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Immunological Evidence for *N*-Acetyltransferase Isozymes in the Rabbit

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SUMMARY

An immunological evaluation of *N*-acetyltransferase (NAT) (EC 2.3.1.5) in liver, duodenum, lung, and kidney of the rabbit is described. Polyclonal antibodies to hepatic NAT isolated from rapid acetylator rabbits were raised in a goat and utilized for immunoblot analyses and enzyme inhibition studies. Immunoblot analyses demonstrated that hepatic and duodenal cytosols from rapid but not slow acetylator rabbits contained an immunoreactive 33-kDa protein. No immunoreactivity was observed for lung or kidney cytosols from either rapid or slow acetylators. The inhibition of sulfamethazine and *p*-aminobenzoic acid acetylation by polyclonal antibodies was investigated using cytosols from rapid and slow acetylator rabbits. With rapid acetylator cytosols, maximal inhibition of hepatic, duodenal, and lung NAT activities was 94.4 ± 9.0%, 92.5 ± 8.5%, and 28.3 ± 2.4%, respectively, for sulfamethazine (500 μM) acetylation and 90.1 ± 8.0%, 80.2 ± 6.4%, and 26.7 ± 3.1%, respectively, for *p*-aminobenzoic acid (500 μM) acetylation. Using 25 μM *p*-aminobenzoic acid as substrate, maximal inhibition of NAT activity was 32.0 ± 2.1% with liver cytosol and 5.8 ± 0.16% with duodenal cytosol, whereas no inhibition of lung NAT activity was observed. Kidney NAT activity was not inhibited by the polyclonal antibodies. With slow acetylator cytosols, no inhibition of NAT activities was observed. It is concluded that at least two NATs are present in liver, duodenum, and lung of rapid acetylator rabbits. Furthermore, the principal NAT in liver and duodenum is immunologically related to the minor form of lung NAT and is antigenically distinct from kidney NAT of rapid acetylators. Hepatic, duodenal, lung, and kidney NAT(s) of slow acetylator rabbits is (are) immunologically distinct from the major hepatic NAT in rapid acetylators. The data support the model in which the hepatic polymorphism in rabbits is caused by the total lack of the major rapid acetylator hepatic NAT in the phenotypic slow acetylator animal. These observations may have significant implications in the organ-specific toxicities of carcinogens that undergo metabolic activation via *N*-acetylation.

Major progress towards characterizing hepatic NAT from humans and several animal species has been made as a result of recent studies into the molecular basis of the acetylation polymorphism. Hepatic NAT from rapid acetylator rabbits (1) and chickens (2) has been purified, a cDNA encoding each NAT isolated and expressed (3–5), and the complete amino acid sequence of chicken liver NAT deduced (3). Moreover, the gene encoding polymorphic hepatic NAT in rapid acetylator rabbits was shown to be absent from the liver of slow acetylator rabbits (5); to date, similar information on monomorphic NAT in rabbit liver has not been published. Recently, Ohsako and Deguchi (6) reported the isolation and expression of three cDNAs for human liver NATs. Two of these differed in only one nucleotide and encoded for polymorphic NATs, whereas the third encoded for monomorphic NAT. Subsequently, Blum *et al.* (7) confirmed that human liver expresses a monomorphic NAT gene (designated *NAT1*) and a polymorphic NAT gene (designated *NAT2*). These authors (8) and others (9) have identified multiple mutant alleles of *NAT2* in human livers. Rat liver contains at least two forms of NAT with different substrate specificities (10), whereas two NAT isozymes have been shown to be present in hamster liver, a monomorphic NAT common to both acetylator genotypes and a polymorphic NAT that exhibited acetylator genotype-related differences (11–13).

Extrahepatic NATs, by comparison, have not been well characterized. NAT polymorphisms have been identified in blood (14), intestine, lung, kidney, bladder (15), and skin (16) of hamsters. Whereas an inverse relationship has been demonstrated between levels of lymphocyte and erythrocyte PABA NAT activity and liver SMZ NAT activity in rabbits (17), the rate of SMZ acetylation by human whole blood *in vitro* cannot be utilized to determine acetylator status (18). In contrast, the

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ABBREVIATIONS: NAT, acetyl coenzyme A:arylamine *N*-acetyltransferase; NAPABA, *N*-acetyl-*p*-aminobenzoic acid; NASMZ, *N*-acetylsulfamethazine; PABA, *p*-aminobenzoic acid; SMZ, sulfamethazine; HPLC, high performance liquid chromatography; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; CoA, coenzyme A.

rate of *N*-acetylation of the arylamine carcinogens 4-aminobiphenyl, 2-aminofluorene, and β-naphthylamine by human bladder cytosol is reportedly polymorphic (19).

In addition, intertissue differences in extrahepatic NATs from animals belonging to a specified acetylator phenotype have been investigated. Immunochemical investigations by Patterson *et al.* (20) have shown that polyclonal antibodies raised against partially purified liver NAT from a rapid acetylator rabbit cross-reacted with duodenal NAT but not with lymphocyte or erythrocyte NAT, whereas kinetic studies of SMZ acetylation from our laboratory (21) showed that K_m values for SMZ or the cofactor acetyl CoA were similar for liver and duodenum, as were those for lung and kidney. However, K_m values for liver and duodenum differed markedly from those for lung and kidney. In hamsters, multiple NAT isozymes have been reported to occur in bladder cytosol (22), peripheral blood lysates (14), and intestine (12).

Apart from their role in drug metabolism, extrahepatic acetyltransferases are known to metabolically activate arylamine and *N*-hydroxyarylamine carcinogens (for a review, see Ref. 15). A major objective of our research is to characterize extrahepatic acetylases using a rabbit model. The present study was undertaken to (i) raise polyclonal antibodies against liver NAT from rapid acetylator rabbits and (ii) determine whether these antibodies cross-react with NAT found in liver of slow acetylator rabbits or with NAT proteins found in duodenum, lung, and/or kidney of rapid or slow acetylator rabbits. An abstract of this work has been presented previously (23).

Experimental Procedures

Materials. Dithiothreitol, acetyl CoA, and phenylmethylsulfonyl fluoride were purchased from Boehringer Mannheim, Gmb H (Mannheim, FRG); bovine serum albumin, PABA sodium, SMZ, and paraoxon were obtained from Sigma Chemical Co. (St. Louis, MO); PABA was from BDH Ltd. (Poole, England); sodiun sulfathiazole was from Nutritional Biochemicals Corporation (Cleveland, OH); and SMZ sodium was from Ramprie Laboratories (Perth, Australia). All other reagents were of analytical grade.

Donkey anti-goat IgG conjugated with horseradish peroxidase was purchased from Silenus Laboratories (Melbourne, Australia). Complete and incomplete Freund's adjuvants were obtained from Commonwealth Serum Laboratories (Melbourne, Australia).

DEAE-Sephacel and Sephadex G-100 were purchased from Pharmacia (Australia) Pty. Ltd. (Sydney, Australia) and a Protein-Pak DEAE 5PW (7.5-mm × 7.5-cm) ion exchange column from Waters Associates (Sydney, Australia). Ultrafiltration membranes (Diaflow PM 10) and microconcentrator units (Centricon 10) were supplied by Amicon (Danvers, MA). Ultrafree-MC filters (with 10,000 NMWL polysulfone membranes) were purchased from Millipore Corporation (Bedford, MA). Nitrocellulose paper (0.45 μm), electrophoresis chemicals, and molecular weight standards were obtained from Bio-Rad (Richmond, CA).

Determination of acetylator status of rabbits. The acetylator phenotype of adult male New Zealand White (Animal Resources Centre, Perth, Australia) and Californian (Western Rabbit Farms, Perth, Australia) rabbits was determined according to the method of Gordon *et al.* (24), with minor modifications. Briefly, rabbits were dosed with SMZ sodium (25 mg of SMZ base/kg, intravenously), and blood samples (0.5 ml) were drawn into heparinized tubes before and 5, 20, 40, 60, 120, 180 and 240 min after the sulfonamide dose. Plasma concentrations of SMZ and NASMZ were determined by HPLC, as previously described (21). Acetylator status was determined from the ratio of NASMZ/(SMZ + NASMZ) in plasma drawn 20 min after dosing and from the SMZ half-life.

Preparation of cytosols. Rabbits were killed with pentobarbital sodium (150 mg/kg, intravenously) and cytosols of liver, lung, kidney, and duodenum (20-cm segment commencing at the pylorus) were prepared as described previously (21), except that tissues were homogenized in 4 volumes of ice-cold 20 mM Tris·HCl buffer, pH 7.4, containing 1 mM EDTA, 2 mM dithiothreitol (buffer A), and 50 μM phenylmethylsulfonyl fluoride. Cytosols were frozen rapidly and stored at −70° until used.

Partial purification of hepatic NAT. A four-step procedure was adopted. First, cytosol was applied to a DEAE-Sephacel column (5 cm × 16 cm) equilibrated with buffer A, and the unadsorbed protein was eluted over 24 hr. Enzyme activity was subsequently eluted at 0.5 ml/min with a linear gradient (500 ml) of NaCl (0–0.5 M). Fractions (20 min each) were collected, and those containing NAT activity were pooled and concentrated by ultrafiltration (Amicon PM 10 membrane, 1.5 atm).

In the second step, the enzyme preparation was separated at a flow rate of 0.25 ml/min on a Sephadex G-100 column (3 cm × 90 cm) equilibrated with buffer A containing 0.02% sodium azide. Fractions (20 min each) containing NAT activity were concentrated by sequential ultrafiltration (Amicon PM 10 membrane to approximately 10 ml, followed by an Amicon Centricon 10 microconcentrator to 1 ml).

The third purification step involved anion exchange chromatography using a HPLC system comprising two solvent delivery pumps (Waters M45 and Waters 6000A), a gradient programmer (Waters M660), a Waters U6K injector, and the column (Waters Protein-Pak DEAE 5PW, 7.5 mm i.d. × 7.5 cm). The column was equilibrated with buffer A at a flow rate of 1 ml/min. Separation was achieved with a 30-min linear NaCl gradient (0 to 0.3 M) followed by a 10-min isocratic elution with 0.5 M NaCl. The eluate was collected in 1-min fractions and NAT-containing fractions were concentrated to 1 ml by ultrafiltration.

The fourth purification step involved rechromatography of the sample, as for step 3. Thereafter, samples for electrophoresis were concentrated.

Assays. NAT-containing fractions collected during the partial purification procedure were identified as follows. Aliquots (100 μl) of fractions were incubated in Eppendorf tubes with SMZ sodium (0.5 mM final concentration), acetyl CoA (0.5 mM final concentration), and buffer A (500-μl final incubation volume), at 37° for 1 hr, and reactions were terminated with trichloroacetic acid (5% final concentration). Samples were centrifuged at 15,000 rpm for 1 min in a TDX microfuge (Abbott Laboratories). Subsequently, aliquots of the supernatant were assayed for parent amine, using the Bratton-Marshall procedure, and the occurrence of acetylation was calculated by the acetyl CoA-dependent disappearance of substrate (25).

Production of polyclonal antibodies to rabbit liver NAT. Partially purified hepatic NAT from rapid acetylator rabbits was subjected to SDS-PAGE with 14% polyacrylamide gels, essentially as described by Laemmli (26). By correlation of NAT activity during the purification procedure with the appearance of a major protein band at M_r 33,000 on SDS-PAGE, NAT was tentatively identified on a gel and excised for homogenization in complete Freund's adjuvant. The resultant emulsion was injected into a goat at multiple subcutaneous sites. Three booster immunizations were administered at monthly intervals, with each booster comprising the NAT protein (emulsified in incomplete Freund's adjuvant) from an entire rabbit liver. Following the final booster, the antibody titer was monitored weekly by immunoblot analysis of both liver cytosol and partially purified hepatic NAT. After the titer had peaked, the goat was bled (500 ml) and an IgG fraction was prepared from the serum (27) and stored at −70°. Similarly, an IgG fraction prepared from preimmune goat serum was stored at −70°.

Electrophoresis and immunoblot analyses. All electrophoresis and blotting apparatus was from Bio-Rad. Cytosols were subjected to SDS-PAGE, with a final polyacrylamide concentration of 12%, as described (26). Immunoblot analyses were performed essentially as described by McManus *et al.* (28), except that the nitrocellulose paper was blocked for 3 hr in phosphate-buffered saline containing 3% bovine

serum albumin and 2% nonfat milk powder, and the primary antibody was diluted 1:500.

Inhibition of cytosolic NAT activities with antibodies to liver NAT. The specific activity of cytosols (liver, duodenum, lung, or kidney prepared from rapid and slow acetylator rabbits) was adjusted to 0.08 nmol of NASMZ formed/min/incubation by dilution with homogenization buffer. Diluted cytosols were preincubated for 24 hr at 4° with varying amounts of either immune or preimmune IgG. In a preliminary study, we showed that there was no loss of cytosolic NAT activity over 24 hr at 4° in the absence of antibodies. The maximal ratios of IgG to cytosolic protein (w/w) studied were 137:1 (liver), 102:1 (duodenum), 142:1 (lung), and 47:1 (kidney), and the concentration of protein in each incubation was kept constant (5 mg of total IgG) by the addition of preimmune IgG. Because dithiothreitol has been shown to reactivate NAT in a concentration-dependent manner (29), 1 mM dithiothreitol (final concentration) was used routinely in preincubations. Subsequently, NAT activity was determined by HPLC with either SMZ (21) or PABA (30) as the substrate. All assays were performed under conditions that were linear with time for at least 1 hr. The limits of detection of NAT activities were 0.03 nmol of NASMZ formed/min/mg of protein and 0.01 nmol of NAPABA formed/min/mg of protein; activities were above these levels in all incubations.

Protein determination. Protein concentrations were determined according to the method of Bradford (31), using bovine serum albumin as a standard.

Statistical analysis. All results are summarized as mean ± standard deviation. Standard deviations associated with percentage of inhibition of NAT activity were propagated according to the method of Wilson (32).

Results

The acetylator status of inbred New Zealand White and Californian rabbits was determined before their use in this study. The plasma ratio of NASMZ/(SMZ + NASMZ) and the SMZ half-life were 0.83 ± 0.01 and 11.0 ± 0.5 min, respectively, for New Zealand White rabbits (n = 17) and 0.046 ± 0.016 and 69.5 ± 4.2 min, respectively, for Californian rabbits (n = 4). According to Gordon *et al.* (24) and in agreement with other studies from our own laboratory,[1] the New Zealand White rabbits phenotyped were rapid acetylators, whereas the Californian rabbits were slow acetylators.

In order to raise polyclonal antibodies against hepatic NAT from rapid acetylator rabbits, the enzyme was partially purified, using a four-step chromatographic procedure (Table 1). Overall, a 563-fold purification of NAT was achieved, with 35% recovery of activity. The partially purified NAT was subjected to SDS-PAGE, and NAT was tentatively identified by correlation of NAT activity during the purification procedure with the appearance of a M_r 33,000 protein band. Fig. 1 shows a representative electrophoretogram of liver cytosol (Fig. 1, *lane A*) and a partially purified M_r 33,000 protein (Fig. 1, *lane B*) from a rapid acetylator rabbit. The M_r 33,000 protein band was excised from the slab gel, emulsified in adjuvant, and utilized as the immunogen.

Hepatic and extrahepatic cytosols from rapid and slow acetylator rabbits, and rapid acetylator rabbit liver NAT that had undergone partial purification through four chromatographic steps, were analyzed by the immunoblot procedure (Fig. 2). Fig. 2, *lane A* shows a single immunoreactive band at M_r 33,000 for partially purified hepatic NAT from a rapid acetylator rabbit. With liver cytosol (Fig. 2, *lane B*) and duodenal cytosol (Fig. 2, *lane D*) from rapid acetylator rabbits, a single band at the same molecular weight was observed. In contrast, no immunoreactivity was observed for either liver cytosol (Fig. 2, *lane C*) or duodenal cytosol (Fig. 2, *lane E*) from slow acetylator rabbits. No detectabe M_r 33,000 band was observed for lung or kidney cytosols prepared from animals of either acetylator phenotype (data not shown). Preliminary studies with rapid acetylator hepatic cytosols demonstrated that the detection limit of the immunoblot method was 5 μg of protein (representing NAT activity of approximately 0.04 nmol of NASMZ/min/mg of protein). To exceed this detection limit, partially purified lung and kidney NATs (100 μg each) were subjected to immunoblot analysis. A faint M_r 33,000 immunoreactive band was observed for partially purified lung NAT from the rapid acetylator rabbit (data not shown). In contrast, no immunoreactivity was detected in partially purified lung NAT from slow acetylators or in partially purified kidney NAT from rapid or slow acetylators.

The ability of NAT antibodies to inhibit acetylase activities in hepatic, duodenal, lung, and kidney cytosols prepared from rapid and slow acetylator rabbits was investigated. Acetyl CoA (500 μM) was utilized as cofactor for the acetylation of SMZ and PABA. Inhibition of SMZ acetylation was investigated at 500 μM, which approximates the apparent K_m estimate of 350 μM for rapid acetylator rabbit liver cytosol (21). The choice of PABA concentrations (500 and 25 μM) was based on K_m values of 475 and 25 μM PABA for rapid and slow acetylator rabbit livers, respectively.[1] These K_m values are in good agreement with those reported by Andres and Weber (33), after allowance has been made for the different acetyl CoA concentrations utilized in the two studies. Our choice of PABA concentrations allowed the differential apparent K_m values for PABA acetylation in rapid and slow acetylator livers to be exploited. Specifically, the existence of rapid and/or slow acetylator NAT in a tissue might be identified, because the antibodies were raised to hepatic NAT from a rapid acetylator rabbit. We predict

[1] P. T. Reeves and K. F. Ilett, unpublished data.

TABLE 1
Purification of NAT from rabbit liver

Purification step	Total protein	Specific Activity	Purification fold	Recovery of activity
	mg	*nmol/min/mg of protein*		%
Cytosol	2002.8	14.1	1	100
DEAE-Sephacel	264.4	110	7.8	102.5
Sephadex G-100	13.4	1104	78.2	52.2
5PW-DEAE, 1	2.40	4782	339	40.6
5PW-DEAE, 2	1.25	7949	563	34.5

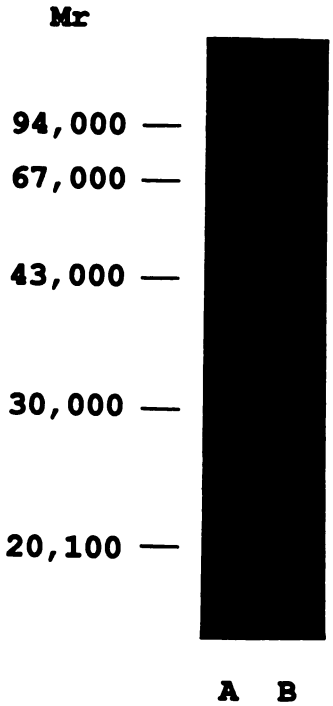

Fig. 1. Electrophoretogram of liver cytosol (10 μg of protein) (*lane A*) and highly purified hepatic NAT (1 μg of protein) (*lane B*) from a rapid acetylator rabbit. Proteins were stained with Coomassie brilliant blue G-250 and the predominant 33-kDa protein band (*lane B*) was excised from a slab gel, emulsified in Freund's adjuvant, and inoculated into an adult female goat.

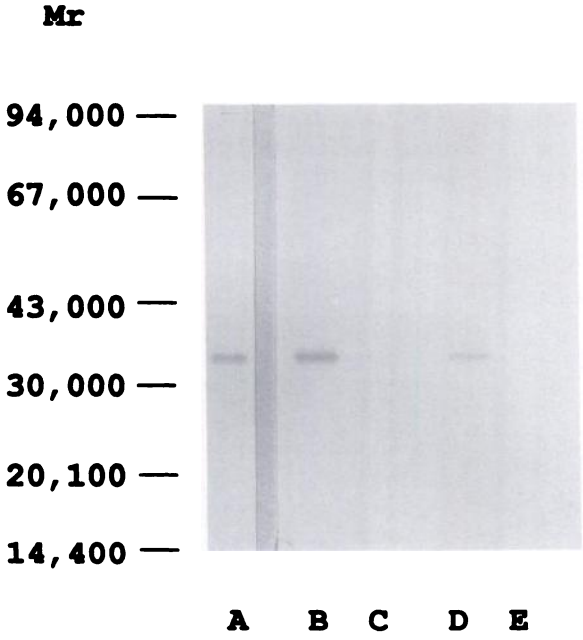

Fig. 2. Immunoblot analysis of rabbit tissue cytosols and highly purified hepatic NAT. Approximately 0.3 μg of highly purified hepatic NAT from a rapid acetylator rabbit was applied to *lane A*. Approximately 100 μg of cytosolic protein were applied to the other lanes as follows: *lane B*, liver cytosol from a rapid acetylator rabbit; *lane C*, liver cytosol from a slow acetylator rabbit; *lane D*, duodenal cytosol from a rapid acetylator rabbit; *lane E*, duodenal cytosol from a slow acetylator rabbit. Following electrophoretic transfer of proteins to nitrocellulose paper, the latter was probed with NAT antibodies diluted 1:500, as described in Experimental Procedures.

marked inhibition of PABA acetylation will occur at the high PABA concentration (500 μM) when NAT from a rapid acetylator is the predominant catalyst. In contrast, minor inhibition of PABA acetylation is predicted at the low PABA concentration (25 μM), should the slow acetylator form of NAT fail to cross-react with our antibodies. Table 2 shows the inhibition data for PABA (25 and 500 μM) and SMZ (500 μM) acetylation with cytosols prepared from rapid and slow acetylator rabbits.

The inhibition profiles for SMZ (500 μM) and PABA (500 μM) acetylation with rapid acetylator cytosols are illustrated in Fig. 3. Following preincubation of rapid acetylator cytosols with antibodies for 24 hr at 4°, maximal inhibition of hepatic and duodenal PABA (500 μM) acetylase activities of 90.1 ± 8.0% and 80.2 ± 6.4%, respectively, was demonstrated (Table 2 and Fig. 3A). By comparison, the NAT antibodies inhibited hepatic and duodenal acetylase activities by 32.0 ± 2.1% and 5.8 ± 0.16%, respectively, when 25 μM PABA was used as substrate (Table 2). With rapid acetylator lung cytosol, PABA NAT activity was maximally inhibited by 26.7 ± 3.1% at 500 μM PABA, whereas no inhibition was observed with 25 μM PABA (Table 2 and Fig. 3B). Maximal antibody inhibition of SMZ acetylation with rapid acetylator cytosols was 94.5 ± 9.0% (liver), 92.5 ± 8.5% (duodenum), and 28.3 ± 2.4% (lung) (Table 2 and Fig. 3). Kidney cytosols prepared from rapid acetylators showed low SMZ and PABA acetylase activities, neither of which was inhibited by antibodies (Table 2 and Fig. 3B). None of the NAT activities towards SMZ or PABA was diminished when preimmune IgG was substituted for immune IgG.

With slow acetylator rabbits, the NAT antibodies did not inhibit SMZ or PABA acetylase activities in any of the cytosols studied (Table 2). At the maximal IgG to cytosolic protein ratio investigated, hepatic NAT activities (as a percentage of control values) were 98.4 ± 4.5% with 500 μM SMZ, 99.8 ± 2.9% with 25 μM PABA, and 98.7 ± 4.7% with 500 μM PABA. The rates of SMZ and PABA acetylation by duodenal, lung, and kidney cytosols in the presence of NAT antibodies ranged from 96.3 ± 6.8% to 101.0 ± 3.5% of control values. Preliminary studies indicated that substrate inhibition of acetylation occurred with 500 μM PABA and that this was marked for cytosols prepared from the slow acetylator phenotype (Table 2).

Esterases reportedly display high activity in some animal species (34), and carboxylesterase has been shown to catalyze *O*-acetylation in the presence of various acetyl donors (35). Because *O*-acetyltransferase and NAT are closely related, if not identical, proteins (10, 29, 36), we wanted to test whether nonspecific esterase activity accounted for the residual acetylation observed in the antibody inhibition studies (Table 2). We, therefore, examined the effect of the esterase inhibitor paraoxon (10^{-4} M) on the acetylation of SMZ and PABA (500 μM each) in cytosols prepared from liver, duodenum, lung, and kidney of rapid and slow acetylator rabbits. No significant effect on either SMZ or PABA acetylation rates was observed with any of the rapid acetylator cytosols. SMZ acetylase activities in duodenal and lung cytosols prepared from slow acetylator rabbits were inhibited by 25.3 ± 3.5% ($p < 0.05$) and 16.8 ± 1.4% ($p < 0.05$), respectively. In contrast, neither SMZ acetylation by liver or kidney cytosols nor PABA acetylation by any of the slow acetylator cytosols was inhibited.

Discussion

Immunoblot analysis using the goat anti-rabbit polyclonal antibodies revealed that rapid acetylator hepatic cytosols con-

TABLE 2

Antibody inhibition of SMZ and PABA NAT activities in cytosols prepared from rapid and slow acetylator rabbits

Cytosols (liver, duodenum, lung, or kidney from a rapid and a slow acetylator rabbit) were preincubated for 24 hr at 4° with NAT antibodies or preimmune IgG. Subsequently, aliquots of the supernatant were incubated with SMZ (500 μM) or PABA (25 and 500 μM) and acetyl CoA (500 μM) for 1 hr at 37°, under linear kinetic conditions. The percentage of inhibition of NAT activity was determined by comparison of the NAT activity in incubations containing NAT antibodies with that in incubations containing preimmune IgG. The concentration of antibodies used was as described in Experimental Procedures. Data are mean ± standard deviation of a triplicate determination on one sample.

Acetylator phenotype	Tissue	Specific activity					
		25 μM PABA		500 μM PABA		500 μM SMZ	
		Without antibody	With antibody	Without antibody	With antibody	Without antibody	With antibody
		nmol/min/mg of protein					
Rapid	Liver	2.67 ± 0.14	1.82 ± 0.05 (32.0 ± 2.1%)[a]	7.95 ± 0.23	0.787 ± 0.066 (90.1 ± 8.0%)	8.07 ± 0.68	0.452 ± 0.021 (94.4 ± 9.0%)
Rapid	Duodenum	4.50 ± 0.10	4.24 ± 0.05 (5.8 ± 0.16%	5.62 ± 0.21	1.11 ± 0.078 (80.2 ± 6.4%)	4.27 ± 0.36	0.320 ± 0.010 (92.5 ± 8.5%
Rapid	Lung	0.470 ± 0.019	0.478 ± 0.02[b]	4.20 ± 0.16	3.08 ± 0.341 (26.7 ± 3.1%)	1.17 ± 0.095	0.839 ± 0.023 (28.3 ± 2.4%)
Rapid	Kidney	0.287 ± 0.010	0.282 ± 0.010[b]	0.214 ± 0.007	0.222 ± 0.014[b]	0.093 ± 0.014	0.093 ± 0.010[b]
Slow	Liver	0.698 ± 0.021	0.696 ± 0.002[b]	0.083 ± 0.005	0.082 ± 0.002[b]	0.138 ± 0.005	0.136 ± 0.005[b]
Slow	Duodenum	1.150 ± 0.016	1.12 ± 0.061[b]	0.141 ± 0.005	0.139 ± 0.002[b]	0.211 ± 0.007	0.213 ± 0.002[b]
Slow	Lung	0.644 ± 0.021	0.650 ± 0.014[b]	0.251 ± 0.003	0.242 ± 0.017[b]	0.418 ± 0.026	0.412 ± 0.012[b]
Slow	Kidney	0.188 ± 0.005	0.183 ± 0.0003[b]	0.059 ± 0.002	0.057 ± 0.014[b]	0.065 ± 0.002	0.063 ± 0.010[b]

[a] Percentage of inhibition shown in parentheses.
[b] No significant inhibition.

tained a prominent immunoreactive M_r 33,000 protein, which co-immunoblotted with hepatic NAT purified 560-fold (Fig. 2). Two major findings were provided by the inhibition studies. First, when SMZ and PABA concentrations (each 500 μM) approximated the apparent K_m estimates for rapid acetylator liver cytosol, maximal inhibition of SMZ and PABA acetylation was 94.4 ± 9.0% and 90.1 ± 8.0%, respectively (Table 2 and Fig. 3A). Inasmuch as the inhibition profiles for SMZ and PABA are essentially identical (Fig. 3A), these data support previously reported evidence that SMZ and PABA acetylase activities are properties of the same major protein in the liver of rapid acetylator rabbits (1, 37). Second, when PABA concentration (25 μM) approximated the apparent K_m estimate for slow acetylator rabbit liver cytosol, maximal inhibition of PABA acetylation was 32.0 ± 2.1% (Table 2). Our data are consistent with the proposition that rapid acetylator liver contains two NAT isozymes, one of which is immunoreactive with the NAT antibodies. Furthermore, the K_m values for PABA of the two proposed NATs differ in a manner that reflects the K_m estimates for "rapid acetylator NAT" and "slow acetylator NAT" in liver cytosols. Although our data offer no direct evidence that the nonimmunoreactive NAT is slow acetylator NAT, the proposal is supported by the finding that the NAT antibodies did not react with hepatic NAT in slow acetylators (Fig. 2 and Table 2).

The finding that slow acetylator hepatic NAT was nonimmunoreactive in the present study (Fig. 2 and Table 2) was of particular interest in light of recent reports. Specifically, a cDNA clone encoding for the principal NAT from liver of rapid acetylator rabbits, has been isolated and expressed (4, 5) and the gene encoding this NAT was shown to be absent from liver of slow acetylator rabbits (5). Hence, the latter study (5) and the current work support the model in which the hepatic polymorphism in rabbits is caused by the total lack of the major rapid acetylator hepatic NAT in the phenotypic slow acetylator animals. The failure to detect a M_r 33,000 immunoreactive band by immunoblot analysis with slow acetylator hepatic cytosol (Fig. 2) suggests also that the polyclonal antibodies do not cross-react with non-NAT proteins.

Our finding that the principal NAT protein in duodenum and liver of rapid acetylator rabbits displays similar antibody cross-reactivity supports the results of other workers (20). Additionally, the immunoblots (Fig. 2) and the inhibition data (Table 2 and Fig. 3A) provide evidence to indicate that the major duodenal NATs from rapid and slow acetylator rabbits are immunologically distinct proteins. With rapid acetylator preparations, NAT antibodies maximally inhibited SMZ and PABA (500 μM each) NAT activity in duodenum by 92.5 ± 8.5% and 80.2 ± 6.4%, respectively. The partial inhibition of duodenal PABA NAT activity raised the possibility of multiple isozymes. Indeed, this was shown to be the case when inhibition studies using 25 μM PABA showed maximal inhibition of only 5.8 ± 0.16% (Table 2). By analogy with liver, there appears to be a second nonimmunoreactive NAT, characterized by a low K_m for PABA, in rapid acetylator duodenal tissue. This finding supports an earlier hypothesis that a second NAT isozyme may contribute to a basal SMZ acetylase activity in duodenum (38). Our inhibition data suggest also that the immunoreactive form of NAT is relatively less abundant in duodenum (maximal inhibition of PABA acetylation, 80.2 ± 6.4% with 500 μM PABA; 5.8 ± 0.16% with 25 μM PABA) than liver (maximal inhibition of PABA acetylation, 90.1 ± 8.0% with 500 μM PABA; 32.0 ± 2.1% with 25 μM PABA). With slow acetylator rabbits, no immunoreactivity of duodenal NAT was demonstrated. Significant inhibition (25.3 ± 3.5%; $p < 0.05$) of SMZ acetylation occurred in the presence of paraoxon (10^{-4} M). Although this finding suggests that an esterase capable of *N*-acetylating SMZ is present in slow acetylator duodenum, further studies are needed to clarify this point.

The present study provides the first evidence for the presence of multiple isozymes of NAT in lung of rapid acetylator rabbits. A faint M_r 33,000 immunoreactive band was observed for partially purified lung NAT on immunoblot analysis (data not shown). Table 2 shows that rapid acetylator lung cytosols acetylated SMZ and PABA (500 μM each) with initial velocities

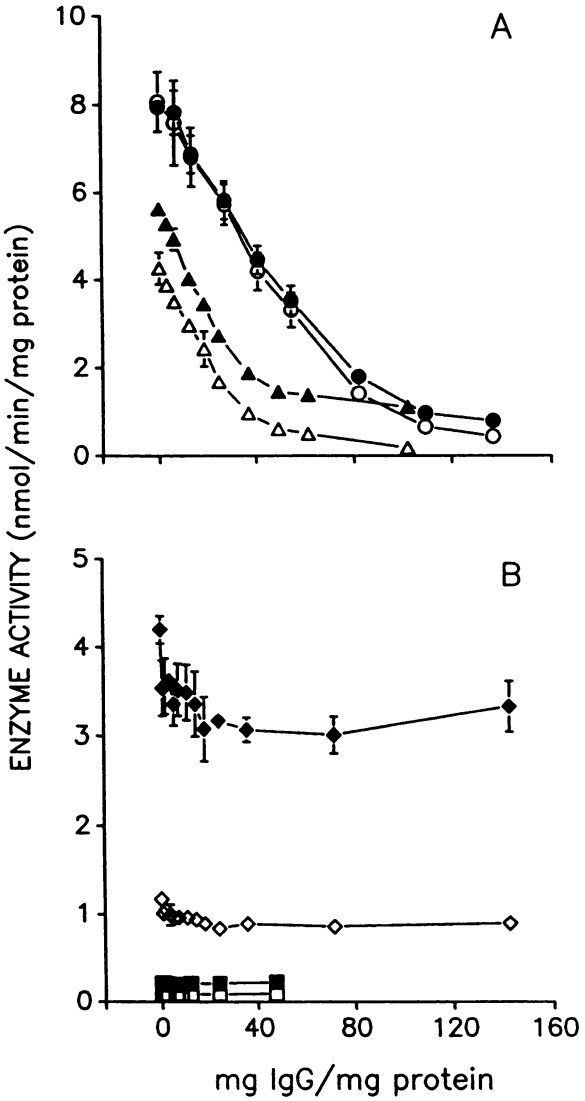

Fig. 3. Inhibition curves for liver (○, SMZ; ●, PABA) and duodenum (△, SMZ; ▲, PABA), (A) and lung (◇, SMZ; ◆, PABA) and kidney (□, SMZ; ■, PABA) (B). Cytosols (liver, duodenum, lung, or kidney from a rapid acetylator rabbit) were preincubated for 24 hr at 4° with NAT antibodies or preimmune IgG. Subsequently, aliquots of the supernatant were incubated with SMZ or PABA (0.5 mM) and acetyl CoA (0.5 mM) for 1 hr at 37°, under linear kinetic conditions. The percentage of inhibition of NAT activity was determined by comparison of the NAT activity in incubations containing NAT antibodies with that in incubations containing preimmune IgG. The concentration of antibodies used was as described in Experimental Procedures. Data are mean ± standard deviation of a triplicate determination on one sample.

of 1.17 ± 0.10 and 4.20 ± 0.16 nmol/min/mg of protein, respectively. These activities were maximally inhibited by 28.3 ± 2.4% (SMZ) and 26.7 ± 3.1% (PABA) in the presence of NAT antibodies. In contrast, the acetylation of 25 μM PABA was not inhibited by antibodies. The observed residual acetylation was shown to be paraoxon insensitive and, therefore, cannot be attributed to nonspecific esterase activities. Although several explanations can be offered to satisfactorily explain these data, the simplest hypothesis is that two isozymes of NAT are present in lung of rapid acetylator rabbits (i) an isozyme that is immunologically related to the major form of hepatic and duodenal NAT and that contributes some 30% of the total pulmonary acetylation capacity at high SMZ and PABA concentrations and (ii) an isozyme that is antigenically distinct from the major form of hepatic and duodenal NAT, has a low K_m for PABA, and comprises the major component of pulmonary NAT. Our earlier enzyme kinetic studies (21) did not provide evidence for the presence of two NAT isozymes in lung. However, in a two-enzyme system the relative magnitudes of the Michaelis-Menten parameters will determine whether the presence of two enzymes will be obvious (39). Although no immunoreactivity of slow acetylator lung NAT was demonstrated in the present study, paraoxon (10^{-4} M) inhibited SMZ acetylation by 16.8 ± 1.4% ($p < 0.05$). The significance of this observation is unclear.

Rapid and slow acetylator kidney cytosols demonstrated low SMZ and PABA NAT activities, which were not significantly inhibited by antibodies (Table 2). This finding cannot be attributed to an artifact associated with low rates of substrate acetylation observed for kidney cytosol, because the specific activity of all cytosols investigated was adjusted routinely to 0.08 nmol of NASMZ formed/min/incubation before antibody inhibition studies (see Experimental Procedures). Rather, the antibody inhibition studies confirm that kidney NAT is antigenically distinct from the major form of hepatic NAT. By inference, these data suggest that, for rapid acetylator rabbit lung and kidney, expression of polymorphic NAT may be controlled at the level of transcription. Other workers (20) have reported that erythrocyte and lymphocyte NATs from rapid and slow acetylator rabbits did not immunoreact with goat antiserum directed against rabbit liver NAT.

In summary, polyclonal antibodies were raised to the major form of hepatic NAT in rapid acetylator rabbits [later referred to as polymorphic NAT (5)]. The present study supports the model of Blum *et al.* (5), in which the hepatic polymorphism in rabbits is caused by the total lack of polymorphic NAT in the phenotypic slow acetylator animals. The data indicate that, in rapid acetylator rabbits, polymorphic NAT or a NAT that is immunologically and kinetically related to polymorphic NAT is the principal form in duodenum and a minor form in lung tissue. At least one other NAT protein that does not cross-react with the antibodies directed to polymorphic NAT is present in minor amounts in liver and duodenum of rapid acetylator rabbits and is the major NAT form in lung. The data in Table 2 indicate that the functional role of the nonimmunoreactive minor form(s) in liver and duodenum can be important and that the contribution of this isozyme to acetylation will depend on the substrate and its concentration. With slow acetylator rabbits, NAT protein in liver, duodenum, lung, and kidney does not cross-react with antibodies directed to polymorphic NAT. Based on these data, the simplest scenario is that only two forms of NAT exist in rabbits. Immunological testing of this hypothesis requires additional antibodies against the NAT that was nonimmunoreactive in the present study. A preliminary report describing molecular biological studies into rabbit NATs suggests that only two forms occur (40). These have been designated NAT1 (monomorphic) and NAT2 (polymorphic) and correspond to the nonimmunoreactive and immunoreactive forms, respectively, described in the present study.

Finally, epidemiological studies have demonstrated a higher incidence of colorectal cancer in rapid acetylator human subjects (41, 42), whereas biochemical studies have shown that rat intestinal cytosols catalyze the acetyl CoA-dependent metabolic activation of *N*-hydroxyarylamine carcinogens to products that

bind covalently to DNA (43). The rabbit may, therefore, be a suitable animal model for investigation of the role of intestinal NATs in the metabolic activation of aromatic amines and heterocyclic arylamines of environmental or dietary origin. The known role of NATs in both drug metabolism and arylamine chemical carcinogenesis suggests that the localization of NAT isozymes may be an important determinant of tissue-specific interaction for such compounds.

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