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IMMUNOCHEMICAL ANALYSIS OF ACETAMINOPHEN COVALENT BINDING TO PROTEINS

PARTIAL CHARACTERIZATION OF THE MAJOR ACETAMINOPHEN-BINDING LIVER PROTEINS*

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Abstract—A sensitive immunoassay for detecting acetaminophen (APAP) bound to proteins was developed using an affinity purified antibody directed against the N-acetylated end of the APAP molecule. Western blots of electrophoretically resolved liver proteins taken from mice given an hepatotoxic dose of APAP demonstrated that nearly 85% of the total detectable protein-bound APAP was covalently associated with proteins of 44 and 58 kD. Pretreatment of liver extracts with the sulfhydryl-specific reagent, *N*-ethylmaleimide (NEM), prior to derivatization with the reactive metabolite of APAP, *N*-acetyl-*p*-benzoquinone imine (NAPQI), greatly reduced immunochemically detectable APAP-protein adducts and indicated that the antibody detects protein-thiol conjugates of APAP. To investigate the basis of the binding selectivity *in vivo*, a variety of systems which yielded APAP-protein adducts were analyzed. Systems which activate APAP enzymatically, as in hepatocyte suspensions or in post-mitochondrial (S9) fractions fortified with an NADPH-regenerating system, resulted in a protein binding profile similar to that produced *in vivo*. Conversely, when extracts or cells were treated with chemically synthesized NAPQI, an alternative protein binding profile was obtained. Two-dimensional electrophoretic analysis of the reduced protein thiol (PSH) content of liver proteins using [^{3}H]NEM labeling revealed that the 58 kD APAP-binding proteins were rich in PSH, whereas the major 44 kD binding protein had virtually no detectable PSH. Many PSH-rich proteins that were not arylated *in vivo* did bind NAPQI *in vitro*. However, the 44 kD proteins were not arylated when chemically synthesized NAPQI was added to homogenates or cell suspensions. The present data further suggest that, in addition to the amount and reactivity of free protein sulfhydryls, the cellular localization with respect to the cytochrome P-450 activation site may influence the susceptibility of proteins to NAPQI binding. These findings signal the need for caution in interpreting studies of APAP mechanisms that rely solely on NAPQI addition.

Acetaminophen (APAP, 4-hydroxyacetanilide, *N*-acetyl-*p*-aminophenol) is a widely used analgesic that is considered safe at therapeutic doses but causes fatal hepatic centrilobular necrosis when administered in excess [2–4]. Although the exact mechanism for the toxicity is still unknown, it is widely accepted that APAP is activated by mixed-function oxidases to a reactive metabolite, *N*-acetyl-*p*-benzoquinone imine (NAPQI) which depletes glutathione and covalently binds to hepatic proteins [4–12]. Good correlation has been demonstrated between the level of APAP covalent binding to proteins and the extent of liver necrosis [3, 13, 14]. However, studies using the 2′- and 3′-hydroxy isomers of APAP have demonstrated that, while both isomers form metabolites which covalently bind to hepatic proteins more avidly than APAP, these compounds do not result in hepatotoxicity [15–17]. This suggests that not all covalent binding of reactive metabolites to proteins need elicit toxicity.

To discern "non-productive" protein binding from that which could lead to cytotoxicity, the specific proteins to which NAPQI binds must be identified and subsequently characterized. Since the isotopic detection of the specific protein adducts would require impractical amounts of radioactive APAP, we have developed an immunoassay for detecting electrophoretically resolved APAP-bound proteins. Using this immunoassay, we have been able to demonstrate that APAP binding to mouse liver proteins *in vivo* is not random but highly selective [18]. The majority of the binding detected following an hepatotoxic dose was found covalently associated with protein bands of 44 and 58 kD. In this paper, we describe the production, isolation and characterization of antibodies which detect APAP-protein adducts and elucidate some of the attributes of the major arylated proteins using two-dimensional gel electrophoresis. In addition, we present evidence that proteins which become arylated following the direct addition of chemically synthesized NAPQI differ from those

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targeted when APAP is enzymatically activated *in situ* and that protein thiol (PSH) content is but one of several determinants directing the selectivity of NAPQI binding to the specific proteins *in vivo*.

MATERIALS AND METHODS

Materials. Uniformly labeled [^{3}H]APAP, *N*-ethyl-2-[^{3}H]maleimide, [^{125}I]-conjugated goat anti-rabbit IgG and the fluorographic reagent, En^{3}Hance were purchased from Dupont New England Nuclear (Boston, MA). Affinity purified horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG was purchased from the Organon Teknika Co. (West Chester, PA). Nitrocellulose blotting membranes (0.45 μm) were obtained from Schleicher & Schuell (Keene, NH). Epoxy-activated Sepharose 6B was a product of Pharmacia (Piscataway, NJ). The ampholines and silver nitrate staining kit were obtained from Bio-Rad (Rockville, NY). Ultra-pure electrophoretic grade Tris–HCl, acrylamide, *N,N'*-methylene-bisacrylamide, glycine and sodium dodecyl sulfate (SDS) were all purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN). HPLC grade acetonitrile was obtained from Fisher Scientific (Springfield, NJ). The 2,6- and 3,5-dimethyl acetaminophen derivatives were synthesized as previously described [19]. The anti-actin monoclonal antibody was provided by Dr. James Lessard (University of Cincinnati, OH). All other reagents were purchased from the Sigma Chemical Co. (St. Louis, MO) and were of the highest purity available.

Animals and treatment procedures. Fasted (18–20 hr), 3-month-old, male mice (Charles River, Crl:CD-1, Wilmington, MA) were used for all experiments. Animals were maintained in stainless steel cages in temperature-controlled quarters with a 12-hr light/dark cycle. Food and water were provided *ad lib.* prior to overnight fasting. Mice were dosed by gavage with APAP (600 mg/kg, p.o.) using a 6% stock drug solution in 50% propylene glycol. Previous assessment of hepatotoxicity through 24 hr utilizing animals of this strain and age indicated that this dosage causes hepatic necrosis, significant covalent binding, and marked elevation of plasma sorbitol dehydrogenase activity [20, 21]. In selected experiments, piperonyl butoxide (600 mg/kg, 5 ml/kg, i.p.) was administered in corn oil 1 hr before APAP dosing to prevent cytochrome P-450 mediated activation of APAP [22]. Control animals were given vehicle only.

Immunogen construction and immunization. To elicit the production of antibodies against APAP-bound to proteins, an immunogen was constructed by first linking diazotized *p*-aminobenzoic acid (PABA) to either arylating carbon atom ortho to the hydroxyl group of APAP. This conjugate was then coupled to amino groups on the carrier protein, keyhole limpet hemocyanin (KLH), through the free carboxyl group of PABA. In this manner, the APAP molecule was covalently attached to PABA through its arylating carbon at the 3′ or 5′ position of the aromatic ring, thereby retaining the natural binding conformation [23, 24]. Moreover, the use of a linker not normally associated with proteins allowed for the specificity of the antibody to be predominantly directed at the parent compound and not at the protein linkage.

The PABA–APAP product was first synthesized by reacting 4 mmol of PABA in 100 ml of 0.1 N HCl with a 1% solution of $NaNO_2$ added dropwise at 4° with continuous stirring. Diazotization was monitored by the release of free nitrous acid using a starch source coated with 50 mM KI solution. Upon completion of this reaction, 4 mmol of [^{3}H]APAP (1.25 μCi/mmol) in 20 ml of 0.5 M carbonate buffer (pH 9.0) was then slowly added turning the bright yellow–green solution to a deep red color. The pH of this reaction mixture was maintained at 9.0 with dropwise addition of 1 N NaOH. This solution was stirred for 2 hr in the cold room and then shell frozen and lyophilized to complete dryness.

The lyophilized [^{3}H]PABA–APAP product was coupled to KLH by a mixed anhydride reaction. The powder was stirred for 30 min at 10° with 9 ml of ice-cold *N,N*-dimethylformamide and 260 μl isobutyl chloroformate prior to the addition of 50 ml of 50 mM $NaHCO_3$ (pH 9.0) containing KLH (1 mg/ml). After mixing for an additional 4 hr, any unreacted [^{3}H]PABA–APAP was removed by dialyzing the protein solution at 4° against five 1-liter changes of 0.8% saline in 10 mM phosphate buffer (PBS), pH 7.4, for 72 hr. By monitoring the extent of isotope binding to KLH, this immunogen preparation was shown to have an epitope density of 37 mol of APAP bound per subunit mol of KLH. It was stored at 4° until further use.

For immunization, 420 μg of the dialyzed KLH–PABA–APAP immunogen was emulsified with an equal volume of complete Freund's adjuvant (500 μl) and injected subcutaneously into 3-month-old male New Zealand white rabbits. A booster injection of the immunogen (200 μg) in incomplete Freund's adjuvant was administered within 4 weeks. Two weeks later blood was collected from the central artery of the ear and allowed to coagulate in sterile glass tubes at 37° for 30 min. After rimming with a thin wooden dowel to allow clot shrinking, the serum was removed, centrifuged at 1000 *g* for 10 min, and stored at −70° in 1-ml aliquots. Titer was maintained through booster injections of the immunogen every 5 months.

Construction of an APAP-Sepharose affinity column. To purify the APAP-specific antibodies an APAP affinity column was constructed. To couple APAP to epoxy-activated Sepharose 6B, 2.5 g of the Sepharose was suspended in 10 ml of HPLC grade water, washed over a sintered glass filter with another 500 ml, and reacted for 16 hr with 62 mg of APAP in 15 ml of 0.1 M NaOH (pH 11.8). The product was first washed on a glass filter with 200 ml of distilled water followed by 100 ml of 0.5 M NaCl in 0.1 M borate buffer, pH 8.0, and finally with 100 ml of 0.1 M acetate buffer, pH 4.0. Unreacted oxirane groups were blocked by mixing with 1.0 M ethanolamine for 4 hr at pH 9.0. Approximately 8 mg of APAP was covalently attached to the Sepharose matrix and accounted for nearly 40% of the total available binding sites. The APAP-epoxy Sepharose matrix was packed in a 10-ml glass chromatography column and stored at 4° in 0.1 M PBS containing 0.01% sodium azide.

Affinity purification of anti-APAP antibodies. Immune serum was precipitated by dropwise addition of saturated $(NH_4)_2SO_4$ solution to a final concentration of 38%. After adjusting the pH to 7.4 with 2 N NaOH and stirring for 30 min at room temperature, the IgG fraction was centrifuged at 1000 *g* for 30 min. To further remove contaminating albumin, the sediment was dissolved in PBS and reprecipitated at a final concentration of 31% $(NH_4)_2SO_4$. The final precipitate was again resuspended in PBS to the original serum volume and dialyzed against PBS until the dialysate was sulfate-negative as determined by reaction with an equal volume of 1% $BaCl_2$.

Aliquots (1 ml) of the ammonium sulfate treated immune serum were passed through the APAP-Sepharose column, and unbound proteins were removed by washing in PBS until negligible absorbance at 280 nm was achieved. Bound anti-APAP IgG was then selectively eluted by the addition of 10 ml of a solution of APAP in PBS (5 mg/ml). Since APAP absorbs UV light strongly at 245 nm, protein elution could no longer be directly monitored spectrophotometrically. Dialysis of the eluted IgG with ten 1-liter changes of PBS over a 2-week period was necessary to remove bound APAP and regain antibody activity. All fractions exhibiting an absorbance at 280 nm were then tested for antibody activity against APAP-bound protein.

NAPQI derivatization of proteins. NAPQI was synthesized by the method of Streeter *et al.* [25] by rapidly stirring 2.65 mmol [^{3}H]APAP (2 μCi/mmol) in 15 ml HPLC grade acetonitrile with 3.45 mmol of Ag_2O and 2.75 mmol of anhydrous Na_2SO_4 for 2 hr at room temperature. A 50-μl aliquot of this solution was directly added to 10 ml (1 mg/ml) of bovine serum albumin (BSA) in PBS (pH 7.4) and stirred for 30 min. The NAPQI derivatized BSA solution was dialyzed overnight against PBS and utilized as the solid phase coating antigen in all competitive and non-competitive HRP-ELISA assays (see below). The amount of APAP bound to BSA was estimated by isotopic measurement of the derivatized protein and was found to be equivalent to 0.79 mol of APAP bound per mol of BSA.

Enzyme-linked immunosorbent assays (ELISA). The titer and specificity of antibodies which detect APAP-protein adducts were assayed using an ELISA with HRP-conjugated goat anti-rabbit IgG. Polystyrene 96-well microtiter plates were coated for 1 hr at 37° with 10 μl of either NAPQI derivatized BSA or native BSA at a concentration of 10 μg protein/ml of 50 mM Na_2CO_3 buffer (pH 9.6). Following five washes with a 50 mM phosphate buffer (pH 7.4) containing 0.05% Tween 20 (PBS-T), 40 μl of immune rabbit serum (serially diluted 1:16 to 1:2048 in PBS-T) was added to each well, and the plates were covered and incubated for 1 hr at 37°. At the end of the incubation period, the plates were again washed five times and then 60 μl of HRP-conjugated goat anti-rabbit IgG (1:200 in PBS-T) was added for an additional hour. After a final wash, 60 μl of a solution containing both the substrate (0.12% hydrogen peroxide in 0.05 M citrate buffer, pH 4.0) and the chromogen [660 μg/ml of 2,2′-azino-di-(3-ethylbenzthiazoline sulfonic acid)] was added to each well. Peroxidase activity was monitored as the rate of change in absorbance/min at 405 nm on a computer-interfaced Artek model 210 plate reader.

The binding specificity of the affinity purified rabbit antibody was characterized by competitive HRP-ELISA using NAPQI-derivatized BSA, native BSA, free APAP, *p*-aminophenol, 3,5-dimethyl APAP and 2,6-dimethyl APAP as potential inhibitors. Compounds which decreased antibody binding to wells coated with NAPQI derivatized BSA were considered to cross-react with the anti-APAP antibody. These compounds (20 μl of 0.12 to 500 μg/ml in PBS-T) were added to separate coated wells and incubated for 1 hr at 37° in the presence of 20 μl of a 1:75 dilution of the affinity purified antibody. The final antibody dilution used (1:150) was within the linear portion of the affinity purified antibody titration curve [18]. All subsequent steps were as described above. The percent inhibition was calculated by comparing the peroxidase activity of wells incubated in the presence and absence of each of these competing agents.

Systems analyzed for binding specificity. Three approaches were utilized to assess the binding specificity of chemically synthesized NAPQI to liver proteins. In the first, livers from untreated mice were perfused with ice-cold PBS and homogenized 1:40 in 0.25 M sucrose, 1 mM $MgCl_2$, 10 mM Tris–HCl, pH 7.4 (STM) buffer using a hand dounce. Five milliliters of the homogenate (2 mg/ml) was reacted for 5 min with 100 μl of the NAPQI solution described above and then fractionated by centrifugation at 9000 *g* for 20 min at 4°. The supernatant fraction obtained was subjected to 105,000 *g* for 60 min, and the resulting cytosolic and microsomal fractions were immunochemically analyzed for bound NAPQI. The second approach involved an assessment of NAPQI binding after blocking exposed protein thiols. In these studies livers were homogenized in STM buffer containing 5 mM NEM and reacted for 20 min at room temperature prior to NAPQI treatment. The final approach involved the direct addition of NAPQI to mouse hepatocyte suspensions isolated by a collagenase perfusion method [26]. The cells were resuspended in Krebs–Henseleit buffer at a density of 2×10^6/ml and then incubated for 5 min at 37° with NAPQI at a final concentration of 500 μM [11, 12]. The treated cells were lysed by nitrogen cavitation and fractionated as described above; then the proteins were analyzed for NAPQI binding.

The specificity of protein binding obtained following the direct addition of NAPQI was compared to profiles produced through *in situ* activation of APAP. Three additional conditions were utilized to ascertain the selectivity of binding to liver proteins of NAPQI resulting from enzymatic activation of APAP. In the first, binding was assessed in fractionated liver homogenates from mice killed 4 hr after receiving an hepatotoxic dose of APAP (600 mg/kg, p.o.). In the second, mouse hepatocyte suspensions incubated for 2 hr at 37° in Krebs–Henseleit medium containing 10 mM APAP were lysed by nitrogen cavitation, fractionated as described above, and then analyzed for binding. In the third condition, post-mitochondrial (S9) supernatant fractions were

prepared from liver homogenates of untreated mice centrifuged at 9000 *g* for 20 min at 4°. Aliquots (1.5 ml) of the supernatant were reacted for 60 min at 37° with 1 mM APAP in an NADPH-generating system containing 0.83 mM NADP, 20 mM glucose-6-phosphate, 4.0 I.U. of glucose-6-phosphate dehydrogenase, and 15 mM $MgCl_2$ [27]. At the end of the incubation, the mixture was then spun at 105,000 *g* for 1 hr at 4°. As a control, parallel incubations were conducted with NADP omitted.

Protein electrophoresis. Liver samples were prepared for one-dimensional analysis as previously described [18]. Proteins were resolved (30 μg/lane) according to molecular weight using the procedure of Laemmli [28] on a discontinuous 10% SDS-PAGE* slab gel system with a 3% stacking layer. The gels were run at a constant current of 20 mA/slab.

For two dimensional electrophoretic separation of APAP-bound proteins, the O'Farrell procedure [29] was followed to first resolve the proteins by isoelectric focusing, then according to molecular weight in the second dimension. Isoelectric focusing gels were cast in 130 × 2 mm glass tubes containing 3.5% polyacrylamide, 2% Bio-Rad ampholines (1.6% of a pH 5–7 range and 0.4% of a 3–10 pH range), 2% Nonidet P-40 in 9.0 M urea. Protein samples, diluted 1:1 in 10 M urea, 2% ampholines, 5% mercaptoethanol and 2% Nonidet P-40, were loaded at 100 μg/tube gel and focused at 400 V for 15 hr and then at 600 V for 1 hr. After completion of the first dimension, the gels were extruded from the glass tubes using a 5-cc tuberculin syringe and equilibrated in 5 ml of buffer containing 0.1 M Tris–HCl (pH 7.0), 2% SDS, 5% mercaptoethanol and 10% glycerol for 15 min at room temperature prior to SDS-PAGE in the second dimension as described above.

Western blotting and immunostaining. Proteins resolved on one- or two-dimensional polyacrylamide gels were electroblotted onto nitrocellulose membranes using a Bio-Rad Transblot apparatus. Proteins were transferred at 80 V for 6 hr in 25 mM Tris/192 mM glycine buffer (pH 8.3) containing 20% methanol [30]. After blotting, membranes were rinsed in Tris-buffered saline (TBS) at pH 7.4 containing 0.05% Tween 20 (TBS-Tween) for 1 hr to remove excess SDS and then blocked overnight in 3% BSA in TBS at 4°. The membranes were then incubated with affinity purified anti-APAP antibody diluted 1:400 in TBS–BSA solution and gently shaken for 3 hr at room temperature. Membranes were rinsed five times for 10 min each in TBS-Tween and then incubated for 90 min with [^{125}I]-conjugated goat anti-rabbit IgG (10 μCi/100 ml TBS–BSA). Following immunolabeling, the nitrocellulose was air dried and exposed at −70° to Kodak XAR-5 film with an intensifying screen. A similar protocol was followed for identifying the position of actin in cytosolic liver extracts from APAP-treated mice resolved in two dimensions. In this experiment, blots were incubated with a 1:1000 dilution of mouse anti-actin antibody. Gels used for electroblotting were stained to determine the quality of transfer; only those gels with complete transfer of proteins were utilized for immunostaining. Single-dimension gels were routinely stained with 0.1% Coomassie brilliant blue in 50% methanol and 3% acetic acid and then destained with 25% methanol and 10% acetic acid. Proteins resolved in two dimensions were visualized using a silver nitrate staining kit.

Protein thiol (PSH) analysis using NEM fluorography. The available thiol content of liver proteins was assessed using [^{3}H]*N*-ethylmaleimide labeling prior to one- or two-dimensional gel electrophoresis [19]. Livers from untreated mice were homogenized 1:9 in STM buffer containing 2.5 mM NEM (45 μCi/μmol) and reacted for 20 min at room temperature. This concentration of NEM represented at least a 2-fold molar excess over both low molecular weight and protein thiols. Homogenates were then centrifuged to provide the 9,000 and 105,000 *g* pellets and cytosol as described above. Each fraction was dialyzed against five 1-liter changes of PBS to remove any unreacted NEM. Samples were resolved by electrophoresis, and the resulting gels were stained with Coomassie brilliant blue as mentioned above. Stained gels were then impregnated with fluorographic En^{3}Hance, dried, and exposed to Kodak XAR-5 film for 7 days at −70°.

RESULTS

Production of antibodies for detecting APAP-protein adducts. To detect the presence of antibodies which recognize APAP-bound proteins, a second APAP-protein conjugate was required which lacked all other antigenic determinants of the immunogen except for the APAP moiety. For this purpose, chemically synthesized NAPQI was directly linked to BSA. Solid phase HRP-ELISA, using 100 ng of NAPQI-derivatized BSA as the coating antigen, demonstrated the presence of antibodies that detect APAP-protein adducts (Fig. 1). Anti-hapten activity

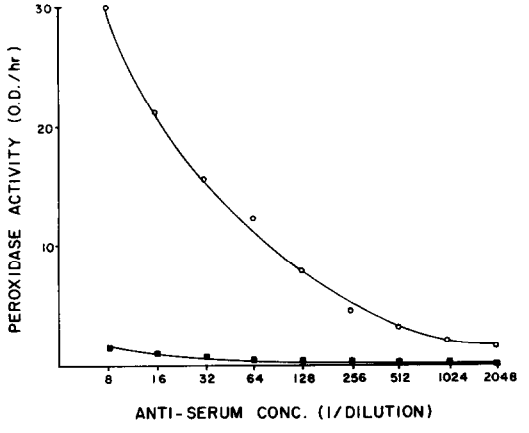

Fig. 1. Detection of antibodies against APAP bound protein using non-competitive HRP-ELISA. Wells of microtiter plates were coated with either 10 μl of a 10 μg/ml solution of NAPQI-derivatized BSA (○) or native BSA (■). Serum from rabbits immunized with APAP-PABA-KLH was serially diluted (1:16 to 1:2048) and added to wells in duplicate. Bound rabbit antibody was detected using peroxidase-conjugated goat anti-rabbit IgG. Peroxidase activity is expressed as optical density/hour.

* Sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

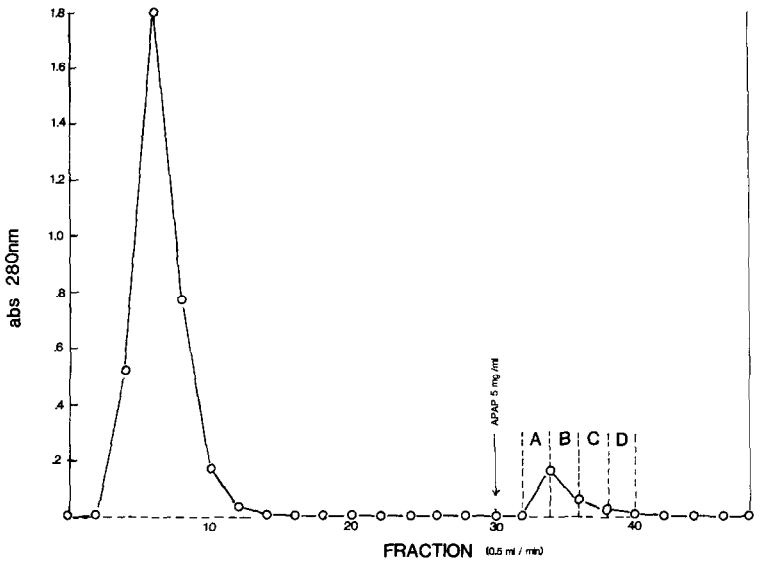

Fig. 2. Affinity purification profile of anti-APAP antibodies. Acetaminophen-specific antibodies were isolated from ammonium sulfate treated immune rabbit serum using an APAP linked column constructed by coupling the hydroxyl group of APAP to oxirane groups on epoxy-activated Sepharose 6B. Bound anti-APAP antibodies were selectively eluted by the addition of 5 mg APAP/ml of PBS (pH 7.4) and collected in fractions designated A, B, C and D. Protein elution was followed spectrophotometrically at 280 nm.

was observed even at the highest antiserum dilution tested (1:2048).

Isolation of anti-APAP antibodies. To minimize non-specific interactions and increase the sensitivity for detecting covalently bound APAP, the APAP-specific antibodies were isolated on an affinity column of APAP coupled to epoxy-activated Sepharose 6B (Fig. 2). The initial flow-through proteins lacked any detectable antibody activity against protein-bound APAP (Table 1). Selective elution of the bound anti-APAP IgG occurred only after the addition of an APAP solution (5 mg/ml) to the column (Fig. 2). The highest specific activities for NAPQI derivatized BSA were detected in the first four fractions collected at the beginning of the APAP elution front (Table 1). These combined fractions resulted in an affinity purified antibody preparation with a specific activity 245-fold greater than the original ammonium sulfate treated antiserum.

Characterization of antibody specificity. The specificity of the affinity purified antibody was characterized using indirect competitive HRP-ELISA. These studies focused on evaluating which determinants of the antigen were involved in antibody recognition. The observation that a preponderance of the anti-APAP antibodies which detect NAPQI-derivatized BSA was retained by the affinity column suggested that neither the protein component of the coating antigen nor the hydroxyl group of APAP, which was involved in ligand formation to the Sepharose matrix, was essential for antibody recognition. This indicated that recognition involved the N-acetyl region of APAP. Hence, the compounds used to analyze the specificity of the antibody had structural similarities to APAP. The relative efficiency of compounds such as APAP; *p*-aminophenol; 2,6-dimethyl APAP; 3,5-dimethyl APAP; and NAPQI-derivatized BSA to diminish antibody binding to the solid phase coating antigen is depicted in Fig. 3. Similar inhibition curves were observed

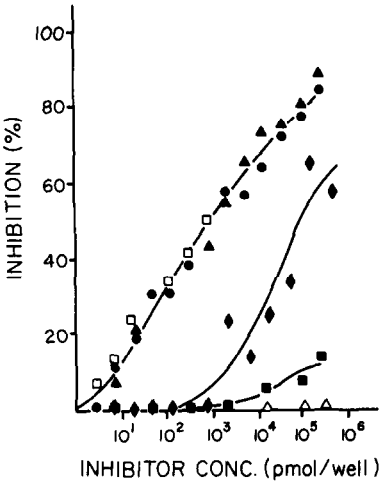

Fig. 3. Specificity of affinity purified rabbit antibodies using competitive HRP-ELISA. A range of concentrations of APAP (●), *p*-aminophenol (◆), 3,5-dimethyl APAP (▲), 2,6-dimethyl APAP (■), NAPQI-derivatized BSA (□) and BSA (△) was added to separate wells coated with 100 ng of the coating antigen. Affinity purified antibodies (final 1:150 dilution) were then incubated in these wells containing the inhibitor for 1 hr at 37°. All subsequent steps were completed as described in Materials and Methods. The percent inhibition was calculated by comparing the peroxidase activity of wells incubated with an inhibitor to wells incubated in the absence of these compounds.

Table 1. Affinity purification of anti-APAP antibodies

Sample	Peroxidase activity (absorbance/hr) (1) BSA coated wells	(2) BSA-APAP coated wells	(2 − 1)	Relative protein content (O.D. at 280 nm)	Specific activity (absorbance/hr) O.D. at 280 nm	Fold purification
$(NH_4)_2SO_4$ cut serum	2.46	25.05	22.59	3.45	6.55	1
Flow through peak	1.17	2.43	1.26	3.21	0.39	—
A	0.54	14.76	14.22	0.015	948	145
B	0.54	15.57	15.03	0.011	1366	209
C	0.30	10.38	10.08	0.004	2520	385
D	0.24	4.98	4.74	0.003	1580	241

Protein containing fractions collected from affinity chromatography were analyzed for activity against APAP-bound protein using a non-competitive HRP-ELISA. Assay wells were coated with 100 ng of either NAPQI-derivatized BSA or BSA, incubated with a 1:16 dilution of ammonium sulfate treated immune serum, the flow through peak, or eluted protein fractions A, B, C and D. Bound rabbit antibodies were detected using peroxidase-conjugated goat anti-rabbit antiserum and expressed as absorbance per hour. The relative protein content was based on the absorbance of appropriate aliquots read at 280 nm. Fold-purification was calculated in comparison to ammonium sulfate treated immune serum.

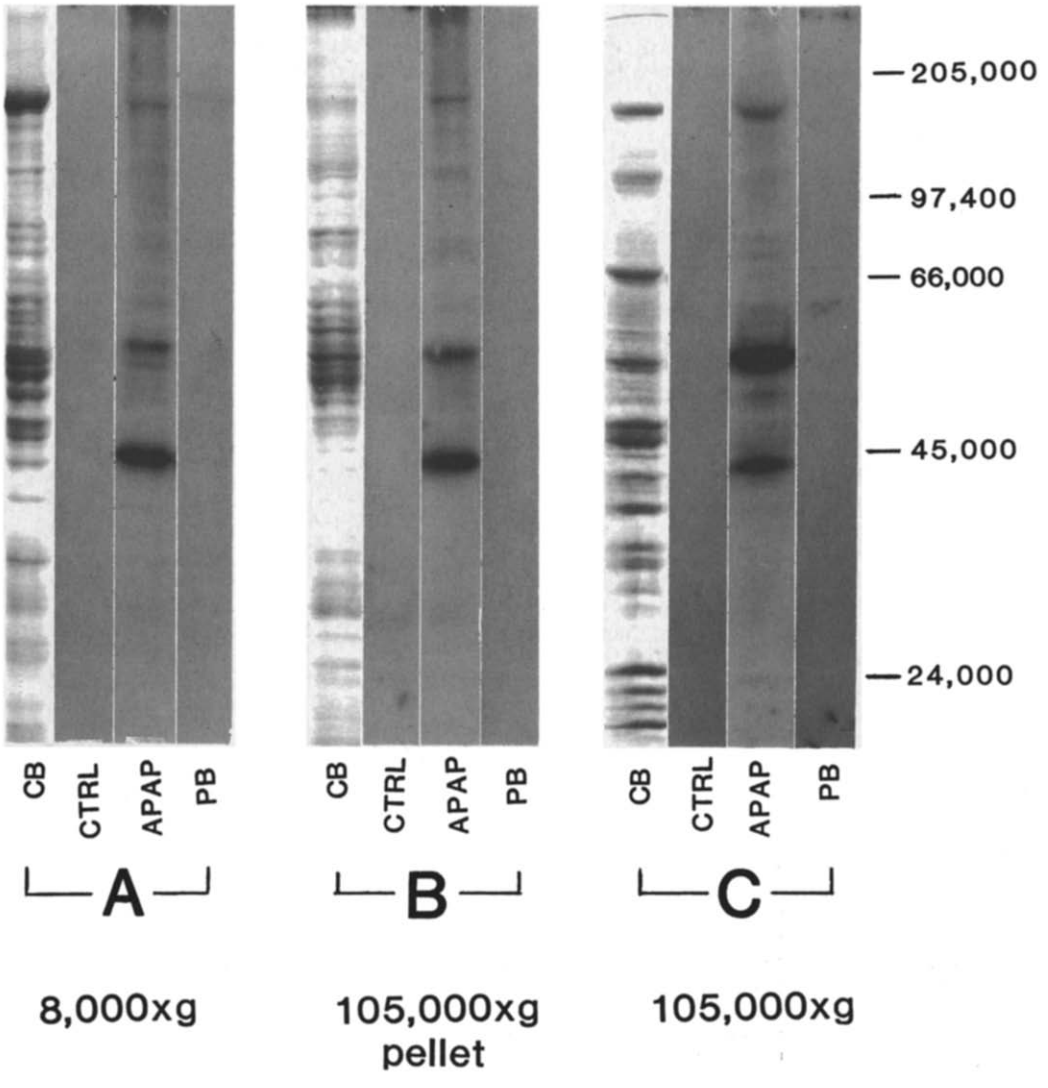

Fig. 4 (*continued on facing page*)

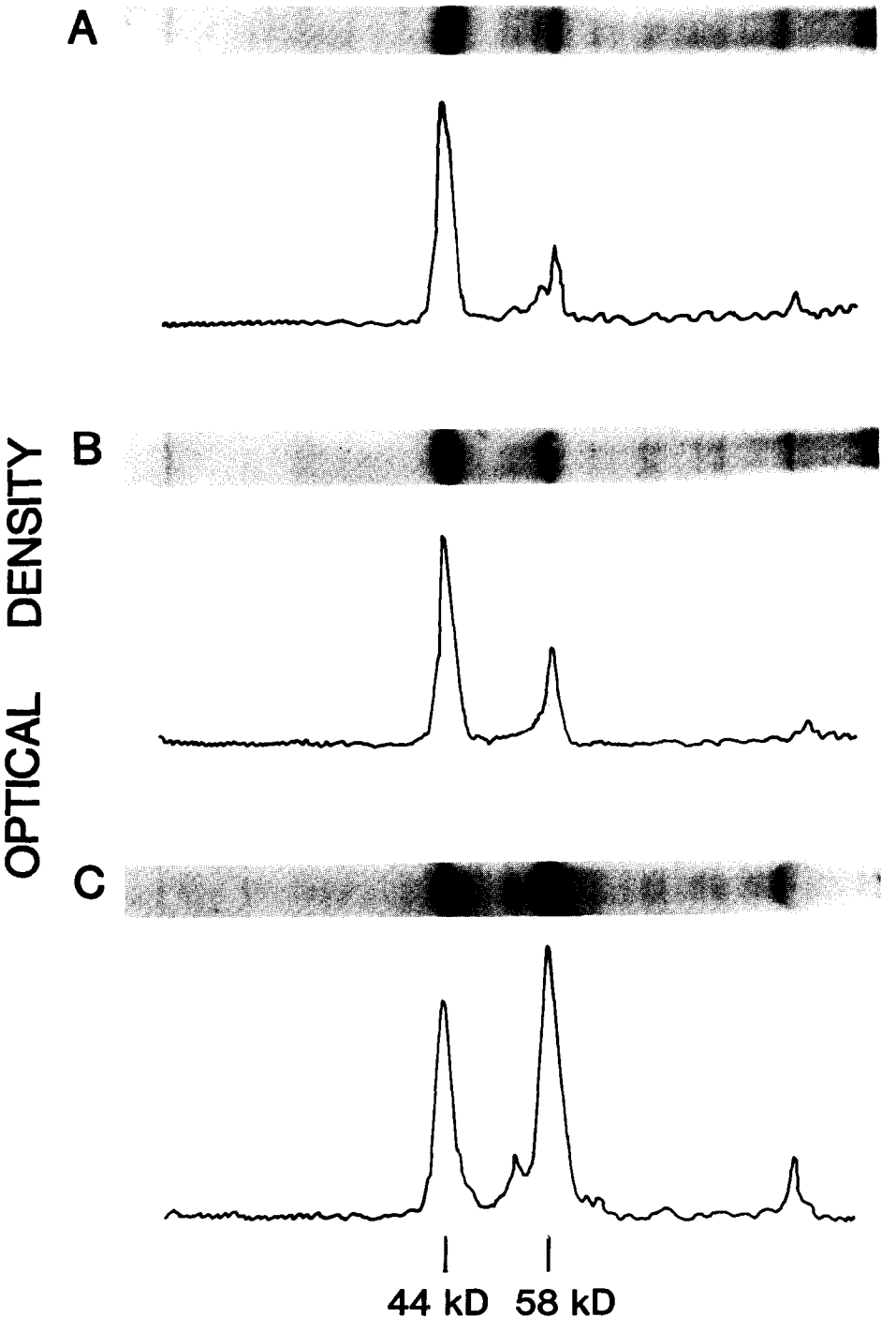

Fig. 4. Left panel: Western blot analysis of APAP-bound liver proteins produced *in vivo*. All lanes were from 10% SDS-PAGE run under reducing conditions. Lanes CB are profiles of liver extracts stained with Coomassie brilliant blue. Lanes CTRL, APAP, and PB are, respectively, Western blot profiles of liver extracts from untreated mice, from mice killed 4 hr after APAP (600 mg/kg, p.o.) dosing, and mice pretreated with piperonyl butoxide prior to APAP dosing. The relative migration rates of M_r standards are indicated. Right panel: Laser densitometry of Western blot profiles of liver fractions taken from mice exposed to an hepatotoxic dose of APAP. The relative intensities of immunochemically detected APAP-bound protein bands visualized by autoradiography were assessed using a laser densitometer. The scanning profiles are of Western blot autoradiographs from the (A) 8,000 *g* pellet, (B) 105,000 *g* pellet and (C) 105,000 *g* supernatant of livers taken from treated CD-1 mice killed 4 hr after APAP dosing. No deflections were observed from laser analyses of control lanes of liver fractions from untreated mice.

with APAP, NAPQI-derivatized BSA, and 3,5-dimethyl APAP which required approximately 800 pmol/well to result in a 50% inhibition of antibody binding. *p*-Aminophenol required a 100-fold higher concentration to result in a similar inhibition. By contrast, the 2,6-dimethyl APAP at 10^5 pmol/well caused less than 20% inhibition, and native BSA did not compete significantly with the purified antibody at any of the concentrations tested (Fig. 3). Steric hindrance by the methyl groups ortho to the N-acetyl moiety on 2,6-dimethyl APAP most likely reduced its recognition by the antibody. Therefore, the antibody should be capable of detecting any APAP-protein conjugate in which the N-acetyl region of APAP remains accessible.

Immunochemical detection of electrophoretically resolved APAP-protein adducts. The utility of the affinity purified anti-APAP antibody to detect electrophoretically resolved APAP-protein adducts was effectively demonstrated using Western blotting techniques. We have reported previously that maximum protein binding *in vivo* is detectable immunochemically 4 hr after administering APAP (600 mg/kg, p.o.) to mice [18]. Therefore, APAP-bound proteins were immunochemically analyzed in subcellular fractions of liver extracts taken from similarly treated mice. Figure 4 (left panel) demonstrates that the specificity of APAP binding to proteins *in vivo* occurred in a very selective manner with a preponderance of the drug being covalently bound to proteins of 44 and 58 kD. As previously noted [18], several other minor protein adducts were also detected. The absence of any detectable binding in liver extracts from untreated mice demonstrated negligible non-specific binding by the affinity purified antibody (lanes CTRL). A comparison of the relative concentration of the resolved proteins using Coomassie blue staining (lanes CB) and the extent of APAP bound to proteins (lanes APAP) indicated that the binding by APAP was not consistently reflective of the protein concentration. Thus, whereas the 58 kD band, which was highly immunostained in the cytosol, corresponded to an intense staining protein band, the 44 kD protein band, which was also highly immunostained, coincided with a lightly stained Coomassie region. Laser densitometry scans of the autoradiographs suggested that more than 85% of the total immunochemically detectable protein-bound APAP was associated with the 44 and 58 kD bands (Fig. 4, right panel). Moreover, these scans also suggested that of the 58 kD adducts the majority appears in the soluble fraction (panel C), whereas nearly equal amounts of the 44 kD adducts were detected in all three subcellular fractions (compare panels A, B and C).

To ascertain whether the APAP-bound liver proteins detected *in vivo* were a consequence of cytochrome P-450 activation, mice were pretreated with the cytochrome P-450 inhibitor, piperonyl butoxide, prior to dosing with APAP. This pretreatment, which inhibits mixed-function oxidase activity, has been shown to prevent the APAP hepatotoxicity in CD-1 mice [22] and virtually abolished the formation of immunochemically detectable APAP-protein adducts (Fig. 4, left panel, lanes PB). Furthermore, it is unlikely that the adducts detected *in vivo* were the result of the activation of the deacetylated product of APAP, *p*-aminophenol, since mice dosed with *p*-aminophenol did not result in any immunochemically detectable adducts (data not shown).

Partial characterization of the major APAP-protein adducts. To better characterize the predominant APAP-binding proteins, cytosolic liver protein extracts from mice killed 4 hr after treatment with 600 mg of APAP/kg were resolved in two dimensions. The 105,000 *g* supernatant fraction was chosen for these studies because it contained a high proportion of both major protein adducts. A silver stain of the two-dimensional profile of cytosolic liver proteins from untreated mice is shown in Fig. 5A. A similar pattern of protein migration was produced with liver extracts from APAP-treated mice (data not shown) which suggested that the migration of protein adducts in either dimension was not altered significantly by APAP binding. Following Western blotting, the most intense immunochemical staining was found associated with protein clusters in the 44 and 58 kD molecular weight range (Fig. 5B). The isoelectric point for the major 44 kD APAP binding protein was shown to be approximately 7.0–7.1 (arrow 1), whereas the 58 kD protein cluster exhibited a pI range of 6.4–6.6 (arrow 2). It should be noted that the 44 kD target does not appear to be actin (arrow 4). In addition to the 44 and 58 kD proteins, a number of other arylated cytosolic proteins with less intense staining were also evident. Consistent with the observations noted for Fig. 4, left panel, comparison of the silver stain and the Western blot profiles in Fig. 5 also demonstrated that very few of the many cytosolic proteins become arylated after APAP exposure *in vivo* (compare panels A and B).

To evaluate qualitatively the PSH content of the major APAP-protein adducts, liver extracts from untreated mice were titrated with [^{3}H]NEM prior to electrophoretic analysis. A fluorogram of the PSH content of the cytosolic proteins resolved in two dimensions is presented in Fig. 5C. It can be shown that the cluster of 58 kD proteins which bind APAP was also among the highest thiol-containing cytosolic proteins detected by NEM labeling (arrow 2). Conversely, the major 44 kD protein which avidly binds APAP was barely detectable by NEM fluorography (arrow 1). Furthermore, other proteins (e.g. arrow 3) which exhibited a high thiol content did not apparently bind APAP.

Comparison of protein adducts produced upon enzymatic activation of APAP with those produced upon addition of chemically synthesized NAPQI. To better understand the biochemical basis for the apparent selectivity of covalent binding to the 44 and 58 kD proteins *in vivo*, comparisons were made among the APAP-protein adducts produced in a number of *in vitro* systems. Figure 6 illustrates the adducts that were detected in the microsomal (M) and cytosolic (C) fractions of systems in which either APAP was enzymatically activated, or chemically synthesized NAPQI was added directly. Western blot analysis demonstrated that the selectivities of binding to the 44 and 58 kD proteins were similar after *in vivo* dosing and under conditions when

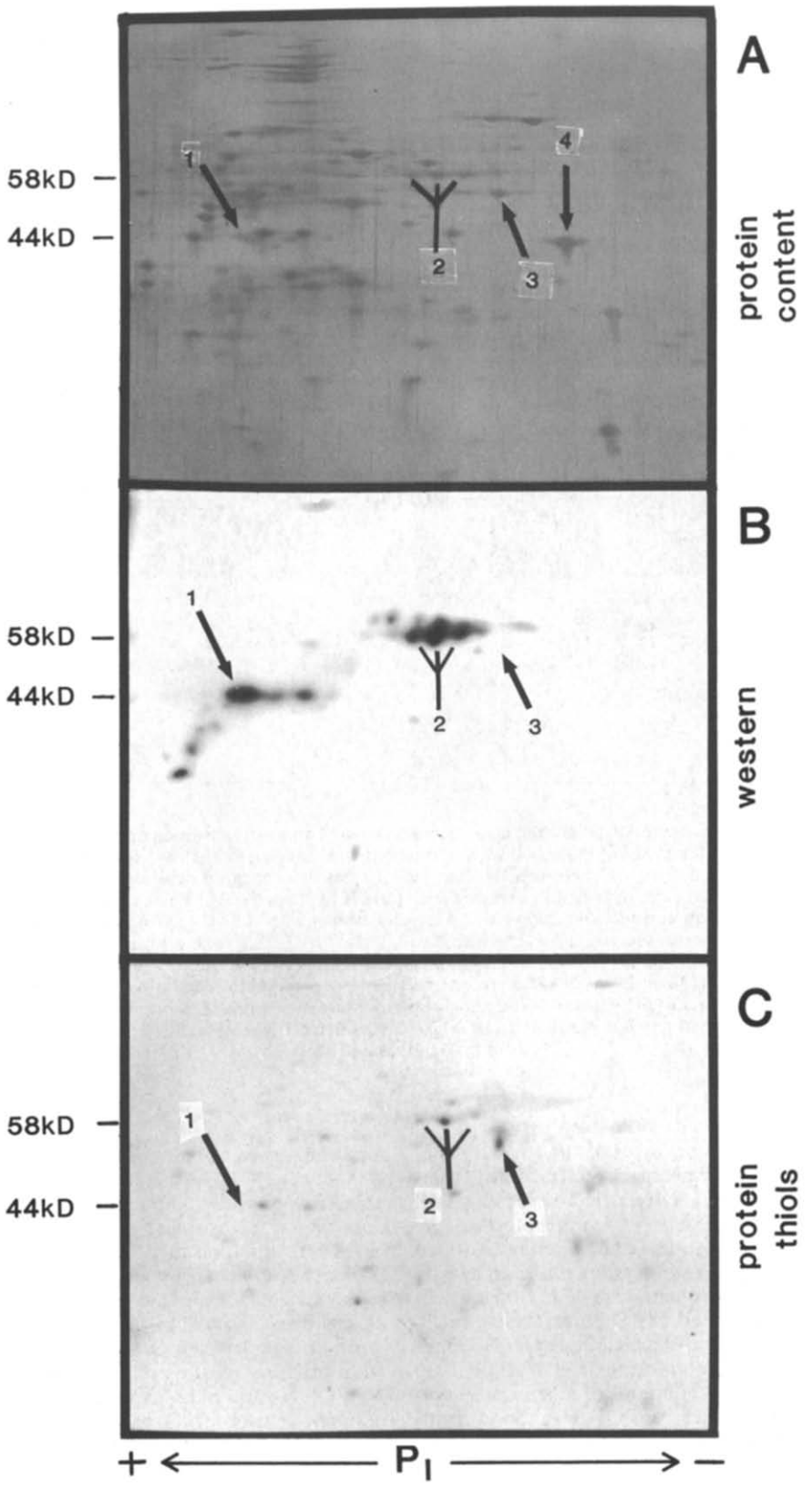

Fig. 5. Two-dimensional analysis of the major APAP-binding liver proteins formed *in vivo*. Liver proteins of the 105,000 *g* supernatant were resolved in the first dimension by isoelectric focusing and then according to molecular weight using SDS-PAGE. Samples from untreated mice were used to produce a silver stain (A), whereas cytosolic proteins from mice killed 4 hr after APAP (600 mg/kg, p.o.) dosing were used for Western blotting analysis (B). To assess the protein thiol content of the soluble fraction, livers from untreated mice were homogenized in STM buffer containing [^{3}H]NEM, fractionated, resolved in two dimensions and visualized by fluorography (C). Arrows are provided to permit direct comparison of the APAP-protein adducts with PSH content and silver stain of proteins with similar mobility. Arrow 4 (panel A) designates the position of actin in resolved liver extracts as determined using an anti-actin monoclonal antibody.

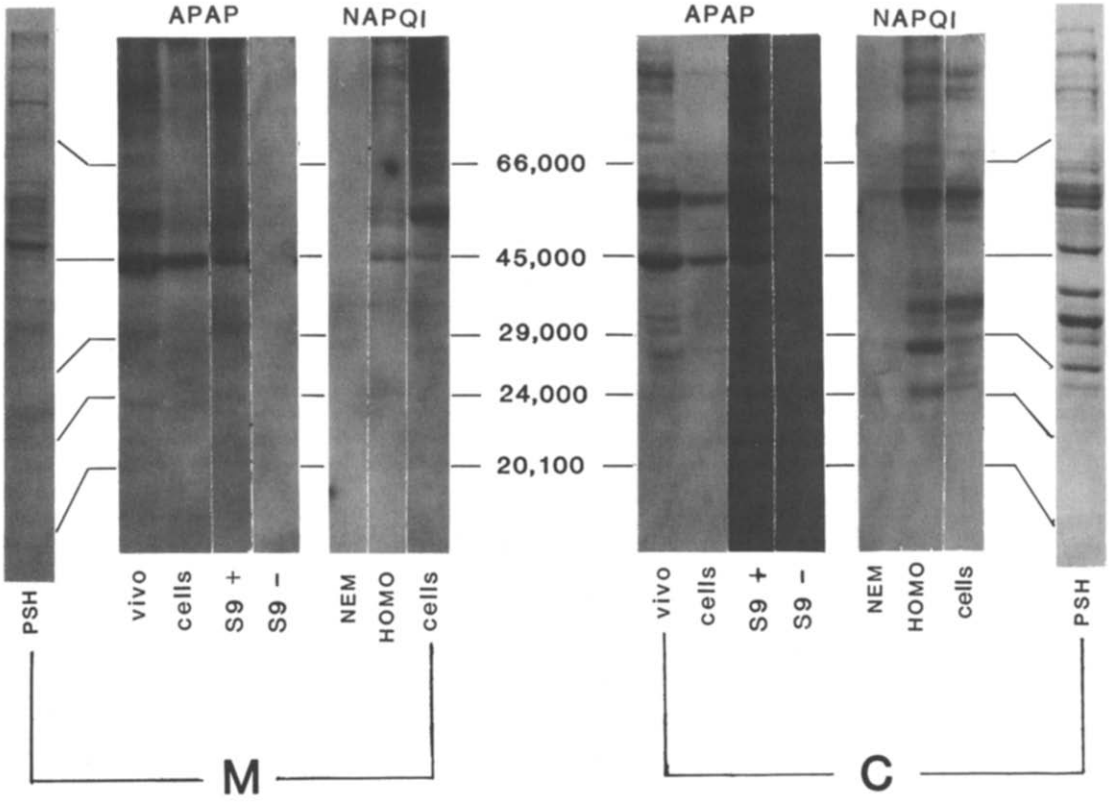

Fig. 6. Comparison of APAP-protein adducts produced via enzymatic activation and by treatment with chemically synthesized NAPQI. Subcellular fractions are designated (M) 105,000 *g* pellet and (C) 105,000 *g* supernatant. All lanes are Western blot profiles, except those designated PSH, which are fluorograms of control liver extracts reacted with [^{3}H]NEM. Lanes APAP are from systems in which APAP was given and include: mice killed 4 hr after dosing with APAP (600 mg/kg, p.o.) (VIVO); isolated hepatocytes incubated for 2 hr with 10 mM APAP (CELLS); and S9 fractions incubated for 60 min with 1 mM APAP, glucose-6-phosphate, glucose-6-phosphate dehydrogenase with (S9+) or without (S9−) NADP. Lanes NAPQI are systems that were exposed to chemically synthesized NAPQI for 5 min. These include extracts of liver homogenates pretreated with NEM prior to NAPQI addition (NEM); liver extracts not pretreated (HOMO); and isolated hepatocytes (CELLS). Details can be found in Materials and Methods.

APAP was activated enzymatically in isolated hepatocytes and in supplemented S9 fractions (lanes APAP). For example, in liver microsomes isolated from mice dosed *in vivo*, from hepatocyte suspensions or from S9 supernatant fractions fortified with an NADPH-generating system, the most heavily arylated protein band migrated at 44 kD, whereas in cytosol both the 44 and 58 kD bands were equally prominent. No immunochemically detectable binding was evident with unfortified S9 fractions. By contrast, the direct addition of chemically synthesized NAPQI (lanes NAPQI) resulted in a different protein binding profile from that produced during enzymatic activation of APAP. Under both conditions the intense binding to the cytosolic 58 kD protein band was readily detected. By contrast, the arylation of the 44 kD band was barely detected in samples analyzed after the direct addition of NAPQI. Furthermore, several proteins that were arylated in liver homogenates or in isolated cells following direct NAPQI addition were not arylated *in vivo*.

Pretreatment of the liver extracts with NEM virtually abolished all binding of directly added NAPQI (Fig. 6, lanes NEM), demonstrating that the binding of NAPQI to proteins appears to be primarily dependent on the accessibility of free cysteine residues. However, arylation of proteins which contain free sulfhydryls was not proportional to their thiol content (compare lanes PSH and NAPQI). This was most clearly demonstrated in the cytosolic fraction. Proteins of 58 kD which contain high PSH coincided with proteins which are also highly arylated by NAPQI (lanes PSH and HOMO). In contrast, another cytosolic protein band of approximately 46 kD was also PSH-rich but did not appear to bind NAPQI (lanes PSH and HOMO). As was the case with the two-dimensional gels, the 44 kD protein band, which was targeted only in the presence of enzymatically activated APAP, exhibited little [^{3}H]NEM labeling indicative of low PSH content (lane PSH). Therefore, although cysteine residues appear to be prominent sites for NAPQI binding, not all NEM-reactive cysteine residues on proteins react equivalently with NAPQI.

DISCUSSION

The covalent binding of APAP to proteins has often been implicated in eliciting hepatotoxicity. However, the biochemical sequence of events leading to the toxicity is still undetermined. As a means to better understand the mechanisms underlying the cellular perturbations, it becomes of primary interest to detect and characterize the specific proteins that become arylated during toxicity. The most widely used method to evaluate the extent of covalent binding of APAP to proteins utilizes radiolabeled drug [3, 12, 13]. As an alternative means of detection, antibodies have been elicited against APAP-protein adducts [18, 31]. Since the isotopic method would have required excessive amounts of radiolabeled APAP to assess the selectivity and importance of APAP binding to specific proteins, we have developed a highly sensitive immunoassay which aided in the detection and partial characterization of electrophoretically resolved APAP-protein adducts. This immunochemical approach, using an affinity purified anti-APAP antibody, had enabled us to detect successfully the protein targets of APAP binding *in vivo* which are associated with hepatotoxic and threshold doses of this drug [18].

In the present study, proteins with molecular weights of 44 and 58 kD are shown to be the major targets that are selectively arylated by APAP *in vivo* and that account for nearly 85% of the total APAP-protein adducts detected immunochemically. APAP-protein adducts of similar molecular weights have been shown to be the earliest detectable protein targets in primary cultures of mouse hepatocytes [1] and also appear to be selectively arylated in extrahepatic tissues such as kidney and lung [32] which undergo necrosis following the administration of an excessive APAP dosage *in vivo* [33]. Two-dimensional analysis of liver cytosol from treated mice revealed that each major APAP-protein band represents clusters of three to four proteins. The isoelectric point of the major 44 kD protein was approximately 7.0–7.1, whereas the 58 kD protein cluster exhibited a pI range of 6.4–6.6. It is not certain at this point whether each cluster may represent isovariant forms of proteins with a common physiological function. The absence of binding in livers from piperonyl butoxide pretreated mice and in S9 fractions not fortified with NADP suggests that the arylated proteins are likely formed via NADPH-linked cytochrome P-450 activation of APAP.

An understanding of the underlying basis for the high extent of arylation as well as the selectivity of APAP binding to these proteins *in vivo* is of fundamental interest from both a biochemical and a toxicological standpoint. Previous studies have determined that cysteine residues on either glutathione or proteins are the primary sites for NAPQI binding [23–25]. Consistent with these observations is the extensive diminution of immunochemically detectable binding in liver extracts treated with NEM prior to NAPQI addition. These data further suggest that the antibody is primarily detecting protein thiol conjugates of APAP. The correlation between APAP binding and the high thiol content of proteins was most readily observed in the 58 kD cluster. All three of the arylated proteins in this cluster appeared to be rich in free PSH. However, *in vivo* many other PSH-rich proteins do not become arylated, indicating that PSH content is not the sole determinant of selective covalent binding. This is not to say that these high thiol-containing proteins are not susceptible to NAPQI binding *in vitro* since many of the same proteins were arylated following direct addition of NAPQI. This argues that protein susceptibility is the result of both PSH reactivity and accessibility to the electrophile. The very high PSH content and apparent high reactivity of the 58 kD proteins for NAPQI make it tempting to speculate on their possible role in protecting cells by sequestering reactive electrophilic species such as NAPQI and other quinones.

Since the major 44 kD protein-adduct appears to contain few free cysteine residues, it is possible that the cellular localization of proteins with respect to the site of cytochrome P-450 activation is also a determinant of APAP binding. This is suggested when the profiles of arylated proteins obtained upon APAP activation *in vivo*, in isolated hepatocytes, or in fortified S9 systems were compared with profiles from intact cells and liver extracts exposed to chemically synthesized NAPQI. The results demonstrated that the specificity of binding produced *in vivo* was conserved in systems which enzymatically activate APAP. By contrast, this binding profile was altered when chemically synthesized NAPQI was added to either hepatocyte suspensions or liver homogenates. In the latter treatment, when NAPQI was added to a homogeneous liver extract, all proteins irrespective of their cellular location should be equally accessible to arylation. Under these *in vitro* conditions the binding to the 58 kD protein band remains one of the most intense cytosolic bands detectable immunochemically. The observation that these sulfhydryl-rich proteins were also arylated upon NAPQI addition to cell suspensions suggests that the reactive metabolite may disrupt the plasma membrane. However, the addition of chemically synthesized NAPQI resulted in the formation of several protein adducts which were not observed *in vivo* as well as in a greatly diminished arylation of the 44 kD protein band. These data indicate that the high extent of binding to the 44 kD proteins which occurs only after enzymatic activation of APAP is likely a result of their cellular localization. Therefore, it is envisaged that proteins with relatively low PSH content but which are near the site of APAP activation may be more susceptible to covalent binding than proteins of higher thiol content which are further from the site of activation. This may explain why *in vivo* the low thiol-containing 44 kD proteins become a selective target for arylation amongst other higher thiol-containing proteins. It is unlikely that these proteins are cytochrome P-450 isozymes because the known mouse liver isozymes are between 50 and 58 kD [34]. The conserved specificity of APAP binding to the 44 kD proteins by the NADPH fortified S9 system suggests that arylation of specific proteins may play a critical role in the ensuing hepatotoxic events. Additional experiments would be required to determine if the protein adducts of 44 and 58 kD detected in each subcellular fraction are functionally

similar proteins. In light of these observations, we feel that results based solely on *in vitro* experimentation with chemically synthesized NAPQI for investigating the relevance of covalent binding to the mechanisms of APAP toxicity should be interpreted cautiously.

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