0026-895X/89/050599-11$02.00/0

MOLECULAR PHARMACOLOGY, 35:599-609



# Purification and Biochemical Characterization of Hepatic Arylamine *N*-Acetyltransferase from Rapid and Slow Acetylator Mice: Identity with Arylhydroxamic Acid *N*,*O*-Acyltransferase and *N*-Hydroxyarylamine *O*-Acetyltransferase

SUSAN S. MATTANO, SUSAN LAND, CHARLES M. KING, and WENDELL W. WEBER

*Department of Pharmacology, University of Michigan Medical School, Ann Arbor, Michigan 48109 (S.S.M., W.W.W.) and Department of Chemical Carcinogenesis, Michigan Cancer Foundation, Detroit, Michigan 48201 (S.L., C.M.K.)*

Received September 15, 1988; Accepted January 31, 1989

**SUMMARY**

An inbred mouse model for the human *N*-acetylation polymorphism has been used to investigate the biochemical basis for the arylamine *N*-acetylation polymorphism and the relationship between the cytosolic enzymes arylamine *N*-acetyltransferase (NAT), arylhydroxamic acid *N*,*O*-acyltransferase, and *N*-hydroxyarylamine *O*-acetyltransferase. Biochemical studies of partially purified NAT from rapid and slow acetylator mice revealed identical molecular weights of 31,500, activation energies of 21,000 cal/mol, equivalent affinities for acetyl coenzyme A, broad pH optima, the presence of an active site sulfhydryl group, and similar behavior during purification with anion exchange, gel filtration, and hydrophobic interaction chromatography. The enzymes differed in inhibition by hydrogen peroxide and dithiobis(2-nitrobenzoic acid). These observations taken in conjunction with previous investigations indicate that the rapid and slow mouse NAT enzymes are isozymes with minimal structural differences.

NATs from rapid and slow acetylator mice were purified more than 10,000-fold by the following sequence of methods: homogenization and fractional centrifugation, protamine sulfate precipitation, and chromatography on DEAE-Trisacryl M, Sephadex G-100, Amethopterin-AH-Sepharose 4B, butyl agarose, and Sephacryl S-200, with a 15–25% recovery. NAT from B6 mice was purified to >95% purity, as judged by silver staining of sodium dodecyl sulfate-polyacrylamide gels. Although only NAT appeared to be subject to a genetic polymorphism as evidenced by *N*-acetylation activities in liver cytosol, the purified NAT protein possessed arylhydroxamic acid *N*,*O*-acyltransterase, *N*-hydroxyarylamine *O*-acetyltransferase, and NAT activities. Thus, the cytosolic *N*-acetyltransferase of mouse liver may catalyze *N*-, *O*-, and *N*,*O*-acetyltransfer reactions through a common acetylated intermediate of a single protein.

The human polymorphism in hepatic NAT is known to affect the metabolism and toxicity of certain hydrazine and arylamine drugs, including isoniazid, procainamide, sulfamethazine, and *P*-aminosalicylic acid (1). A relationship between this acetylation polymorphism and the induction of bladder and colon cancer has been reported (2, 3). Family and population studies of the polymorphic metabolism of isoniazid in humans established the autosomal monogenic inheritance of the trait, with the rapid aceteylator allele exhibiting dominance (4). Acetylation polymorphisms have subsequently been demonstrated in animal models, including rapid and slow acetylator rabbits, hamsters, and mice (5–9). However, it is not known whether a single enzyme exists that differs qualitatively or quantitatively between rapid and slow acetylators or whether multiple NAT isozymes are expressed by rapid and slow acetylators.

Three different cytosolic acetylation reactions are involved in the bioactivation of arylamine carcinogens (Fig. 1). *N*-Acetylation can produce either arylacetamides or arylhydroxamic acids (1). AHAT catalyzes the metabolic activation of arylhydroxamic acids by intramolecular transfer of the acetyl group from *N* to *O*, forming an unstable *N*-acetoxyarylamine (10–14). Furthermore, the arylhydroxamic acid can serve as an acetyl donor in the *N*-acetylation of arylamines (14, 15). The structure of both the aryl and acyl moieties can influence these reactions (16–18). Both *N*-acetyl transfer and *N*,*O*-acyl transfer reactions are catalyzed through an ordered ping-pong mechanism, and both activities were determined to be catalyzed by a single

These studies were supported by United States Public Health Service Grants CA 23386, CA 39018, and GM 27028.

**ABBREVIATIONS:** NAT, acetyl coenzyme; A:arylamine *N*-acetyltransferase; A, A/J; AHAT, arylhydroxamic acid *N*,*O*-acyltransferase; B6, C57BL/6J; DTNB, 5,5′-dithiobis(2-nitrobenzoate); DTT, dithiothreitol; MOPS, 3-(*N*-morpholino)propanesulfonic acid; N-OH-DMABP, *N*-hydroxy-3,2-dimethyl-4-aminobiphenyl; N-OH-AAF, *N*-hydroxyacetylaminofluorene; OAT, *N*-hydroxyarylamine *O*-acetyltransferase; PABA, *p*-aminobenzoic acid; PCMB, *p*-chloromercuribenzoate; PMSF, phenylmethylsulfonylfluoride; SDS, sodium dodecyl sulfate; CoASAc, acetyl coenzyme A.

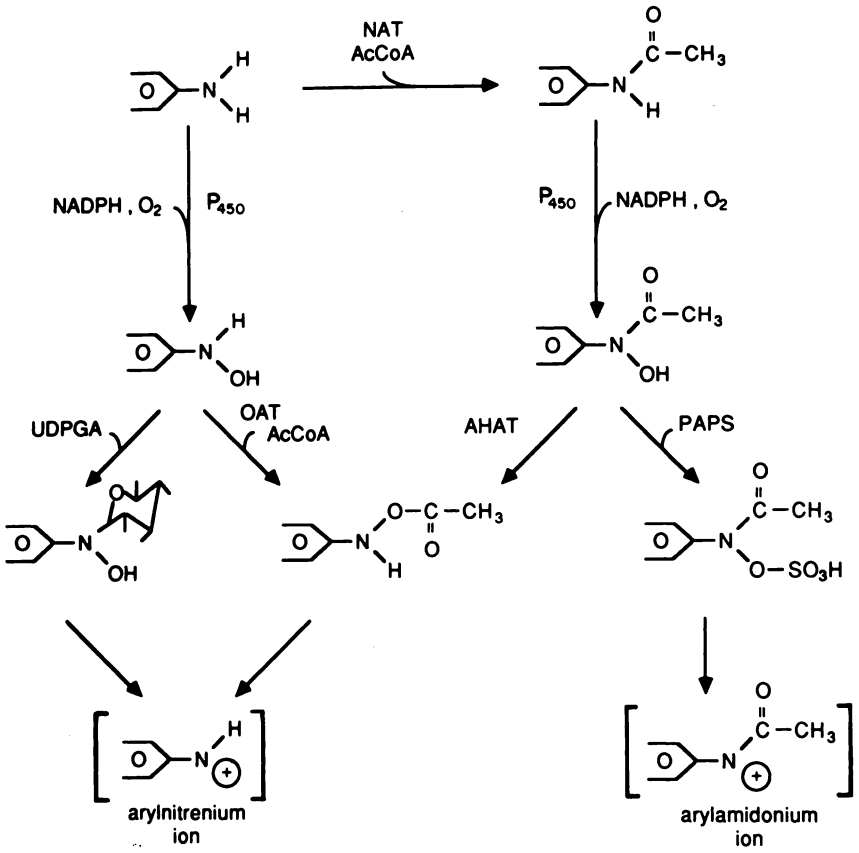


**Fig. 1.** Bioactivation pathways for arylamine carcinogens.

enzyme, in rabbit liver, that is subject to wide genetic variation (19). Evidence for direct *O*-acetylation was provided by experiments with N-OH-DMABP (20). The acetohydroxamic acid derivative of this hydroxylamine cannot serve as an acetyl donor in either *N*-acetylation or *N,O*-acetyltransfer reactions (20). A single enzyme of hamster liver has been reported to be capable of both *N*- and *O*-acetylation (21). In order to clarify the role of acetylation in the production of toxic effects by xenobiotics subject to metabolism by acetylation, it is first necessary to establish the relationship between these three activities. Of fundamental importance is whether these reactions are catalyzed by a single protein and whether the reactions exhibit similar genetic polymorphisms.

Rapid and slow acetylator inbred mouse strains have been identified by the use of *N*-acetylation assays, and several biochemical characteristics have been compared in the A (slow acetylator) and B6 (rapid acetylator) strains (9, 22–26). The arylamine NAT enzymes differ qualitatively, as demonstrated by differences in $K_m$ for 2-aminofluorene and differential inhibition by the solvent dimethylsulfoxide (24, 25). In the present study, we have extended these investigations in A and B6 mice to explore the biochemical basis for the acetylation polymorphism in the A and B6 strains. In addition, we report that the rapid acetylator enzyme has been purified to yield an identifiable single enzyme that can catalyze all three acetylation reactions, i.e., *N*-, *O*-, and *N,O*-acetylation.

## Materials and Methods

B6 and A mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and were at least 35 days old when used. Animals were housed at 25° on a 12-hr light/dark cycle and were given water and Purina Mouse Chow 9F 5020 *ad libitum*. PABA, DTT, Tris base, bovine serum albumin, acetyl carnitine, carnitine acetyltransferase, EDTA, leupeptin, PMSF, protamine sulfate, Sephadex G-100, Sephacryl S-200, butyl-agarose, MOPS buffer, sodium phosphate monobasic and dibasic, DTNB, and PCMB were from Sigma Chemical Company (St. Louis, MO). 2-Aminofluorene was purchased from K and K Laboratories (Plainview, NY) and recrystallized from 70% methanol. CoASAc was obtained from Pharmacia/P-L Biochemicals, Inc. (Milwaukee, WI). DEAE-Trisacryl was purchased from LKB Instruments, Inc. (Gaithersburg, MD). The 4-iodoaniline was from Aldrich Chemical Co. (Milwaukee, WI). Amethopterin-AH-Sepharose 4B was prepared as previously described (27). Acrylamide was from FMC Bioproducts (Rockland, ME). Diaflo PM10 ultrafiltration membranes were from Amicon Corp. (Danvers, MA). [*ring*-$^3$H]-N-OH-AAF (10–30 mCi/mmol) (17) and [*ring*-$^3$H]-N-OH-DMABP (23.2 mCi/mmol) (28) were prepared and characterized as previously described.

**Acetyltransferase assays.** NAT activity was measured spectrophotometrically with the recently described recycling system (29). PABA and CoASAc were used at a concentration of 0.1 mM. The following components were present in the assay system in a total volume of 90 μl; 15 mM acetyl carnitine, 2 units/ml carnitine acetyltransferase, 2 mM EDTA, 2 mM DTT, and 50 mM Tris·HCl, pH 7.5. One unit of enzyme activity was defined as the acetylation of 1 mmol of substrate/min at 37°. Protein concentrations were determined with the method of Bradford (30).

AHAT activity was determined using a tRNA trapping assay (10, 14) with [*ring*-$^3$H]N-OH-AAF (0.042 μmol) as the substrate, with a specific activity of 15 μCi/μmol. tRNA was recovered by filtration, and binding of substrate was determined by liquid scintillation counting.

OAT activity was determined using a nucleic acid trapping assay (20) with [*ring*-$^3$H]N-OH-DMABP (0.015 μmol) as the substrate, with a specific activity of 24 μCi/μmol. tRNA ($15A_{260}$ units), rather than DNA, was used as the trapping agent. The tRNA was recovered by filtration, and binding of substrate was determined by liquid scintillation counting, as in the AHAT assay.

**Purification of *N*-acetyltransferase.** The enzymes were purified by an extension of a previously published procedure (58). All steps were carried out at 4°.

Step 1. Crude extract. Fresh livers (80 g) obtained after cervical dislocation were trimmed, washed, and homogenized in 5 volumes of 20 mM Tris·HCl, pH 7.5, 2 mM DTT, 1 mM EDTA, 50 μM PMSF, 10 μM leupeptin, in a Waring blender on high speed for 1 min. The homogenate was centrifuged at 20,000 × *g* for 30 min, the supernatant was filtered through cheesecloth, and its pH was adjusted to 7.8 with a few drops of 1 M Tris base.

Step 2. Protamine sulfate precipitation. A 1% protamine sulfate solution (15–20 ml) was added dropwise over 30 min with a peristaltic pump while the supernatant preparation was stirred vigorously. Precipitated material was pelleted by centrifugation at 20,000 × *g* for 20 min and discarded.

Step 3. DEAE-Trisacryl M ion exchange chromatography. The supernatant fraction from the previous step was allowed to bind batchwise for 15 min to 40–50 ml of DEAE-Trisacryl M that was equilibrated with 20 mM Tris·HCl, pH 7.5, 2 mM DTT, 1 mM EDTA. The gel was washed in a sintered glass funnel with several volumes of that buffer until the protein concentration in the effluent was no more than 50 μg/ml, as determined with the Bradford reagent. The gel was packed into a 2.5 × 20 cm column and rinsed with 1 column volume of 20 mM Tris·HCl, pH 7.5, 2 mM DTT, 1 mM EDTA, at 90 ml/hr. The enzyme was eluted with a linear salt gradient (160 ml, 0–500 mM NaCl in 20 mM Tris·HCl, pH 7.5, 2 mM DTT, 1 mM EDTA), and 2.5-min fractions were collected. The peak fractions were pooled and concentrated to 10 ml by ultrafiltration through an Amicon PM10 membrane with 1.5 atmospheres of $N_2$ gas.

Step 4. Sephadex G-100 chromatography. The concentrated enzyme was applied to a 5 × 50 cm Sephadex G-100 column, equilibrated with 20 mM Tris·HCl, pH 7.5, 2 mM DTT, 1 mM EDTA, 0.02% sodium azide, and was eluted at a flow rate of 20 ml/hr, with 7.5-min fractions being collected. The peak fractions were pooled and concentrated to 5 ml by ultrafiltration.

Step 5. Methotrexate chromatography. The concentrated enzyme was applied at a flow rate of 6.0 ml/hr to a 1 × 12 cm column filled with 8 ml of amethopterin-AH-Sepharose 4B. The column was washed with 5 volumes of 20 mM Tris·HCl, pH 7.5, 2 mM DTT, 1 mM EDTA, the enzyme was eluted with a linear salt gradient (0–200 mM NaCl in 20 mM Tris·HCl, pH 7.5, 2 mM DTT, 1 mM EDTA), and 10-min fractions were collected. The peak fractions were pooled and concentrated to 10 ml with ultrafiltration.

Step 6. Butyl-agarose hydrophobic interaction chromatography. An equal volume of 4 M ammonium sulfate, 20 mM Tris·HCl, pH 7.5, 2 mM DTT, 1 mM EDTA, was added dropwise with stirring to the concentrated enzyme from step 5, over a period of 30 min, with a peristaltic pump. Precipitated material was pelleted by centrifugation at 20,000 × *g* for 20 min and discarded. The supernatant was applied to an 8-ml, 1 × 12 cm, column filled with butyl-agarose equilibrated with 2 M ammonium sulfate, 20 mM Tris·HCl, pH 7.5, 2 mM DTT, 1 mM EDTA. The column was washed with 2 bed volumes of 2 M ammonium sulfate, 20 mM Tris·HCl, pH 7.5, 2 mM DTT, 1 mM EDTA. NAT was eluted at a flow rate of 10 ml/hr with a gradient of decreasing ammonium sulfate and increasing glycerol concentration (100 ml, final concentrations 0 and 10%, respectively), and 12-min fractions were collected. The fractions containing activity were pooled and dialyzed overnight against 2 liters of 20 mM Tris·HCl, pH 7.5, 2 mM DTT, 1 mM EDTA. The sample was then concentrated by ultrafiltration to 5 ml.

Step 7. Sephacryl S-200 chromatography. Concentrated enzyme from step 6 was run twice through two 1.5 × 120 cm Sephacryl columns arranged in tandem and pre-equilibrated with 20 mM Tris·HCl, pH 7.5, 2 mM DTT, 1 mM EDTA, at a flow rate of 12 ml/hr; 6-min fractions were collected. Peak fractions were collected but not pooled after the second Sephacryl elution.

**Molecular weight determination with SDS-polyacrylamide gel electrophoresis.** NAT that was purified through Sephacryl S-200 was run on 10% SDS polyacrylamide gels (acrylamide/acrylaide/bisacrylamide, 32:1:0.25, according to FMC Bioproducts technical note T-17 (1985). Fixation and silver stain were performed using the procedure of Paine (31). Molecular weight was determined by plotting the logarithm of molecular weight of standard proteins versus migration distance. Molecular weight markers were obtained from Bio-Rad, Richmond, CA.

**Amino acid composition.** Amino acid composition analysis was performed, as previously described (32), at the University of Michigan Sequencing Facility.

**Enzyme stability.** Acetyltransferase that was purified through the Sephadex G-100 step was diluted 5-fold in 10 mM Tris, MOPS, or $KHPO_4$ buffer, pH 7.5, and incubated at 4° for 10 days. Activity was tested with 0.1 mM PABA and CoASAc on days 0, 4, 7, and 10. On day 7, 1 mM DTT was added to each sample in an attempt to restore activity.

**pH optima.** Acetyltransferase that was purified through Sephadex G-100 was diluted into 5 mM MOPS buffer, pH 7.5, 1 mM DTT, and 1 mM EDTA. Fifty microliters of this solution was added to 20 μl of a mixture containing 4-iodoaniline, DTT, EDTA, acetylcarnitine, and carnitine acetyltransferase that had been clarified by centrifugation. The reaction was started by the addition of 20 μl of CoASAc in MOPS buffer, at the specified pH values, containing amounts of NaCl needed to adjust each to a constant salt concentration. This procedure was necessary because of NAT sensitivity to inhibition by salts (33). The final 90-μl assay mixture contained 0.1 mM CoASAc, 0.2 mM 4-iodoaniline ($pK_a$ = 3.78), 5 mM acetylcarnitine, 200 milliunits/ml carnitine acetyltransferase, 40 mM MOPS buffer (pH 6.5–7.9), 200 mM NaCl, 1 mM DTT, and 1 mM EDTA. The enzyme reactions were carried out at 37° and otherwise were performed as previously described (29).

**Determination of $K_m$ for CoASAc.** The $K_m$ for CoASAc was determined with A and B6 liver 9000 × *g* supernatants that were diluted 20-fold in 20 mM Tris·HCl, pH 7.5, 1 mM DTT, 1 mM EDTA, 50 μM PMSF, 10 μM leupeptin. The reaction mixture contained 0.2 mM PABA and 0.5 to 2.0 mM CoASAc. Kinetic constants were calculated with the Cleland HYPER program (34), which performs linear regression using a least squares method.

**Activation energies.** Acetyltransferase that was purified through Sephadex G-100 was diluted in 20 mM $KHPO_4$ pH 7.5, 1 mM DTT, 1 mM EDTA. Activity was determined with 0.1 mM CoASAc. Enzyme saturation was confirmed at each temperature with 0.10, 0.15, and 0.20 mM PABA. Activation energies were calculated from the Arrhenius equation by the method of least squares.

**Sensitivity to inhibition by sulfhydryl reagents.** The sensitivity of acetyltransferase to inhibition by sulfhydryl reagents was carried out with enzyme purified through the G-100 gel filtration step and diluted into 20 mM Tris·HCl, pH 7.5, 1 mM EDTA. The assay system contained 0.1 mM PABA and 0.1 mM CoASAc. For inhibition with DTNB, the reagent was added at time 0 to a concentration of 100 μM. Attempts to reactivate NAT were made by adding 4.5 mM DTT after 4 min of incubation. Reversal of inhibition with 0.002% $H_2O_2$ was attempted by the addition of DTT at concentrations of up to 40 mM after 20 min of incubation with the peroxide. After incubation with 1 μM PCMB, an attempt to restore acetyltransferase activity was made by adding 10 mM DTT and allowing 20 min for reactivation.

## Results

***N*-Acetyltransferase purification.** The purification procedure developed for mouse NAT was based on a procedure recently reported for liver NAT purified from rapid acetylator rabbits (33). Tables 1 and 2 summarize typical purification schemes for A and B6 liver NAT. Both rapid and slow enzymes eluted from DEAE-cellulose with 250–300 mM NaCl, with 4–5-fold purifications. To maximize recovery at this step, it was necessary to use twice as much gel as reported for rabbit liver

TABLE 1

**Purification of liver NAT from A mice**

| Step | Total protein | Total activity | Specific activity | Purification | Recovery |
|---|---|---|---|---|---|
| | *mg* | *units* | *milliunits/mg* | *fold* | *%* |
| 1. 20,000 × g supernatant | 6,042 | 7.3 | 1.2 | 1.0 | |
| 2. Protamine sulfate precipitate | 4,194 | 8.4 | 2.0 | 1.6 | 100 |
| 3. DEAE-Trisacryl M | 731 | 5.8 | 8.0 | 6.5 | 79 |
| 4. Sephadex G-100 | 43 | 3.2 | 75 | 61 | 44 |
| 5. Methotrexate | 2.5 | 2.7 | 1,098 | 900 | 37 |
| 6. Butyl-agarose | 0.14 | 3.1 | 21,458 | 17,588 | 42 |
| 7. Sephacryl S-200 | 0.05 | 1.1 | 22,449 | 18,401 | 15 |

[a] NAT activity was determined with 0.1 mM PABA and CoASAc.

TABLE 2

**Purification of liver NAT from B6 mice**

| Step | Total protein | Total activity | Specific activity | Purification | Recovery |
|---|---|---|---|---|---|
| | *mg* | *units* | *milliunits/mg* | *fold* | *%* |
| 1. 20,000 × g supernatant | 12,080 | 32.2 | 2.67 | 1.0 | |
| 2. Protamine sulfate precipitate | 9,564 | 28.6 | 2.99 | 1.12 | 89 |
| 3. DEAE-Trisacryl M | 2,682 | 25.6 | 9.56 | 3.6 | 74 |
| 4. Sephadex G-100 | 331 | 23.6 | 71.4 | 26.7 | 73 |
| 5. Methotrexate | 19.6 | 15.6 | 796 | 298 | 48 |
| 6. Butyl-agarose | 0.72 | 11.0 | 15,321 | 5,738 | 34 |
| 7. Sephacryl S-200 | 0.28 | 7.9 | 28,082 | 10,518 | 24 |

[a] NAT activity was determined with 0.1 mM PABA and CoASAc.

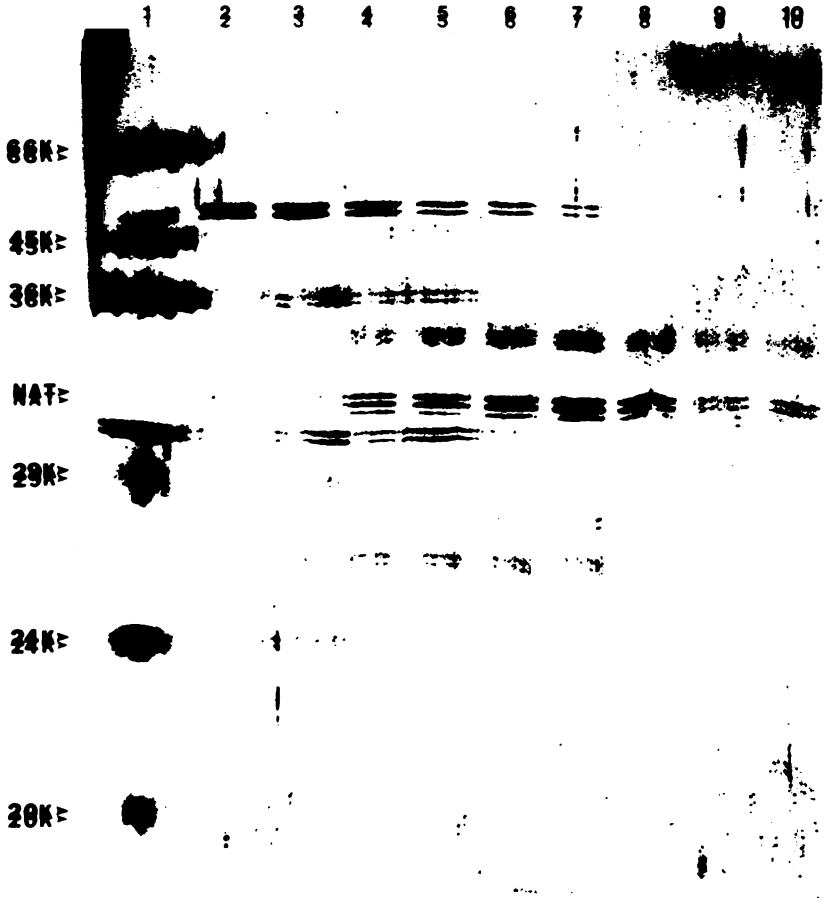


**Fig. 2.** SDS-polyacrylamide gel electrophoresis and silver stain of A Sephacryl fractions. Sephacryl fractions 1–9 are in *lanes 2–10*, respectively. *Lane 1* contains a mixture of the following molecular weight markers: bovine serum albumin (66,000), ovalbumin (45,000), glyceraldehyde-3-phosphate dehydrogenase (36,000), carbonic anhydrase (29,000), trypsinogen (24,000), and trypsin inhibitor (20,100).

NAT, due to different binding properties of mouse NAT. Gel filtration on Sephadex resulted in an 8–10-fold purification of both enzymes. Methotrexate bound to ε-aminohexane Sepharose 4B agarose was introduced as an affinity chromatographic step, and was found to be effective, but not specific for NAT. Both rapid and slow NATs eluted with 100–150 mM NaCl. This step replaced the hydroxylapatite gel used in purifying rabbit NAT, because hydroxylapatite yielded a poor (1.5–2-fold) purification factor with mouse NAT. After the methotrexate step, butyl-agarose (hydrophobic interaction) and two Sephacryl (gel filtration) steps were necessary for the most effective purification. PABA bound to agarose through the carboxyl group

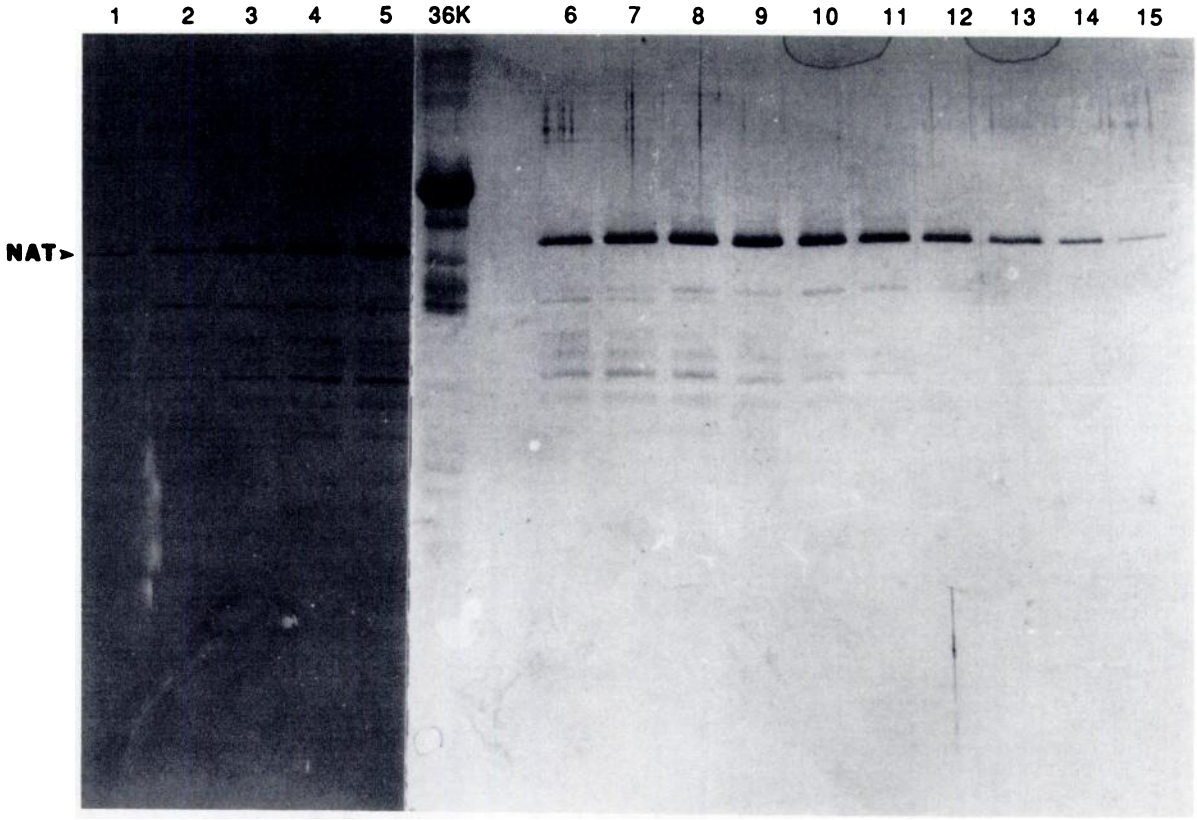


**Fig. 3.** SDS-polyacrylamide gel electrophoresis and silver stain of B6 Sephacryl fractions. Sephacryl fractions 1–15 are in *lanes 1–5* and *6–15*, respectively. Marker lane contains glyceraldehyde-3-phosphate dehydrogenase (36,000) (*36K*).

TABLE 3

**Amino acid compositions of hepatic NAT from rapid acetylator mouse, rabbit, and pigeon, in comparison with a mean composition of 81 proteins**

| Amino Acid | B6 mouse | III/J rabbit[a] | Pigeon[b] | Mean composition[c] |
|---|---|---|---|---|
| | | *residue/mol* | | |
| Ala | 16.5 | 6.9 | 5.0 | 25.8 |
| Cya[d] | 8.6 | 5.0 | 3.9 | 7.3 |
| Asx | 26.4 | 26.7 | 26.9 | 32.7 |
| Glx | 35.2 | 37.3 | 30.0 | 29.7 |
| Phe | 12.8 | 14.0 | 10.4 | 11.2 |
| Gly | 32.0 | 20.5 | 18.3 | 22.9 |
| His | 6.9 | 5.7 | 8.1 | 6.3 |
| Ile | 13.6 | 19.0 | 14.4 | 16.5 |
| Lys | 16.5 | 14.5 | 18.4 | 20.5 |
| Leu | 25.3 | 34.5 | 31.2 | 21.9 |
| Met | 4.9 | 5.1 | 7.6 | 4.9 |
| Pro[e] | 9.7 | 10.1 | 9.4 | 11.2 |
| Arg | 12.9 | 16.4 | 13.1 | 15.7 |
| Ser | 20.1 | 19.1 | 14.7 | 20.5 |
| Thr | 18.8 | 16.8 | 12.6 | 17.6 |
| Val | 14.6 | 19.2 | 21.9 | 20.5 |
| Tyr[f] | 12.7 | 15.1 | 15.8 | 12.7 |
| Trp[g] | | 4.7 | 3.8 | 3.9 |
| Total residues | 289 | 291 | 270 | 288 |
| Molecular weight | 31,000 | 33,500 | 32,870 | 33,510 |

[a] Data from Andres *et al.* (33).
[b] Data from Andres *et al.* (27).
[c] Data from Holmquist (43).
[d] Cysteine and cystine were determined as cysteic acid.
[e] 4-Hydroxyproline was detected at a level of 2.1 residue/mol in the mouse enzyme.
[f] The tyrosine value for the mouse enzyme was estimated from the Leu/Tyr ratio determined for the rabbit enzyme.
[g] Tryptophan was not determined for the mouse.

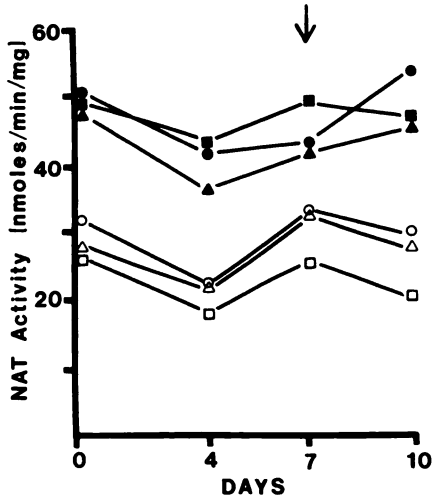


**Fig. 4.** Stability of B6 and A liver NAT at 4°. On day 7, 1 mM dithioerythritol was added to each sample as indicated by the *arrow*. NAT activity was measured with 0.1 mM PABA and CoASAc ●, B6 enzyme in 10 mM MOPS, pH 7.5, 1 mM dithioerythritol, 1 mM EDTA. ■, B6 enzyme in 10 mM $KHPO_4$, pH 7.5, 1 mM dithioerythritol, 1 mM EDTA. ▲, B6 enzyme in 10 mM Tris, pH 7.5, 1 mM dithioerythritol, 1 mM EDTA. ○, A enzyme in 10 mM MOPS, pH 7.5, 1 mM dithioerythritol, 1 mM EDTA. □, A enzyme in 10 mM $KHPO_4$ pH 7.5, 1 mM dithioerythritol, 1 mM EDTA. △, A enzyme in 10 mM Tris, pH 7.5, 1 mM dithioerythritol, 1 mM EDTA.

(United States Biochemical, Cleveland, OH) was examined as a possible affinity gel. However, the enzyme was not absorbed to the gel in the presence or absence of CoASAc. In addition, chromatofocusing chromatography (Pharmacia) was attempted without success. In this system, NAT bound to the gel but

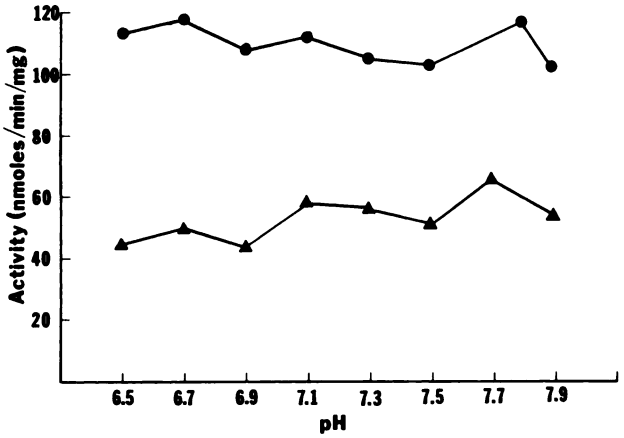


**Fig. 5.** pH optimum profiles of B6 and A hepatic NAT. NAT activity was measured with 0.1 mM CoASAc and 0.2 mM 4-iodoaniline. ●, B6; ▲, A NAT activity.

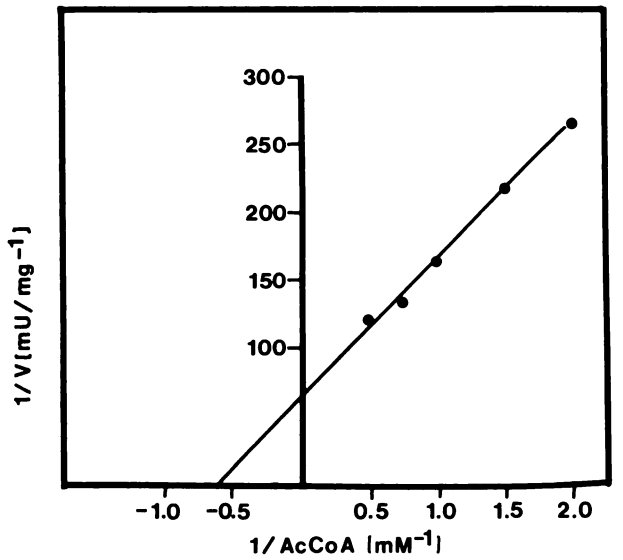


**Fig. 6.** Lineweaver-Burk double-reciprocal plot of A hepatic NAT activity as a function of CoASAc concentration.

could not be recovered in an active form, possibly due to precipitation of the enzyme at its pI. For final purification, two elutions through tandem Sephacryl columns were required. The purity of each fraction eluted from the second Sephacryl step was evaluated by SDS-polyacrylamide gel electrophoresis with silver staining (Figs. 2 and 3). For the B6 enzyme (Fig. 3), the fractions with the greatest NAT activity, i.e., 7–10, were essentially pure, with only traces of unknown smaller peptides as contaminants. The overall purification ranged from 10,000- to 18,000-fold, depending on the amount of activity lost in the final step, with an overall 15–24% recovery. Final specific activities for both rapid and slow enzymes were approximately 20 units/mg of protein. The molecular masses of A and B6 NAT were 31,500 D, as determined by SDS-polyacrylamide gel electrophoresis.

**Amino acid composition.** Amino acid composition analysis was performed on the purified B6 enzyme and compared with the reported compositions of the rabbit and pigeon enzymes, as well as with a "mean composition" reported for 81 proteins (Table 3). Because the sample was contaminated with some residual Tris, the tyrosine content could not be determined.

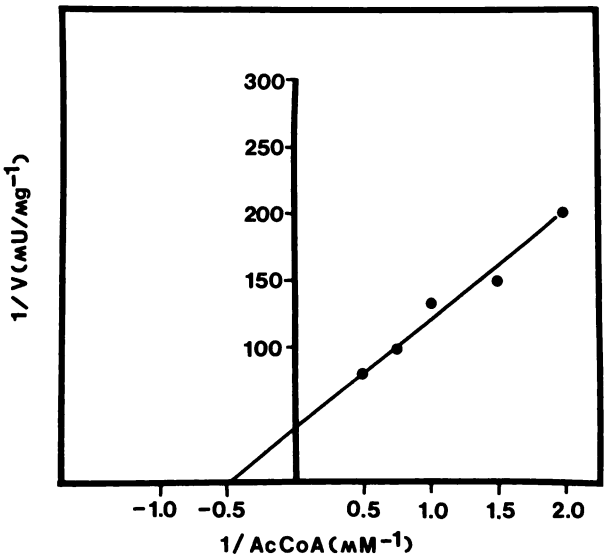


**Fig. 7.** Lineweaver-Burk double-reciprocal plot of B6 hepatic NAT activity as a function of CoASAc concentration.

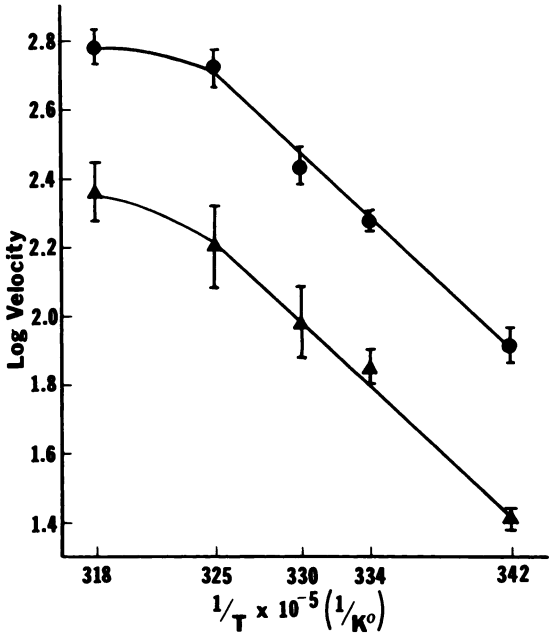


**Fig. 8.** Activation energy determination of Arrhenius plot of B6 and A hepatic NAT. ●, B6 NAT (mean ± standard deviation; three experiments) $E_a$ = 21.2 kcal/mol, corr. = 0.997. ▲, A NAT (mean ± standard deviation; three experiments) $E_a$ = 21.1 kcal/mol, corr. = 0.995.

Therefore, the tyrosine content was estimated using the Leu/Tyr ratio determined in rabbit (33). Tryptophan content was not determined, but all other amino acids were present, as well as 4-hydroxyproline, which was not reported for the pigeon or rabbit enzymes (27, 33).

**Enzyme stability.** Stability of A and B6 enzymes purified through the Sephadex G-100 step was monitored in either 10 mM Tris, MOPS, or $KHPO_4$ buffers at pH 7.5 over 10 days at 4°, by use of an NAT-PABA assay (Fig. 4). After 4 days, both enzymes lost 20% of their original activity in all buffers. The addition of 1 mM DTT on day 7 restored the activities to their original levels, and these levels were maintained through day 10. Increasing the buffer concentration to 50 mM inhibited both

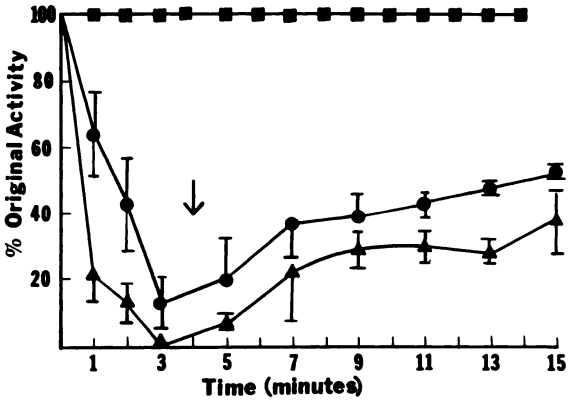


**Fig. 9.** Reversible inactivation of B6 and A liver NAT with 100 μM DTNB (mean ± standard deviation; three experiments). After 4 min, 4.5 mM DTT was added to reactivate (at *arrow*). ■, enzymes without DTNB addition; ●, B6 NAT; ▲, A NAT.

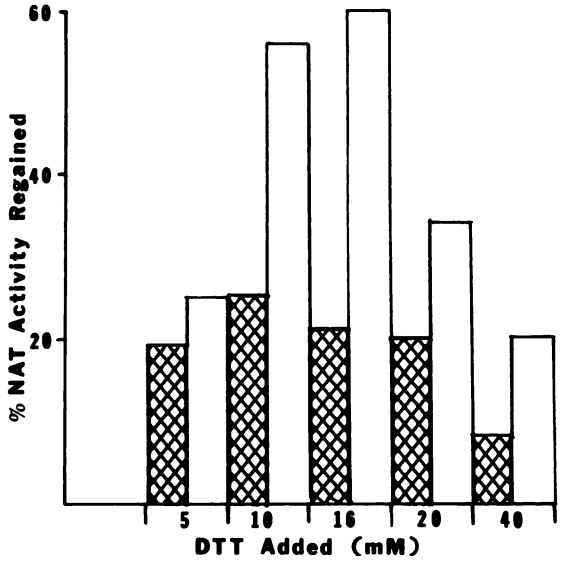


**Fig. 10.** Reversible inactivation of B6 and A liver NAT with 0.002% $H_2O_2$. After the addition of $H_2O_2$, both enzymes were completely inactivated. The enzymes were reactivated by 20-min incubation with increasing concentrations of DTT. □, B6 NAT, ■, A NAT.

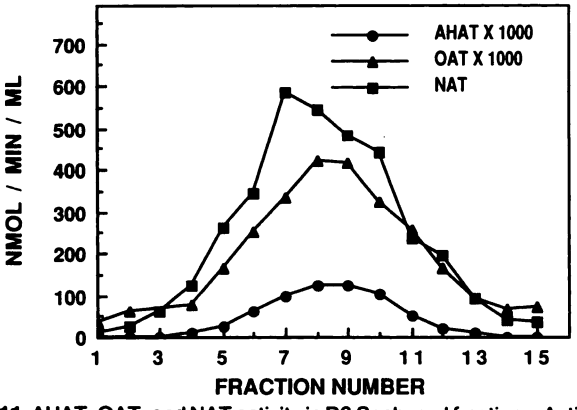


**Fig. 11.** AHAT, OAT, and NAT activity in B6 Sephacryl fractions. Activities were determined in fractions whose SDS-polyacrylamide gel electrophoresis profiles are shown in Fig. 3.

enzymes an average of 30% (data not shown). Either finally or partially purified mouse liver NAT was stable for at least 1 year when stored in 5% (v/v) glycerol at −70°, as judged by the use of PABA as an NAT substrate. No differences in stability have been noted between the two mouse NATs, regardless of storage in Tris, MOPS, or $KHPO_4$ buffer.

**pH optima.** Because of the sensitivity of *N*-acetyltransferase to changes in ionic strength, the maintenance of constant ionic strength at each pH level was necessary. This was accomplished by the addition of NaCl to a concentration of 200 mM, to overwhelm the changes in ionic strength produced when titrating the buffer to each pH value. Determinations of pH optima with pigeon and rabbit NAT (27, 44) have also shown that pH optimum profiles change dramatically with the degree of ionization of the arylamine substrate. For this reason, 4-iodoaniline ($pK_a = 3.78$) was chosen because its degree of ionization would not change significantly throughout the pH range studied. Under these conditions, broad pH optima were observed for NATs from both mouse strains, with no indication of activity peaks between pH 6.5 and 7.9 (Fig. 5).

**$K_m$ for CoASAc.** $K_m$ values for the A and B6 liver enzymes were not found to differ significantly, using 0.2 mM PABA (Figs. 6 and 7). For the B6 enzyme, the $K_m$ for CoASAc was 1.75 ± 0.25 mM (mean ± SE, four experiments), whereas that for the A enzyme was 1.90 ± 0.20 mM (mean ± SE, four experiments). Both enzymes are saturated at the concentration of PABA used to determine the $K_m$ for CoASAc (25).

**Activation energies.** PABA, having a very low $K_m$ for both enzymes (25), was chosen as the arylamine substrate. The two NATs were found to have equal activation energies of 21,000 cals/mol with PABA (Fig. 8), both showing some inhibition at the highest temperature, 40°.

**Inhibition by sulfhydryl reagents.** Partially purified NATs from both strains could be inhibited by several reagents that react with sulfhydryl groups. DTNB (100 μM) inactivated A NAT by 100% and B6 NAT by 87% in 3 min. The addition of 4.5 mM DTT reactivated A by nearly 40% and B6 by 50% in 15 min (Fig. 9). Similarly, 0.002% hydrogen peroxide inactivated A and B6 NAT completely in 10 min. Maximal reactivation occurred with 10–16 mM DTT in 20 min; A NAT regained 20–25% activity and B6 NAT regained 55–60% activity (Fig. 10). In contrast, 1 μM PCMB irreversibly inactivated both A and B6 NAT in 8 min. neither enzyme recovered activity with 10 mM DTT in 20 min.

**AHAT and OAT activity associated with NAT.** Fractions containing the peak NAT activity from B6 liver were collected from the final gel filtration column and were analyzed for AHAT and OAT activity (Fig. 11). All three acetylation activities were present, although the *N*-acetylation activity was 1000-fold greater than either the AHAT or OAT activity. In addition, all three activities had similar elution profiles from the final gel filtration column. With the 6% experimental error involved in the determination of PABA NAT activity taken into account, the NAT activities in fractions 7 and 8 do not differ significantly from each other. Furthermore, the three activities coincide with a single protein band of 31,500 D, as visualized by SDS-polyacrylamide gel electrophoresis of the B6-sephacryl fractions (Fig. 3).

**Acetylation activities in liver cytosol.** AHAT, OAT, and NAT activities were compared in 105,000 × *g* liver cytosol from B6 and A mice, to investigate whether the genetic polymor-

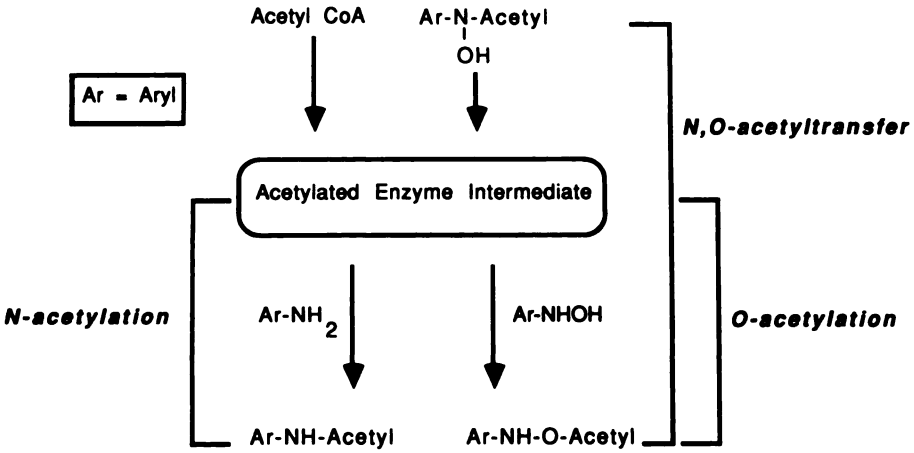


**Fig. 12.** *N*-, *O*-, and *N*,*O*-acetyl transfer probably involves a common acetylated enzyme intermediate in the mouse.

TABLE 4

**Comparison of NAT, AHAT, and OAT activities in A and B6 mouse liver cytosols**

Numbers represent mean ± standard error from four experiments. NAT activity was determined with 0.1 mM CoASAc and 0.1 mM PABA. AHAT and OAT assays were as described in Materials and Methods.

| | PABA NAT Activity | N-OH-AAF AHAT Activity | N-OH-DMABP OAT Activity |
|---|---|---|---|
| | | *pmol/min/mg of protein* | |
| A | 1470 ± 60 | 3.0 ± 0.5 | 28.3 ± 3.1 |
| B6 | 3150 ± 250 | 2.5 ± 0.2 | 26.9 ± 1.6 |

phism in NAT activity expressed between the strains is also reflected in a polymorphism in AHAT or OAT activities. A 2.5-fold difference in NAT activity was observed in the two strains, whereas no difference in AHAT or OAT activity was seen (Table 4).

## Discussion

Hepatic NAT has been purified from slow (A) and rapid (B6) acetylator inbred mouse strains. The rapid and slow enzymes were purified through identical procedures, and a single NAT enzyme was found in both animals. An overall 10,000- to 18,000-fold purification was achieved with a 15–24% yield (Tables 1 and 2; Figs. 2 and 3). Purified enzyme stored in 20% glycerol under $N_2$ was stable at −70° for at least 1 year, as judged by the acetylation of PABA. Addition of the protease inhibitors PMSF and leupeptin, the chelating agent EDTA, and the reducing agent DTT to the liver homogenization buffer greatly increased the overall recovery of NAT activity.

The purification procedure reported here is based on that developed for rabbit hepatic NAT (33). The rabbit and mouse NAT enzymes behaved similarly in several chromatographic systems, including anion exchange, gel filtration, and hydrophobic interaction chromatography. In addition, earlier studies demonstrated that rat (36, 37) and rabbit liver (19) acetyltransferases exhibited similar behavior with respect to ion exchange and gel filtration chromatography. Methotrexate columns were found to be useful in purifying mouse and pigeon liver NAT, although they were not very effective in purifying rabbit liver NAT (27). Previous studies have demonstrated that methotrexate is a noncompetitive inhibitor of PABA acetylation by rabbit, pigeon, and mouse liver NAT (27, 38).[1] Differences in the purification of rabbit liver NAT with methotrexate have been attributed to salt concentration dependence of the $K_i$ of the rabbit enzyme for methotrexate (33).

The purest B6 enzyme preparation was found to contain limited quantities of smaller peptides, but these were judged to represent less than 5% of the total protein content. The lower molecular weight proteins seen in the B6 preparation (Fig. 3) have not been identified; they could be NAT proteolysis products or additional proteins with similar biochemical characteristics. Further study will be necessary to characterize the contaminants. We were, however, unable to purify the A NAT to the same extent as the B6 NAT, using the same conditions. The presence of several major contaminating proteins in the A preparation indicates that A mouse liver contains proteins that are biochemically similar to NAT and that are not common to the B6 liver. This is not surprising, because the A and B6 strains have been reported to differ in many traits in addition to NAT (35, 39–41). In order to isolate NAT differences from other genetic polymorphisms in the A and B6 strains, we have developed two congenic mouse strains (42). If the contaminating proteins in the A liver are not related to NAT, they should not be present in the B6.A-Nat$^a$ strain, in which the A NAT gene has been transferred into the B6 mouse. Purification of the A NAT from the B6.A-Nat$^a$ strain should either produce homogeneous NAT or should give information about additional proteins associated with NAT in the A animal.

Amino acid compositions of hepatic NAT from pigeon and rapid acetylator rabbits (27, 33) are compared with that from the rapid acetylator mouse and a mean composition of 81 proteins, determined by Holmquist (43), in Table 3. Andres *et al.* (33) proposed that a low alanine content was a common feature of NAT enzymes. In the mouse enzyme, the alanine composition was lower than the mean alanine composition reported by Holmquist but higher than that of either rabbit or pigeon. In addition, the mouse enzyme had increased levels of glycine, as compared with the rabbit and pigeon. As a result, the mouse enzyme is slightly less hydrophobic than the rabbit enzyme, composed of 34.3 mol% hydrophobic residues, compared with 39.4 mol% hydrophobic residues in the rabbit enzyme. The tryptophan value is not included in the calculation of mol% hydrophobicity, but based on the low Trp value observed in pigeon and rabbit NATs, it would be expected to contribute little to the overall calculation of mol% hydrophobicity.

In the previously described partial purification of A and B6 NAT (24), specific activities of the two enzymes purified 30-

[1] H. H. Andres and W. W. Weber, unpublished observations.

fold were reported to be about 4-fold higher than in the present study. This difference is due to the higher concentration of CoASAc used to determine NAT activity in the previous study, compared with the present study (2.2 versus 0.1 mM). The present study employed a recycling system in which the concentration of CoASAc in the assay system is maintained by the transacetylation of CoASH by an auxiliary enzyme (29). This procedure avoids the accumulation of CoASH, which can inhibit NAT, and allows NAT activity determination at low physiological concentrations of CoASAc. When the activity of B6 NAT, purified 20-fold with the present procedure, was determined with 2.2 mM CoASAc, its specific activity increased from 230 nm/min/mg to 1215 nm/min/mg, which is comparable to the 795 nm/min/mg reported earlier (see Ref. 24; Table 1).

The A and B6 mouse strains were originally chosen as an animal model for the *N*-acetylation polymorphism based on differences in the *N*-acetylation rate of several substrates *in vitro* (9, 22). Subsequent studies of A and B6 hepatic NAT demonstrated that the enzymes have different $K_m$ values with 2-aminofluorene (25) and different $K_i$ values with methotrexate and folic acid (39) and they are inhibited to different extents by the solvent dimethylsulfoxide (25). In this series of studies, directed toward identifying additional biochemical characteristics that distinguish rapid from slow acetylator mouse liver NAT, no outstanding biochemical differences were noted. The A and B6 liver enzymes have the same molecular weights. They do not differ in stability over time while stored at 4° in Tris, MOPS, or $KHPO_4$ buffers. They have broad pH optima, extending from pH 6.5 to 7.9 with 4-iodoaniline, whose degree of ionization remained constant throughout the pH range chosen. Their affinity for CoASAc did not differ significantly. In addition, the enzyme reactions share the same rate-limiting step, as demonstrated by identical activation energies. Consistent with rat and rabbit liver acetyltransferases (14, 44) both enzymes have active site sulfhydryl groups that are inhibited by the reagents PCMB and DTNB and the oxidizing agent $H_2O_2$. The enzymes were shown to differ in response to the inhibitors DTNB and $H_2O_2$, and in subsequent reactivation by DTT. Collectively, these properties suggest that the two mouse enzymes have minimal structural differences.

In addition to qualitative differences, an examination of the lower yield of protein in the A purification scheme, as compared with that of B6 (Tables 1 and 2), raises the possibility that the enzymes differ quantitatively. The specific activity of the A enzyme following the last column step is nearly equivalent to that of the B6 enzyme, which is purified to a much greater extent. Thus, one would anticipate that further purification of the A enzyme might reveal a higher specific activity than the B6 enzyme, in much lower quantity. We have previously demonstrated that the A enzyme has a higher affinity for 2-aminofluorene than the B6 enzyme, and we may postulate that the "slow" *N*-acetylator enzyme might be more kinetically efficient than the "rapid" enzyme but present in lower quantity. Further investigation will establish whether the mouse hepatic NAT polymorphism results from both quantitative and qualitative enzyme differences.

Purified B6 hepatic arylamine NAT was analyzed for AHAT and OAT activity. The evidence presented here indicates that the *N*-, *O*-, and *N*,*O*-acetyltransfer reactions occurring in mouse liver are catalyzed by a single protein (Figs. 3 and 11). Previous investigations of mouse liver cytosol detected little AHAT activity (45–47). In the present study we found that, although AHAT activity in cytosol is low, when A and B6 cytosols were purified by protamine sulfate precipitation and DEAE chromatography the AHAT activity became more readily measurable. Previously reported difficulties in detecting mouse liver cytosolic AHAT activity may have been due to the presence of inhibitory factors in cytosol or to technical limitations, such as the use of radiolabeled substrates with lower specific activities. NAT isozymes with different substrate specificities have been characterized in hamster liver (48, 49) and in mouse liver (26). It is possible that additional OAT and/or AHAT activities are present in mouse liver cytosol but did not copurify with the activities in the present study, thus accounting for the relatively low final specific activities of AHAT and OAT as compared with NAT. Alternatively, because measuring AHAT and OAT activities involves the production of reactive products that can inactivate enzymes through covalent interactions, it is possible that the more pure preparations lead to proportionately greater inactivation of the enzyme due to a relative increase in adduct formation with the enzyme. Further examination of this system will be needed to address the possibility of other acetylation activities in mouse liver cytosol.

The *N*-, *O*-, and *N*,*O*-acetyltransfer reactions are catalyzed in a two-step process, with the first step involving transfer of the acetyl group from the acetyl donor to the enzyme, forming an acetylated enzyme intermediate (12, 14, 15, 44). Thus, for the mouse enzyme that catalyzes *O*-, *N*-, and *N*,*O*-acetyltransfer reactions, we can postulate that the enzyme can be acetylated by either CoASAc or arylhydroxamic acid substrates, forming an acetylated enzyme intermediate. This intermediate may then catalyze the acetylation of arylamine or N-OH-arylamine substrates, resulting in *N*-acetylated or *O*-acetylated products (Fig. 12).

NAT and AHAT activities have been attributed to a single protein in rabbit liver, and both activities are subject to the same genetic polymorphism, showing large variations in activity between rapid and slow acetylators (19). However, in a recent study OAT activity was not observed to vary between A and B6 liver cytosols (26), and in the present study neither hepatic AHAT nor OAT activity was observed to vary between rapid and slow acetylator mice (Table 4). Studies presented here, along with earlier findings (24, 25), suggest that the rapid and slow mouse liver NAT enzymes are qualitative variants. Therefore, the possibility exists that the two enzymes differ in active site configurations such that arylhydroxamic acids, which serve initially as acetyl donors and subsequently as acetyl acceptors, exhibit minor phenotypic differences because of these more complex roles, in contrast to aromatic amines, which serve only as acetyl acceptors. Alternatively, large differences in NAT activity have been observed to be masked *in vitro* when the rapid rabbit liver enzyme is not saturated with respect to arylamine substrates such as PABA and *p*-aminosalicylic acid (50). Kinetic studies of the mouse liver enzymes with N-OH-DMABP may clarify this point.

The importance of the human NAT polymorphism in determining individual susceptibility to drug toxicities, and possibly to disorders including bladder and colorectal cancer associated with arylamine exposure (1), underscores the need for a better understanding of the biochemical basis for the polymorphism. Experiments in *Salmonella typhimurium* (51–53) and rats (18, 54) have provided biochemical and biological evidence that the

expression of the mutagenic and carcinogenic properties of aromatic amines is often related to the formation of *O*-acetoxyarylamine derivatives. *O*- and *N,O*-acetyltransferase activities have been demonstrated in human tissues (55–57); however, the role of these metabolic pathways in the development of adverse biological effects in humans exposed to aromatic amines is not clear. Further efforts to identify the relative importance of these activities in arylamine activation in different tissues and to establish the relationship of these metabolic activities to acetylator phenotype will be important in understanding aromatic amine-induced carcinogenesis in humans.

**Acknowledgments**

We wish to thank Herbert Andres for valuable suggestions and Linda Johnson for performing the amino acid composition analysis of the mouse enzymes.

**References**

1. Weber, W. W. *The Acetylator Genes and Drug Response*. Oxford University Press, New York, (1987).
2. Cartwright, R. A., R. W. Glashan, H. J. Rogers, R. A. Ahmad, D. Barham-Hall, E. Higgens, and M. A. Kahn. Role of *N*-acetyltransferase phenotypes in bladder carcinogenesis: a pharamcogenetic epidemiological approach to bladder cancer. *Lancet* **2**:842–845 (1982).
3. Lang, N. P., D. Z. J. Chu, C. F. Hunter, D. C. Kendall, T. J. Flammang, and F. F. Kadlubar. Role of aromatic amine acetyltransferase in human colorectal cancer. *Arch. Surg.* **121**:1259–1261 (1986).
4. Evans, D. A. P., K. A. Manley, V. A. McKusick. Genetic control of isoniazid metabolism in man. *Br. Med. J.* **2**:485–491 (1960).
5. Frymoyer, J. W., and R. F. Jacox. Clinical and experimental investigation of the genetic control of sulfadiazine and isoniazid metabolism in the rabbit. *J. Lab. Clin. Med.* **62**:891–904 (1963).
6. Frymoyer, J. W., and R. F. Jacox. Studies of genetically controlled sulfadiazine acetylation in rabbit liver: possible identification of the heterozygous trait. *J. Lab. Clin. Med.* **62**:905–909 (1963).
7. Hearse, D. J., and W. W. Weber. Multiple *N*-acetyltransferases and drug metabolism. *Biochem. J.* **132**:519–526 (1973).
8. Hein, D. W., J. G. Omichinski, J. A. Brewer, and W. W. Weber. A unique pharmacogenetic expression of the *N*-acetylation polymorphism in the inbred hamster. *J. Pharmacol. Exp. Ther.* **220**:8–15 (1982).
9. Glowinski, I. B., and W. W. Weber. Genetic regulation of aromatic amine *N*-acetylation in inbred mice. *J. Biol. Chem.* **257**:1424–1430 (1982).
10. King, C. M., and B. Phillips. Enzyme catalyzed reactions of the carcinogen *N*-hydroxy-2-fluorenylacetamide with nucleic acid. *Science (Wash. D. C.)* **159**:1351–1353 (1968).
11. King, C. M., and B. Phillips. *N*-Hydroxy-2-fluorenylacetamide: reaction of the carcinogen with guanosine, ribonucleic acid, deoxyribonucleic acid, and protein following enzymatic deacetylation or esterification. *J. Biol. Chem.* **244**:6209–6216 (1969).
12. Bartsch, H., M. Dworkin, J. A. Miller, and E. C. Miller. Electropilic *N*-acetoxyaminoarenes derived from carcinogenic *N*-hydroxy-*N*-acetyl-aminoarenes by enzymatic deacetylation and transacetylation in liver. Biochim. Biophys. Acta **286**:272–298 (1972).
13. Bartsch, H., M. Dworkin, J. A. Miller, and E. C. Miller. Formation of electrophilic *N*-acetoxyarylamines in cytosols from rat mammary gland and other tissues by transacetylation from the carcinogen *N*-hydroxy-4-acetylaminobiphenyl. *Biochim. Biophys. Acta* **304**:42–55 (1973).
14. King, C. M. Mechanism of reaction, tissue distribution, and inhibition of arylhydroxamic acid acyltransferase. *Cancer Res.* **34**:1503–1516 (1974).
15. Booth, J. Acetyl transfer in arylamine metabolism. *Biochem. J.* **100**:745–753 (1966).
16. Yeh, H.-M., and P E. Hanna. Arylhydroxamic acid bioactivation via acyl group transfer: Structural requirements for transacylating and electrophile-generating activity of *N*-(2-fluorenyl)hydroxamic acids and related compounds. *J. Med. Chem.* **25**:842–845 (1982).
17. Weeks, C. E., W. T. Allaben, N. M. Tresp, S. C. Louie, E. J. Lazear, and C. M. King. Effects of structure of *N*-acyl-*N*-2-fluorenylhydroxylamines on arylhydroxamic acid acyltransferase, sulfotransferase, and deacylase activities and on mutations in *Salmonella typhimurium* TA 1538. *Cancer Res.* **40**:1204–1211 (1980).
18. Shirai, T., J. M. Fysh, M.-S. Lee, J. B. Vaught, and C. M. King. Relationship of metabolic activation of *N*-hydroxy-*N*-acylarylamines to biologic response in the liver and mammary gland of the female CD rat. *Cancer Res.* **41**:4346–4353 (1981).
19. Glowinski, I. B., W. W. Weber, J. M. Fysh, J. B. Vaught, and C. M. King. Evidence that arylhydroxamic acid *N,O*-acyltransferase and the genetically polymorphic *N*-acetyltransferase are properties of the same enzyme in rabbit liver. *J. Biol. Chem.* **255**:7883–7890 (1980).
20. Flammang, T. J., and F. F. Kadlubar. Acetyl coenzyme A-dependent metabolic activation of *N*-hydroxy-3,2′-dimethyl-4-aminobiphenyl and several carcinogenic *N*-hydroxyarylamines in relation to tissue and species differences, other acyl donors, and arylhydroxamic acid-dependent acyltransferases. *Carcinogenesis (Lond.)* **7**:919–926 (1986).
21. Saito, K., A. Shinohara, T. Kamataki, and R. Kato. *N*-Hydroxyarylamine *O*-acetyltransferase in hamster liver: identity with arylhydroxamic acid *N,O*-acyltransferase and arylamine *N*-acetyltransferase. *J. Biochem. (Japan)* **99**:1689–1697 (1986).
22. Tannen, R. H., and W. W. Weber. Rodent models of the human isoniazid acetylator polymorphism. *Drug Metab. Dispos.* **7**:274–279 (1979).
23. Tannen, R. H., and W. W. Weber. Inheritance of acetylator phenotype in mice. *J. Pharmacol. Exp. Ther.* **213**:480–484 (1980).
24. Glowinski, I. B., and W. W. Weber. Biochemical characterization of genetically variant aromatic amine *N*-acetyltransferases in A/J and C57BL/6J mice. *J. Biol. Chem.* **257**:1431–1437 (1982).
25. Mattano, S. S., and W. W. Weber. Kinetics of arylamine *N*-acetyltransferase in tissues from rapid and slow acetylator mice. *Carcinogenesis (Lond.)* **8**:133–137 (1987).
26. Hein, D. W., A. Trinidad, T. Yerokum, R. J. Ferguson, W. G. Kirlin, and W. W. Weber. Genetic control of acetyl coenzyme A-dependent arylamine *N*-acetyltransferase, hydrazine *N*-acetyltransferase, and *N1-hydroxy-arylamine O*-acetyltransferase enzymes in C57BL/6J, A/J, AC57F$_1$, and the rapid and slow acetylator A.B6 and B6.A congenic inbred mouse. *Drug Metab. Dispos.* **16**:341–347 (1988).
27. Andres, H. H., H. J. Kolb, and L. Weiss. Purification and physical-chemical properties of acetyl-CoA: arylamine *N*-acetyltransferase from pigeon liver. *Biochim. Biophys. Acta* **746**:182–192 (1983).
28. Wang, C. Y., H. Yamada, K. C. Morton, K. Zukowski, M.-S. Lee, and C. M. King. Induction of repair synthesis of DNA in mammary and urinary bladder epithelial cells by *N*-hydroxy derivatives of carcinogenic arylamines. *Cancer Res.* **48**:4227–4232 (1988).
29. Andres, H. H., A. J. Klem, S. M. Szabo, and W. W. Weber. New spectrophotometric and radiochemical assays for acetyl CoA: arylamine *N*-acetyltransferase applicable to a variety of arylamines. *Anal. Biochem.* **145**:367–375 (1985).
30. Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. *Anal. Biochem.* **72**:248–254 (1976).
31. Paine, P. L. Diffusive and nondiffusive proteins *in vivo*. *J. Cell. Biol.* **99**:188s–195s (1984).
32. Tarr, G. E. In *Methods of Protein Microcharacterization* (J. E. Shively, ed.). Humana Press, Clifton, NJ, 155–194 (1986).
33. Andres, H. H., R. S. Vogel, G. E. Tarr, L. Johnson, and W. W. Weber. Purification, physicochemical, and kinetic properties of liver acetyl CoA: arylamine *N*-acetyltransferase from rapid acetylator rabbits. Mol. Pharmacol. **31**:446–456 (1987).
34. Cleland, W. W. The statistical analysis of enzyme kinetic data. *Adv. Enzymol.* **29**:1–32 (1967).
35. Liu, S. J., and R. P. Erickson. Genetic differences among the A/J × C57BL/6J recombinant inbred mouse lines and their degree of association with glucocorticoid induced cleft palate. *Genetics* **113**:745–754 (1986).
36. Allaben, W. T., and C. M. King. The purification of rat liver arylhydroxamic acid *N,O*-acyltransferase. *J. Biol. Chem.* **259**:12128–12134 (1984).
37. Land, S., W. T. Allaben, and C. M. King. Aromatic amine metabolism: immunochemical relationships of *N*-acetyltransferase and *N,O*-acyltransferase. *Fed. Proc.* **45**:1726 (1986).
38. Hultin, T. A., and W. W. Weber. Genetic differences in inhibition of 2-aminofluorene *N*-acetyltransferase activity between C57BL/6J and A/J mice. *Biochem. Pharmacol.* **35**:1214–1216 (1986).
39. Karolyi, I. J., S. Liu, and R. P. Erickson. Susceptibility to phenytoin-induced cleft lip with or without cleft palate: many genes are involved. *Genet. Res. Camb.* **49**:43–49 (1987).
40. Nesbitt, M. N., and E. Skamene. Recombinant inbred mouse strains derived from A/J and C57BL/6J: a tool for the study of genetic mechanisms in host resistance to infection and malignancy. *J. Leukocyte Biol.* **36**:357–364 (1984).
41. Hultin, T. A., and W. W. Weber. Genetic variability in deacetylation of 2-acetylaminofluorene and *N*-hydroxy-2-acetylaminofluorene in inbred strains of mice. *Carcinogenesis (Lond.)* **8**:1939–1941 (1987).
42. Mattano, S. M., R. P. Erickson, M. N. Nesbitt, and W. W. Weber. Linkage of *Nat* and *Es-1* in the mouse and development of strains congenic for *N*-acetyltransferase. *J. Hered.* **79**:430–433 (1988).
43. Holmquist, R. Evaluation of compositional nonrandomness in proteins. *J. Mol. Evol.* **11**:349–360 (1978).
44. Weber, W. W., and S. N. Cohen. *N*-Acetylation of drugs: isolation and properties of an *N*-acetyltransferase from rabbit liver. *Mol. Pharmacol.* **3**:266–273 (1967).
45. King, C. M., and C. W. Olive. Comparative effects of strain, species, and sex on the acyltransferase and sulfotransferase-catalyzed activations of *N*-hydroxy-*N*-2-fluorenylacetamide. *Cancer Res.* **35**:906–912 (1975).
46. Morton, K. C., C. M. King, and K. P. Baetcke. Metabolism of benzidine to *N*-hydroxy-*N,N′*-diacetylbenzidine and subsequent nucleic acid binding and mutagenicity. *Cancer Res.* **39**:3107–3113 (1979).
47. Glowinski, I. B., L. Savage, M.-S. Lee, and C. M. King. Relationship between nucleic acid adduct formation and deacylation of arylhydroxamic acids. *Carcinogenesis (Lond.)* **4**:67–75 (1983).
48. Hein, D. W., T. J. Flammang, W. G. Kirlin, A. Trinidad, and F. Ogolla.

Acetylator genotype-dependent metabolic activation of carcinogenic *N*-hydroxyarylamines by *S*-acetyl coenzyme A-dependent enzymes of inbred hamster tissue cytosols: relationship to arylamine *N*-acetyltransferase. *Carcinogenesis (Lond.)* **8**:1767–1774 (1987).

49. Kato, R., and Y. Yamazoe. *N*-Hydroxyarylamine *O*-acetyltransferase in mammalian livers and *Salmonella*, in *Carcinogenic and Mutagenic Responses to Aromatic Amines and Nitroarenes* (C. M. King, L. J. Romano, and D. Schuetzle, eds), Elsevier, New York 125–136 (1988).
50. Andres, H. H., and W. W. Weber. *N*-Acetylation pharmacogenetics: Michaelis-Menten constants for arylamine drugs as predictors of their *N*-acetylation rates *in vivo*. *Drug Metab. Dispos.* **14**:382–385 (1986).
51. Saito, K., Y. Yamazoe, T. Kamataki, R. Kato. Mechanism of activation of proximate mutagens in Ames tester stains: the acetyl CoA-dependent enzyme in *Salmonella typhimurium* TA98 deficient in TA98/1,8-DNP$_6$ catalyzes DNA-binding as the cause of mutagenicity. *Biochem. Biophys. Res. Commun.* **116**:141–147 (1983).
52. Saito, K., A. Shinohara, T. Kamataki, and R. Kato. Metabolic activation of mutagenic *N*-hydroxyarylamines by *O*-acetyltransferase in *Salmonella typhimurium*. *Arch. Biochem. Biophys.* **239**:286–295 (1985).
53. Staiano, N., L. C. Erickson, C. L. Smith, E. Marsden, and S. S. Thorgeirsson. Mutagenicity and DNA damage induced by arylamines in the *Salmonella*/hepatocyte system. *Carcinogenesis (Lond.)* **4**:161–167 (1983).
54. Allaben, W. T., C. E. Weeks, C. C. Weis, G. T. Burger, and C. M. King. Rat mammary gland carcinogenesis after local injection of *N*-hydroxy-*N*-acyl-2-aminofluorenes: relationship to metabolic activation. *Carcinogenesis (Lond.)* **3**:233–240 (1982).
55. Flammang, T. J., Y. Yamazoe, F. P. Guengerich, and F. F. Kadlubar. The *S*-acetyl coenzyme A-dependent activation of the carcinogen *N*-hydroxy-2-aminofluorene by human liver cytosol and its relationship to the aromatic amine *N*-acetyltransferase phenotype. *Carcinogenesis (Lond.)* **8**:1967–1970 (1987).
56. Flammang, T. J., D. W. Hein, G. Talaska, and F. F. Kadlubar. *N*-Hydroxyarylamine *O*-acetyltransferase and its relationship to aromatic amine *N*-acetyltransferase polymorphism in the inbred hamster and in human tissue cytosol, in *Carcinogenic and Mutagenic Responses to Aromatic Amines and Nitroarenes* (C. M. King, L. J. Romano, and D. Schuetzle, eds.). Elsevier, New York, 137–148 (1988).
57. Land, S., K. Zukowski, M. S. Lee, M. Debiec-Rychter, C. M. King, and C. Y. Wang. Metabolism of aromatic amines: relationships of *O*-acetylation, *N*,*O*-acyltransfer, *N*-acetylation, and deacetylation in human liver. *Proc. Am. Assoc. Cancer Res.* **29**:122 (1988).
58. Light, H. C., J. J. Hubert, P. D. Josephy, S. S. Mattano, and W. W. Weber. Benzidine activation in the Ames test: roles of hepatic *N*-acetyltransferase and other cytosolic and microsomal factors. *Carcinogenesis* **8**:139–143 (1987).

**Send reprint requests to:** Wendell W. Weber, Department of Pharmacology, 6322 Medical Science I, University of Michigan, Ann Arbor 48109-0626.