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Purification, Physicochemical, and Kinetic Properties of Liver Acetyl-CoA:Arylamine *N*-Acetyltransferase from Rapid Acetylator Rabbits

HERBERT H. ANDRES, R. SCOTT VOGEL, GEORGE E. TARR, LINDA JOHNSON, and WENDELL W. WEBER

Departments of Pharmacology (H.H.A., R.S.V., W.W.W.) and Biochemistry (G.E.T., L.J.), The University of Michigan Medical School, Ann Arbor, Michigan 48109

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SUMMARY

Cytosolic liver acetyl-CoA:arylamine *N*-acetyltransferase (EC 2.3.1.5) from homozygous rapid acetylator rabbits (strain III/J) was purified to homogeneity as judged by gel filtration sodium dodecyl sulfate-polyacrylamide disc gel electrophoresis and isoelectrofocusing. The isoelectric point was estimated to be 5.2. The molecular weight was determined to be 33,500 by sodium dodecyl sulfate-polyacrylamide disc gel electrophoresis and 33,000 by Sephacryl S-200 gel filtration. The amino acid composition is reported and 16 tryptic peptides were sequenced by Edmann degradation, including a peptide from which a very specific oligonucleotide probe can be synthesized. The enzyme contained neither amino sugars nor cofactors. A broad pH optimum from pH 5.9 to 8.6 was observed. *N*-Acetyltransferase activity showed a strong dependency on the salt concentration. From the influence of the basicity of the acceptor amine on the maximum velocity, it was concluded that the formation of the covalent acetyl-enzyme intermediate is the rate-limiting step in the *N*-acetyltransferase-catalyzed acetylation of amines. The covalent intermediate reacts, then, in a fast step with the acceptor amine, when using aniline derivatives with pK_a values ranging from 5.65 to 1.74. However, with the weakly basic 4-nitroaniline, the acetyltransfer from the catalytic intermediate to the amine seems to be rate-limiting. A structure-activity study of 30 aniline derivatives that differ in hydrophobicity, position, size, charge, and number of substituents showed that some *ortho*-substituted derivatives were not acetylated.

N-Acetylation is an important step in the biotransformation of arylamines, hydrazines, and biogenic amines of aromatic amino acids (1–3). Pharmacokinetic studies of the acetylation of isoniazid, sulfamethazine, and sulfadiazine in rabbit and man showed bimodal frequency distribution histograms (4–8). Therefore, an individual may be classified as either a rapid or slow acetylator. Further studies demonstrated that the ability of individuals to acetylate arylamines and hydrazines *in vivo* was genetically determined. The mode of inheritance followed simple autosomal Mendelian genetics. Acetylation polymorphisms have also been shown in rabbit, mouse, hamster, and rat (8–11). The slow acetylator phenotype has been correlated with numerous drug-induced as well as with some spontaneous disorders. Furthermore, it has been argued that slow acetylators carry a higher risk for arylamine-induced cancer of occupational origin (12).

The rabbit has often been used as the animal model for studies of the molecular basis for the acetylation polymorphism because the *in vivo* *N*-acetylation patterns in rabbits are so similar to those in man. In early studies on this animal model, a cytosolic liver enzyme was identified, which catalyzed the *N*-acetylation of arylamines and hydrazines using CoASAc as the physiological acetyl donor (6). More detailed kinetic studies showed that the CoASAc-dependent *N*-acetylation of amines followed ping-pong kinetics (13, 14). The observation of ping-pong kinetics is consistent with the occurrence of a covalent, catalytic intermediate. Therefore, the enzyme-catalyzed *N*-acetylation was described as an ordered reaction in which CoASAc first acetylates the enzyme, forming a covalent acetyl-enzyme intermediate. In a second step, the acetyl moiety is transferred from the enzyme to the acceptor amine. Furthermore, comparative kinetic studies with different acceptor amines using partially purified liver-*N*-acetyltransferase from rapid and slow acetylator rabbits showed that the *in vivo* acetylation pattern was reflected in the catalytic properties of the respective liver enzyme (15).

Yet, major problems remained to be solved. First, there was no purification procedure published which would allow the

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ABBREVIATIONS: CoASAc, reduced acetylcoenzyme A; EDTA, ethylenediaminetetraacetate; HPLC, high performance liquid chromatography; DMED, dimethylethylenediamine; SDS, sodium dodecyl sulfate; CoASH, reduced coenzyme A; MES, 3-(*N*-morpholino)ethanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid.

isolation of a sufficient amount of homogeneous, catalytically active enzyme. Therefore, not a great deal could be learned about the physicochemical properties of the enzyme, such as its molecular weight, isoelectric point, amino acid analysis, sequence information, and sugar and cofactor analysis. Second, it was not known whether the *N*-acetylation of a broad spectrum of amines was catalyzed by a set of isozymes or by a single enzyme with a broad substrate specificity.

In this paper, we describe a procedure for the purification of active, homogeneous liver acetyl-CoA:arylamine *N*-acetyltransferase (EC 2.3.1.5) (*N*-acetyltransferase) from an inbred rapid acetylator rabbit (strain III/J) and the investigation of some physical-chemical properties of the enzyme. With a structure-activity study using about 30 different aniline derivatives, we demonstrate that the isolated enzyme is able to catalyze the *N*-acetylation of a broad spectrum of amines. The aniline derivatives were chosen to study the effect of hydrophobicity, position, size, charge, and number of substituents on K_m and V_{max} values. Furthermore, the influence of the pK_a of the acceptor amine on the kinetic parameters is described. We report on the pH optimum of the enzyme and the effect of different salts on the enzyme activity.

Materials and Methods

Mature New Zealand White inbred rabbits (strain III/J) were obtained from The Jackson Laboratory. Sephadex G-100 and G-50, Sephacryl S-200, cellulose Sigma cell type 50, butyl-agarose, protamine sulfate from salmon (negative Millon test, histone free), Trisma base, dithioerythritol, EDTA, phosphotransacetylase (EC 2.3.1.8), acetylphosphate, dimethylaminobenzaldehyde, leupeptin (synthetic), phenylmethylsulfonyl fluoride, and formic acid were purchased from Sigma Chemical Co. CoASAc was obtained from P-L Biochemicals, Inc. Ultrafiltration membranes (Diaflo PM10) were obtained from Amicon. [2-^{3}H]CoASAc was obtained from New England Nuclear. DEAE-Trisacryl M, Ampholine Pagplate, pH 3.5–9.5, and TSK-G 3000SW size exclusion column were purchased from LKB Instruments. Hydroxylapatite Bio-Gel HT, preweighed acrylamide/*N,N'*-methylene-bisacrylamide (37.5:1 mixture), *N,N,N',N'*-tetramethylethylene diamine, ammonium persulfate, and the marker proteins for molecular weight determination were obtained from Bio-Rad. Four M methanesulfonic acid/0.2% tryptamine was purchased from Pierce Chemical Co. Na^+ citrate/chloride, $[Na^+]$ = 1.0 N, pH 7.4 buffer was purchased from Pickering Laboratory. The HPLC cation exchange column was obtained from Interaction Chemicals. Trypsin-TPCK was purchased from Worthington.

Purification of Acetyl-CoA:Arylamine *N*-Acetyltransferase

All steps were carried out at 4°. The following buffers were used: buffer I (20 mM Tris-HCl, pH 7.5, 2 mM dithioerythritol, 1 mM EDTA, 50 μM phenylmethylsulfonyl fluoride, 10 μM leupeptin), buffer II (20 mM Tris-HCl, pH 7.5, 2 mM dithioerythritol, 1 mM EDTA), buffer III (20 mM Tris-HCl, pH 7.5, 2 mM dithioerythritol, 1 mM EDTA, 0.02% sodium azide), buffer IV (4 M ammonium sulfate, 20 mM Tris-HCl, pH 7.5, 2 mM dithioerythritol, 1 mM EDTA), buffer V (2 M ammonium sulfate, 20 mM Tris-HCl, pH 7.5, 2 mM dithioerythritol, 1 mM EDTA).

Step 1. *Crude extract.* Frozen rabbit liver (200 g; stock-frozen in liquid nitrogen, stored at −70° or in liquid nitrogen) was homogenized in a blender for 45 sec under nitrogen in 1 liter of buffer I. The homogenate was centrifuged at 20,000 × *g* for 30 min and the supernatant preparation was filtered through cheese-cloth. The pH of the supernatant preparation was adjusted to 7.5 with a few drops of 1 M Tris-base.

Step 2. *Protamine sulfate precipitation.* A protamine sulfate solution [1% protamine sulfate (w/v)] was added to the 20,000 × *g* supernatant preparation with vigorous stirring over a period of 30 min using a peristaltic pump. The pump was equipped with a thin polypropylene tube to produce very small drops. The amount of protamine sulfate solution needed to give a particle-free supernatant preparation without loss of activity varied slightly from lot to lot. Typically, between 60 and 70 ml of protamine sulfate solution were used. After the addition of the protamine sulfate solution, the suspension was stirred for 15 min. The precipitated material was pelleted by centrifugation of 20,000 × *g* for 20 min and discarded.

Step 3. *DEAE-Trisacryl M ion exchange chromatography.* The supernatant preparation from step 2 was added to 100 ml of DEAE-Trisacryl M equilibrated with buffer II. Shaking the suspension gently, the enzyme was bound to the resin within 15 min. The gel was washed on a Buchner funnel with 2–3 liters of buffer II in several aliquots until the protein concentration in the effluent was less than 50 μg/ml, determined with the Bradford reagent (16). The gel was packed into a column (2.5 × 20 cm) and rinsed with 1 bed volume of buffer I. The enzyme was eluted twice at a flow rate of 25 ml/cm²/hr by a linear salt gradient (500 ml, 0–500 mM NaCl in buffer II). The peak fractions were pooled and concentrated, if necessary, to 60–80 ml by ultrafiltration (Amicon PM10 membrane, 1.5 atm).

Step 4. *Sephadex G-100 chromatography.* The enzyme solution was applied to a Sephadex G-100 column (5 × 50 cm) equilibrated with buffer III. The enzyme was eluted at a flow rate of 5 ml/cm²/hr. All fractions containing activity were pooled.

Step 5. *Bio-Gel HT hydroxylapatite chromatography.* Hydroxylapatite (20 ml) was mixed with 40 ml of swollen, fine-free cellulose, packed in a column (2.5 × 12 cm), and equilibrated with buffer II. The activity containing pool from the previous step was applied at a flow rate of 20 ml/cm²/hr. The column was washed with about 3 bed volumes of buffer II. The enzyme was eluted twice at the same flow rate by a linear salt gradient (500 ml, 0–150 mM NaH_2PO_4/NaOH, pH 7.5, in buffer II). The peak fractions were pooled and concentrated to about 10 ml by ultrafiltration.

Step 6. *Hydrophobic chromatography on butyl-agarose 4B.* Over a period of 30 min, an equal volume of buffer IV was added with vigorous stirring to the enzyme solution from the previous step. A peristaltic pump was used, equipped with a thin polypropylene tube to produce very small drops. After stirring for an additional 10 min, the precipitated protein was pelleted by centrifugation at 20,000 × *g* for 20 min and discarded. The supernatant preparation was applied to a butyl-agarose 4B column (7 ml, 0.6 × 12 cm) equilibrated in buffer V at a flow rate of 15 ml/cm²/hr. The column was washed with 10 bed volumes of buffer V. The enzyme was then eluted by a gradient of decreasing ammonium sulfate and increasing glycerol concentration (final concentrations 0% and 10%, respectively) in a total volume of 160 ml of buffer II. The activity-containing fractions were pooled, dialyzed against 2 liters of buffer II, and concentrated by ultrafiltration to about 1 ml (Amicon PM10 membrane, 1.5 atm).

Step 7. *Sephacryl S-200 chromatography.* The concentrated enzyme solution was applied onto a Sephacryl S-200 column (two 1.5 × 120 cm columns in a tandem arrangement) equilibrated with buffer III. Homogeneous *N*-acetyltransferase was eluted at a flow rate of 5 ml/cm²/hr. Peak fractions were pooled and concentrated by ultrafiltration (Amicon PM10 membrane, 1.5 atm).

Trypsin Digestion and Separation of Peptides

Homogeneous *N*-acetyltransferase (4 mg) in a total volume of 500 μl of buffer was precipitated in a 2-ml polypropylene tube by the addition of 200 μl of 20% trichloroacetic acid and centrifuged. The pellet was washed four times with 500 μl of ice-cold 80% (v/v) acetone. The pellet was redissolved in 400 μl of 6 M guanidine/HCl, 100 mM Tris-HCl, 10 mM dithioerythreitol, 1 mM EDTA (pH 8.2) buffer which was filtered, degassed, and saturated with nitrogen prior to use. The protein was reduced and denatured under nitrogen for 2 hr at room temperature. The total sulfhydryl concentration was measured with Ellman's reagent. A 2-fold molar excess of iodoacetic acid over the total

sulfhydryl concentration was added using a 500 mM iodoacetic acid/100 mM Tris-HCl, pH 9.0, stock solution. Incubating in the dark, at room temperature, under anaerobic conditions, the sulfhydryl groups were carboxymethylated after 4 hr as judged by Ellman's reagent. The sample was then extensively dialyzed against water, by covering the 2-ml polypropylene tube with a dialysis membrane fixed with a rubber band, and inverting it. After removal of the guanidine, the denatured, reduced, and alkylated protein precipitated and was collected by centrifugation for 5 min in a Microfuge. The carboxyl groups were amidated with DMED by the method of Tarr (16) as follows. The protein pellet was dissolved in 200 μl of hexafluoroacetone. One hundred μl of 4 M DMED/HCl, pH 3.7, were added and mixed. Fifty μl of 4 M DMED/HCl, pH 3.7, were used to dissolve (as much as possible) fresh ethyldimethylaminopropyl carbodiimide. The solution was rapidly mixed with the protein. After 1 hr at room temperature, the mixture was diluted with 500 μl of 0.1% trifluoroacetic acid/water. The derivatized protein was purified by reverse phase HPLC (Synchropak RP-P, 25 cm × 4.1 mm). Solvent A consisted of 0.1% trifluoroacetic acid/water and solvent B consisted of 0.07%. trifluoroacetic acid/acetonitrile. A gradient was run 0–100% solvent B in 100 min at a flow rate of 1 ml/min. The solvent was evaporated from the protein-containing fraction. The protein was redissolved in 100 μl of water. One hundred μl of 200 mM ammonium hydrogen carbonate, pH 8.2, were added, and the protein was digested with TPCK-treated trypsin (trypsin/protein = 1:50) incubating for 16 hr at room temperature. The reaction mixture was centrifuged and the peptides in the supernatant were purified by reverse phase HPLC (Synchropak RP-P, C_{18}, 10 μm, 300 nm). Solvent A consisted of 0.1% trifluoroacetic acid/water and solvent B consisted of 0.07% trifluoroacetic acid/acetonitrile. A gradient was run 0–100% solvent B in 200 min at a flow rate of 0.7 ml/min. The peptides were repurified under the same conditions using a Waters Bondapak phenyl column.

Sequence Determination

The peptides and the protein were sequenced using the manual Edman sequencing system described by Tarr (17), separating the peptide from the 5-anilinothiazolinone derivative by the partitioning method, and analyzing the phenylthiohydantoin on a Waters Nova-Pak column.

Fluorometric Analysis of Amino Sugars

N-Acetyltransferase (50 μg) was desalted as described under amino acid analysis. The desalted protein pellet was hydrolyzed in 6 N HCl at 100° for 3 hr. The hydrolysate was evaporated under a stream of nitrogen at 40° and dried *in vacuo*. Amino sugars were separated and detected fluorometrically as described by Perini and Peters (18).

Isoelectrofocusing using Thin Layer Polyacrylamide Gel

Flat-bed isoelectrofocusing on thin layer polyacrylamide gel (Ampholine Pagplate, pH 3.5–9.5) was performed using the LKB 2117 Multiphor chamber essentially as described by the manufacturer. The anode-electrode solution consisted of 1 M H_3PO_4, the cathode-electrode solution was 1 M NaOH. The temperature was kept at 8°. The pH gradient was established by prefocusing for 30 min at 10 W, 1000 V, 50 amp. The enzyme was directly applied onto the polyacrylamide gel near the cathode. The molarity of buffer, in which the enzyme was applied, was 20 mM or less. Isoelectrofocusing of the protein was accomplished by focusing for 1.5 hr at the setting described above. Measurement of the pH gradient, fixing, staining, and destaining were carried out in accordance to the practical information from the manufacturer.

SDS-Polyacrylamide Slab Gel Electrophoresis

SDS-polyacrylamide slab gel electrophoresis was carried out in a Bio-Rad slab gel apparatus. Gels were prepared by the method of Weber and Osborn (19), using the Tris-glycine buffer system. The stacking gel (10 × 14 cm) was 0.75 mm thick and the final acrylamide concentration was either 10 or 12.5%. Protein samples (50 μl) were reduced and denatured with 10 μl of a mixture of β-mercaptoethanol/20% (w/v) SDS/glycerol (2:5:3). The protein concentration was 1–5 μg for each protein to be visualized on the gel. The reduced and denatured protein was applied onto the stacking gel in a maximum of 20 μl. Electrophoresis was performed at a constant current of 25 mamp/slab gel and the temperature was maintained at 25°. The gels were stained for at least 2 hr with a Coomassie Brilliant Blue R-250 solution consisting of 0.125 g of Coomassie Brilliant Blue R-250, 63 ml of isopropanol, 25 ml of acetic acid, and 162 ml of water. Gels were destained with the same solution omitting the dye. The gel was preserved by drying it with a Bio-Rad model 1125B slab gel dryer.

Protein Determination

Protein was determined either by the dye binding assay of Bradford (16) using bovine serum albumin as standard or by amino acid analysis (17). The amino acid analysis gave about 10–15% higher values for homogeneous *N*-acetyltransferase. Protein in SDS-containing samples was determined by first hydrolyzing it, followed by the quantification of the free amino acids with the dimethyl sulfoxide ninhydrin reagent of Moore (20) as follows. One hundred μl of a 1 N NaOH solution were added to 1–10 μg of protein in a total volume of 100 μl. The samples were vortexed, centrifuged, and dried at 110°. To the still warm polypropylene tubes (1.5 ml), 100 μl of 15% (v/v) acetic acid were added. After the addition of 500 μl of dimethyl sulfoxide-ninhydrin reagent, the mixture was incubated for 20 min at 110°. Then it was rapidly cooled in an ice/sodium chloride mixture. The reaction mixture (500 μl) was diluted with 500 μl of ice-cold 50% (v/v) ethanol/water. The absorbance at 578 nm was immediately measured. Values were corrected for a blank, using the SDS-containing buffer alone. Protein was determined from a linear bovine serum albumin standard curve.

Molecular Weight Determination

The molecular weight of denaturated enzyme was estimated from SDS-polyacrylamide disc electrophoresis (19) using marker proteins. The molecular weight of the native enzyme was determined by gel filtration on Sephacryl S-200 (1.5 × 100 cm) and with an HPLC size exclusion column (TSK-G 3000 SW, 7.5 × 600 mm) according to the method of Andrews (21) using appropriate marker proteins.

Enzyme Assay

For the purification, *N*-acetyltransferase activity was measured as described before, recycling the inhibitory product CoASH with the acetylphosphate/phosphotransacetylase system (22). 4-Methylaniline was used as acceptor amine at a final assay concentration of 0.2 mM. *N*-Acetyltransferase activity was measured by quantifying the disappearance of 4-methylaniline as reflected by decreasing Schiff's base formation with dimethylaminobenzaldehyde. A molecular extinction coefficient for the Schiff's base at 450 nm of 5.25×10^4 $M^{-1}cm^{-1}$ was used (22). One unit was defined as the acetylation of 1 μmol of acceptor amine/min at 37°.

Amino Acid Analysis

Ninety μg (150 μl) of *N*-acetyltransferase were placed in a 6 × 50 mm Pyrex tube, precipitated with 20 μl of 20% trichloroacetic acid, and centrifuged in an Eppendorf/Brinkman centrifuge model 3200. The pellet was desalted by four washes with 100 μl of ice-cold 70% acetone. After each addition of acetone, the sample was vortexed, kept on dry ice/ethanol for 5 min, and centrifuged. The supernatant was then discarded. The desalted protein was dried *in vacuo*, redissolved in 160 μl of 88% formic acid, divided into three 50-μl aliquots (in 6 × 50 mm Pyrex tubes), and dried again. One aliquot was hydrolyzed with 6 M HCl/0.1% phenol at 150° for 1 hr and analyzed for amino acids (17). To determine cysteine and cystine as cysteic acid, the second aliquot was oxidized with 200 μl of performic acid [9.5 ml of 88% formic acid and 0.5 ml of hydrogen peroxide, 30%, prereacted for 1 hr at room temperature (23)] for 1 hr at room temperature. The oxidized protein was dried *in vacuo* and analyzed for amino acids after hydrolysis (17).

The third aliquot was used for the determination of tryptophan as previously described (24). The sample was hydrolyzed with 50 μl of 4 M methanesulfonic acid/0.2% tryptamine at 115° for 18 hr. Forty-two μl of 3.5 N NaOH and 900 μl of water were added to give pH 2.3. The amino acids (100-μl aliquots) were separated on an Interaction ion exchange column at 65° equilibrated with 1.0 N Na^+ citrate/chloride pH 7.4 buffer. The amino acids were eluted with a flow rate of 0.5 ml/min, post column derivatized with *o*-phthalaldehyde, and detected fluorometrically. Tryptophan was determined from the arginine/tryptophan ratio after correction for a 75% yield of tryptophan. Tryptophan yields were determined from control experiments with lysozyme and free tryptophan.

pH Optimum

N-Acetyltransferase activity was measured by the "recycling assay system" described by Andres *et al.* (22). The concentrations in the assay mixture (90 μl) were: 0.1 mM CoASAc, 0.2 mM 4-iodoaniline, 5 mM D,L-acetylcarnitine, 200 milliunits/ml carnitine acetyltransferase (EC 2.3.1.7), 40 mM sodium phosphate (pH 5.0–8.6), 200 mM NaCl, 1 mM dithioerythritol, and 1 mM EDTA. The assay was carried out as follows. To 50 μl of an enzyme solution in 5 mM sodium phosphate, pH 7.5, 1 mM dithioerythritol, 1 mM EDTA, 20 μl of a mixture of 4-iodoaniline dithioerythritol, EDTA, carnitine acetyltransferase, and D,L-acetylcarnitine/HCl were added, after it was neutralized with solid Na_2HOP_4. Carnitine acetyltransferase is commercially available as ammonium sulfate suspension. To remove the salt, the suspension was centrifuged and the pellet was redissolved in the neutralized 4-iodoaniline, dithioerythritol, EDTA, and D,L-acetylcarnitine mixture. The enzymatic reaction was started by CoASAc dissolved in phosphate buffer having different pH values containing the necessary amount of sodium chloride. The pH values of the phosphate buffer were adjusted so that the pH values in the reaction mixture corresponded to those given in Fig. 1. Termination of the enzymatic reaction and indicator reaction were carried out as described (22).

Structure-Activity Study

The arylamines used in the structure-activity study were purified as described (22) and stored under nitrogen at −20°. This was necessary because the commercially available preparations contained contaminations, mainly oxidized arylamine derivatives, which hindered the determination of kinetic parameters. *N*-Acetyltransferase activity was measured by the method described by Andres *et al.* (22). This assay maintains CoASAc at low physiological concentrations by a recycling system which simulates those *in vivo* (25). It also prevents product inhibition caused by CoASH. K_m and V_{max} values were thus determined for the acceptor amine under conditions like those *in vivo*.

Concentrations in the assay mixture (90 μl) were: 0.1 mM CoASAc, 100 mM Tris-HCl, pH 7.5 (at 37°), 2 mM dithioerythritol, 2 mM EDTA, 8 mM acetylphosphate, and 5 units/ml phosphotransacetylase. At least four different acceptor amine concentrations were used for the K_m determination. The absolute concentrations varied depending upon the final K_m value for a given acceptor amine. Typically, acceptor amine concentrations around the K_m were used. The enzyme concentration was adjusted such that no more than 25% of a given acceptor amine concentration was converted to its acetylated product during a standard incubation time of 10 min at 37°. K_m and some V_{max} values (see Table 4) were derived from Lineweaver-Burk plots using regular linear regression analysis ($r \geq 0.95$). The molecular extinction coefficient for the Schiff's base between a particular acceptor amine and dimethylaminobenzaldehyde was taken from Andres *et al.* (22).

The *ortho*-substituted compounds which were not acetylated (see Table 4) were tested with 0.2 mM acceptor amine and 100-fold more enzyme than that needed to acetylate aniline. In addition, the incubation time was increased to 60 min.

Results

Stability of *N*-acetyltransferase. There are several reports in the literature concerning the relative instability of *N*-acetyltransferases (7, 26–31). However, in our hands the enzyme was quite stable. Liver, kidney, and spleen *N*-acetyltransferase activity was not affected when organs were shock-frozen in liquid nitrogen and stored at −70° or in liquid nitrogen. A decrease in activity by about 15% was observed after storing the tissues for 1 year at −70°. *N*-Acetyltransferase has an essential sulfhydryl group.[1] Therefore, all buffers used during its isolation should be degassed and contain 1 mM EDTA and 1–3 mM dithioerythritol or dithiothreitol. The sulfhydryl reagents could not be replaced by another reagent such as β-mercaptoethanol. The stability of the enzyme in the crude homogenate was dramatically increased by using a combination of a serine and a cysteine protease inhibitor (50 μM phenylmethylsulfonyl fluoride or diisopropylfluorophosphate and 10 μM leupeptin). The homogeneous enzyme was stable in several buffer systems, e.g., phosphate, Tris-HCl, MES/NaOH, MOPS/NaOH from pH 6.0 to 8.5. The pH range between 7.0 and 7.8 proved to be optimal. At 4°, homogeneous *N*-acetyltransferase lost about 10% activity per week, whereas no loss of activity was observed at 37°, after incubating for 1 hr. For long-time storage of homogeneous enzyme, 5% glycerol (v/v) was added to the buffer. The enzyme solution was frozen at pH 7.0–7.8 in liquid nitrogen and stored at −70° without loss of activity for a year. Repeated freezing and thawing led to inactivation.

Purification of *N*-acetyltransferase. A large-scale purification procedure for rabbit liver enzyme was developed based on the purification scheme described for *N*-acetyltransferase from pigeon liver (32). In order to obtain a particle-free, 100,000 × *g*-like supernatant preparation from the crude homogenate, the 20,000 × *g* supernatant preparation was treated with protamine sulfate. The enzymatically inactive precipitate was pelleted by centrifugation. For further purification DEAE-Trisacryl M ion exchange chromatography was used. This gel proved to be superior to DEAE-A-25 Sephadex [used in earlier purification procedures (7,27,32)] having a 10-fold higher binding capacity for *N*-acetyltransferase. Elution of a comparatively small column yielded a more concentrated enzyme. Consequently, the enzyme could be applied directly onto the following gel filtration column without concentrating it by ammonium sulfate precipitate, which has shown very poor results in the past (13). The gel filtration step proved to be very reliable, with a good yield as well as a good purification factor. Attempts were made to further purify rabbit liver *N*-acetyltransferase by affinity chromatography on (±)-amethopterin-AH-Sepharose 4B, which was the most effective step in purifying pigeon liver *N*-acetyltransferase (32). Unfortunately, the K_i of amethopterin (*N*-[*p*-(*N*-methyl,*N'*-2,4-diaminopteridyl-6-methyl)aminobenzoyl] glutamic acid) for the rabbit liver enzyme proved to be salt dependent (an increase in salt concentration would increase the K_i), so that washing of the column at higher ionic strength resulted in eluting the enzyme together with the bulk of the proteins. Eluting the affinity gel with a linear amethopterin gradient without salt wash gave only about a 3- to 5-fold purification. Other possible affinity columns were examined

[1] H. H. Andres, A. J. Klem, L. M. Shopfer, J. K. Harrison, and W. W. Weber, submitted for publication.

under several experimental conditions: Cibacron Blue F3GA- and Procion Red HE-3B-agarose, normally very effective in binding enzymes requiring adenylyl-containing cofactors (33), adenosine 3′,5′-diphosphate-agarose (attached through the N^6-amino group), and CoASH-CH-Sepharose 4B (attached as a thioester). These resins did not bind the enzyme. In contrast, using 2-aminofluorene-epoxy-Sepharose 6B, the enzyme could only be eluted under drastic conditions with poor yield. Experiments have shown that the interaction of N-acetyltransferase with this resin was neither specific nor at the active site. Rather, it was of hydrophobic nature. Therefore, two conventional steps, hydroxylapatite and hydrophobic interaction chromatography on butyl-agarose, were used instead. The final purification was achieved with Sephacryl S-200 gel filtration. A long column had to be used to separate a major contaminating protein with a molecular weight of 37,000, which co-purified with the enzyme through six steps.

Table 1 and Fig. 1 summarize a typical purification for liver *N*-acetyltransferase from rapid acetylator rabbits. About 2–3 mg of electrophoretically homogeneous enzyme could be isolated from 200 g of frozen livers. The purification factor was between 800- and 1700-fold and the yield between 10 and 17%. The specific activity varied from 2 units/mg of protein to 6 units/mg of protein.

Homogeneity measurements. Homogeneity of the purified enzyme was established using several analytical methods. On repeated gel filtration, the enzyme was eluted from Sephacryl S-200 as a single peak with constant specific activity in each fraction. In addition, SDS-polyacrylamide disc electrophoresis and polyacrylamide isoelectrofocusing were carried out. With both methods, only one protein band was observed after staining (Fig. 2). The isoelectric point was estimated to be 5.2. Finally, *N*-terminal analysis with Edman degradation did not show any contamination.

Molecular weight determination. The molecular weight of reduced, denatured *N*-acetyltransferase was determined by SDS-polyacrylamide disc electrophoresis calibrated with standard proteins. From a linear standard curve obtained by plotting the logarithm of the molecular weight of the molecular weight markers versus migration distance, an apparent molecular weight of 33,500 was obtained for *N*-acetyltransferase (Fig. 2). The molecular weight of native *N*-acetyltransferase was estimated by gel filtration on Sephacryl S-200 and on HPLC TSK-G 3000 SW. From the Sephacryl G-200, the enzyme was eluted between α-chymotrypsinogen (M_r = 25,700) and ovalbumin (M_r = 45,000). From the linear relation between the logarithm of the molecular weight of the marker proteins and the elution volume, a molecular weight of 33,000 was determined for *N*-acetyltransferase. The molecular weight of the enzyme on the HPLC size exclusion column was determined with several buffer systems. Different apparent molecular weights were obtained with different buffer systems, despite the fact that a linear standard curve could be obtained with each buffer system.

Absorption spectrum. An absorption spectrum of homogeneous *N*-acetyltransferase was monitored from 260 to 400 nm. A symmetrical peak with a maximum at 275 nm was observed, which is typical for a protein without a cofactor.

Amino acid-, sugar-, and *N*-terminal analysis. The results of the amino acid analysis are given in Table 2. All amino acids are present. Quite striking is the low amount of alanine compared with a mean amino acid composition from 81 sequenced proteins (34). This seems to be a common feature of *N*-acetyltransferases (32). The enzyme contains 39.4 mol % hydrophobic and 49.6 mol % hydrophilic amino acids. Therefore, *N*-acetyltransferase from rabbit liver is slightly more hydrophobic than an "average" protein (36.9 mol % hydrophobic and 53 mol % hydrophilic amino acids).

No amino sugar could be detected with the method of Perini and Peters (18). Determination of the *N*-terminal amino acid was attempted by manual Edman degradation using phenylisothiocyanate (17). No *N*-terminal amino acid residue was detected. As a positive control, diisopropylfluorophosphate-treated trypsinogen was added at 1/50 of the NAT amount. The *N*-terminal amino acid of trypsinogen, valine, was readily detected. Preincubation of NAT with pyroglutamate aminopeptidase prior to sequencing did not change the result. This makes it likely that the *N*-terminal amino acid is blocked by an acetyl group (35).

Tryptic peptides. Homogeneous *N*-acetyltransferase was reduced, denatured, and alkylated as described under Materials and Methods. After removing the reagents by dialysis, the protein precipitated and proved to be resistant to digestion with trypsin despite its flocculent nature. Therefore, the insoluble protein was dissolved in hexafluoroacetone and its carboxylic groups were amidated with DMED (17) yielding a positively charged, water-soluble product, which was then digested by trypsin. During digestion the solution became turbid. The sample was centrifuged and, from the supernatant, 16 soluble peptides were purified by reverse phase HPLC (Synchropak RP-P, C_{18}) and sequenced (see Table 3). The pellet consisting of hydrophobic peptides was redissolved in formic acid and attempts were made to purify those on a less hydrophobic μBondapak CN column. Low yield and poor resolution prevented us from obtaining sequence information.

Salt effects. During the kinetic studies of *N*-acetyltransferase, a strong salt effect was observed and studied systematically. With sodium and potassium chloride, 50% inhibition was seen

TABLE 1

Purification of liver acetyl-CoA:arylamine *N*-acetyltransferase from rapid acetylator rabbits (III/J)

Step	Volume	Total protein	Total activity	Specific activity	Purification	Recovery
	ml	*mg*	*units*	*milliunits/mg protein*	*-fold*	%
1. 20,000 × *g* supernatant preparation	930	27,200	102.0	3.8	1	100
2. Protamine sulfate precipitation	935	16,952	90.8	5.4	1.4	89
3. DEAE-Trisacryl M	138	1,592	65.4	41.1	11	64
4. Sephadex G-100	150	122	51.6	423	111	51
5. Hydroxylapatite	41	31	38.7	1,248	328	38
6. Butyl-agarose	32	6.2	25.2	4,064	1,069	25
7. Sephacryl S-200	16	2.9	17.1	5,896	1,551	17

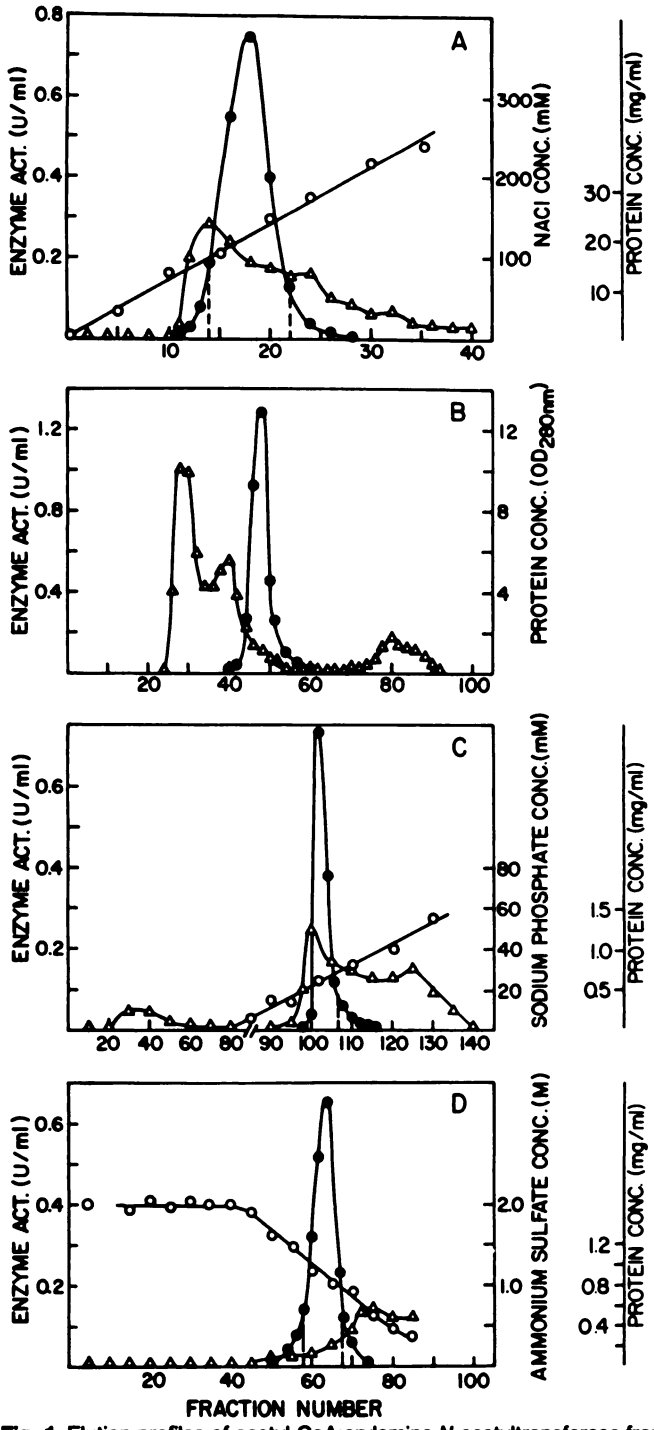

Fig. 1. Elution profiles of acetyl-CoA:arylamine *N*-acetyltransferase from a DEAE-Trisacryl M column (A), a Sephadex G-100 column (B), a Bio-Gel HT hydroxylapatite column (C), and a butyl-agarose column (D). The experimental details are given in the text. ●, enzyme activity (units/ml); △, protein concentration; ○, salt gradient.

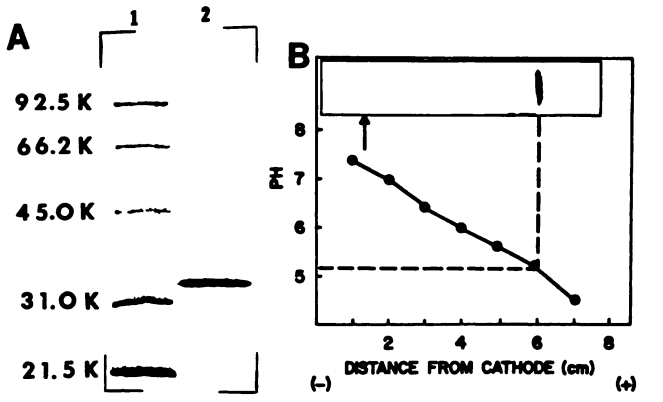

Fig. 2. SDS-polyacrylamide disc gel electrophoresis. Polyacrylamide slab gels (10%) were prepared and run as described under Materials and Methods. *Lane 1* contains molecular weight markers as follows: phosphorylase B (M_r = 92,500), bovine serum albumin (M_r = 66,200), ovalbumin (M_r = 45,000), carbonic anhydrase (M_r = 31,000),and soybean trypsin inhibitor (M_r = 21,500). *Lane 2* contains 15 μg of purified *N*-acetyltransferase. B. Thin layer polyacrylamide isoelectrofocusing on Ampholine Pagplate, pH 3.5–9.5. A 10-μg sample of *N*-acetyltransferase was applied near the cathode indicated by ↑. (For further experimental details, see Materials and Methods.)

TABLE 2

Amino acid analysis of liver *N*-acetyltransferase from rapid acetylator rabbit and pigeon in comparison with a mean composition of 81 proteins

Amino acid	Rabbit III/J	Pigeon[a]	Mean composition[b]
		residue/mol	
Ala	6.9	9.6	25.8
Cys[c]	5.0	3.7	7.3
Asx	26.7	26.9	32.2
Glx	37.3	30.0	29.7
Phe	14.0	10.4	11.2
Gly	20.5	18.3	22.9
His	5.7	8.1	6.3
Ile	19.0	14.4	16.5
Lys	14.5	18.4	20.5
Leu	34.5	31.2	21.9
Met	5.1	7.6	4.9
Pro	10.1	9.4	11.2
Arg	16.4	13.1	15.7
Ser[d]	19.1	14.7	20.5
Thr[e]	16.8	12.6	17.6
Val	19.2	21.9	20.5
Tyr	15.1	15.8	12.7
Trp[f]	4.7	3.8	3.9
Total residues	291	270	288
Molecular weight	33,500	32,870	33,510

[a] Data from Andres *et al.* (31).
[b] Data from Holmquist (34).
[c] Cysteine and cystine were determined as cysteic acid. Values are corrected for a 90% yield.
[d] Serine values are corrected for a 72% yield.
[e] Threonine values are corrected for a 91% yield.
[f] Tryptophan was determined from the Arg/Trp ratio after hydrolysis with methanesulfonic acid. Values are corrected for a 75% yield of Trp.

at about 300 mM and 350 mM, respectively (Fig. 3A). The inhibition could be partially reversed by increasing the CoASAc concentration. The divalent cations, magnesium and calcium, proved to be much stronger inhibitors, inhibiting *N*-acetyltransferase by 50% at about 10 mM (Fig. 3C). Peculiar inhibition curves were found when testing different sulfate salts. With an increase in the salt concentration, the activity initially decreased. By increasing the salt concentration further, activation was observed (Fig. 3B). We ensured that the observed curves were not artifacts produced by nonsaturating concentrations of the acceptor amine or by a sacrificed recycling system or by a changed molecular extinction coefficient of the indicator molecule (Schiff's base of dimethylaminobenzaldehyde and 4-methylaniline).

To rule out whether the observed curves were due to an ionic effect or due to the increased osmolarity of the assay solution,

TABLE 3
Tryptic peptides of liver *N*-acetyltransferase from rapid acetylator rabbits (III/J) organized by increasing hydrophobobicity

1.	Ile-Gly-Try-Lys	
2.	Thr-Tyr-Asn-Tyr-Lys	
3.	Glu-Asn-Thr-Asp-Leu-Val-Glu-Phe-Lys	
4.	Val-Leu-Thr-Glu-Glu-Glu-Val-Glu-Gly-Val-Leu-Lys	
5.	Leu-Ala-Ile-Glu-Ala-Gly-Phe-Arg	
6.	Asn-Tyr-Ile-Val-Asp-Ala-Gly-Phe-Lys	
7.	Gln-Gln-His-Val-Pro-Asp-Gln-Glu-Phe-Leu-Asn-Ser	incomplete
8.	Glu-Glu-Gly-Glu-Thr-Cys-Tyr-Leu-Asp-Asp-Ile-Arg	
9.	Asp-Gln-Pro-Gln-Val-Pro-Ser-Ile-Phe-Arg	
10.	Leu-Tyr-Cys-Phe-Thr-Leu-Gln-Pro-Arg	
11.	Thr-Ile-Glu-Glu-Phe-Glu-Ser-Ala-Asn-Thr-Tyr-Leu-Gln-Glu-Ser-Pro-Asp	incomplete
12.	Ser-Ile-Lys-Ser-Leu-Gln-Thr-Pro-Glu-Gly-Val-His-Trp-Leu-Val-Gly-Leu-Thr	incomplete
13.	Ser-Tyr-Gln-Met-Trp-Gln-Pro-Val-Glu-Leu-Ile-Ser-Gly-Lys	
14.	Thr-Ile-Phe-Asn-Ile-Ser-Leu-Gly-Lys	
15.	Leu-Asp-Leu-Glu-Ser-Leu-Thr-Asp-Ile-Phe-Gln-His-Gln-Ile-Arg	incomplete
16.	Val-Ala-Ser-Ile-Ser-Leu-Pro-Thr-Ser-Gly	

we measured *N*-acetyltransferase activity in the presence of up to 500 mM sucrose. No inhibition of the activity was observed.

pH optimum. There are several problems associated with the determination of the pH optimum of *N*-acetyltransferase. First, the acceptor amine, as a base, becomes more and more protonated when one lowers the pH of the reaction mixture. In order to study the pH effect on the enzyme rather than the effect of acceptor amine protonation, we chose the weak base 4-iodoaniline as the acceptor amine. With a pK_a value of 3.78, only 5% of the molecules are protonated at the lowest pH value tested. Furthermore, this compound saturated the enzyme at low μM concentrations (see Table 4). The latter reason made us decide against 4-trifluoromethyl-, 4-cyano-, and 4-nitroaniline, these being much weaker bases than 4-iodoaniline but having much higher K_m values (see Table 4). In addition, we showed that 4-iodoaniline saturated the enzyme over the entire pH range.

Second, the activity of *N*-acetyltransferase is strongly dependent on ionic strength (see Fig. 3). When titrating a buffer for the determination of the pH optimum, not only is the pH value changed, but also the ionic strength. Therefore, we kept the buffer concentration low (40 mM) and added 200 mM sodium chloride. By this procedure, the change in ionic strength when titrating the buffer is negligible.

Third, the enzyme is very unstable on storage at pH values lower than 6. Therefore, a stock solution of the enzyme was diluted appropriately with 5 mM buffer, pH 7.5. The enzymatic reaction was started by CoASAc dissolved in buffer having different pH values (pH 5.0–8.6) containing the necessary sodium chloride (see Materials and Methods). Under those conditions, the activity was essentially independent of pH over a wide pH range (pH 5.8–8.6) (Fig. 4).

Kinetic constants for 4-substituted aniline derivatives with pK_a values ranging from 5.86 to 1.11. In the first set of experiments, we studied the effects of the pK_a of the acceptor amine on the apparent K_m and the apparent V_{max} values. To do this, we used aniline derivatives (pK_a 5.65–1.11) with relatively small, uncharged substituents in the 4-position to minimize steric as well as electrostatic effects. As seen in Table 4, Group A, the apparent V_{max} changed little with the change in the basicity from pK_a 5.65 to 1.74. The average specific activity was 2.25 ± 0.16 units/mg of protein (mean ± SD). The one exception was 4-nitroaniline, with a specific activity of 0.25 unit/mg of protein, having the lowest pK_a of all the aniline derivatives (pK_a 1.11). The apparent K_m values in group A ranged from 11 (4-methoxyaniline) to 650 μM (4-cyanoaniline). The compounds in the pK_a range from 5.65 to 3.78 had apparent K_m values in the range from 10 μM to 100 μM. Acceptor amines with pK_a values of 2.54 and lower (4-trifluoromethyl-, 4-cyano-, and 4-nitroaniline) showed significantly higher K_m values (210–650 μM). These data indicate that apparent K_m and basicity of the acceptor amine are roughly correlated. A linear regression was calculated with a correlation coefficient of 0.76.

Besides the basicity of the acceptor amine, other physicochemical parameters can be examined for correlation with the kinetic data. For example, there are several examples in the literature in which the hydrophobicity of a low molecular weight compound appears to play an important role in its interaction with proteins (36–38). In our study, we used as a measure for the hydrophobicity of the aniline derivatives the free energy-related substitution constant π (39). This is constant is defined as

$$\pi = \log P_x - \log P_H$$

where P_H is the partition coefficient of the unsubstituted molecule between 1-octanol and water and P_x is that of the derivative. As seen in Table 4, Group A, no correlation was found between the hydrophobicity of the acceptor amines and their apparent K_m values.

Kinetic constants for methyl and ethyl ring-substituted isomers of aniline. V_{max} and K_m values were determined for 2-, 3-, and 4-methylaniline. All aniline derivatives had K_m values in the low μM range (5–43 μM; Table 4, Group B). As judged by the V_{max}, all three methylanilines were readily acetylated, even the *ortho*-substituted isomer.

From the three ethyl aniline derivatives, only two derivatives—the 3- and 4-ethylanilines—were acetylated, whereas the *ortho*-substituted 2-ethylaniline was not. Both acetylated derivatives showed very low K_m values (3-ethylaniline: <5 μM; 4-ethylaniline: 6 μM) combined with rather high V_{max} values.

Kinetic constants for dimethyl ring-substituted isomers of aniline. Judged by the K_m and V_{max} values, the dimethyl-substituted arylamines (Table 4, Group C) can be divided into three groups. Dimethylanilines with one methyl group in the *ortho*-position had lower V_{max} values than aniline

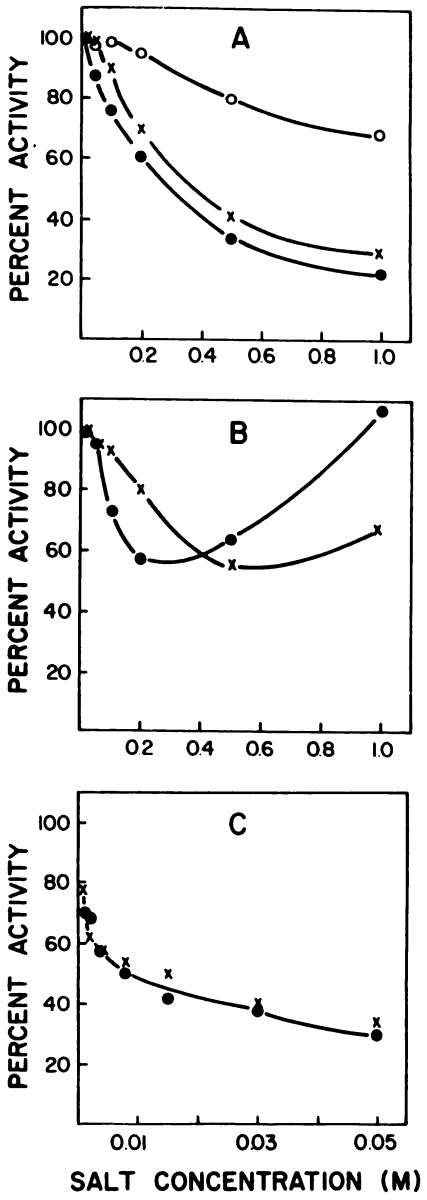

Fig. 3. Salt effects on *N*-acetyltransferase. A. *N*-Acetyltransferase activity in the presence of sodium and potassium chloride was measured as shown: O, sodium chloride, measured with 2.0 mM CoASAc; ×, sodium chloride, measured with 1.0 mM CoASAc; and ●, potassium chloride, activity measured with 0.1 mM CoASAc. B. *N*-Acetyltransferase activity in the presence of magnesium and calcium chloride measured with 0.1 mM CoASAc. C, *N*-Acetyltransferase activity in the presence of ammonium and sodium sulfate measured with 0.1 mM CoASAc.

in addition to relatively high K_m values (260–700 μM). The double *ortho*-substituted 2,6-dimethylaniline did not undergo an acetylation even with high enzyme concentrations and prolonged incubation times. The dimethylanilines with no methyl group in the *ortho*-position had V_{max} values slightly higher than aniline and showed K_m values close to or below the detection limits.

Kinetic constants for aniline derivatives with charged substituents (—COOH, —SO_3H) in the 2-, 3-, and 4-positions. The results obtained with the aniline derivatives of Group D (Table 4) can be summarized as follows. A charged substituent in the *ortho*-position—whether it is a carbonyl or a sulfonate group—prevented acetylation of the amino groups.

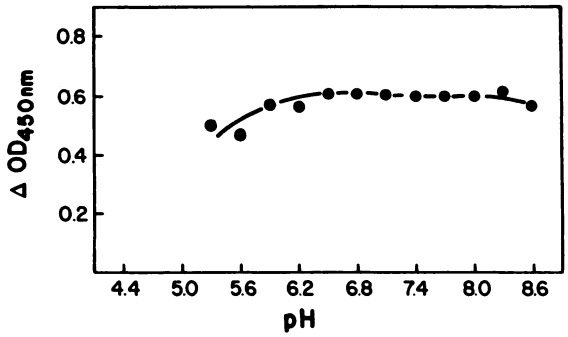

Fig. 4. pH optimum for *N*-acetyltransferase. (For experimental details see Materials and Methods.)

TABLE 4

Kinetic and physical-chemical constants for different aniline derivatives

Aniline derivatives	Apparent V_{max}	Apparent K_m	pK_a[a]	Hydrophobicity[b]
	units/mg protein	μM		π
Aniline	1.95 ± 0.07	120 ± 50	4.6	0.0
Group A				
4-Hydroxy	2.42 ± 0.05	80 ± 50	5.65	−0.9
4-Methoxy	2.29 ± 0.04	11 ± 6	5.34	0.01
4-Methyl	2.20 ± 0.12	39 ± 10	5.10	0.48
4-Fluoro	2.28 ± 0.13	100 ± 30	4.60	0.21
4-Chloro	2.29 ± 0.05	67 ± 12	3.98	0.93
4-Bromo	2.20 ± 0.09	11 ± 8	3.86	1.13
4-Iodo	1.93 ± 0.07	29 ± 3	3.78	2.40
4-Trifluoromethyl	2.15 ± 0.11	210 ± 40	2.54	1.05
4-Cyano	2.5 @[c]	650 ± 200	1.74	0.4
4-Nitro	0.26 ± 0.04	270 ± 50	1.11	0.5
Group B				
2-Methyl	2.19 ± 0.12	43 ± 6	4.44	0.37
3-Methyl	2.54 ± 0.06	5 ± 2	4.68	0.48
4-Methyl	2.20 ± 0.12	39 ± 10	5.10	0.48
2-Ethyl	no act		4.37	0.89
3-Ethyl	2.79 ± 0.08	<5	4.70	0.97
4-Ethyl	2.99 ± 0.01	6 ± 2		
Group C				
2.3-Dimethyl	1.42 ± 0.09	210 ± 40	4.72	0.77
2.4-Dimethyl	1.52 ± 0.09	220 ± 30	4.84	0.85
2.5-Dimethyl	1.5 @	700 ± 150	4.57	0.89
2.6-Dimethyl	no act		3.89	0.97
3.4-Dimethyl	2.34 ± 0.14	7 ± 2	5.22	0.72
3.5-Dimethyl	2.31 ± 0.11	<5	4.91	0.86
Group D				
2-COOH	no act			0.27
3-COOH	0.54 ± 0.02	220 ± 70	3.73	−0.74
4-COOH	3.54 @	310 ± 100	3.80	−0.32
2-SO_3H	no act		2.46	
3-SO_3H	0.18 ± 0.01	380 ± 120	3.74	
4-SO_4H	0.53 ± 0.01	260 ± 80	3.22	−4.76

[a] Data from Fujita *et al.* (46) and Hart (47).
[b] Data from Leo *et al.* (39).
[c] @, estimated from the *y* intercept of the double reciprocal plots.

The same substituents in the 3-position affected the V_{max} values in a negative fashion showing 0.54 unit/mg of protein for 3-amino benzoic acid and 0.18 unit/mg of protein for 3-amino-sulfonic acid. *p*-Aminobenzoic acid had the highest V_{max} of all compounds studied (3.54 units/mg of protein). Its sulfonated analogue had a rather low maximal velocity with 0.54 units/mg of protein. All four isomers had relatively high K_m values (220–380 μM).

Discussion

One of the major questions in *N*-acetyltransferase research has been whether the CoASAc-dependent acetylation of a wide spectrum of amines in rabbit liver was catalyzed by a set of isozymes or by a single enzyme with broad substrate specificity. Because the enzyme(s) could not be purified to homogeneity, indirect methods like heat inactivation studies (7), co-purification of *N*-acetyltransferase activity measured with different acceptor amines (7), and isoelectrofocusing experiments in granulated gels (7) were used to investigate this issue. The accumulated data have tended to support the second hypothesis.

The purification procedure described in this paper yielded a homogeneous preparation of *N*-acetyltransferase from homozygous rapid acetylator rabbits, which showed a single protein band on both SDS-polyacrylamide electrophoresis and isoelectrofocusing. Using this homogeneous enzyme preparation, we have found that many aniline derivatives (Table 4) with diverse chemical features served as the acceptor amine in the *N*-acetylation reaction. Therefore, a single enzyme with a broad substrate specificity seems to have all the catalytic properties reported earlier (12). This suggests that the liver of slow acetylator rabbits contains also only one *N*-acetyltransferase and is supported by the genetic analysis of the *N*-acetylation polymorphism which shows a simple Mendelian trait with two alleles at a common locus (6). Immunochemical studies have shown that polyvalent antibodies raised against the liver enzyme from the rapid acetylator genotype cross-reacted with the enzyme from the slow acetylator genotype (41) and that rapid and slow acetylator liver contains the same amount of *N*-acetyltransferase protein. Based on these immunotitration studies (40) and on qualitatively different kinetic data (15, 41), the differences between rapid and slow liver *N*-acetyltransferase cannot be due to changes in the synthesis or breakdown of the enzyme.

This supports the hypothesis that a small change in the primary structure accounts for the catalytic differences which are reported for liver *N*-acetyltransferase from rapid and slow acetylator rabbits (15, 41). Furthermore, from kinetic studies where "intermediate" activities were observed in heterozygous animals (41), one can conclude that the difference between the two homozygous genotypes is due to differences in the encoding gene rather than differences in the processing of the protein. In the latter case, no "intermediate" activities would be observed in heterozygous animals.

The molecular weight of native *N*-acetyltransferase (33,000) was identical to that of the denatured enzyme (33,500) within the experimental error. Thus, *N*-acetyltransferase has a monomeric subunit structure. The measured molecular weight of rabbit liver *N*-acetyltransferase is in the same range as that of pigeon liver *N*-acetyltransferase (32,900) (32). Using the HPLC size exclusion column, we obtained different apparent molecular weights with different buffer systems. Finding an elution volume larger than expected (which translates into a lower apparent molecular weight) indicates that the protein may interact with this packing material. Therefore, the apparent molecular weights determined with this particular size exclusion column cannot be considered to be valid.

Sequence information was obtained despite the unexpected solubility properties of the denatured enzyme. Some regions of the protein seemed to consist of very hydrophobic amino acids, resulting in an insoluble, nondegradable product after reduction and carboxymethylation. Therefore, the protein was derivatized to yield a soluble, degradable product. Nevertheless, the protein solution became turbid when the digest proceeded, indicating that some hydrophobic regions did not contain modifiable aspartic or glutamic acid residues. Preliminary experiments have shown that the active site cysteine is located within one of those hydrophobic peptides. This is not too surprising considering the hydrophobic nature of the acceptor amine substrates.

It is obvious from these results that the combination of small amounts of starting material and the solubility problems with a major fraction of the peptides will make it very difficult to reveal the differences in the primary structure between the rapid and the slow isozyme on the protein level. Fortunately, the reported partial sequence may provide an approach to this problem by standard recombinant DNA techniques. From the protein sequence Tyr-Gln-Met-Trp-Gln-Pro- (peptide 13), the sequence for a very specific synthetic, only 8-fold degenerate 17-mer oligonucleotide probe can be derived (UA_C CA_G AUG UGG CA_G CC). With this probe, genomic and liver cDNA libraries can be screened for the *N*-acetyltransferase gene. In addition, the other protein sequences that we report will aid in unambiguous identification of positive clones. Furthermore, this probe could be used to learn more about the relationship of *N*-acetyltransferases in different tissues, such as the gut mucosa, peripheral blood cells (15, 40), and pineal gland (31).

In addition to these reports of rapid liver NAT physicochemical properties, we also determined some of its kinetic properties. Quite pronounced salt effects were found: mono- and divalent ions proved to be inhibitory, the divalent ions being the stronger ones. The divalent sulfate anion seemed to stimulate *N*-acetyltransferase activity at high concentrations. Different inhibition curves were obtained with different sulfate salts, reflecting the influence of the cation. The inhibitory effect of high salt concentrations was less pronounced, when the enzyme was measured with 2.0 mM CoASAc. For this reason, the salt effect was not noticed before, because the older assay procedure used 2.2 mM CoASAc (41). The observed "protection" against salt inactivation at higher CoASAc concentrations can be explained if one assumes that high salt concentrations increase the K_m values for CoASAc, which leads in our "recycling" assay to a decrease in the apparent rate.

An early systemic structure-activity study for an *N*-acetyltransferase was carried out by Riddle and Jencks (42), using partially purified *N*-acetyltransferase from pigeon liver. With the nonphysiological acetyl donor, *p*-nitroacetanilide, an identical maximum velocity was observed for five substituted anilines of different basicity. They concluded from those and other data that the enzyme-catalyzed reaction proceeds through the rate-determining formation of a common acetyl-enzyme intermediate which reacts with the acceptor amine in a fast step. Substituting *p*-nitroacetanilide with *p*-nitrophenyl acetate, an acetylating reagent of great acetyltransfer potential, they showed that the maximum velocity increased when different acceptor amines of increasing basicity were used. Above a certain pK_a, the same maximum velocity was obtained for all strongly basic acceptor amines. These results suggested that with a weakly basic amine the rate-limiting step is the acetyltransfer from the covalent acetyl-enzyme intermediate to the

acceptor amine, whereas with strongly basic amines the first step—the acetylation of the enzyme—is rate-limiting.

When we conducted a similar study using the physiological acetyl donor CoASAc and ten 4-substituted aniline derivatives with pK_a values ranging from 5.65 to 1.11, the maximum velocities for acceptor amine with pK_a values from 5.65 to 1.74 were in the same range (2.25 ± 0.16 units/mg of protein). Only the V_{max} value for *p*-nitroaniline was dramatically lower with 0.25 unit/mg of protein. By analogy with the results of Riddle and Jencks (42), our data suggest that a change in rate-limiting step occurs between pK_a 1.74 and 1.11 and the formation of the covalent acetyl-enzyme intermediate (30) is the rate-limiting step when using acceptor amines with a pK_a higher than 1.74. This assumes that special properties of *p*-nitroaniline do not account for the low V_{max} value. Unfortunately, there were no other 4-substituted aniline derivatives in the pK_a range of interest (1.74–1.11) to use in further study of the pK_a-dependent decrease in the V_{max}. The *N*-acetyltransferase-catalyzed reaction (13)[2] can be rewritten as follows:

1. CoASAc + ESH ⇋ [ESH:CoASAc]
2. [ESH:CoASAc] → [ESAc:CoASH]
3. ESAc:CoASH] ⇋ ESAc + CoASH
4. ESAc + Amine ⇋ [ESAc:amine]
5. [ESAc:amine] → [ESH:Ac amine]
6. [ESH:Ac amine] ⇋ ESH + Ac amine

It follows that one of the first three reactions could be rate-limiting when using an aniline derivative with a pK_a above 1.74.

From studies on the 2-substituted aniline derivatives, only the 2-methylaniline was acetylated. Surprisingly, neither the K_m nor the V_{max} value indicated a steric problem when compared with aniline or the 4-substituted derivatives of Group A with pK_a values ranging from 5.65 to 3.78. In contrast, *N*-acetyltransferase failed to catalyze the acetylation of 2-ethylaniline, *ortho*-aminobenzoic acid, and *ortho*-aminosulfonic acid. Steric factors seem to be responsible. The pK_a is not a problem because the pK_a of *ortho*-aminosulfonic acid (pK_a 2.54, the least basic of the three derivatives) is considerably higher than that of the 4-nitroaniline (1.11), which is still acetylated. Furthermore, in the case of *ortho*-aminobenzoic acid and *ortho*-aminosulfonic acid, the negative charge in general—resulting in the reduction of the hydrophobicity of the derivatives as judged by the π-values—cannot be the reason why these two compounds are not acetylated. 4-Aminosulfonic acid with a π-value as low as −4.76 is readily acetylated.

That *N*-acetyltransferase from rabbit liver was unable to catalyze the acetylation of 2-aminobenzoic acid is in contrast to the results obtained with pigeon liver *N*-acetyltransferase (43).

All six possible dimethyl ring-substituted analine derivatives were acetylated except the double *ortho*-substituted 2,6-dimethylaniline. Because this compound is readily acetylated in organic-chemical reaction using different acetylation reagents, one can conclude that steric hindrance prevented the enzymatic acetylation. The three derivatives with a methyl group in the 2-position showed relatively high K_m values and considerably lower V_{max} values when compared with aniline, whereas 3,4- and 3,5-dimethylaniline had very low K_m and high V_{max} values. Because the acetylation of none of the mono-substituted methylaniline derivatives showed any steric hindrance, the negative effect on the kinetic parameters of the 2-substituted dimethylanilines may be attributed the second substituent in the 3-, 4-, 5-, or 6-position. This second substituent causes the substrates to interact with the active site in a way that promotes interference with the methyl group in 2-position.

An arylamine with a charged substituent such as a carboxy or a sulfonate group, in general, had lower activity than an uncharged substituent such as an ethyl group in the same location. The exception was 4-aminobenzoic acid which had an exceptionally high specific activity compared to any of the aniline derivatives used. The high K_m values could be due to unfavorable charge interactions in the active site. Previous investigations using large uncharged arylamines such as benzidine and naphthylamine (44) have shown that these compounds have very low K_m values. The negative charge of the aniline derivatives in Group D could be adjacent to some negatively charged amino acids or some hydrophobic residues causing a repulsion from the active site.

This structure-activity study for the rapid acetylator isozyme will be of particular interest in comparison with an identical study using the slow acetylator isozyme. Preliminary studies in our laboratory with partially purified slow acetylator isozyme showed that there were dramatic differences between the K_m values of rapid and slow *N*-acetyltransferase when using negatively charged acceptor amines (45). In contrast, K_m values for uncharged acceptor amines seemed to be in the same range. A detailed study will follow the purification of *N*-acetyltransferase from slow acetylator rabbits.

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Send reprint requests to: Dr. Wendell W. Weber, Department of Pharmacology, The University of Michigan Medical School, M6322, Medical Science Building I, Ann Arbor, MI 48109–0010.