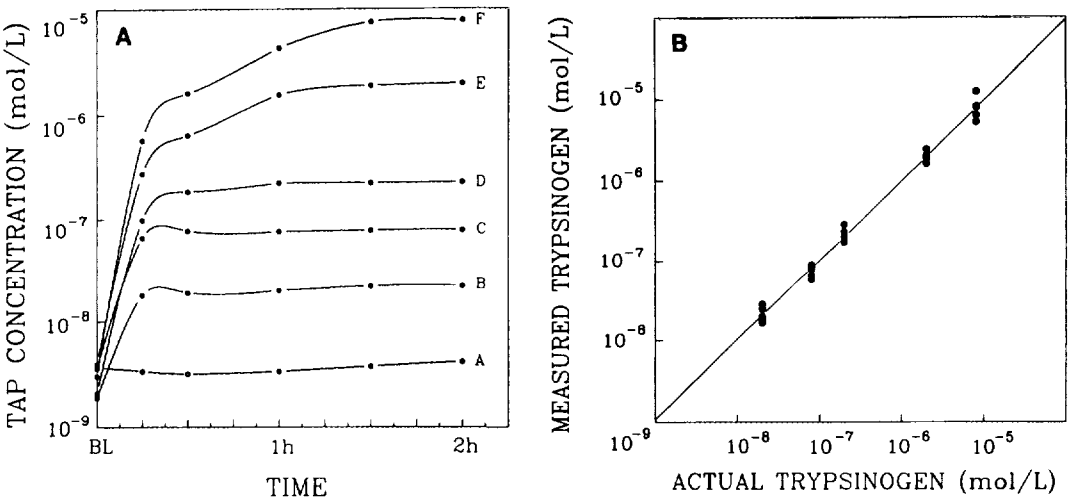

FIG. 1. (A) Generation of trypsinogen activation peptide in incubations containing trypsinogen alone at 8×10^{-6} M (A), or 2×10^{-8} M (B), 8×10^{-8} M (C), 2×10^{-7} M (D), 2×10^{-6} M (E), or 8×10^{-6} M (F) trypsinogen incubated with 10 U/ml enterokinase. Curves represent spline curves of mean values at each time point. (B) Levels of trypsinogen measured by free TAP following enterokinase activation (TRG_m) plotted against actual concentration of trypsinogen contained in each incubation (TRG_a). The solid line represents curve of best fit (calculated regression: $TRG_m = 0.97 \times TRG_a + 0.29$).

of trypsin inhibitor were included in the incubation mixtures. The positive correlation of actual and measured trypsinogen concentration observed in the present study demonstrates that trypsin inhibition successfully prevents the possible proteolytic effect of trypsin while enterokinase activity and TAP generation are not affected. Furthermore, the observed lack of TAP immunoreactivity in incubations containing 8 μmol/liter trypsinogen attests to the high specificity of the $(Asp)_4$-Lys fragment and confirms the C-terminal direction of the antibodies as observed by Hurley *et al.* (9).

Summary

Measurement of trypsinogen by quantitative immunosorbent assay of the highly specific and inert trypsinogen activation peptide following complete activation of trypsinogen by enterokinase offers a simple and sensitive method that provides reliable results over a wide range of trypsinogen concentrations. This method offers a significant advantage in being applicable to complex biological tissues.

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Commercial [^{3}H]Glutamate Contains a Contaminant That Labels Tubulin Covalently

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Tubulin, the major protein constituent of microtubules, is subjected to various types of posttranslational modifications including polyglutamylation, a process which adds a variable number of glutamates to specific

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glutamate residues in the α- and β-subunits via a peptide bond (1–4). Glutamate at high concentrations is also used to induce polymerization of tubulin into microtubules (5) and to stabilize tubulin (6).

Recently, in a study of the effect of glutamate on microtubule dynamics, it was reported that tubulin is covalently modified by glutamate and that incorporation of [^{3}H]glutamate occurred during microtubule assembly of microtubule protein (tubulin preparation containing microtubule associated proteins) in 1 M glutamate (7). This raised the possibility that the enzyme(s) responsible for polyglutamylation of tubulin could be present in such preparations. We have now found that an apparent incorporation of glutamate also occurs in tubulin devoid of associated proteins and into bovine serum albumin. Based on a number of experiments, we now conclude that the apparent incorporation of glutamate is due to a contaminant in commercial [^{3}H]glutamate. This should be a concern for investigators conducting studies of glutamate binding to various proteins and receptors.

MATERIALS AND METHODS

Materials. Bovine brain microtubule protein and purified tubulin were prepared by temperature-dependent assembly–disassembly (8) and phosphocellulose-Biogel P-10 chromatography (9). Bovine serum albumin (BSA)[1] was obtained from Sigma Chemical Company. [^{3}H]Glutamic acid was purchased from American Radiolabeled Chemicals, Inc. and Dupont NEN.

Labeling by [^{3}H]glutamate. Microtubule protein (5 mg/ml) or tubulin (2 mg/ml) was polymerized at 37°C for 30 min in a 0.3-ml volume containing buffer A (0.1 M Pipes, 1 mM ethylene glycol bis(β-aminoethyl ether) *N,N'*-tetraacetic acid (EGTA), 1 mM $MgSO_4$, pH 6.9), 0.5 mM GTP, and 1 M glutamate containing 10 μCi of [^{3}H]glutamate. Polymerization was also done in the absence of 1 M glutamate but with the addition of 25 μM paclitaxel to stimulate assembly. The glutamate concentration due to the [^{3}H]glutamate was 1.3 μM. Microtubules were pelleted after polymerization by centrifugation at 35,000*g* for 5 min. The protein pellet was resuspended in buffer A and precipitated with perchloric acid (10% final concentration). The protein precipitate was washed repeatedly with water until no tritium was detected in the wash solution, solubilized in 0.1 M NaOH, and assayed for protein content using the Bradford method (10) and radioactivity using liquid scintillation counting. Alternatively, assembled microtubules were pelleted by centrifugation (35,000*g*) through 50% sucrose in buffer A, and resuspended microtubule pellets were centrifuged through a 1-ml Sephadex G-25 gel filtration column. In some cases the protein was precipitated by 50% ethanol instead of perchloric acid. BSA (25 mg/ml) was also incubated with 1 M [^{3}H]glutamate and 0.5 mM GTP in buffer A for 30 min at 37°C and precipitated with PCA, washed, and analyzed as described above.

SDS–PAGE. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed using the buffer system described by Laemmli (11). Protein bands were visualized with Coomassie blue, cut out, dissolved by incubation with 30% H_2O_2 at 65°C (12), and assayed for radioactivity.

RESULTS AND DISCUSSION

Microtubule protein was polymerized in the presence of 1 M glutamate containing [^{3}H]glutamate and, after precipitation with perchloric acid followed by thorough washing of the pellet, a fraction (0.085%) of the label was found to be associated with the protein. Assuming that all the tritium in commercial [^{3}H]glutamic acid is present in glutamate, this corresponds to an incorporation of 189 nmol of glutamate per milligram of microtubule protein (Table 1). The extent of incorporation was not reduced by omission of GTP from the solution, lessening the possibility that the nucleotide serves as an energy source in an enzyme-catalyzed glutamylation reaction. To further exclude the participation of an enzymatic microtubule-associated protein in this reaction, tubulin free of microtubule-associated proteins was polymerized in a similar fashion. This led to the incorporation of 0.11% of the label and an apparent molar ratio of 50 glutamate residues per tubulin dimer (Table 1). The labeling of tubulin did not depend on the structural integrity of the protein; in fact, labeling increased about 6-fold in 8 M urea (Table 1). The labeling was also not specific for tubulin; a high apparent incorporation also occurred with BSA (Table 1). Although only one experiment for the labeling of BSA is shown in Table 1, several experiments were done with this protein under different conditions and in all cases extensive labeling occurred. Labeled tubulin and BSA were electrophoresed by SDS–PAGE and tritium was found in the corresponding protein bands. Several arguments can be made to support the view that the ^{3}H label was attached to tubulin and BSA in a covalent fashion. These include the fact that the label remained with the protein after gel filtration, sucrose gradient centrifugation, dialysis against an 8 M urea solution containing 0.1 M glutamate, precipitation with per-

[1] Abbreviations used: BSA, bovine serum albumin; Pipes, 1,4-piperazinediethanesulfonic acid; PCA, perchloric acid; DTT, dithiothreitol.

TABLE 1

Apparent Covalent Labeling by Glutamate[a]

Protein	Glutamate concentration	Incorporation		
		% of total ^3H	nmol Glu/mg protein	mol Glu/mol protein
Microtubule protein	1 M	0.085 ± 0.011 $n = 2$	189 ± 24	—
Tubulin	1 M	0.11 ± 0.017 $n = 11$	500 ± 100	49.8 ± 10.4
Tubulin + 8 M urea	1 M	0.65 ± 0.035 $n = 2$	2740 ± 30	274 ± 3
Bovine serum albumin	1 M	0.42 $n = 1$	168	11.1
Tubulin	1.3 μM	0.14 ± 0.03 $n = 2$	—	—
Tubulin[b] (DTT pretreatment)	1 M	0.02 ± 0.008 $n = 2$	117 ± 47	11.7 ± 4.7
Tubulin[b] (NaOH pretreatment)	1 M	0.033 ± 0.002 $n = 2$	194 ± 12	19.4 ± 1.2
Tubulin[b] (BSA pretreatment)	1 M	0.044 ± 0.006 $n = 2$	217 ± 26	21.7 ± 2.6

[a] Procedures are given under Material and Methods. All reactions contained 10 μCi of [^3H]glutamate.

[b] [^3H]Glutamate (20 μCi) was incubated with 10 mM DTT or 0.1 M NaOH for 5 h at 37°C before incubation with tubulin and 1 M unlabeled glutamate. The [^3H]glutamate was also incubated with 5 mg/ml BSA in buffer A for 16 h at 37°C. The [^3H]glutamate was recovered by centrifugation through a Millipore Ultrafree-MC Centricon filter (m_r cutoff = 10,000) and incubated with tubulin and 1 M unlabeled glutamate.

chloric acid or ethanol followed by extensive washing of the pellets, and SDS–PAGE. The label could not be removed by a 4-h treatment with a 10,000-fold molar excess of hydroxylamine, indicating the absence of an ester linkage.

Although it appears that incubation of tubulin and BSA with glutamate leads to glutamylation of the protein, several lines of evidence indicate that the incorporated ^3H species is not glutamate. First, there is no theoretical basis for covalent modification of a protein by glutamate; glutamate is not expected to be reactive with functional groups on proteins. Second, ^3H incorporation was found to be independent of glutamate concentration. Tubulin incubated with 10 μCi of [^3H]glutamate incorporated approximately the same percentage of label in the absence or presence of 1 M unlabeled glutamate (Table 1). The fact that 10 μCi of [^3H]glutamate corresponds to a concentration of 1.3 μM means that an increase in unlabeled glutamate concentration of 10^6-fold did not reduce the level of tritium incorporation. Third, the large number of glutamates incorporated per mole of tubulin should have been detected by a shift in molecular mass detected by SDS–PAGE and certainly by amino acid analysis. However, modified tubulin and unmodified tubulin migrated together on SDS–PAGE, and there were no changes in glutamate content determined by amino acid analysis after incubation with 1 M glutamate. In addition, the amino acid analysis showed no detectable radioactivity associated with glutamate.

The results suggest that a contaminant in the [^3H]glutamic acid is responsible for the covalent labeling. In an attempt to remove any reactive ^3H species, [^3H]glutamate was preincubated with dithiothreitol (DTT), NaOH, or BSA before labeling. Preincubation of [^3H]glutamate with either DTT or NaOH prior to addition of tubulin reduced the ^3H incorporation by 81 and 69%, respectively (Table 1). Incubation of [^3H]glutamate with BSA for 16 h resulted in an incorporation of 0.7% of the label into BSA and a 60% reduction of subsequent ^3H labeling of tubulin.

It is concluded that a reactive ^3H-labeled species is present in commercial [^3H]glutamate and that this contaminant is responsible for the apparent covalent modification of tubulin by glutamate reported previously (7). The contaminant is capable of covalent binding to tubulin, bovine serum albumin, and presumably, other proteins. The percentage of total tritium which is incorporated is quite low and is well below the stated content of impurities in commercial [^3H]glutamate. For example, the [^3H]glutamate used for most of this work was from American Radiochemical Company (ARC) and was purchased in 1992. At that time the radiochemical purity was listed as >99%. We also purchased [^3H]glutamate with a purity of 99.9% from Dupont NEN. Initially

the latter product produced an apparent incorporation of glutamate into tubulin which was about 10% of that found with the ARC product. However, when tested again 3 months later, the extent of labeling increased 4.5-fold. Thus, it appears that the reactive species is produced as a result of radioactive decay of the [^{3}H]glutamate. According to ARC this decay occurs at the rate of less than 1% per month. Although the percentage of ^{3}H incorporation is very low, if unlabeled glutamate is present and the radioactivity is attributed to glutamate, the calculated number of glutamate residues incorporated is substantial.

The presence of reactive contaminants in radiolabeled preparations is not a new occurrence. It had been previously reported, using [^{3}H]glycerol, that glycerol associates with tubulin in a nonexchangeable manner (13). However, a subsequent investigation demonstrated that the labeling was due to a contaminant in the glycerol (14). Studies of glutamate binding to proteins and receptors is fairly common. It is important for investigators involved in such studies to be aware of the artifact described in this report.

Finally, it should be emphasized that the results presented in this report do not affect conclusions regarding polyglutamylation of tubulin because the existence of such a posttranslational modification was demonstrated by direct chemical analysis of isolated tubulin (1–4).

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Technical Considerations for the Use of Ethidium Bromide in the Quantitative Analysis of Nucleic Acids

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The fluorometric assay of nucleic acids with ethidium bromide (EtBr)[3] is used both for qualitative (verification of nucleic acid extractions in molecular biology) and quantitative (for estimating growth rates of fish using RNA:DNA ratios) analyses. For quantitative analysis, the method of Karsten and Wollenberger (1) is perhaps the most widely used. We utilized this method for the analysis of RNA:DNA ratios in fish and sought to test our analytical accuracy by means of a series of samples spiked with nucleic acids. The unexpected finding that fluorescence in unspiked samples exceeded those of all spiked samples forced us to examine the method for the source of this differential fluorescence. We subsequently found that the assay conditions must be controlled much more stringently than is generally realized; the ratio of nucleic acids to EtBr must be within rather narrow limits. Failure to optimize the nucleic acid:EtBr ratio, either in standard curves or in the determination of unknowns, can lead to erroneous results. This optimization is less important for qualitative analysis, although low fluorescence due to suboptimal conditions could lead to conclusions of poor nucleic acid extraction when extraction was, in fact, thorough. In this report, we identify several technical considerations in the quantitative assay of nucleic acids with EtBr. Although this note deals exclusively with DNA, these findings also apply both to the assay of RNA with EtBr and to the assay of

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[3] Abbreviation used: EtBr, ethidium bromide.

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