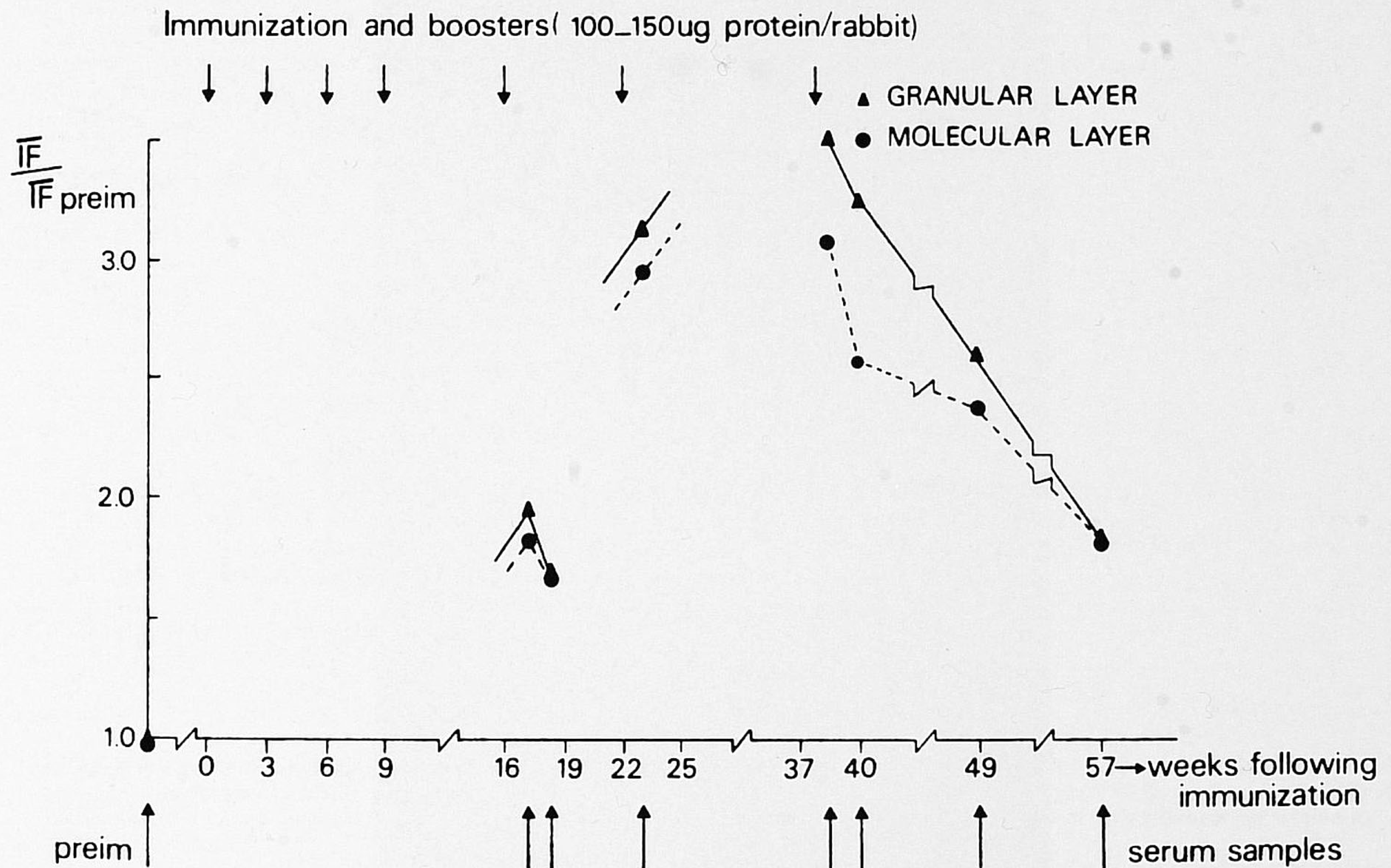

FIG. 1. Development of immunofluorescence of serum samples taken from rabbits after repeated immunization with B-50. Arrows on top: immunization schedule; Arrows at bottom; serum sampling. The sera were diluted 1:80 and fluorescence was measured on sagittal sections of quick-frozen cerebellum. The ratio of the average immunofluorescence (=$\overline{IF}$) of 20 measurements in the granular (▲) or molecular (●) layer with test serum and with preimmune serum (=$\overline{IF}$ preim) is shown as a function of time after immunization. The SEM was less than 3%.

fluorescence in the molecular (=M) and granular (=G) layers (Fig. 2). The antisera did not stain white matter (W). Control sections, incubated with preimmune serum or serum of a non-immunized rabbit, showed some background fluorescence with bright yellow autofluorescent granules in the cytoplasm of the Purkinje cells (=P) (data not shown). The increasing yield of immunofluorescence during the immunization period suggested that indeed antibodies had been formed. These antibodies reacted with components of the cerebellar tissue. When cryostat sections of cerebellum were treated with the γ-globulin fraction isolated from the antiserum with the highest yield of immunofluorescence (antiserum of 7th bleeding taken after 38 weeks), a comparable immunofluorescence staining was found (not shown). The fluorescence yield, however, was only increased by about 20% relative to the unfractionated antiserum.

Test for the Presence of Specific Antibodies Against B-50

Ouchterlony double diffusion analysis with the antisera raised against B-50 failed to show any precipitation lines with added B-50 (up to about 10 μg; data not shown). This suggested that if antibodies specific for B-50 were present in the immunofluorescent positive test samples they might only be present at low concentration. Therefore, a more sensitive method was tried, i.e. the SGIP method introduced by van Raamsdonk *et al.* [20]. When the immunoperoxidase staining of B-50 in thin gel sections was performed with serum samples collected after the 3rd to 6th booster, this resulted in staining of the whole gel section. After incubation of the gel section with preimmune serum, such brown staining was not observed (not shown).

The background staining could be removed by prior exposure of the serum to blank gel sections. Thus background staining was probably due to a reaction of antibodies to acrylamide, which could be expected to be present as contaminant in the immunogen. Taking this precaution, the antiserum diluted 1:20 (7th immunization, 38 weeks) reacted with the isolated band B-50 (Fig. 3, B1), in SPM with B-50 (Fig. 3, B2) and probably to a minor extent with some other proteins present in SPM in the same MW range [24]. This test indicated that the antiserum after the 7th immunization contained specific antibodies to B-50 proteins of SPM. Some

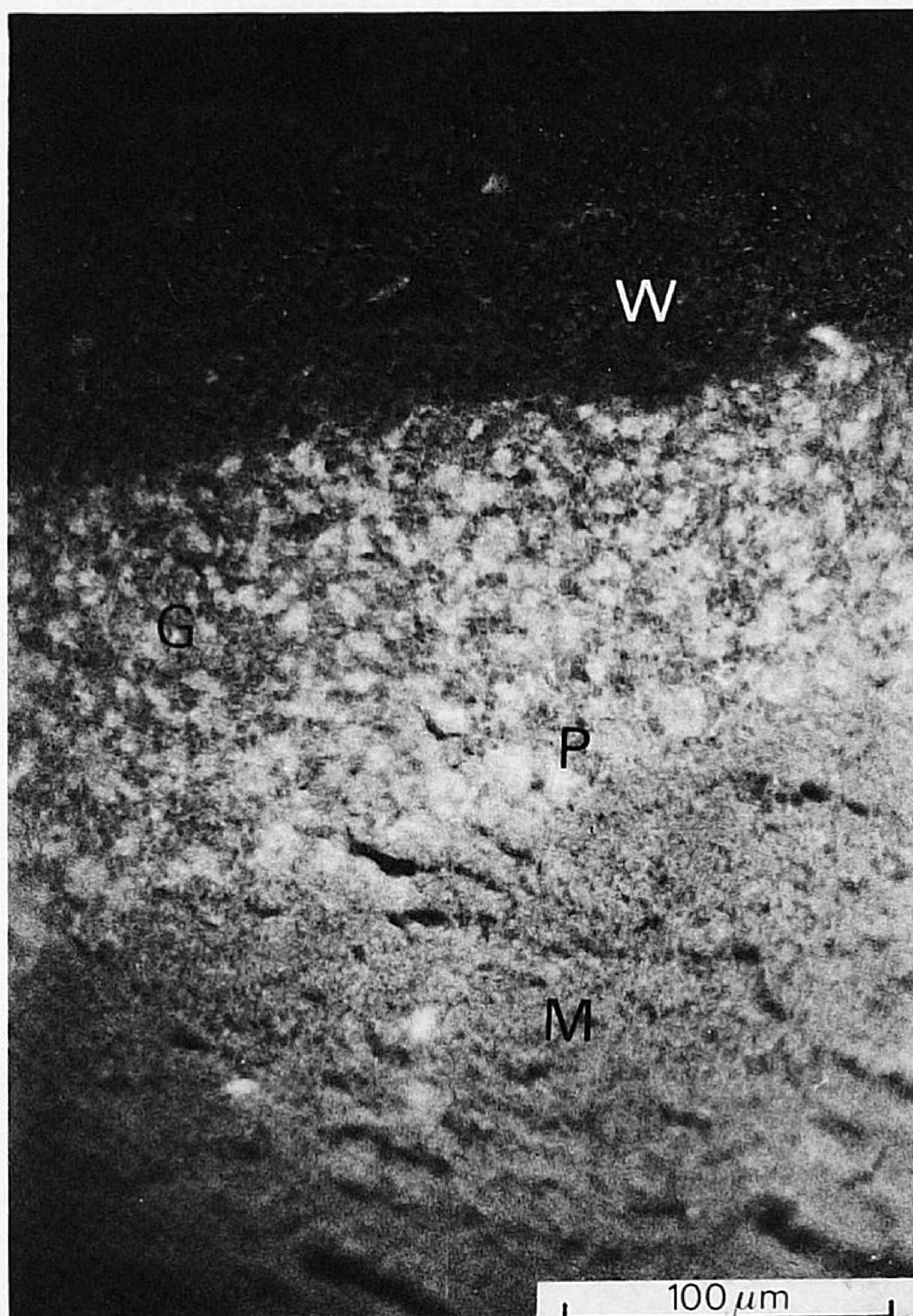

FIG. 2. Immunofluorescence of sagittal sections of rat cerebellum incubated with antiserum raised against B-50, followed by reaction with fluoresceinated horse anti-rabbit immunoglobulin (serum after 38 weeks of immunization).

staining of certain rat serum proteins was observed in sections incubated with dilutions of antiserum, but also with preimmune and normal serum (Fig. 3 B3 and C3). Since incubation with any serum sample resulted in staining of the rat serum proteins, it seemed likely that this staining was non-specific. It did not interfere with the immunocytochemical analysis, also because the serum proteins had been removed from the brain by the perfusion procedure used for the preparation of the tissue sections.

Immunocytochemical Localization of B-50-like Material in Glutaraldehyde-Formaldehyde Fixed Tissue: Detection with the Peroxidase-Anti-Peroxidase (PAP) Method

The antiserum obtained after the 7th injection, used in a dilution of 1:800 and 1:400, yielded an intense staining of the molecular layer of rat cerebellum, sectioned sagitally (Fig. 4a). In some experiments the deep folium of the molecular layer showed more staining than the regions near the surface. There was also some staining in the granular layer, and very little in the white matter. When sections of the same series were incubated with preimmune serum, no staining was seen (Fig. 4b). Figure 5 shows at higher magnification that the immunocytochemical reaction did not include the cell bodies of the Purkinje cells and the trunk of the Purkinje dendritic tree. The staining of the granular layer was localized around cells and between them. This staining may be attributed to reaction of the antibody with the large synaptic complexes, in which mossy fibers axon rosettes make contact with the dendrites of granule cells, the so-called cerebellar glomeruli.

Figure 6 shows the immunostaining of sagittal sections of the hippocampus. The neuropil layers CA_1, CA_2, CA_3, CA_4 and dentate gyrus were stained, whereas the cell bodies of the pyramidal and granular cells retained little stain. The white matter of fimbria and alveus was not stained [8]. Similar results as shown in Fig. 4–6 were obtained with the crude immunoglobulin fraction of the antiserum, used in dilution 1:1600.

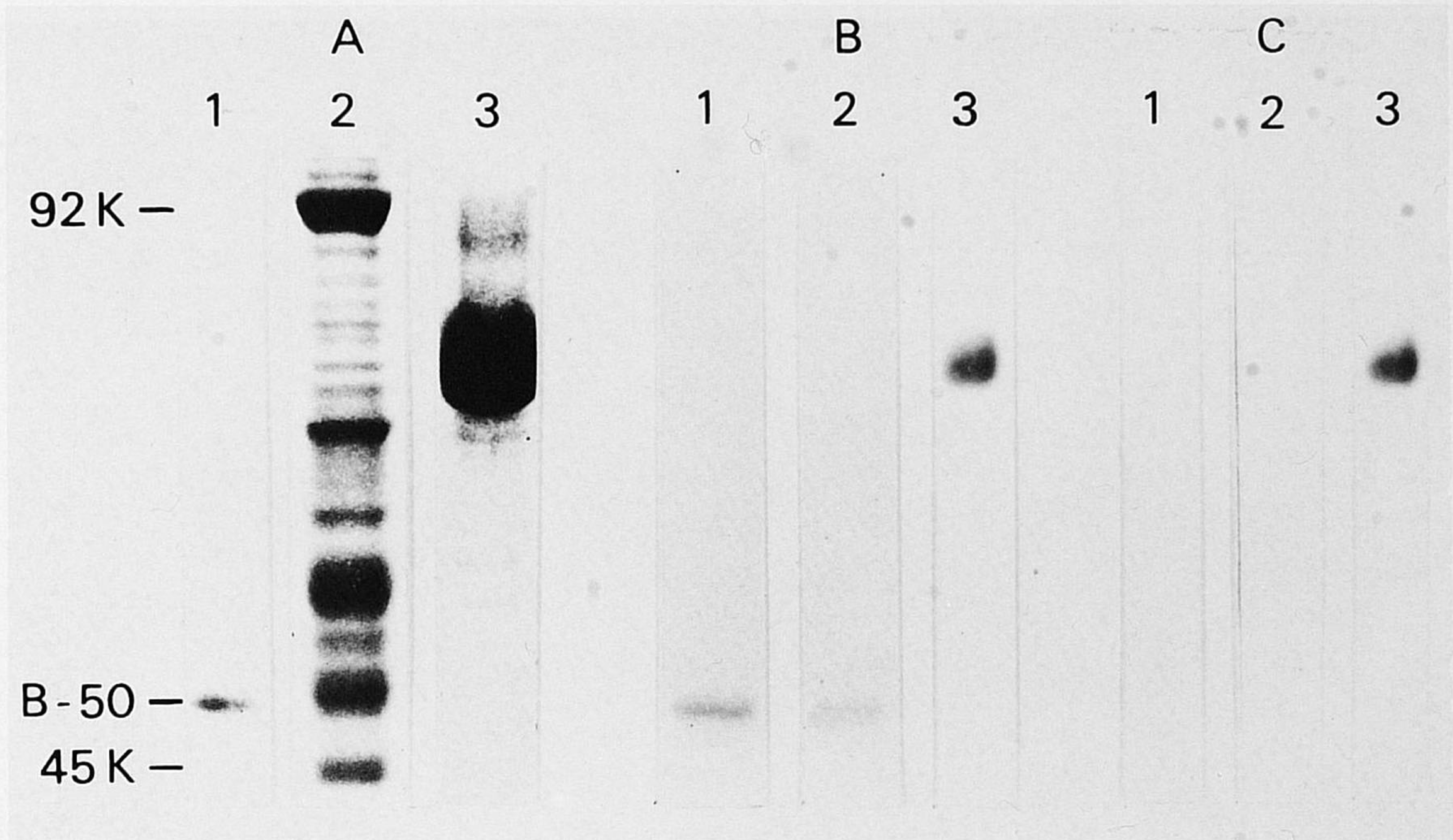

FIG. 3. Identification of antibodies to B-50 on slab gels using the SGIP method. The samples tested are: B-50 (track 1), SPM (track 2) and rat serum proteins (track 3). (A) Protein pattern after Fast Green staining; MW markers phosphorylase 92 K, ovalbumin 45K. (B) Incubation of a longitudinal gel section in antiserum to B-50 (after 7 immunizations). (C) Incubation of a longitudinal gel section in preimmune serum.

DISCUSSION

In this report we have described the production of an antiserum raised in rabbits against a phosphoprotein band (B-50) extracted from SPM and isolated after SDS-polyacrylamide gel electrophoresis. SDS-extracted and denatured proteins have successfully been used to raise a number of different antibodies [9,18]. Stumph *et al.* [18] studied in rabbits the production of antibodies to non-histone chromosomal proteins and to bovine serum albumin, both dissolved in 0.1% SDS. These antibodies were not directed against a random denaturated SDS complex, since they failed to cross-react with other proteins dissolved in SDS. In addition, the antibodies produced against the native proteins reacted with the corresponding SDS-denatured proteins. This was demonstrated by Ouchterlony double diffusion and by SDS-electrophoresis, followed by incubation of longitudinal gel pieces containing the separated proteins in the primary antiserum and subsequently in anti-rabbit immunoglobulin conjugated to FITC. The last procedure, the detection of the antibody-antigen complexes in gels, has been adapted by van Raamsdonk *et al.* [20] to the elegant and more versatile SGIP method.

The study cited above indicates that SDS treatment of proteins does not necessarily interfere with immunological properties, although it may cause changes in composition of antigenic determinants of a protein which are active under certain conditions [9]. The SGIP method [20] which we used to determine the specificity range or our antiserum, has as a fundamental aspect the cross-reactivity of antiserum to a native protein with its SDS-denaturated homologue.

In our hands, at least 6 injections with low doses of antigen were required to induce the formation of antibodies which reacted with brain tissues components as judged from the immunofluorescence. Unfortunately, the Ouchterlony double diffusion analysis failed to reveal precipitation lines of the antiserum with the antigen. The SGIP method showed that the serum which was the most active in the immunofluorescence histochemical assay, reacted with B-50 and possibly also with a few other SPM proteins in the same MW range (Fig. 3). The slight cross-reactivity with some rat serum proteins was present in both preimmune and immune serum and clearly did not interfere with the immunohistochemical studies as the preimmune serum showed very little if any staining at all. These immunohistochemical studies (Figs. 4 and 5) revealed that in cerebellar sections anti-B-50 combined strongly with tissue components of the molecular and granular layers, whereas the cell bodies of Purkinje cells and the white matter showed very little or no immunostaining. In the hippocampus, a strong immunostaining was found in regions rich in neuropil, like CA 1–4 and dentate gyrus (Fig. 6; 8).

Recently, Livett [10] reviewed the immunocytochemical

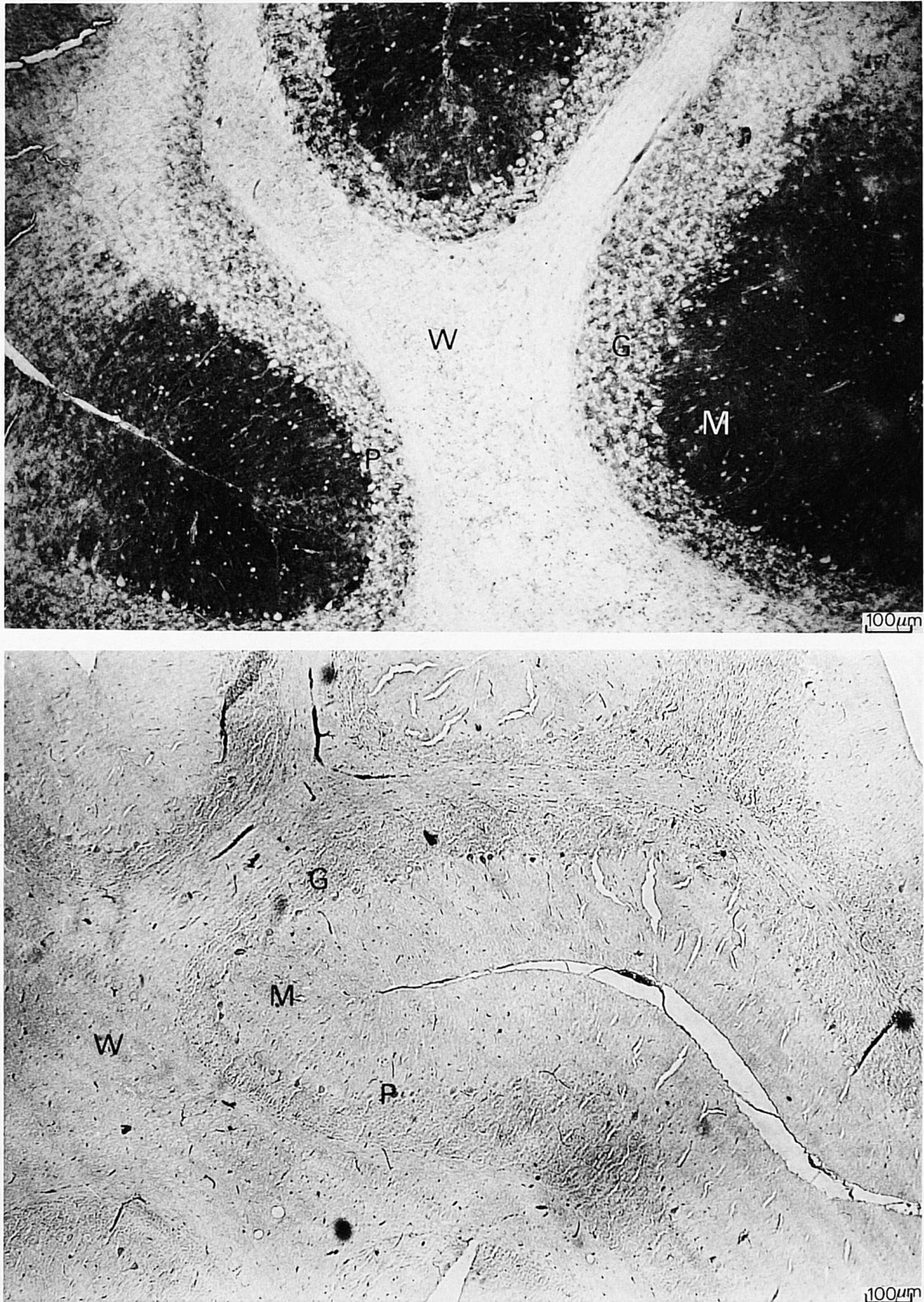

FIG. 4. Immunocytochemical localization of B-50-like material in sagittal sections of rat cerebellum incubated with antiserum to B-50 and stained by the peroxidase-anti-peroxidase (PAP) method, at low magnification (15.3×). (A) antiserum to B-50 (1:800 dil.). Note: Dark staining of the molecular layer (M) and speckled staining of the granular layer (G). Purkinje cells (P) and white matter (W) were not stained. (B) Preimmune serum (1:800 dil.). Complete absence of specific staining.

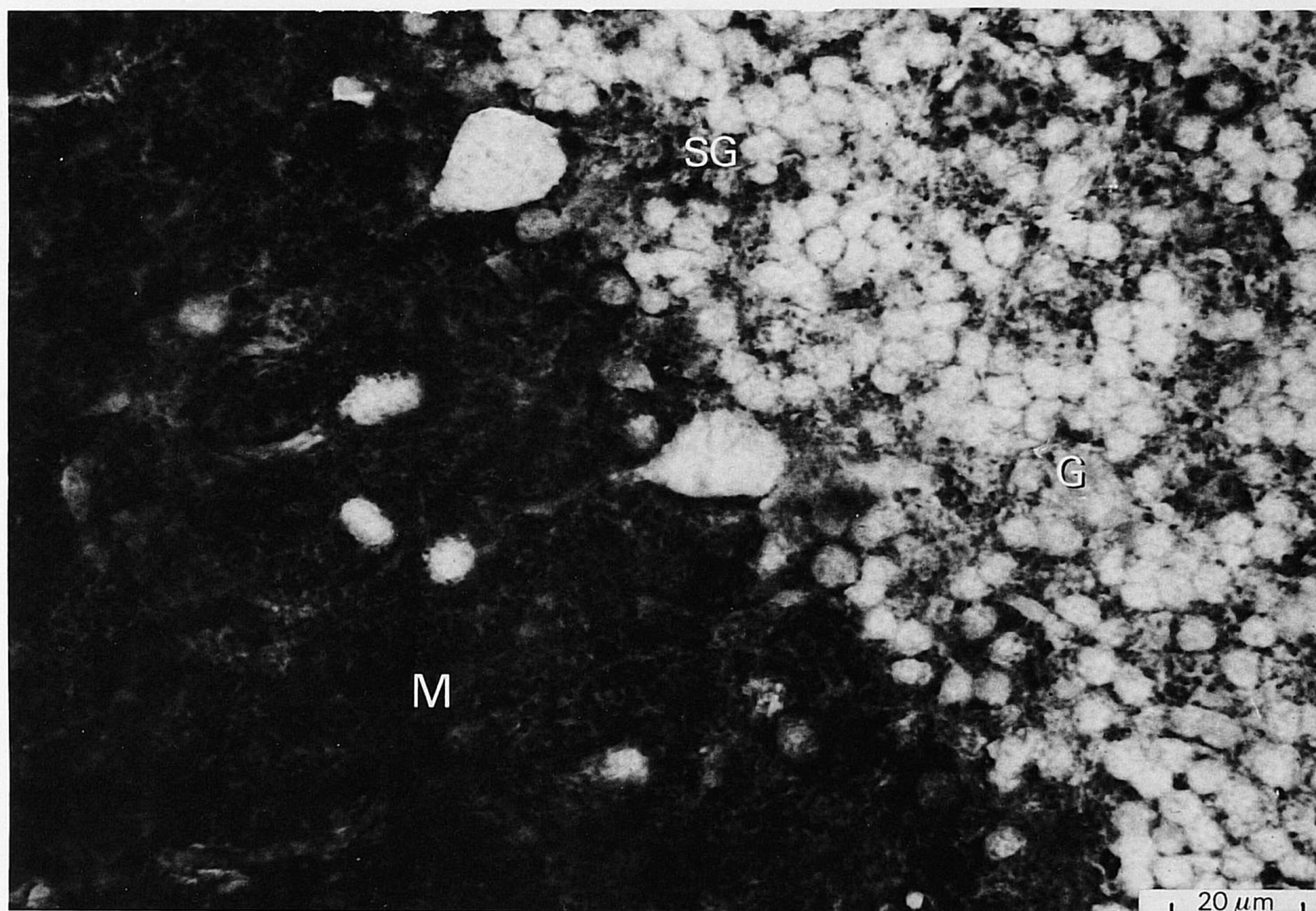

FIG. 5. Sagittal section of rat cerebellum stained by the PAP method with anti-B-50 (1:400 dil.) at high magnification (180×). The synaptic contacts of the axonal arborizations of climbing and parallel fibers with the dendritic trees of Purkinje, Basket and Golgi cells in the molecular layer (M) were stained. In the granular layer (G) the antiserum may have reacted with the synaptic glomeruli (SG).

localization studies conducted with polyspecific antisera to isolated native nerve ending fractions, SPM and synaptic vesicle membranes. The evidence of these studies [11, 13, 15] indicates that the anti-SPM sera yielded, in immunofluorescent preparations of frozen cerebellum, a highly specific staining of the molecular and granular layer. This staining was attributed to a reaction with the neuropil of the molecular layer. Since in the granular layer a great similarity existed in size and distribution of the immunostaining to mossy fibers nerve endings as revealed by a classical bouton stain [13], this immunostaining was ascribed to a reaction with the synaptic glomeruli. An irregular and speckled immunostaining between the granular cells in the granular layer (compare Fig. 6) has been described by Goridis *et al.* [7] in a study conducted with an antiserum to isolated synaptic glomeruli of rat cerebellum. When we compare our immunocytochemical data with the evidence of these studies [7,10] we suggest that the antiserum to B-50 reacted with components of the neuropil of the hippocampus and the cerebellum and with the synaptic glomeruli of the cerebellum.

The finding that the antiserum to B-50 reacted most strongly with sites known to contain many synaptic contacts is in accordance with the source of the antigen, namely synaptic plasma membranes (SPM). Indeed, the ACTH-sensitive protein kinase and its substrate have previously been detected throughout the rat brain, with the highest endogenous activity in the cortex cerebrum [21]. With the two-dimensional protein separation method as described by Zwiers *et al.* [24], we have obtained preliminary evidence to suggest that B-50 is not present in membrane fractions of liver and adrenal cortex membranes (Zwiers, unpublished). It is remarkable that a recent study [1] on the immunocytochemical localization of protein I, the endogenous substrate of the cAMP-dependent and Ca^{2+}-dependent protein kinases of brain membranes, reports a distribution of immunoreactivity in cerebellum and hippocampus of the rat, which resembles closely our findings here with phosphoprotein B-50, another endogenous synaptic protein [14].

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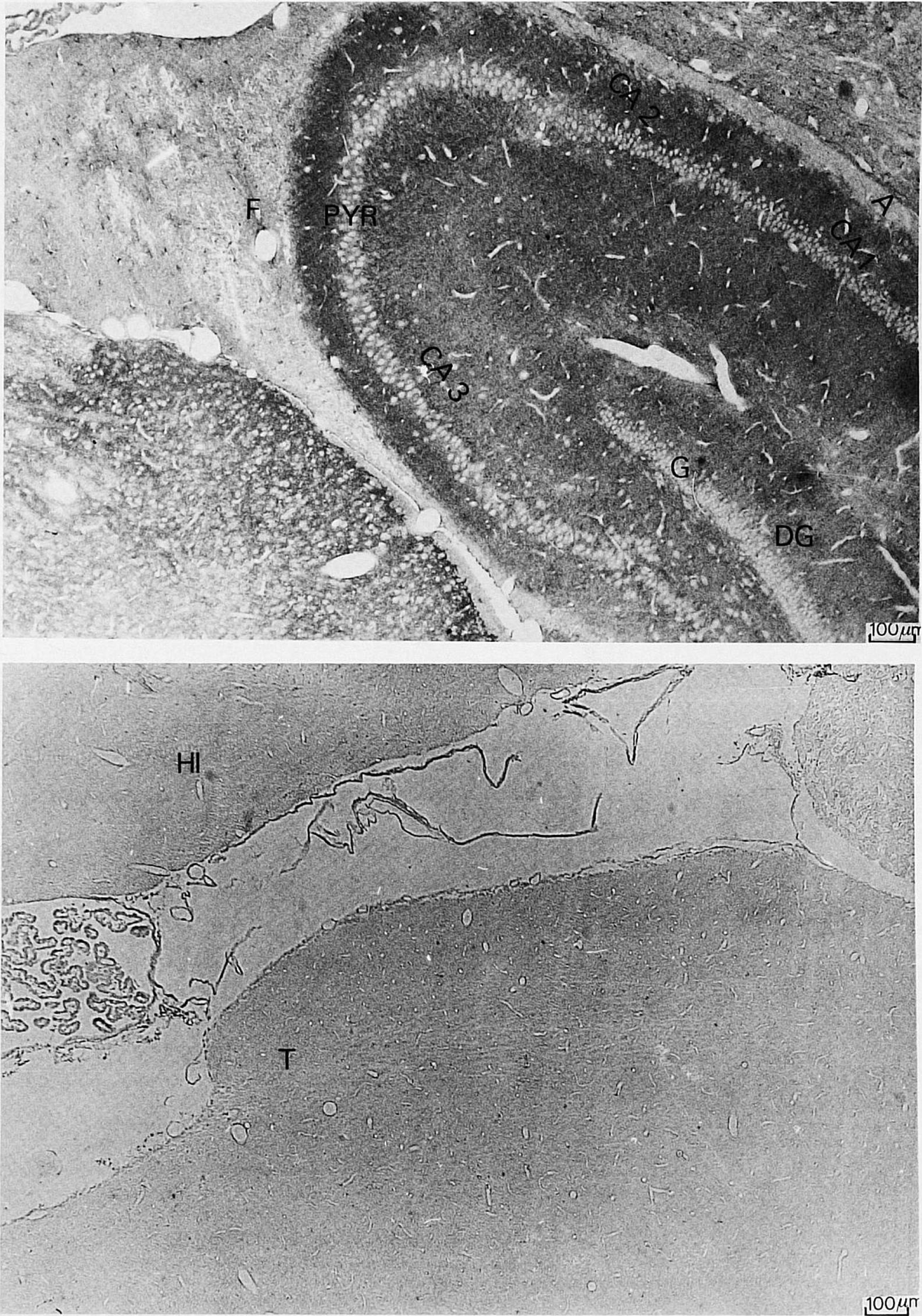

FIG. 6. Sagittal sections of rat hippocampus incubated with anti-B-50 (1:400 dil.) and stained by the PAP method. (A) section cut approx. 2100 μm parasagittal. Note staining in CA1, CA2 and CA3 and dentate gyrus (DG). Little staining was found in the cell bodies of the pyramidal cells (PYR), in the granular cells (G), in the fimbria (F) and in alveus (A). (B) Section cut 400-600 μm parasagittal, incubated with preimmune serum (1:400 dil.). HI=Hippocampus, T=Thalamus

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