0026-895X/91/010036-09$03.00/0

MOLECULAR PHARMACOLOGY, 40:36-44

Purification, Immunochemical Characterization, and Immunohistochemical Localization of Rat Hepatic Aryl Sulfotransferase IV

MICHAEL W. DUFFEL, THOMAS P. BINDER,[1] LYNN HOSIE,[2] HOLLY A. BADEN, JILL A. SANDERS, SHIRLEY A. KNAPP, and JEFFREY BARON

Division of Medicinal and Natural Products Chemistry, College of Pharmacy [M.W.D., T.P.B., L.H., H.A.B., J.A.S.], and Department of Pharmacology, College of Medicine [S.A.K., J.B.], University of Iowa, Iowa City, Iowa 52242

Received January 2, 1991; Accepted April 1, 1991

SUMMARY

Aryl sulfotransferases catalyze the formation of sulfuric acid esters from a diverse group of endogenous and xenobiotic organic chemicals. The isoenzyme of aryl sulfotransferase in livers of male Sprague-Dawley rats that exhibits the most varied substrate specificity is aryl sulfotransferase IV. A new method for the purification to homogeneity of aryl sulfotransferase IV was developed that, when compared with previously described procedures, provided a >10-fold increase in total yield of enzyme/g of tissue. Homogeneous aryl sulfotransferase IV was used to prepare polyclonal antibodies in male New Zealand White rabbits. Results of immunochemical analyses demonstrated that these antibodies reacted with only a single protein in rat hepatic 100,000 × *g* supernatant fractions and, further, that the immunoreactive protein had the isoelectric point and subunit molecular mass characteristic of aryl sulfotransferase IV. Immunohistochemical analyses demonstrated that aryl sulfotransferase IV is present in hepatocytes throughout the liver, although centrilobular cells contain a significantly greater ($p < 0.01$) amount of aryl sulfotransferase IV than do either midzonal or periportal cells, in which similar levels of the enzyme are found.

Aryl sulfotransferase IV catalyzes the PAPS-dependent formation of sulfuric acid esters from a wide variety of hydroxyl-containing organic chemicals. The enzyme has been considered as being both an isoenzyme of aryl sulfotransferase and a tyrosine ester sulfotransferase (EC 2.8.2.9). Although aryl sulfotransferase IV is one of at least five isoenzymes of aryl sulfotransferase that have been purified from livers of male rats (1–6), the range of chemical classes serving as substrates for this enzyme is more extensive than that for any of the other aryl sulfotransferases. Examples of these substrates include phenols, tyrosine esters, catechols, benzylic alcohols, arylhydroxamic acids, and arylhydroxylamines. Aryl sulfotransferase IV is involved in the detoxication of drugs and other xenobiotics and catalyzes the formation of sulfuric acid esters that, in general, are chemically stable under physiological conditions, are water soluble, and are excreted more readily than are the parent chemicals from which they are derived. However, aryl sulfotransferases can also catalyze the formation of chemically reactive sulfuric acid esters from benzylic alcohols (7–9), arylhydroxylamines (10), and arylhydroxamic acids (11), and the generation of these sulfate esters can give rise to cellular necrosis, chemical carcinogenesis, and other toxic responses (12). The substrate specificity of aryl sulfotransferase IV is consistent with an important role for this isoenzyme in the formation of these chemically reactive metabolites. Indeed, there is evidence that aryl sulfotransferase IV is primarily responsible for catalyzing the cytosolic *N*-hydroxy-2-acetylaminofluorene sulfotransferase activity in livers of male rats (13).

Attempts to conclusively demonstrate the involvement of aryl sulfotransferase IV in the toxicity of phenols, benzylic alcohols, arylhydroxylamines, and arylhydroxamic acids have often been hindered by difficulties in obtaining the purified enzyme in sufficient amounts for both conducting of extensive investigations on its catalytic mechanism and its ability to catalyze the sulfation of these chemicals and preparation of antibodies for use in immunochemical and immunohistochemical analyses. This report describes a purification procedure for aryl sulfotransferase IV with a significantly improved yield of the enzyme/g of liver. Furthermore, polyclonal antibodies to the homogeneous rat hepatic aryl sulfotransferase IV were

This investigation was supported by United States Public Health Service Grant CA38683, awarded by the National Cancer Institute, Department of Health and Human Services.

[1]Current address: Archer Daniels Midland Co., Clinton, IA 52732.

[2]Current address: Sandoz Chemicals Corp., Charlotte, NC 28266.

ABBREVIATIONS: PAPS, 3′-phosphoadenosine-5′-phosphosulfate; IEF, isoelectric focusing; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

prepared in rabbits. The specificity of these antibodies was evaluated by Western blot analysis of homogeneous aryl sulfotransferase IV and of 100,000 × *g* supernatant fractions from rat liver, both before and after preparative IEF. Immunohistochemical studies were then performed using these antibodies, thereby allowing determinations of the localization and intralobular distribution of the enzyme in rat liver.

Experimental Procedures

Materials

Sucrose (grade I), ATP (grade I), and ATP-agarose (ATP attached through the ribose hydroxyls to 4% beaded agarose, with a six-carbon spacer) were obtained from Sigma Chemical Co. (St. Louis, MO). Enzyme research grade ammonium sulfate was from GIBCO (Grand Island, NY), and hydroxylapatite (BioGel HT) was obtained from Bio-Rad Laboratories (Richmond, CA). DE-52 and CM-52 were obtained from Whatman (Clifton, NJ), and the glycerol used in all purification buffers was AR grade from Mallinckrodt (Paris, KY). PAPS was prepared by chemical synthesis (14). All other reagents and materials were of the highest purity commercially available.

Assay of Aryl Sulfotransferase Activity

Aryl sulfotransferase IV activity was determined using the standard phenol sulfotransferase assay described by Sekura and Jakoby (1). This method employs the solubility in chloroform of the ion pair formed between 2-naphthylsulfate and methylene blue. Assays for aryl sulfotransferase IV activity were conducted at 37°, using reaction mixtures containing 0.25 mM 2-naphthol, 0.2 mM PAPS, 5 mM 2-mercaptoethanol, aryl sulfotransferase, and 0.25 M sodium acetate, pH 5.5, in a total volume of 0.4 ml. For assay of aryl sulfotransferase I and II activities in chromatographic fractions, reaction mixtures contained 0.25 mM 2-naphthol, 0.2 mM PAPS, 5 mM 2-mercaptoethanol, aryl sulfotransferase, and 0.25 M sodium phosphate, pH 7.4, in a total volume of 0.4 ml at 37°. Reactions were started by the addition of enzyme, and incubations were carried out for 10 min. Enzyme units are expressed as nmol of 2-naphthylsulfate formed/min.

Evaluation of Enzyme Purity

The purity of aryl sulfotransferase IV preparations was evaluated by means of both SDS-PAGE and analytical IEF. Protein samples were prepared for SDS-PAGE by heating for at least 5 min at 100° in the presence of 2% (w/v) SDS and 5% (v/v) 2-mercaptoethanol. SDS-PAGE was performed on 12% (w/v) polyacrylamide slab gels using the buffer system described by Laemmli (15). Analytical IEF was carried out in 4.8% (w/v) polyacrylamide gels using a final ampholyte (Servalyt 4–7; Serva Biochemicals, Westbury, NY) concentration of 2.0% (w/v).

Enzyme Purification

Male Sprague-Dawley rats (375–425 g) were obtained from BioLabs (St. Paul, MN) and were given standard lab chow and water *ad libitum* for at least 1 week before use. Rats were killed by decapitation, and the livers were immediately removed and placed in 0.25 M sucrose at 4°. Unless otherwise indicated, all purification steps were carried out at 4°. All buffers used in the purification steps were prepared at 25°, the pH was adjusted at that temperature, and the buffers were then chilled to 4°. Protein concentrations were determined using a modified Lowry procedure (16), with bovine serum albumin as standard.

Step 1. Preparation of the 100,000 × *g* supernatant fraction. Four rat livers (66 g, total wet weight) were homogenized in 0.25 M sucrose (4:1, v/w, homogenate) in a Potter-Elvejhem homogenizer. The homogenate was subjected to centrifugation at 100,000 × *g* for 90 min, and the supernate was then decanted.

Step 2. Ammonium sulfate fractionation. Solid ammonium sulfate, (17.6 g/100 ml) was slowly added to the 100,000 × *g* supernatant fraction, and the resulting mixture was gently stirred for 20 min. After centrifugation at 30,000 × *g* for 20 min, the supernate was decanted. Solid ammonium sulfate (16.0 g/100 ml) was slowly added to this supernate, and the mixture was gently stirred for 20 min. The mixture was then centrifuged at 30,000 × *g* for 20 min. After the supernate was decanted, the pellet was resuspended in 66 ml of buffer A (0.01 M Tris·HCl, pH 7.4, containing 0.25 M sucrose, 10%, v/v, glycerol, and 1.0 mM dithiothreitol). The protein solution was dialyzed overnight against two 1.5-liter changes of buffer A. The precipitate remaining after dialysis was removed by centrifugation for 25 min at 20,000 × *g*, and the pellet was discarded.

Step 3. DEAE-cellulose chromatography. The dialyzed ammonium sulfate fraction was applied to a column (4 × 20 cm) of DE-52 that had been equilibrated with buffer A. The column was washed with buffer A until all unbound protein was eluted. The various isoenzymes of aryl sulfotransferase were then eluted with a 1600-ml linear gradient formed between 800 ml of buffer A and 800 ml of buffer A containing 0.3 M sodium chloride. Fractions of 15 ml were collected. Aryl sulfotransferases I and II eluted at approximately 0.1 M sodium chloride, and aryl sulfotransferase IV eluted at approximately 0.14 M sodium chloride. Fractions containing aryl sulfotransferase IV activity were combined and concentrated in a stirred ultrafiltration cell (Amicon PM-10 membrane) to a final volume of approximately 50 ml. The preparation was then divided into two portions, which were carried through the remaining steps separately. The enzyme could be stored at this point in buffer A at −70° for at least 30 days without loss of activity.

Step 4. ATP-agarose chromatography 1. Concentrated DE-52 eluate was added to an equal volume of buffer B (0.1 M sodium acetate, pH 5.5, containing 0.25 M sucrose, 10%, v/v, glycerol, and 1.0 mM dithiothreitol) and applied, at a flow rate of approximately 0.2 ml/min, to a column (1 × 5 cm) of ATP-agarose that had been previously equilibrated with buffer B. The column was washed with buffer B until all unbound protein was removed. Aryl sulfotransferase IV was eluted from the column with a linear gradient formed between 17.5 ml of buffer B and 17.5 ml of buffer B containing 20 mM ATP; 4-ml fractions were collected. Aryl sulfotransferase IV was obtained in the first three protein-containing fractions after initiation of the gradient. Fractions containing aryl sulfotransferase IV activity were pooled, concentrated to approximately 5 ml in a stirred ultrafiltration cell (Amicon PM-10 membrane), and then dialyzed against two 1.0-liter changes of buffer C (5 mM sodium phosphate, pH 7.0, containing 0.25 M sucrose, 10%, v/v, glycerol, and 1.0 mM dithiothreitol).

Step 5. Hydroxylapatite chromatography. The dialyzed protein was applied to a column (1 × 4.5 cm) of hydroxylapatite that had been previously equilibrated with buffer C. After washing of the column with buffer C in order to remove unbound protein, the column was eluted with a linear gradient formed between 20 ml of buffer C and 20 ml of buffer C containing 0.3 M sodium phosphate (pH 7.0); 2-ml fractions were collected. Aryl sulfotransferase IV was obtained as the first chromatographic peak of sulfotransferase activity after the start of the salt gradient. Fractions containing aryl sulfotransferase IV were pooled and concentrated to an approximate volume of 4 ml, using a stirred ultrafiltration cell (Amicon PM-10 membrane). The concentrated fractions were dialyzed overnight against 1 liter of buffer B.

Step 6. ATP-agarose chromatography 2. The dialyzed aryl sulfotransferase IV was applied to a column of ATP-agarose (1 × 2 cm) that had been equilibrated at 25° with buffer B. Although this chromatographic step was carried out at 25°, the eluate fractions were placed on ice immediately after collection. The column was thoroughly washed with buffer B until all unbound protein was eluted. After fitting of the column with a flow adapter to minimize the dead-volume at the top of the column, the direction of eluant flow was inverted, and the column was eluted with 40 ml of 0.04 mM PAPS in buffer B. Aryl sulfotransferase IV activity was observed in the first protein-containing fractions obtained with PAPS elution. Fractions with aryl sulfotransferase IV activity (4–6-ml total volume) were combined and concen-

trated in an Amicon stirred ultrafiltration cell (PM-10 membrane) to a final volume of 1–2 ml.

Antibody Preparation

Homogeneous aryl sulfotransferase IV was emulsified with complete Freund's adjuvant and administered in multiple subcutaneous injections to New Zealand White rabbits (4 kg); each rabbit received a total of 100 μg of the purified protein. After 10 days, each rabbit was given multiple subcutaneous booster injections containing a total of 75 μg of aryl sulfotransferase IV emulsified with incomplete Freund's adjuvant. This was repeated 7 days later. Blood was obtained via the ear vein at 8 and 14 days after the second booster injection, and antiserum was isolated by centrifugation at 1000 × *g* for 30 min.

Immunochemical Analysis of Antibody Specificity

The specificity of the rabbit antiserum to rat hepatic aryl sulfotransferase IV was examined using standard Western blot procedures. SDS-PAGE was carried out in 12% gels (Bio-Rad Mini-Protean II apparatus) for purified aryl sulfotransferase IV, cytosol, and fractions of intermediate purity obtained from the DEAE-cellulose column. The fractions of intermediate purity included a concentrated fraction containing aryl sulfotransferases I and II (no isoenzyme IV was present, as judged by assay with 2-naphthol at pH 5.5) and a concentrated fraction containing aryl sulfotransferase IV. After SDS-PAGE, proteins were electrophoretically transferred to a nitrocellulose membrane. Immunochemical analysis was carried out with the following modification of a published procedure (17): 0.15% (v/v) Tween 20 was added to all wash solutions and antibody incubation mixtures. Nitrocellulose membranes containing the transferred proteins were exposed to either immune or normal (nonimmune) rabbit serum (1:20,000 dilution) and then to horseradish peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad) (1:5000 dilution). Color development was obtained by incubation with 4-chloro-1-naphthol and H_2O_2.

Antibody specificity with respect to isoenzymes III and IV was evaluated using preparative IEF, followed by assay of enzymatic activity and SDS-PAGE/Western blot analysis for each fraction obtained by IEF. Preparative IEF of 5 ml of freshly prepared rat hepatic cytosol was carried out in a Rotofor preparative IEF cell (Bio-Rad), using 2% (v/v) ampholytes (Bio-Lyte 5–7; Bio-Rad) in 0.25 M sucrose. IEF was conducted at constant power (12 W) for 5.5 hr. Twenty 2.7-ml fractions were collected, and the pH of each was immediately determined and adjusted to 7.0 by the addition of 0.2–0.4 ml of a buffer containing 1.0 M potassium phosphate (pH 7.0), 10 mM dithiothreitol, and 10% (v/v) glycerol. The fractions were kept on ice until assayed for sulfotransferase activity as described above. The protein concentration of each fraction was determined using the modified Lowry procedure (16).

SDS-PAGE of each IEF fraction was carried out using 12% gels (Bio-Rad Mini-Protean II apparatus). Fifty microliters of each fraction were added to 25 μl of buffer containing SDS and 2-mercaptoethanol. After boiling for 5 min, 20 μl were applied to SDS-polyacrylamide gels. After SDS-PAGE, proteins were electrophoretically transferred to a nitrocellulose membrane (Bio-Rad), and immunochemical analysis was carried out as described above, using a 1:5000 dilution of primary antibody and a 1:2500 dilution of the second antibody.

Immunohistochemical Localization of Aryl Sulfotransferase IV

Male Holtzman rats (180–220 g) were fasted for 24 hr before sacrifice by decapitation. Blocks of tissue (approximately 5 × 3 × 1 mm in thickness) were obtained from the median lobe of each liver, fixed by immersion in a solution containing 0.35% *p*-benzoquinone and 0.02 M $CaCl_2$ in 0.2 M sodium cacodylate buffer, pH 7.5, and then embedded in paraffin, as described previously (18). After fixation, 7-μm-thick serial sections were prepared, and aryl sulfotransferase IV was localized at the light microscope level by means of avidin-biotin-peroxidase staining (19, 20). Control sections were exposed to normal (nonimmune) rabbit serum. Other serial sections were stained with hematoxylin and eosin.

Quantitative Analysis of Immunochemical Results

Quantitation of antiaryl sulfotransferase IV binding to centrilobular, midzonal, and periportal hepatocytes was obtained, after avidin-biotin-peroxidase staining, by means of microdensitometry, using a Bioquant image analysis system with a video counting and microdensitometry (VCMTE) program (R & M Biometrics, Inc., Nashville, TN). The extent of antibody binding was calculated by subtracting the mean integrated absorbance of cells in sections exposed to nonimmune rabbit serum from that of corresponding cells in serial sections exposed to the antiserum to aryl sulfotransferase IV. Studies requiring quantitative analysis of Western blots were carried out using the same image analysis instrumentation. The extent of antiaryl sulfotransferase IV binding to protein bands transferred to nitrocellulose in the Western blot procedure was determined by subtracting the mean integrated absorbance of unstained areas above the bands from that of the peroxidase-stained bands.

Results

Results obtained with the new procedure for the purification of aryl sulfotransferase IV are summarized in Table 1. This method, which differs from previously described purification procedures (2, 3) in the substitution of an ammonium sulfate fractionation for Affi-Gel Blue chromatography and in the use of an ATP-agarose affinity chromatography step with ATP elution, resulted in a yield of 6.6 mg of homogeneous aryl sulfotransferase IV from the livers of four male Sprague-Dawley rats. Although several steps in the procedure were important for achieving this improved yield of aryl sulfotransferase IV, the stability of the enzyme during early and intermediate steps in the purification process was a critical factor. In order to examine the stability of aryl sulfotransferase IV during the initial steps of purification, the fraction precipitating with ammonium sulfate at 30–55% saturation (the second precipitation in step 2) was resuspended in buffer containing 0.01 M Tris·HCl, 0.25 M sucrose, and 1 mM dithiothreitol, pH 7.4. After overnight dialysis against 2 liters of this buffer, aliquots were taken for 30-day stability studies at both 4° and −70°. Glycerol, at a final concentration of either 10 or 20% (v/v), was added to separate aliquots. Storage for 30 days at 4° without glycerol resulted in a 48% loss of aryl sulfotransferase IV activity, whereas the addition of 10% glycerol resulted in only a 35% loss of activity over 30 days. The use of 20% glycerol did not result in any increase in the stability of aryl sulfotransferase IV over that seen with 10% glycerol. Aryl sulfotransferase IV did not lose activity upon storage for 30 days at −70°, regardless of whether glycerol was added to the Tris·HCl, sucrose, dithiothreitol buffer. Therefore, as a result of its effect on enzyme stability at 4°, 10% glycerol was added to the sucrose and dithiothreitol used in all subsequent buffers for the purification of aryl sulfotransferase IV.

TABLE 1

Purification of aryl sulfotransferase IV

Data given for steps 4–6 represent the sum of both halves of the aryl sulfotransferase IV-containing fraction after chromatography on DEAE-cellulose

Purification step	Volume	Activity	Protein	Specific activity
	ml	*units*	*mg*	*units/mg*
1. 100,000 × *g* supernatant	220	60,500	3,590	16.9
2. Ammonium sulfate	69	49,130	1,660	29.7
3. DEAE-cellulose	52	32,000	486	66.0
4. ATP-agarose 1	14	13,600	29.5	464
5. Hydroxylapatite	9.9	12,600	18.8	668
6. ATP-agarose 2	3.9	6,600	6.6	989

Chromatography on DEAE-cellulose was the first step in the purification procedure at which aryl sulfotransferase IV was separated from isoenzymes I and II. Further removal of trace amounts of aryl sulfotransferases I and II was accomplished in subsequent purification steps. A representative elution profile for the DEAE-cellulose chromatography step is shown in Fig. 1. As previously described (1–3), aryl sulfotransferase isoenzymes I and II can be distinguished from isoenzymes III and IV by assay of DEAE-cellulose eluates with 0.2 mM 2-naphthol at pH 7.4 and pH 5.5; aryl sulfotransferases I and II are much more active at pH 7.4 than at pH 5.5, whereas aryl sulfotransferases III and IV are considerably more active at pH 5.5 than at pH 7.4. It has been suggested that part of the basis for this difference might be substrate inhibition of aryl sulfotransferase IV by 0.2 mM 2-naphthol at pH 7.4 (21).

Chromatography on ATP-agarose (step 4) was useful for removing those proteins that do not interact with ATP, as well as those that bind to ATP-agarose with very high affinity. Aryl sulfotransferase IV was eluted in the first protein-containing fractions after the beginning of the linear concentration gradient of ATP. This step resulted in a 7-fold increase in specific activity and a 40% recovery of enzyme.

Chromatography on hydroxylapatite resulted in a 40% increase in specific activity, together with a 90% recovery of the enzyme, which was eluted from the hydroxylapatite column in the first protein-containing fractions after initiation of the sodium phosphate gradient. It was important to quickly concentrate fractions containing aryl sulfotransferase IV, in order to minimize the loss of enzymatic activity. Rapid concentration of the enzyme after determination of sulfotransferase activity was also necessary after chromatography on ATP-agarose with PAPS elution (step 6).

Preparations of aryl sulfotransferase IV eluted by PAPS from the ATP-agarose column usually displayed a single band on SDS-PAGE. When detected by SDS-PAGE, contaminants were removed by a repeat of the ATP-agarose chromatography (step 6). It is noteworthy, however, that a few preparations exhibited very small amounts of contaminant proteins that were detected only by means of IEF. As seen in Fig. 2, the minor contaminant proteins all had pI values below 5.8, the isoelectric point of aryl sulfotransferase IV. None of these

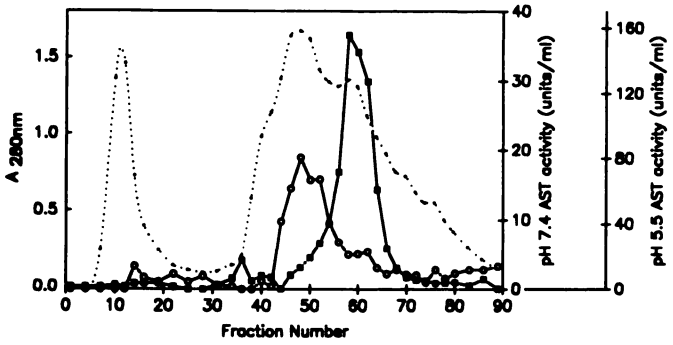

Fig. 1. DEAE-cellulose chromatography during purification of aryl sulfotransferase IV. A representative elution profile is shown for DEAE-cellulose chromatography of aryl sulfotransferases (*AST*), using a linear gradient formed between 500 ml of buffer A and 500 ml of buffer A containing 0.3 M sodium chloride. Aryl sulfotransferases I and II were located by assay of column fractions at pH 7.4 (O——O), whereas aryl sulfotransferases III and IV were located by assay of column fractions at pH 5.5 (■——■). The elution of protein is represented by the absorbance at 280 nm (· · · ·).

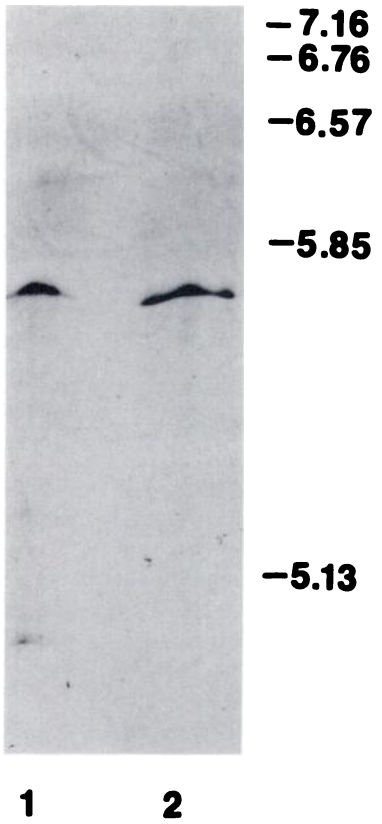

Fig. 2. IEF of aryl sulfotransferase IV. *Lane 1*, sample of aryl sulfotransferase IV that exhibits minor impurities after step 6; *lane 2*, sample of the same preparation of aryl sulfotransferase IV after CM-cellulose chromatography. *Numbers at the right*, positions and isoelectric points of the following standard proteins: myoglobin (7.16, 6.76), human carbonic anhydrase I (6.57), bovine carbonic anhydrase II (5.85), and β-lactoglobulin A (5.13).

isoelectric points correspond to those known for aryl sulfotransferases I, II, or III.

Minor contaminant proteins possessing low isoelectric points could be removed by CM-cellulose chromatography, which was carried out as follows. The concentrated aryl sulfotransferase IV preparation was dialyzed against 1 liter of buffer D (5 mM sodium succinate, pH adjusted to 5.5 with HCl, 0.25 M sucrose, 10%, v/v, glycerol, 1 mM dithiothreitol) and applied to a column (0.5 × 5.0 cm) of CM-52 that had been previously equilibrated with buffer D. After washing of the column with buffer D, aryl sulfotransferase IV was eluted with a linear gradient formed between 15 ml of buffer D and 15 ml of buffer D containing 0.2 M sodium succinate. Aryl sulfotransferase IV was the only protein that bound to the CM-cellulose in buffer D and the only protein that eluted in the sodium succinate gradient. Fractions containing aryl sulfotransferase IV were pooled and concentrated in a stirred ultrafiltration cell (Amicon PM-10 membrane) to a final volume of approximately 1.0 ml. Although chromatography on CM-cellulose at pH 5.5 was effective in removing trace amounts of proteins with low isoelectric points, the resulting specific activity of the aryl sulfotransferase IV was approximately the same as that obtained after ATP-agarose chromatography. Furthermore, CM-cellulose chromatography was accompanied by a loss of approximately 50%, in total units, of aryl sulfotransferase IV. This loss of enzyme units was due to inactivation of aryl sulfotransferase IV, be-

cause the unbound protein fractions contained no sulfotransferase activity. However, after CM-cellulose chromatography, the aryl sulfotransferase IV preparation displayed a single band on both IEF (pI = 5.8) and SDS-PAGE (M_r = 33,500).

Aryl sulfotransferase IV that was shown to be homogeneous by both SDS-PAGE and IEF was used to elicit the production of polyclonal antibodies in rabbits. The resulting antibodies, however, did not inhibit the catalytic activity of homogeneous aryl sulfotransferase IV, as determined by preincubation of the enzyme in Tris-buffered saline (0.02 mM Tris·HCl, pH 7.5, containing 0.5 M NaCl) for 30 min at 25° with antiserum at dilutions of 1:100, 1:1,000, and 1:10,000. The specificity of the antibodies was then characterized by Western blot analysis of rat hepatic cytosol and of two intermediate fractions obtained during the purification of aryl sulfotransferase IV. As seen in Fig. 3, antibodies to aryl sulfotransferase IV bound to a single protein band in cytosol (Fig. 3, *lane B4*), which corresponds to the band observed for the purified aryl sulfotransferase IV (Fig. 3, *lane B1*). There was no cross-reactivity of the antibodies with the DEAE-cellulose fraction that was enriched in aryl sulfotransferases I and II (Fig. 3, *lane B3*). The aryl sulfotransferase IV-containing fraction from the DEAE-cellulose chromatography step did, however, react strongly with the antibodies (Fig. 3, *lane B2*). Control experiments (not shown) demonstrated that normal (nonimmune) rabbit serum did not react with any proteins in rat hepatic cytosol. The relatively weak cross-reactivity with a smaller protein (M_r = 27,500) in the aryl sulfotransferase IV preparation after DEAE-cellulose chromatography is most likely due to a degradation product of aryl sulfotransferase IV. Although this band was not seen in Western blots of fresh rat liver cytosol (even at much higher protein concentrations), it was observable in cytosolic fractions that had been stored at 4° for several days (data not shown). Indeed, the degradation product at M_r = 27,500 can constitute as much as 40–50% of the immunoreactive protein in rat liver cytosol that has been stored at 4° for 1 month.

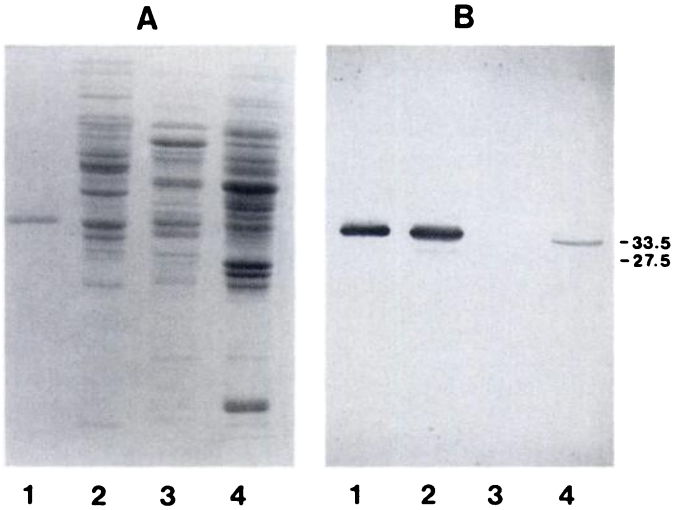

Fig. 3. Western blots of aryl sulfotransferase IV. Coomassie-stained SDS-PAGE (A) and corresponding nitrocellulose blots after immunochemical staining (B). In each panel, *lane 1*, 0.75 μg of purified aryl sulfotransferase IV (M_r = 33,500); *lane 2*, 15 μg of protein from the DEAE-cellulose fractions enriched in aryl sulfotransferase IV (second half of the major activity peak obtained by assay at pH 5.5); *lane 3*, 15 μg of protein from the DEAE-cellulose fractions enriched in aryl sulfotransferases I and II (first half of the major activity peak obtained by assay at pH 7.4); *lane 4*, 25 μg of protein from freshly prepared rat liver 100,000 × *g* supernate. The values of $M_r \times 10^{-3}$ for aryl sulfotransferase IV and its degradation product are shown on the *right*.

Additional evidence for the specificity of the antiaryl sulfotransferase IV in rat liver cytosol was obtained using preparative IEF followed by SDS-PAGE and Western blotting. As seen in Fig. 4, preparative IEF of rat liver 100,000 × *g* supernate provided a method for correlating enzyme activity with isoelectric point and antibody reactivity. Although aryl sulfotransferase isoenzyme III has a subunit molecular weight identical to that of aryl sulfotransferase IV (3), the two isoenzymes differ in their isoelectric points. As seen in Fig. 4, the antibodies to aryl sulfotransferase IV did not bind to any of the proteins in fraction 11 (pH 6.4 in the IEF gradient, corresponding to the pI of aryl sulfotransferase III). Likewise, the antiaryl sulfotransferase IV did not bind to proteins focused at pH 8.1 and pH 6.9, consistent with the lack of cross-reactivity of the antiserum with aryl sulfotransferases I and II in DEAE-cellulose fractions (Fig. 3).

Immunohistochemical staining demonstrated that aryl sulfotransferase IV is present in hepatocytes throughout the lobule (Fig. 5). However, staining for the enzyme was not of uniform intensity across the lobule. Microdensitometric analysis of immunohistochemical staining intensity revealed that the greatest amount of aryl sulfotransferase IV is present in centrilobular hepatocytes, with midzonal and periportal hepatocytes containing slightly lower levels of the enzyme (Table 2). The relative ratio of aryl sulfotransferase IV content in hepatocytes was found to be 1.0:0.8:0.7 (centrilobular/midzonal/periportal). Differences between the intensity of staining of centrilobular

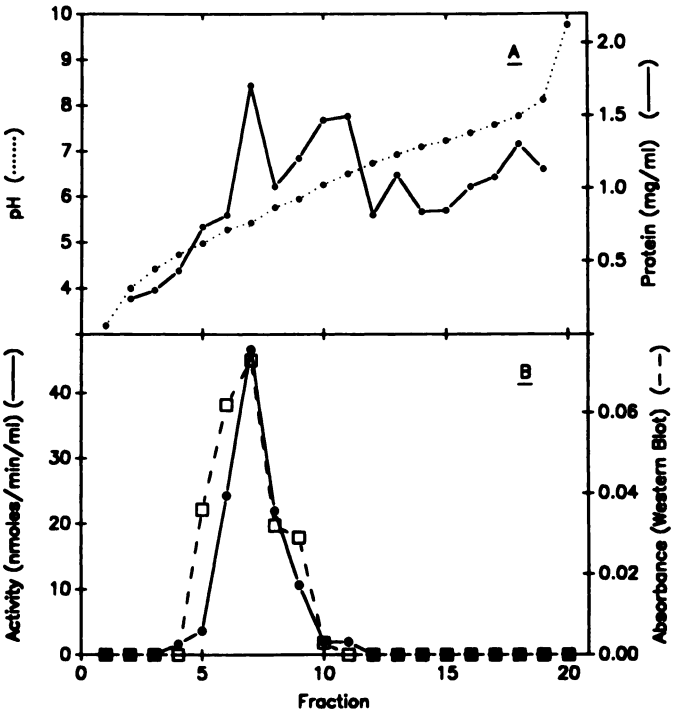

Fig. 4. Quantitative immunochemical analysis of rat hepatic 100,000 × *g* supernate after preparative IEF. Preparative IEF was carried out as described in Experimental Procedures. A, pH gradient and profile of protein concentration for the preparative IEF. B, Sulfotransferase activity (2-naphthol as substrate at pH 5.5) and the absorbance values obtained for immunochemical staining of Western blots of each fraction obtained from IEF. The only protein band reacting with the rabbit antiserum to rat hepatic aryl sulfotransferase IV corresponded to a M_r of 33,500 and was seen only in fractions 5–10.

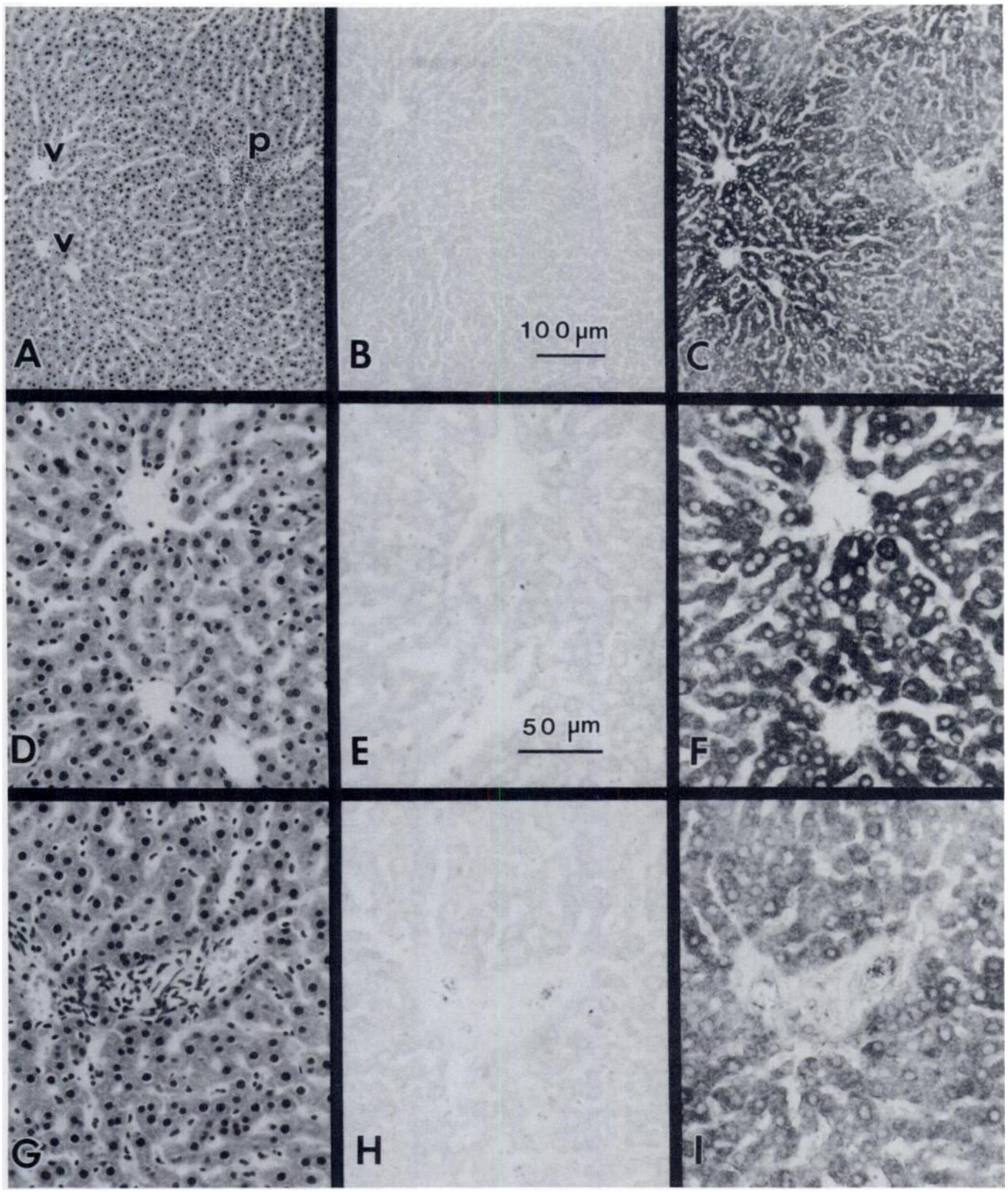

Fig. 5. Immunoperoxidase staining for aryl sulfotransferase IV in rat liver. A–C, Same area of a lobule in 7-μm-thick serial sections from the liver of an untreated rat; D–F, centrilobular regions; G–I, periportal regions. A, D, and G, areas in a section stained with hematoxylin and eosin (in A, *v* = central vein and *p* = portal triad); B, E, and H, areas in a section exposed to normal rabbit serum; C, F, and I, areas in a section exposed to rabbit antiserum to aryl sulfotransferase IV.

TABLE 2

Binding of antibody to aryl sulfotransferase IV to centrilobular, midzonal, and periportal hepatocytes in rat liver

Antibody binding values given represent the mean ± standard error of at least 20 microdensitometric determinations made after completion of avidin-biotin-peroxidase staining, using sections obtained from 10 untreated rats, and are expressed in terms of mean integrated absorbance units. The extent of antiaryl sulfotransferase IV binding to centrilobular, midzonal, and periportal hepatocytes was calculated by subtraction of the mean integrated absorbance of cells in sections exposed to normal rabbit serum from that of corresponding cells in serial sections exposed to rabbit antiaryl sulfotransferase IV serum.

Hepatocytes	Antiaryl sulfotransferase IV binding	Relative extent of antiaryl sulfotransferase IV binding[a]
	absorbance units	
Centrilobular	0.284 ± 0.038[b]	1.00
Midzonal	0.226 ± 0.032	0.80
Periportal	0.200 ± 0.030	0.70

[a] Determined by assignment of a value of 1.0 to the extent of antiaryl sulfotransferase IV binding to centrilobular hepatocytes.

[b] Significantly greater than values for antiaryl sulfotransferase IV binding to midzonal and periportal hepatocytes, $p < 0.01$.

hepatocytes and both midzonal and periportal hepatocytes was statistically significant ($p < 0.01$), whereas no statistically significant difference was detected in aryl sulfotransferase IV content between midzonal and periportal hepatocytes.

Discussion

In comparison with previously described methods for the purification of rat hepatic aryl sulfotransferase IV (2, 3), the procedure presented here provides a >10-fold improvement in the yield of homogeneous aryl sulfotransferase IV; that is, the present method yields approximately 100 μg of aryl sulfotransferase IV/g of rat liver, whereas previously reported procedures result in the purification of only about 6 μg of aryl sulfotransferase IV/g of liver. Several improvements in the purification procedure might have contributed to the greatly increased yield of catalytically active aryl sulfotransferase IV. For instance, the use of both sucrose and glycerol, as well as of dithiothreitol, in all buffers resulted in increased stability of the protein during early steps in the purification protocol. Stabilization of enzymatic activity during the early stages of purification was critical, because loss of aryl sulfotransferase IV activity occurred much more rapidly with partially purified preparations than with the purified enzyme. It is noteworthy that, whereas the use of 10% (v/v) glycerol and 0.25 M sucrose resulted in the stabilization of aryl sulfotransferase IV, glycerol has been reported (1) to depress the activity of aryl sulfotransferases I and II. Another modification made early in the purification procedure that improved the overall yield of aryl sulfotransferase IV was the replacement of Affi-Gel Blue chromatography (2, 3) with ammonium sulfate fractionation (step 2 in the present procedure). Although both Affi-Gel Blue chromatography and ammonium sulfate fractionation give rise to comparable increases in the specific activity of the enzyme, the recovery of total units of aryl sulfotransferase IV after ammonium sulfate fractionation is 81%, whereas that from Affi-Gel Blue chromatography is only about 24% (2, 3). On the other hand, this modification did mean that continuation of the earlier purification method did not result in the generation of a homogeneous aryl sulfotransferase IV preparation. The inclusion of ATP-agarose affinity chromatography with ATP elution (step 4) efficiently solved this problem, however.

Rat hepatic aryl sulfotransferase IV obtained employing this improved purification procedure was used to generate polyclonal rabbit antibodies that were specific for aryl sulfotransferase IV. The lack of cross-reactivity of the antiaryl sulfotransferase IV with aryl sulfotransferases I and II is consistent with previously reported findings (2), which showed that there was no cross-reactivity between aryl sulfotransferase IV and an antibody that was raised to purified rat hepatic aryl sulfotransferase II. Thus, when the findings of the present study are combined with those previously reported (1–3), it is clear that aryl sulfotransferase IV is both antigenically and catalytically distinct from aryl sulfotransferases I and II. Furthermore, results obtained when preparative IEF was coupled with SDS-PAGE and Western blotting revealed that the antiaryl sulfotransferase IV did not cross-react with any protein in rat hepatic cytosol that exhibited an isoelectric point (6.4) characteristic of aryl sulfotransferase III.

In addition to the lack of cross-reactivity between the antiaryl sulfotransferase IV and aryl sulfotransferases I, II, and III, Western blots of rat hepatic cytosol demonstrated that there was no cross-reactivity with any protein possessing a relative molecular mass (70,000) corresponding to that of the phenol sulfotransferase purified by Borchardt and Schasteen (4). Furthermore, Western blots of rat hepatic cytosol also revealed that the antiaryl sulfotransferase IV did not bind to any proteins having the relative molecular mass (35,000) reported for the subunits of minoxidil sulfotransferase (6). Thus, the rabbit polyclonal antibodies prepared against homogeneous rat hepatic aryl sulfotransferase IV were specific for this protein in rat liver cytosol.

After determination that the antibodies were specific for aryl sulfotransferase IV, immunohistochemical studies were conducted in order to localize the enzyme in rat liver. Immunohistochemical staining, together with microdensitometric analysis of immunoperoxidase staining intensity, demonstrated that aryl sulfotransferase IV was present in hepatocytes across the lobule. However, centrilobular hepatocytes were found to contain a significantly greater amount of aryl sulfotransferase IV than did hepatocytes in either the midzonal or periportal regions of the lobule. This was a particularly interesting observation, because results of biochemical studies conducted on hepatocytes isolated from microdissected periportal and centrilobular regions have led to conclusions ranging from a predominantly periportal localization for the sulfation of acetaminophen (22) and 4-methylumbelliferone (23) to a more uniform intralobular distribution for harmol sulfation (24). Indirect evidence for the concentration of sulfation activity in the periportal region was obtained from studies on hepatic necrosis induced by *N*-hydroxy-2-acetylaminofluorene (25). Results of investigations utilizing comparisons between anterograde and retrograde perfusions of rat liver (26–29) have also indicated that the sulfation of many xenobiotics may occur predominantly in the periportal region of the lobule. In each of these latter studies, however, it was recognized that other factors, in addition to the concentration of aryl sulfotransferase(s), could be important in determining the overall extent of sulfation by hepatocytes in the different regions of the lobule. Indeed, our immunohistochemical findings on aryl sulfotransferase IV, together with results of previous studies on the purified enzyme, isolated hepatocytes, and perfused liver, strongly suggest that enzyme content is only one of a number of factors that deter-

mine the overall rate and the extent of xenobiotic sulfation. For instance, both the concentration and rate of regeneration of PAPS are clearly critical for the catalytic activities of the aryl sulfotransferases. The concentration of PAPS available to a sulfotransferase might potentially be regulated by the concentrations and/or activities of the enzymes that are involved in PAPS biosynthesis (i.e., ATP sulfurylase and adenosine 5′-phosphosulfate kinase). The rates of product formation catalyzed by these enzymes might, in turn, be regulated by either the concentration of inorganic sulfate (30) or the availability of ATP for PAPS biosynthesis (31, 32). Because the K_m of purified aryl sulfotransferase IV for PAPS is 58 μM (33) and because the concentration of PAPS in liver homogenates has been reported to be approximately equal to this K_m value (34, 35), it is also reasonable to speculate that intralobular differences in PAPS concentration might contribute to differences in sulfation activity across the lobule. It is clear, however, that determination of such differences in the intralobular distribution of PAPS must be made *in situ* in order to substantiate this potential mechanism for the regulation of sulfotransferase activity.

In addition to the probable regulation of sulfation by PAPS concentration, previous determinations of the effect of pH on aryl sulfotransferase activity (2, 3, 21, 33) suggested that differences in the pH of microenvironments near an aryl sulfotransferase could significantly affect its catalytic activity. The existence of such *in situ* intralobular differences, however, has not yet been investigated with respect to aryl sulfotransferase IV. Furthermore, whereas effects of pH on substrate inhibition of aryl sulfotransferase IV have been noted (2, 3, 21, 36), these effects have not been examined with a sufficiently broad range of substrates and pH values to be of use in guiding interpretation of previously reported findings on isolated hepatocytes and perfused liver, in relation to the immunohistochemical findings of the present investigation.

In conclusion, our immunohistochemical findings on aryl sulfotransferase IV provide a basis for further studies on the regulation of xenobiotic sulfation within cells and tissues that should lead to a much more comprehensive understanding of the parameters regulating the *in vivo* formation of sulfuric acid esters from xenobiotics. The ability to obtain homogeneous rat hepatic aryl sulfotransferase IV in sufficient quantities for conducting detailed kinetic and mechanistic studies, as well as the availability of specific antibodies to the enzyme that are suitable for immunohistochemical and other immunochemical analyses, will likewise facilitate these investigations.

Acknowledgments

The authors are grateful to John A. Gattera and Jon P. Burke for excellent technical assistance.

References

1. Sekura, R. D., and W. B. Jakoby. Phenol sulfotransferases. *J. Biol. Chem.* **254**:5658–5663 (1979).
2. Sekura, R. D., and W. B. Jakoby. Aryl sulfotransferase IV from rat liver. *Arch. Biochem. Biophys.* **211**:352–359 (1981).
3. Sekura, R. D., M. W. Duffel, and W. B. Jakoby. Aryl sulfotransferases. *Methods Enzymol.* **77**:197–206 (1981).
4. Borchardt, R. T., and C. S. Schasteen. Phenol sulfotransferase. I. Purification of a rat liver enzyme by affinity chromatography. *Biochem. Biophys. Acta* **708**:272–279 (1982).
5. Wu, S.-C. G., and K. D. Straub. Purification and characterization of *N*-hydroxy-2-acetylaminofluorene sulfotransferase from rat liver. *J. Biol. Chem.* **251**:6529–6536 (1976).
6. Hirshey, S. J., and C. N. Falany. Purification and characterization of rat liver minoxidil sulfotransferase. *Biochem. J.* **270**:721–728 (1990).
7. Boberg, E. W., E. C. Miller, and J. A. Miller. Strong evidence from studies with brachymorphic mice and pentachlorophenol that 1′-sulfooxysafrole is the major ultimate electrophilic and carcinogenic metabolite of 1′-hydroxysafrole in mouse liver. *Cancer Res.* **43**:5163–5173 (1983).
8. Kedderis, G. L., M. C. Dyroff, and D. E. Rickert. Hepatic macromolecular covalent binding of the hepatocarcinogen 2,6-dinitrotoluene and its 2,4-isomer *in vivo*: modulation by the sulfotransferase inhibitors pentachlorophenol and 2,6-dichloro-4-nitrophenol. *Carcinogenesis (Lond.)* **5**:1199–1204 (1984).
9. Okuda, H., H. Nojima, K. Miwa, N. Watanabe, and T. Watabe. Selective covalent binding of the active sulfate ester of the carcinogen 5-(hydroxymethyl)chrysene to the adenine residue of calf thymus DNA. *Chem. Res. Toxicol.* **2**:15–22 (1989).
10. Kadlubar, F. F., J. A. Miller, and E. C. Miller. Hepatic metabolism of *N*-hydroxy-*N*-ethyl-4-aminoazobenzene and other *N*-hydroxy arylamines to reactive sulfuric acid esters. *Cancer Res.* **36**:2350–2359 (1976).
11. Lai, C.-C., J. A. Miller, E. C. Miller, and A. Liem. *N*-Sulfooxy-2-aminofluorene is the major ultimate electrophilic and carcinogenic metabolite of *N*-hydroxy-2-acetylaminofluorene in the livers of infant male C57BL/6J × C3H/HeJ F_1 (B6C3F_1) mice. *Carcinogenesis (Lond.)* **6**:1037–1045 (1985).
12. Miller, E. C., J. A. Miller, E. W. Boberg, K. B. Delclos, C.-C. Lai, T. R. Fennell, R. W. Wiseman, and A. Liem. Sulfuric acid esters as ultimate electrophilic and carcinogenic metabolites of some alkylbenzenes and aromatic amines in mouse liver, in *Carcinogenesis* (E. Huberman, ed.), Vol. 10. Raven Press, New York, 93–107 (1985).
13. Ringer, D. P., T. R. Norton, and B. A. Howell. 2-Acetylaminofluorene-mediated alteration in the level of liver aryl sulfotransferase IV during rat hepatocarcinogenesis. *Cancer Res.* **50**:5301–5307 (1990).
14. Sekura, R. D. Adenosine 3′-phosphate 5′-phosphosulfate. *Methods Enzymol.* **77**:413–415 (1981).
15. Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. *Nature (Lond.)* **227**:680–685 (1970).
16. Bensadoun, A., and D. Weinstein. Assay of proteins in the presence of interfering materials. *Anal. Biochem.* **70**:241–250 (1976).
17. Johnson, D. A., J. W. Gautsch, J. R. Sportsman, and J. H. Elder. Improved technique utilizing nonfat dry milk for analysis of proteins and nucleic acids transferred to nitrocellulose. *Gene Anal. Techn.* **1**:3–8 (1984).
18. Baron, J., J. M. Voigt, T. T. Kawabata, and J. A. Redick. Immunohistochemistry, in *Regulation of Hepatic Metabolism: Intra- and Intercellular Compartmentation* (R. G. Thurman, F. C. Kauffman, and K. Jungermann, eds.). Plenum, New York, 87–118 (1986).
19. Hsu, S. M., L. Raine, and H. Fanger. Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques: a comparison between ABC and unlabeled antibody (PAP) procedures. *J. Histochem. Cytochem.* **29**:577–580 (1981).
20. Baron, J., and J. M. Voigt. Localization, distribution, and induction of xenobiotic-metabolizing enzymes and aryl hydrocarbon hydroxylase activity within lung. *Pharmacol. Ther.* **47**:419–445 (1990).
21. Binder, T. P., and M. W. Duffel. Sulfation of benzylic alcohols catalyzed by aryl sulfotransferase IV. *Mol. Pharmacol.* **33**:477–479 (1988).
22. Araya, H., T. Mizuma, T. Horie, M. Hayashi, and S. Awazu. Heterogeneous distribution of the conjugation activity of acetaminophen and *p*-nitrophenol in isolated rat liver cells. *J. Pharmacobio.-Dyn.* **9**:218–222 (1986).
23. El Mouelhi, M., and F. C. Kauffman. Sublobular distribution of transferases and hydrolases associated with glucuronide, sulfate, and glutathione conjugation in human liver. *Hepatology* **6**:450–456 (1986).
24. Araya, H., T. Mizuma, T. Horie, M. Hayashi, and S. Awazu. Heterogeneous distribution of conjugation activities of harmol in isolated rat liver cells. *J. Pharmacobio.-Dyn.* **7**:624–629 (1984).
25. Groothius, G. M. M., D. K. F. Meyer, and M. J. Hardonk. Methodological studies on acinar damage by *N*-hydroxy-2-acetylaminofluorene and carbon tetrachloride. *Naunyn-Schmeideberg's Arch. Pharmacol.* **322**:298–308 (1983).
26. Pang, K. S., and J. A. Terrell. Retrograde perfusion to probe the heterogeneous distribution of hepatic drug metabolizing enzymes in rats. *J. Pharmacol. Exp. Ther.* **216**:339–346 (1981).
27. Pang, K. S., H. Koster, I. C. M. Halsema, E. Scholtens, and G. J. Mulder. Aberrant pharmacokinetics of harmol in the perfused rat liver preparation: sulfate and glucuronide conjugations. *J. Pharmacol. Exp. Ther.* **219**:134–140 (1981).
28. Dawson, J. R., J. G. Weitering, G. J. Mulder, R. M. Stillwell, and K. S. Pang. Alteration of transit time and direction of flow to probe the heterogeneous distribution of conjugating activities for harmol in the perfused rat liver preparation. *J. Pharmacol. Exp. Ther.* **234**:691–697 (1985).
29. Conway, J. G., F. C. Kauffman, S. Ji, and R. G. Thurman. Rates of sulfation and glucuronidation of 7-hydroxycoumarin in periportal and pericentral regions of the liver lobule. *Mol. Pharmacol.* **22**:509–516 (1982).
30. Levy, G. Sulfate conjugation in drug metabolism: role of inorganic sulfate. *Fed. Proc.* **45**:2235–2240 (1986).
31. Thurman, R. G., F. C. Kauffman, and J. Baron. Biotransformation and zonal toxicity, in *Regulation of Hepatic Metabolism: Intra- and Intercellular Compartmentation* (R. G. Thurman, F. C. Kauffman, and K. Jungermann, eds.). Plenum, New York, 321–382 (1986).
32. Aw, T. Y., and D. P. Jones. ATP concentration gradients in cytosol of liver cells during hypoxia. *Am. J. Physiol.* **249**:C385–C392 (1985).

33. Duffel, M. W., and W. B. Jakoby. On the mechanism of aryl sulfotransferase. *J. Biol. Chem.* **256**:11123–11127 (1981).
34. Wong, K. P., and T. Yeo. Assay of adenosine 3′-phosphate 5′-sulphatophosphate in hepatic tissues. *Biochem. J.* **181**:107–110 (1979).
35. Hazelton, G. A., J. J. Hjelle, R. L. Dills, and C. D. Klaassen. A radiometric method for the measurement of adenosine 3′-phosphate 5′-phosphosulfate in rat and mouse liver. *Drug Metab. Dispos.* **13**:30–34 (1985).
36. Nakamura, J., T. Mizuma, T. Horie, M. Hayashi, and S. Awazu. Aryl sulfotransferase in rat liver: multiplicity and substrate specificity. *J. Pharmacobio.-Dyn.* **10**:736–742 (1987).

Send reprint requests to: Michael W. Duffel, Division of Medicinal and Natural Products Chemistry, College of Pharmacy, University of Iowa, Iowa City, IA 52242.