Analytical Biochemistry **274,** 18–26 (1999)
Article ID abio.1999.4260, available online at http://www.idealibrary.com on IDEAL®

Quantitative Immunoblots of Proteins Resolved from Brain Homogenates: Underestimation of Specific Protein Concentration and of Treatment Effects

James P. O'Callaghan,* Hideki Imai,† Diane B. Miller,* and Aaron Minter*

**Centers for Disease Control and Prevention, National Institute for Occupational Safety and Health, Morgantown, West Virginia 26505-2888; and †National Institute for Environmental Studies, Ibaraki, Japan*

Received November 30, 1998

Estimation of the concentration of a specific protein in a biological sample often is obtained by analysis of immunoblots. We used this technique to estimate the concentration of three proteins present in homogenates of brain: glial fibrillary acidic protein (GFAP), myelin basic protein (MBP), and synapsin I. Homogenates prepared from rat brains known to contain more than 6-fold increases in GFAP, based on a GFAP enzyme-linked immunosorbent assay (ELISA), showed only small relative increases in this protein when the same samples were subjected to immunoblot analysis with polyclonal or monoclonal anti-GFAP; quantification was based on PhosphorImager analysis of [^{125}I] protein A bound to the antibodies. Estimates of GFAP in the GFAP-enriched samples approached the expected 6-fold increase when the total protein load per gel lane was reduced from 30 to 1 μg. Pure GFAP run as standard was not affected by 10-fold increases in protein load, but spiking brain homogenates with pure GFAP "quenched" the values obtained for the standard run alone. Examination of the quenching potential of pure brain tubulin, a protein that nearly comigrates with GFAP on SDS gels, showed that it may be one component of brain homogenates that contributes to masking of immunodetection of GFAP. The effect of total brain homogenate proteins on the signal obtained for a specific protein was not limited to GFAP; similar effects were observed for MBP and synapsin I. The data indicate that estimates of the concentration of a specific protein, whether as a function of its relative amount in a given protein mixture or its relative amount in one mixture compared to another, are influenced by other homogenate proteins present in the mixture.

Analysis of the relative abundance of a particular protein in a complex mixture often is a requirement in biochemical studies. Commonly, this is achieved by resolution of the protein mixture by SDS–PAGE, followed by electrophoretic transfer to a solid support membrane. Specific proteins on the membrane can then be detected with monoclonal or polyclonal antibodies that in turn are bound by reagents detectable by photometric, colorometric, fluorometric, or radiometric analysis. Estimates of the amount of a specific protein bound to the membrane can be obtained by comparing the levels of signal observed for the resolved band to the levels of signal observed for standards of known purity. Where absolute values are not required, a common practice is to compare the amount of signal obtained under one condition with that obtained from another (e.g., control vs treated), with data being expressed in arbitrary units of immunoreactivity. Since the introduction of the technique of electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets nearly two decades ago (1), quantitative versions of the immunoblot procedure have gained widespread acceptance in the biological sciences.

Previous work in our laboratory examined neuron- and glia-localized proteins as indices of toxicant-induced brain injury (2). Quantitative data obtained in these studies relied primarily on enzyme-linked immunosorbent assay (ELISA)[1] technology and solid-phase immunoassays, rather than immunoblots. Recently, when we used both ELISA and quantitative immunoblot analysis to determine the specific protein content of brain homogenates prepared from control and toxicant-exposed rats, the two methods gave strikingly different results. Here we report that standard immunoblots greatly underestimate the concentration of spe-

[1] Abbreviations used: ELISA, enzyme-linked immunosorbent assay; TMT, trimethyltin hydroxide; NC, nitrocellulose; PVDF, polyvinylidene fluoride; GFAP, bovine glial fibrillary acidic protein; MBP, myelin basic protein; EAE, experimental allergic encephalomyelitis.

0003-2697/99 $30.00

cific neuron and glial proteins in brain homogenates due to masking of the protein signal. Moreover, we show that large differences between the specific protein content of homogenates prepared from different treatment conditions, as assessed by ELISA, appear to be dramatically reduced when the same samples are subjected to immunoblot analysis using low to moderate protein loads run under standard conditions (3). Our observations indicate that immunoblot data can be affected by a number of variables associated with the technique, most notably, the amount of total protein subjected to analysis.

MATERIALS AND METHODS

Materials. Trimethyltin hydroxide (TMT) was obtained from K&K Laboratories (a division of ICN Biomedicals, Cleveland, OH). SDS (for preparing tissue homogenates and for gel electrophoresis) and all other electrophoresis reagents were purchased from Bio-Rad Laboratories (Hercules, CA). BCA protein assay reagent was purchased from Pierce Chemical Co. (Rockford, IL). Bovine serum albumin was obtained from Sigma Chemical Co. (St. Louis, MO). Nitrocellulose (NC) and polyvinylidene fluoride (PVDF) were purchased from Schleicher & Schuell (Keene, NH). Bovine glial fibrillary acidic protein (GFAP) standard was purchased from American Research Products (Belmont, MA). Rabbit anti-bovine GFAP and rabbit anti-mouse immunoglobins were obtained from Dako Corp. (Carpenteria, CA). Rabbit myelin basic protein (MBP) standard was purchased from Calbiochem-Behring Corp. (La Jolla, CA). Monoclonal anti-porcine GFAP and monoclonal anti-monkey MBP antibodies were obtained from Boehringer-Mannheim (Indianapolis, IN). Bovine synapsin I standard and monoclonal anti-bovine synapsin I (c1022) antibodies were the generous gifts of Dr. P. Greengard (Rockefeller University, New York, NY). Rat brain tubulin was obtained from twice-cycled microtubules (4) and purified by preparative gel electrophoresis. [^{125}I] r-protein A and [^{125}I]-Bolton–Hunter reagent were obtained from NEN Life Science Products (Boston, MA). Fluorescein-linked secondary antibodies (Vistra reagents) were purchased from Amersham/Molecular Dynamics (Sunnyvale, CA).

Animals. Male Long-Evans rats (200–250 g), purchased from Charles River Breeding Laboratories (Wilmington, MA) were housed individually in a temperature-controlled (22 ± 2°C) and humidity-controlled (50 ± 10%) colony room maintained on a 12-h light: 12-h dark cycle beginning at 0600 h. Rats were administered TMT (8.0 mg/kg) or its vehicle (0.9% saline) by injection into a lateral tail vein; dosages of TMT are expressed as the free base and were administered in a volume of 1.0 ml/kg body wt. Animals were sacrificed by decapitation at various postdosing intervals; most data correspond to the 21-day time point, the time of peak induction of GFAP as assessed by solid-phase immunoassay (2) or ELISA (Fig. 1).

Tissue preparation. For histology, brains were immersion-fixed in 10% neutral-buffered Formalin, paraffin-embedded, sectioned in 6.0 μM sections, and stained with cresyl violet. For immunoassays and immunoblots, the brains were immediately removed following sacrifice and placed on a cold plate (Model TCP-2, Thermoelectrics Unlimited, Wilmington, DE). With the aid of curved forceps, the hippocampal region was dissected free-hand, weighed, and homogenized by sonification (Kontes cell disrupter, Vineland, NJ) in 10 vol of hot (90–95°C) 1% (w/v) SDS and stored frozen at −80°C until analysis. Homogenates of liver, spleen, heart, and adrenal gland were prepared identically to homogenates of brain.

Total protein assay. Total protein concentration in the SDS homogenates was determined by the method of Smith *et al.* (5). Bovine serum albumin was used as the standard.

GFAP ELISA. GFAP was assayed according to the method of O'Callaghan (6). Briefly, a rabbit polyclonal antibody to GFAP was coated on the wells of Immulon-2 microtiter plates (Dynatech Laboratories, Chantilly, VA). After blocking nonspecific binding with nonfat dry milk (7), aliquots of the SDS homogenates were diluted in sample buffer and added to the wells of the plate. After appropriate blocking and washing steps, a mouse monoclonal antibody to GFAP was added to "sandwich" GFAP between the two antibodies. An alkaline phosphatase-linked antibody directed against mouse IgG was then added, and a colored reaction product was obtained by subsequent addition of enzyme substrate. Quantification was achieved by spectrometry at 405 nm using a microplate reader (UV Max running on a Soft Max program, Molecular Devices, Menlo Park, CA). This assay of GFAP has been cross-validated with another immunoassay (6) and with densitometric analysis of Coomassie blue-stained GFAP resolved by two-dimensional electrophoresis (2). Prior to this study we had not attempted to measure GFAP tissue content using immunoblot analysis.

[^{125}I] protein-A immunoblots of GFAP, MBP, and synapsin I. Aliquots of brain homogenates alone (1–30 μg total protein), GFAP, MBP, or synapsin I standards (1–1000 ng) or brain homogenates plus GFAP, MBP, or synapsin I were diluted in Laemmli sample buffer (8), boiled, and loaded on 6 or 10% SDS–polyacrylamide gels. In some cases, homogenates of liver, spleen, heart, adrenal gland, or pure rat brain tubulin were substituted for the brain homogenate. Large-format (18 × 16 × 0.15 cm) gels with 15 sample wells (6 × 1.5-mm slots/well) (Hoeffer Scientific Instruments, San Francisco, CA) were used for all experiments. Proteins then were resolved and transferred to NC (0.1 μM

porosity) or PVDF (0.2 μM porosity) membranes using the procedure of Towbin (1). Following the transfer, the proteins were subjected to immunoblot analysis according to the general procedure of Burnette (9) where the amount of specific protein bound to the membrane is proportional to the amount of [^{125}I] protein-A-bound antibody. We followed the step-by-step immunobinding protocol previously described by O'Callaghan (6). Briefly, the fixed membranes were washed in Tris-buffered saline, blocked in 0.5% gelatin, and then incubated with an antibody solution containing blocking solution plus 0.1% Triton X-100. Each antibody was diluted to 1:500, except the synapsin I monoclonal, which was used at 1:2500. The blots were washed and, if a monoclonal antibody had been used initially, a second incubation with link antibody (rabbit anti-mouse immunoglobins) (1:500) was used. The blots were then blocked again, incubated in [^{125} I] protein A in blocking solution (200 cpm/μl) containing 0.1% Triton X-100 and then washed overnight in TBS plus Triton X-100. None of the solutions were reused. The membranes were dried and the amount of bound [^{125} I] protein A was quantified by PhosphorImager analysis of the band volume using ImageQuaNT with "object average" background (Molecular Dynamics, Sunnyvale, CA). The arbitrary volume units obtained from the PhosphorImager were linear over at least a 10,000-fold concentration range of [^{125}I] protein A, as determined by direct application of [^{125}I] protein A to the membranes. In some cases, fluorescein-tagged secondary antibodies (Vistra fluorescence, Amersham Life Science and Molecular Dynamics, Sunnyvale, CA) and subsequent quantification by Storm (Model 860, Molecular Dynamics) using ImageQuanNT software were used as an alternate means for signal analysis.

Iodination of GFAP. GFAP (1 mg/ml after reconstitution) was iodinated prior to SDS–PAGE with [^{125}I]-Bolton–Hunter reagent according to the original procedure described by Bolton and Hunter (10). [^{125}I] GFAP alone (5–100 ng) or in combination with 20 μg of brain homogenate was resolved by SDS–PAGE, transferred to NC, and subjected to a "mock" immunoblot procedure by omission of [^{125}I] protein A. The amount of [^{125}I] GFAP bound to the NC then was determined by PhosphorImager.

Statistics. Data were subjected to analysis of variance (ANOVA) followed by *post hoc* comparison of treatment means (SAS Institute, Inc., 1986). Differences were considered to be significant at the $P < 0.05$ level.

RESULTS

Administration of the known organometallic neurotoxicant, TMT, resulted in the expected hippocampal pathology (2) at 3 weeks postdosing (Fig. 1). As with trauma, neurological disease states, and other chemically induced lesions of the brain, the damage caused by TMT results in hypertrophy of astrocytes at the site of injury (11, 12). The hallmark of astrocyte hypertrophy is the accumulation of glial filaments enriched in GFAP (13). Thus, TMT-induced damage of the hippocampus resulted in a large increase (650% of control) in the concentration of GFAP in this structure (Fig. 1), as assessed by sandwich ELISA of hippocampal homogenates. We planned to examine these hippocampal homogenates for the expression of other proteins using [^{125}I] protein A -based immunoblots with subsequent protein quantification by Phosphorimager. When the same samples assayed for GFAP by ELISA were immunoblotted onto NC and quantified by PhosphorImager, the effect of TMT on GFAP was reduced to a level that did not differ significantly from saline controls (Fig. 1). Different modes of background correction or contributions of minor cross-reacting bands did not significantly change the control vs TMT relationship (data not shown). Samples immunoblotted from 10% gels (Fig. 1) or 6% gels (data not shown) gave the same results. The use of fluorescein-linked secondary antibodies instead of [^{125}I] protein A gave the same results (data not shown). Immunoblotted gels stained with Coomassie blue or subjected to silver staining did not show residual levels of protein at a molecular mass below 200 kDa (data not shown). The use of polyclonal anti-GFAP or monoclonal anti-GFAP gave the same results, as did the use of NC or PVDF support membranes (data not shown). Typically, as a first attempt at analysis of a specific brain protein, we loaded 20–30 μg total homogenate protein on the gels for subsequent immunoblot analysis (e.g., see Fig. 1), i.e., total protein loads considered to be in the low range of acceptable values for the load surface employed in this study (3). Subsequently, the possibility that the amount of total protein subjected to immunoblot analysis might affect the amount of detectable GFAP was assessed by loading 1–30 μg of total homogenate protein per lane (Fig. 2). Increasing the amount of total homogenate protein above 10 μg, for samples obtained from either TMT-treated or saline-treated rats, did not result in a linear increase in GFAP (Fig. 2, top). Moreover, increasing the protein load appeared to decrease the effect of TMT on GFAP (expressed as a percentage of saline controls) (Fig. 2, bottom). Only at a protein load of 1 μg did the effect of TMT ($\sim$550% of control) approach that observed by ELISA (Fig. 1). To determine if total homogenate proteins were influencing the detection and quantification of GFAP, we spiked homogenates prepared from saline- and TMT-treated rats with pure GFAP (Fig. 3). In general, loading 5–100 ng GFAP per well resulted in linear increases in GFAP PhosphorImager values (r = 0.95–0.98 for the three GFAP alone curves; see Fig. 3). Linear increases in GFAP values ($r > 0.90$) were obtained for 5–200 ng of GFAP transferred to NC and 5–500 ng GFAP transferred to PVDF

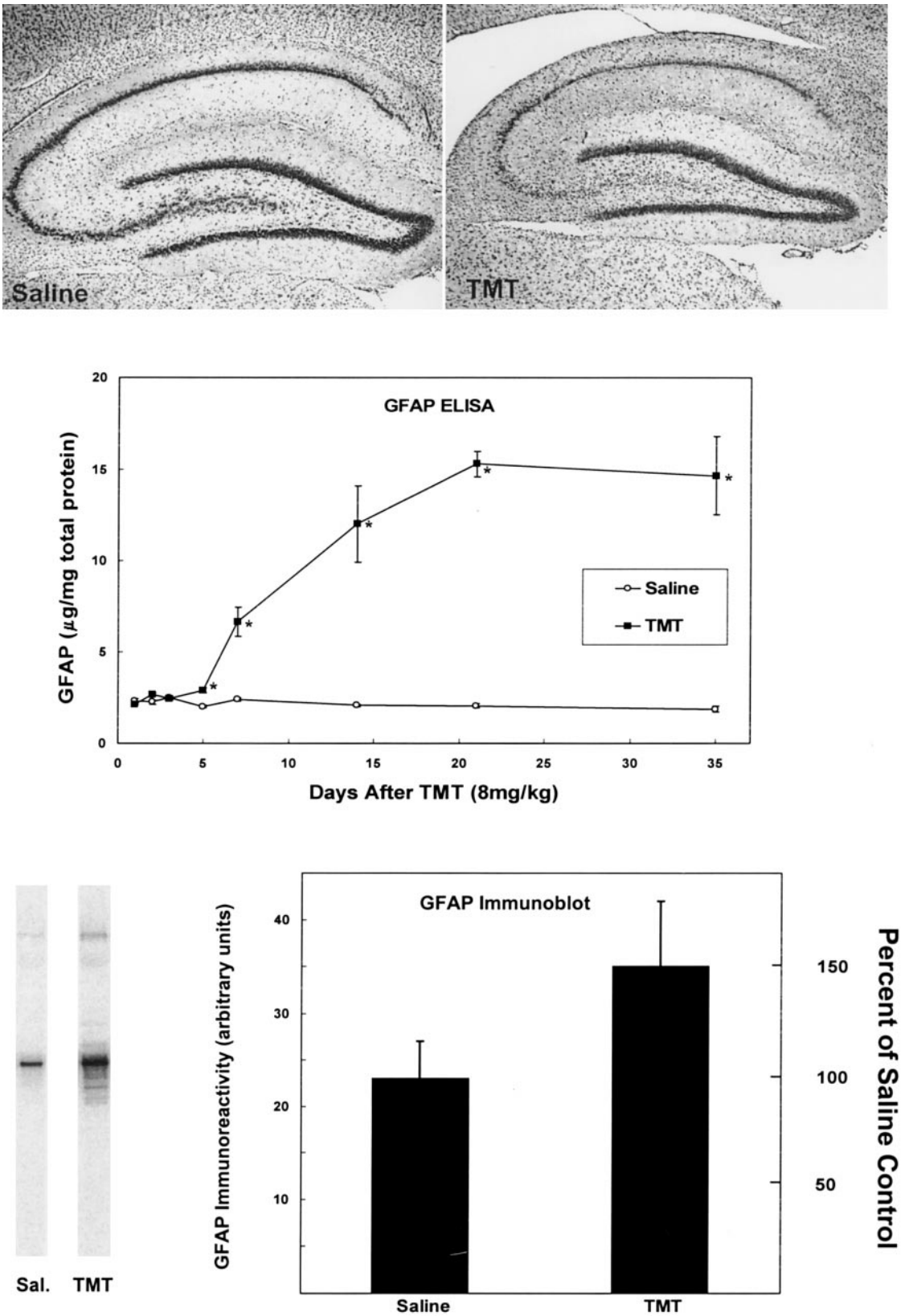

FIG. 1. Sandwich ELISA, but not quantitative immunoblots reveal large induction of GFAP associated with TMT-induced neuronal damage. (Top) Cresyl violet staining of dorsal hippocampus 3 weeks after saline or TMT (8 mg/kg). (Middle) Time course of GFAP induction by TMT as assessed by ELISA of hippocampal homogenates. (Bottom) Immunoblots of GFAP using polyclonal antibodies: typical blot—PhosphorImage obtained from homogenates of hippocampus at 3 weeks postdosing (left); PhosphorImager quantification of GFAP in homogenates (30 μg total protein) of hippocampus at 3 weeks postdosing (right). *Significantly different from saline controls, $P < 0.05$. A different photomicrograph of the tissue section labeled TMT appeared previously (2).

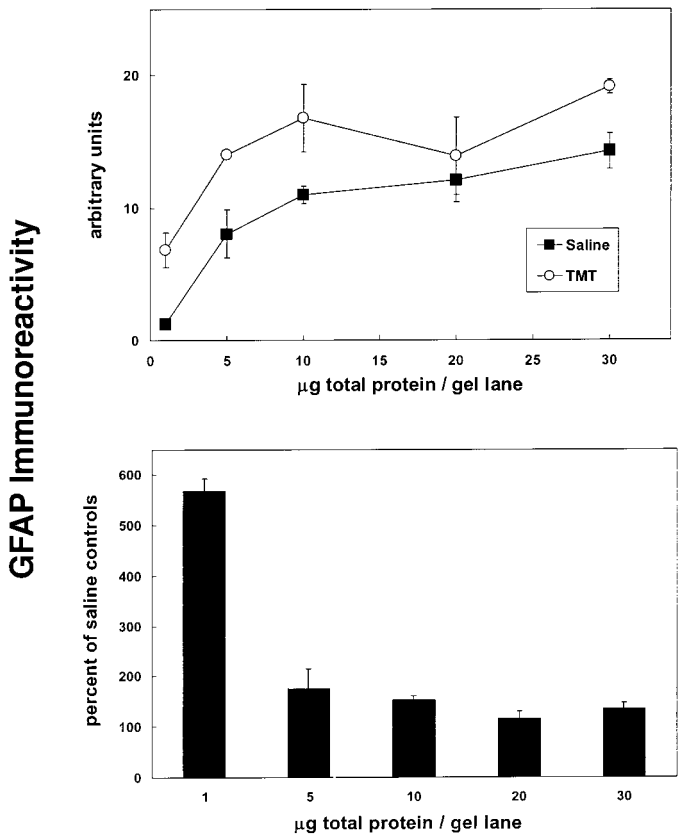

FIG. 2. Immunoblot analysis of GFAP varies as a function of total homogenate protein loaded on the gel. PhosphorImager quantification of GFAP in homogenates of hippocampus at 3 weeks postdosing as a function of total amount of homogenate protein loaded on the gel. Data are expressed in arbitrary units of GFAP immunoreactivity (top) or as a percent of saline controls (bottom).

(data not shown). Adding the same increments of pure GFAP to 20 μg of total homogenate protein obtained from a saline-treated rat "quenched" the predicted GFAP values at loads above 50 ng (Fig. 3, top). Increasing the relative abundance of GFAP in 20 μg of total protein homogenate by using a sample obtained from a TMT-treated rat resulted in further quenching of the predicted GFAP signal (Fig. 3, middle). Although the GFAP values for the 20 μg TMT homogenate were off the GFAP standard curve (Fig. 3, middle), this cannot account for the quenching effect because the same phenomenon is observed when the sample values fall within the linear range of the standard curve run on PVDF (data not shown). Moreover, when the load of this total protein homogenate was reduced to 1 μg, actual GFAP values approached but still did not reach predicted values over the entire range of pure GFAP added to the homogenate (Fig. 3, bottom). Predicted GFAP values in the above experiments may not have been obtained because binding sites on the membrane were overloaded, the transfer was inefficient, or the protein washed off the membrane during the various incubations. To test this possibility, [^{125}I] GFAP alone (5–100 ng), or in the presence of 20 μg of total homogenate protein, was subjected to a mock immunoblot analysis by omitting [^{125}I] protein A from the procedure. Increasing loads of [^{125}I] GFAP resulted in linear increases in GFAP bound to the NC membranes (r = 1.0); repeating the experiment in the presence of total homogenate protein also resulted in linear (r = 1.0), but also greater, increases in GFAP bound to the NC membranes (Fig. 4). These data were not indicative of greater transfer [^{125}I] GFAP from the gel because no radioactivity was detected in the gel following transfer with or without homogenate protein (data not shown). Despite greater binding of GFAP in the presence of homogenate proteins, underestimation of GFAP signal may be due to the presence of large amounts of other proteins on the blot. We first examined this possibility by analyzing the "quenching" potential of homogenates of liver, spleen, heart, and adrenal gland. The addition of 30 μg of liver, spleen, heart, and adrenal homogenate did not affect the pure GFAP signal (data for adrenal shown in Fig. 5). Nonneural tissue homogenates may not contain a high abundance of proteins migrating with or near GFAP on SDS gels. Therefore, we examined the effects of brain tubulin, a high-abundance brain protein with an electrophoretic mobility only slightly less than GFAP. The addition of pure tubulin (15 μg) to the pure GFAP standard resulted in as much as a 50% masking of the GFAP signal (Fig. 6); lower loads of tubulin were without effect (data not shown). Because the results of the previous experiments indicated the importance of analyzing very low loads of total protein (Figs. 1–3) and the need for a protein carrier (Fig. 4), we reassayed the GFAP content of the samples previously subjected to immunoblot and ELISA analysis (Fig. 1) using 1 μg total protein and a GFAP standard run in the presence of 1 μg liver homogenate carrier. The GFAP values obtained (Fig. 7) were comparable (726% of control) to those observed by GFAP ELISA (Fig. 1). It was possible that the quenching phenomenon observed for GFAP may not apply to other proteins. To assess this possibility we subjected two additional proteins to immunoblot analysis: synapsin I, a synaptic vesicle-localized (neuronal) protein with a molecular mass (~85 kDa) higher than the molecular mass of GFAP (~50 kDa), and MBP, a myelin-localized (glial) protein with a molecular mass (~18 kDa) lower than GFAP. Both MBP and synapsin I showed the same phenomenon observed for GFAP: addition of pure protein to 20 μg total homogenate proteins resulted in quenching of the predicted signal (Fig. 8).

DISCUSSION

When using the immunoblot technique to quantify a specific protein, typically it is assumed that the signal

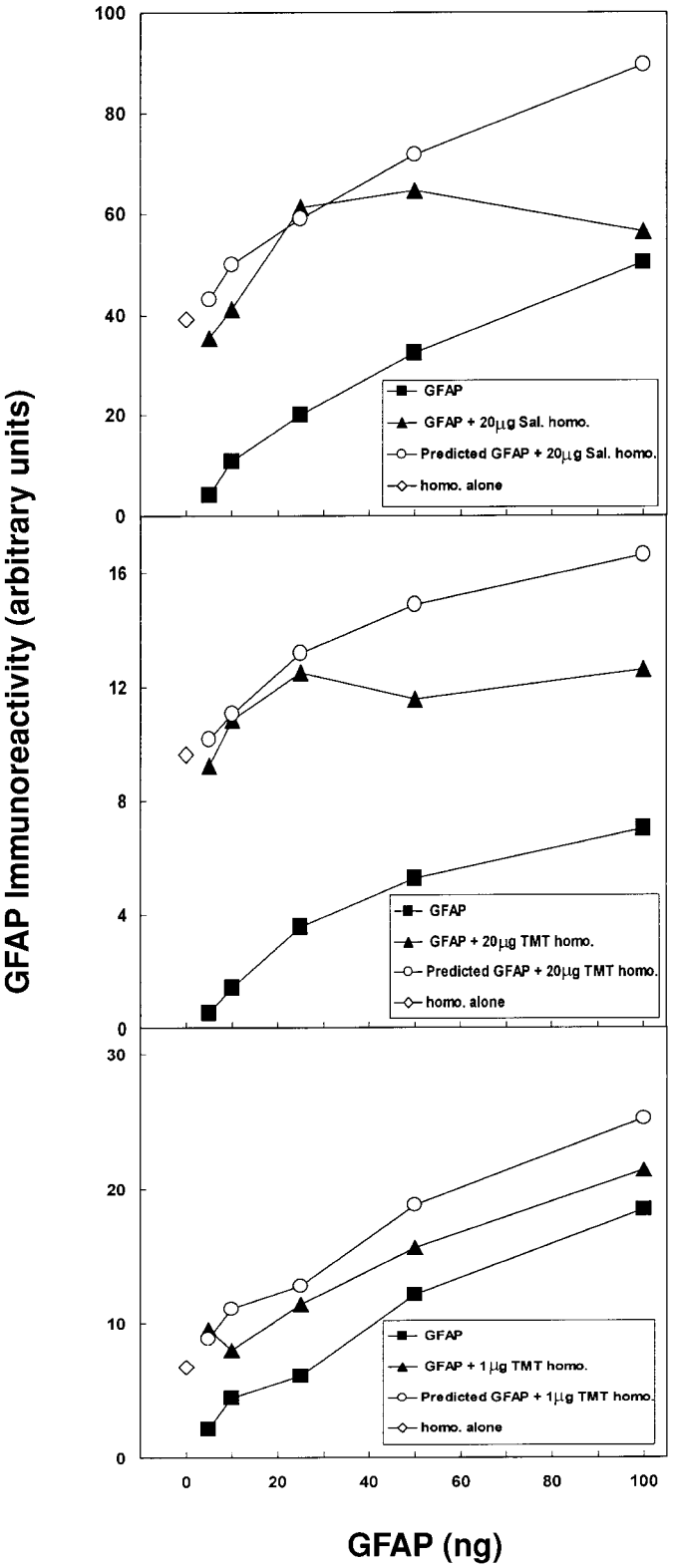

FIG. 3. Brain homogenate proteins "quench" pure GFAP signal. PhosphorImager analysis of increasing amounts of pure GFAP alone or in the presence of 20 μg protein from control homogenate (top), 20 μg protein from TMT homogenate (middle), or 1 μg protein from TMT homogenate (bottom). Predicted GFAP values were obtained by adding the values obtained for the pure protein with those obtained from the homogenate.

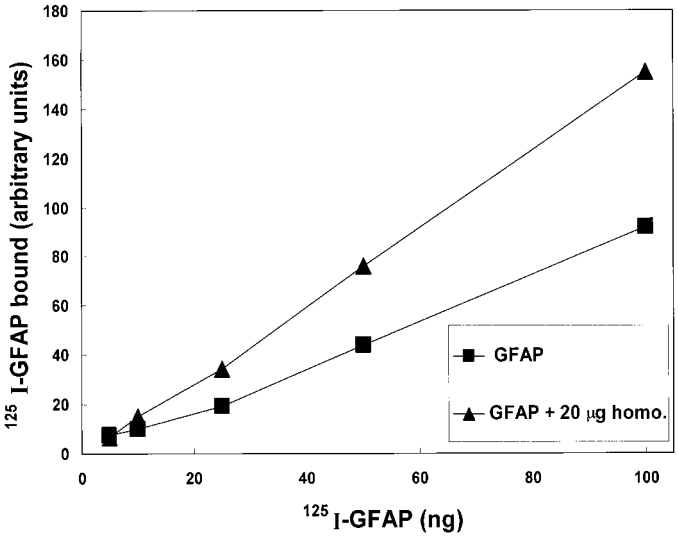

FIG. 4. Binding of [^{125}I] GFAP to NC shows a linear increase with increasing protein loads and is enhanced in the presence of brain homogenate proteins.

obtained is (i) representative of the relative abundance of the protein in the mixture and (ii) representative of the difference in protein content between mixtures that result from the effects of specific treatments. When brain homogenates are subjected to immunoblot analysis at low to moderate protein loads under standard conditions (3), our findings demonstrate that neither of these assumptions is valid for analysis of three neural proteins, GFAP, MBP, and synapsin I. Thus, when aliquots of a brain homogenate known to contain greater than six-fold increases in GFAP were loaded on large-format gels at a total protein load of 30 μg, subsequent quantification of GFAP from immunoblots failed to reveal an increase in this protein above control levels. It is unlikely that the results obtained for GFAP by immunoblot analysis are representative of the "true" condition because of the following observations: (i) neuronal degeneration in general (11) and TMT-induced neuronal degeneration in particular (see Fig. 1) are known to increase GFAP (2); (ii) a GFAP ELISA gave the expected result (Fig. 1); (iii) GFAP ELISA data are in concordance with GFAP data obtained by solid-phase immunoassay or densitometric analysis of this protein resolved by 2-D PAGE (2); and (iv) TMT-induced neuronal degeneration in the rat (2) or mouse (Dr. K. Reuhl, personal communication) is known to increase GFAP immunoreactivity in tissue sections.

The most likely explanation for our results is masking of the GFAP signal by other proteins in the homogenate. This argument is consistent with the previous qualitative findings of Poon *et al.* (14) and is supported by several observations. First, decreasing the amount of total protein loaded on a gel lane (see Figs. 2 and 7) brings the quantitative immunoblot data in line with

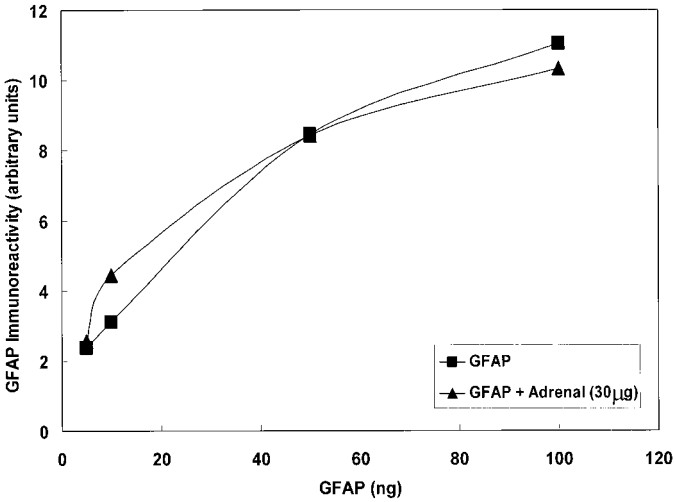

FIG. 5. Rat adrenal homogenate does not "quench" pure GFAP signal. PhosphorImager analysis of increasing amounts of pure GFAP alone or in the presence of 30 μg rat adrenal homogenate. Predicted GFAP values were obtained by adding the values obtained for the pure protein with those obtained from the adrenal homogenate.

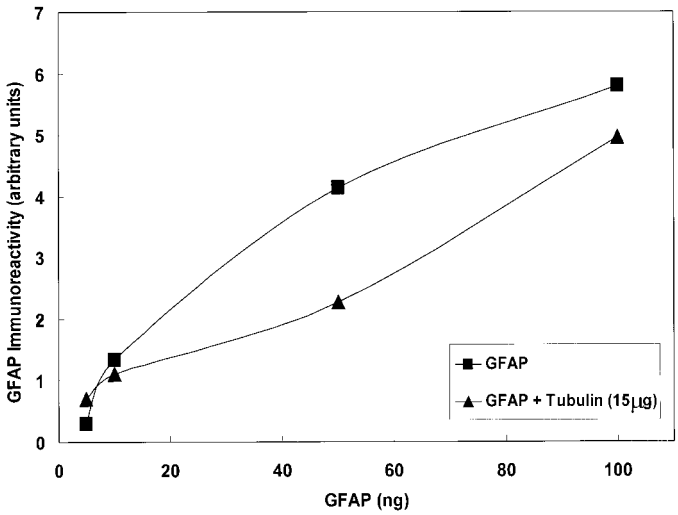

FIG. 6. Rat brain tubulin "quenches" pure GFAP signal. PhosphorImager analysis of increasing amounts of pure GFAP alone or in the presence of 15 μg purified tubulin. Predicted GFAP values were obtained by adding the values obtained for the pure protein with those obtained from the tubulin preparation.

the expected values based on the results of other assays. Second, increasing the amount of total homogenate protein loaded per gel lane, whether from control or treated tissues, does not result in a linear increase in signal. Finally, increasing the amount of pure GFAP loaded per gel lane results in a linear increase in signal, but this signal is masked when pure GFAP is combined with homogenates prepared from control brain tissue, GFAP-enriched brain tissue or a brain protein (tubulin) that migrates close to GFAP on SDS gels.

Apparent quenching of GFAP signal was not observed by the addition of nonneural tissue to pure GFAP. The addition of "carrier" protein to pure GFAP enhances the amount of binding to nitrocellulose (Fig. 4); however, this raises the possibility that nonneural tissue resulted in quenching of GFAP signal that was not apparent due to enhanced binding. Given that only high levels of exogenous brain tubulin caused quenching of GFAP, and that these proteins have nearly identical molecular mass on SDS gels, suggests that endogenous proteins of very similar or identical molecular mass in a given homogenate are the main contributors to the quenching problem. Thus, it is likely that quenching of endogenous proteins also occurs in nonneural tissues and, therefore, the phenomenon reported here is not specific to brain.

Several factors associated with technical aspects of the immunoblot technique are not likely to play a role in the effects we observed. Specifically, reductions in the signal cannot be linked to the use of a specific support matrix because nitrocellulose and PVDF gave similar results. Second, our observations cannot be attributed to the use of monoclonal antibodies because both monoclonal and polyclonal antibodies were susceptible to false signal reporting. Finally, reductions in the signal, due to loss of the protein from the blot, or due to an inefficient transfer, can be ruled out because antigen (GFAP) is retained on the support matrix to a greater degree when it is resolved with other homogenate proteins as opposed to being run alone. It also is

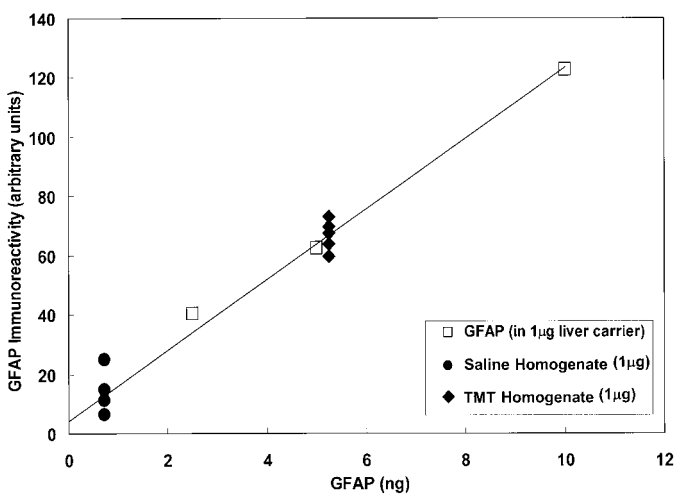

FIG. 7. Immunoblot values for pure GFAP (in 1 μg liver homogenate carrier) and brain homogenates (1 μg total protein) prepared from saline- or TMT-treated rats. The brain homogenates were the same ones subjected to analysis previously at a total protein load of 30 μg (Fig. 1, bottom). Liver homogenate (1 μg) did not quench pure GFAP signal (data not shown). The GFAP PhosphorImager values for the TMT homogenates were significantly greater than the corresponding saline homogenate values, $P < 0.05$.

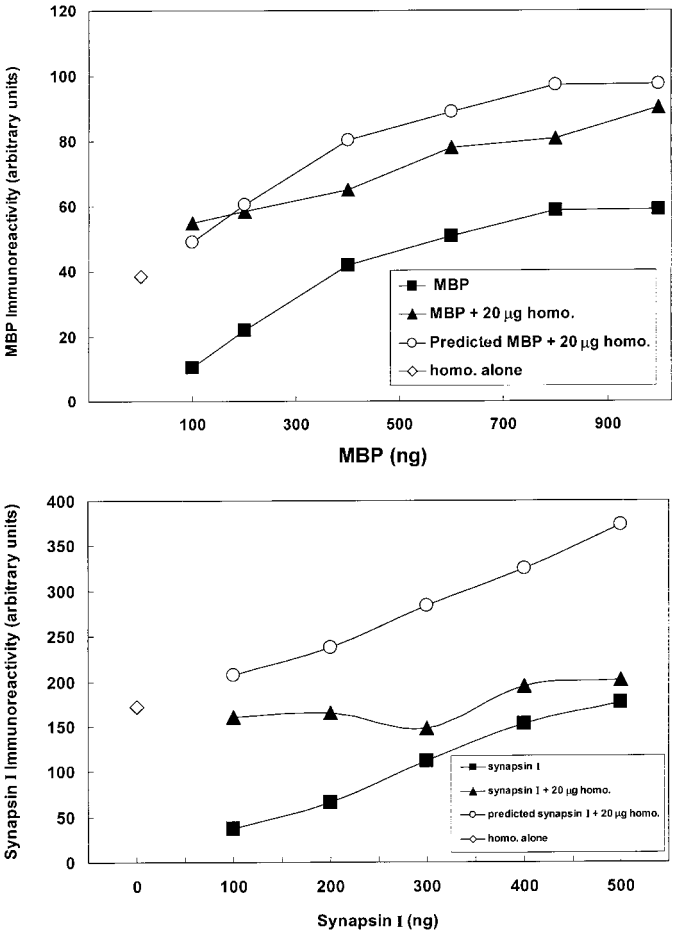

FIG. 8. Brain homogenate proteins "quench" pure MBP and synapsin I signals. (Top) PhosphorImager analysis of increasing amounts of pure MBP alone or in the presence of 20 μg protein from control homogenate. (Bottom) PhosphorImager analysis of increasing amounts of pure synapsin I alone or in the presence of 20 μg protein from control homogenate. Predicted values were obtained by adding the values obtained for the pure protein with those obtained from the homogenate.

doubtful that our findings are linked to the analysis of a single brain protein because our observations could be extended to two additional proteins present in the homogenate (MBP and synapsin I) and similar results previously were obtained for extracts of fibroblasts (14). Thus, our observations may be indicative of a general phenomenon associated with analyzing specific protein content in heterogeneous mixture of proteins.

The implications of signal quenching in homogenates might be considered benign if one were only interested in determining the concentration of a given protein in a homogenate relative to a pure standard. The end result of this situation would be an underestimation of the "true" amount of protein in the homogenate. Of much greater concern is the situation where the relative content of a specific protein in a homogenate is determined for samples prepared from control vs treated tissue. Our data clearly demonstrate that large differences between the control and treated condition can be obscured by the signal masking phenomenon. With respect to GFAP alone, reports in the literature indicate that this is a non-trivial issue. For example, a long-standing controversy exists concerning the induction of GFAP in experimental allergic encephalomyelitis (EAE) (13, 15), an experimental model for studying multiple sclerosis (13, 15). In the EAE model, GFAP immunoreactivity is increased when assessed by immunohistochemistry but not when evaluated by quantification of GFAP from immunoblots (13, 15). Similarly, the expected induction of GFAP in brain tissue obtained from victims of Parkinson's disease or in brain tissue obtained from mice exposed to the neurotoxicant, TMT, was not observed when the samples were analyzed by immunoblots (16, 17). In these studies, the immunoblot data likely are in error because EAE, multiple sclerosis, and Parkinson's disease are associated with the induction of gliosis (characterized by an increase in GFAP) (13, 15, 18). Likewise, in brain homogenates from TMT-treated mice, GFAP (assessed by immunoassay) also is increased (J. P. O'Callaghan and D. B. Miller, data not shown). We cannot rule out the possibility that discrepancies between immunohistochemical and immunoblot assessments of GFAP may be related to exposure of more epitopes (immunohistochemistry) on glial filaments of activated astrocytes rather than to an actual increase in the amount of GFAP (15).

Together, our observations indicate that data obtained from quantitative analysis of immunoblots of brain proteins are subject to a number of influences, most notably the total protein load subjected to examination. Utilization of this technique requires, at a minimum, determination of the dynamic working range for each protein analyzed. If these parameters are not established, the immunoblot values obtained will likely underestimate the true tissue content of a given protein or the true effects of a specific treatment or condition on a given protein.

ACKNOWLEDGMENTS

Excellent technical assistance was provided by Ms. Elizabeth H. Boykin and Ms. Lisa R. Bishop. The authors thank John W. Haycock for useful discussions.

REFERENCES

1. Towbin, H., Staehelin, T., and Fordon, J. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 4350–4354.
2. Brock, T. O., and O'Callaghan, J. P. (1987) *J. Neurosci.* **7,** 931–942.
3. Harlow, E., and Lane, D. (1988) *in* Antibodies: A Laboratory Manual, pp. 471–510, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
4. Shelanski, M. L., Gaskin, F., and Cantor, C. R. (1973) *Proc. Natl. Acad. Sci. USA* **70,** 765–768.

5. Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J., and Klenk, D. C. (1985) *Anal. Biochem.* **150,** 76–85.
6. O'Callaghan, J. P. (1991) *Neurotoxicol. Teratol.* **13,** 275–281.
7. Johnson, D. A., Gautsch, J. W., Sportsman, J. R., and Elder, J. H. (1984) *Gene Anal. Technol.* **1,** 3–8.
8. Laemmli, U. K. (1970) *Nature* **227,** 680–685.
9. Burnette, W. N. (1981) *Anal. Biochem.* **112,** 195–203.
10. Bolton, A. E., and Hunter, W. M (1973) *J. Biochem.* **133,** 529–539.
11. O'Callaghan, J. P., Jensen, K. F., and Miller, D. B. (1995) *Neurochem. Int.* **26,** 115–124.
12. Ridet, J. L., Malhorta, S. K., Privat, A., and Gage, F. H. (1997) *Trends Neurosci.* **20,** 570–577.
13. Eng, L. F., and Lee, Y. L. (1995) *in* Neuroglia (Kettenmann, H., and Ransom, B. R., Eds.), pp. 650–667, Oxford Univ. Press, New York.
14. Poon, R., Toyoshima, H., and Hunter, T. (1996) *J. Immunol. Methods* **199,** 155–158.
15. Tani, M., Glabinski, A. R., Tuohy, V. K., Stoler, M. H., Estes, M. L., and Ransohoff, R. M. (1996) *Am. J. Pathol.* **148,** 889–896.
16. Girault, J.-A., Raisman-Vozari, R., Agid, Y., and Greengard, P. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 2493–2497.
17. Dey, P. M., Graff, R. D., Lagunowich, L. A., and K. R. Reuhl. (1994) *Toxicol. Appl. Pharmacol.* **126,** 69–74.
18. Langston, J. W., Koller, W. C., and Giron, L. T. (1992) *in* The Scientific Basis for the Treatment of Parkinson's Disease (Olanow, E. W., and Lieberman, A. N., Eds.), pp. 33–58, Parthenon, Park Ridge, NJ.