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New Spectrophotometric and Radiochemical Assays for Acetyl-CoA: Arylamine *N*-Acetyltransferase Applicable to a Variety of Arylamines

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Simple and sensitive spectrophotometric and radiochemical procedures are described for the assay of acetyl-CoA:arylamine *N*-acetyltransferase (NAT; EC 2.3.1.5), which catalyzes the reaction acetyl-CoA + arylamine → *N*-acetylated arylamine + CoASH. The methods are applicable to crude tissue homogenates and blood lysates. The spectrophotometric assay is characterized by two features: (i) NAT activity is measured by quantifying the disappearance of the arylamine substrate as reflected by decreasing Schiff's base formation with dimethylaminobenzaldehyde. (ii) During the enzymatic reaction, the inhibitory product CoASH is recycled by the system acetyl phosphate/phosphotransactylase to the substrate acetyl-CoA. The radiochemical procedure depends on enzymatic synthesis of [^{3}H]acetyl-CoA in the assay using [^{3}H]acetate, ATP, CoASH, and acetyl-CoA synthetase. NAT activity is measured by quantifying *N*-[^{3}H]acetylarylamine after separation from [^{3}H]acetate by extraction. Product inhibition by CoASH is prevented in this system by the use of acetyl-CoA synthetase. © 1985 Academic Press, Inc.

KEY WORDS: *N*-acetyltransferase; ping-pong Bi–Bi mechanism; acetylation polymorphism; rabbit; mouse.

N-Acetylation is an important step in the biotransformation of arylamines, hydrazines, and biogenic amines of aromatic amino acids. These chemical classes include drugs such as sulfamethazine, sulfadiazine, *p*-aminobenzoic acid, *p*-aminosalicylic acid, isoniazid and hydralazine (1), aromatic amine carcinogens like 2-aminofluorene, benzidine, and β-naphthylamine (2), and neurotransmitters like serotonin (3). Investigators have demonstrated in several animal species including man (4), rabbit (5), rat (6), mouse (7), and hamster (8) that individuals can be classified as either "slow" or "rapid" acetylators due to variation in the activity of the liver *N*-acetyltransferase. The *N*-acetylation polymorphism has been associated with variations in drug toxicity and may be a determinant in arylamine-induced cancer of occupational origin (9).

The existence of an enzyme which catalyzes the reaction

was first demonstrated by Chou (10). The enzyme reaction follows a ping-pong Bi–Bi mechanism (11). In reactions such as these, families of parallel lines are obtained when Lineweaver–Burk plots are constructed with varying concentrations of one substrate at different, fixed concentrations of a second substrate, and vice versa.

These kinetic patterns mean that an increase in the concentration of one substrate, in our case acetyl-CoA, results in an increase in the V_{max} of the reaction and the K_m for the second substrate, the acceptor amine. Therefore, the "true" K_m and V_{max} can only

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be obtained by extrapolating the apparent Michaelis constants, determined experimentally, to infinite concentrations of both substrates; however, a technical difficulty arises in routine measurements of enzymes which follow ping-pong kinetics in that "infinite" substrate concentrations are not achievable in experimental systems. Therefore, when determining kinetic constants *in vitro* it would be desirable to use acetyl-CoA concentrations which are in physiological range to approximate kinetic constants occurring *in vivo*. Measurements of acetyl-CoA:arylamine *N*-acetyltransferase (NAT[2]; EC 2.3.1.5) at such low acetyl-CoA concentrations with assays which are currently available are not feasible, however, because the supplies of acetyl-CoA are depleted very rapidly.

Another problem that has been encountered with available assays is the strong product inhibition of NAT activity by CoASH. This results in a nonlinear NAT assay with either increasing enzyme concentration or increasing time. CoASH inhibition appears to be a common feature of all *N*-acetyltransferases characterized, including serotonin *N*-acetyltransferase from pineal gland (12) and histone *N*-acetyltransferase (13).

Several spectrophotometric assays have been described to measure NAT activity. Lynen measured this reaction by following the disappearance of the thioester bond of acetyl-CoA at 232 nm (14). Measurement in whole homogenates was hampered because all proteins absorb strongly at that wavelength. In addition, thioesterases which are present in such preparations reduce the specificity of this technique.

Weber and Cohen monitored isoniazid acetylation at 303 nm at pH 9.0 (11). Limitations of this method include protein absorbance at this wavelength, use of a pH which does not coincide with the pH optimum of the enzyme, and a detection method that can only be used for isoniazid.

The technique that has been used most commonly by many investigators involves measuring the rate of disappearance of the arylamine substrate using the method of Bratton and Marshall (15). The principle of the detection reaction involves the conversion of unacetylated arylamine to its diazo cation by sodium nitrite. Subsequently, the reaction of the diazo cation with *N*-1-(naphthyl)ethylendiamine forms a colored diazo compound which is quantified at 540 nm.

Despite its broad applicability, there are several problems associated with this method. First, the procedure involves several time-consuming steps, leaving room for significant errors. Second, nitrite trapping agents like urea are present in some crude homogenates, which can prevent the formation of the diazo cation. Third, the stability of the diazo cation depends to a remarkable extent on other substituents of the arylamine. For example, electron-withdrawing substituents favor phenol formation with the release of nitrogen, in which case no further diazo coupling occurs. Finally, some diazo cations are sterically restricted and do not react with *N*-1-(naphthyl)ethylendiamine.

Thus, we have sought to develop alternative assay systems for NAT assay. The methods we describe allow the use of low acetyl-CoA concentrations, prevent inhibition by CoASH, and include a detection system applicable to a broad spectrum of arylamines.

MATERIALS AND METHODS

Phosphotransacetylase (EC 2.3.1.8), acetyl-CoA synthetase (EC 6.2.1.1), ATP, CoASH, acetylphosphate, Trizma Base, bovine serum albumin, *p*-dimethylaminobenzaldehyde, dithioerythritol, EDTA, and dimethyl sulfoxide were purchased from Sigma Chemical Company, St. Louis, Missouri. Acetyl-CoA was obtained from P–L Biochemicals, Inc., Milwaukee, Wisconsin. Acetonitrile was purchased from Fisher Scientific Company, Pittsburgh, Pennsylvania, and Omnifluor and

[2] Abbreviations used: NAT, acetyl-CoA:arylamine *N*-acetyltransferase (*N*-acetyltransferase); AAF, acetylaminofluorene; AF, aminofluorene; ATP, adenosine triphosphate; DTE, dithioerythritol; DMSO, dimethyl sulfoxide; PABA, *p*-aminobenzoic acid; PTA, phosphotransacetylase.

[^{3}H]acetate were from New England Nuclear, Boston, Massachusetts.

The arylamines were purchased from several companies and purified by distillation or recrystallization (Table 1).

Arylamine stock solutions (100 mM) were prepared in degassed acetonitrile, methanol, or acetone and stored under nitrogen at −20°C. Under these conditions, no oxidation was observed.

Animals

Mature New Zealand White inbred rabbits (strain III/J), and A/J and C56BL/6J mice were obtained from the Jackson Laboratory, Bar Harbor, Maine. Additional mature New Zealand White rabbits were obtained from Langshaw Rabbit Farm, Kalamazoo, Michigan. The B6.AF$_1$ mice (offspring of a mating between C57BL/6J and A/J) were bred and maintained by Dr. Robert P. Erickson in the Department of Human Genetics. Mice were housed two to eight per standard shoe box cage with food (sulfa-free Purina Mouse Chow) and water ad libitum. Bedding consisted of cedar wood shavings which were changed weekly. They were maintained at temperatures between 68 and 74°F on a 12-h light–dark cycle. Mice were 18 weeks of age when used.

Enzyme Sources

Liver *N*-acetyltransferase from the New Zealand White inbred rabbit strain III/J was purified to electrophoretic homogeneity

TABLE 1

PURIFICATION METHOD AND SUPPLIER OF ARYLAMINES

Compound	Source	Purification
Aniline	Fisher	Distilled
4-Fluoroaniline	Sigma	Distilled
4-Chloroaniline	Aldrich	Ethanol
4-Bromoaniline	Sigma	None
4-Iodoaniline	Aldrich	Water
4-Aminobenzonitrile	Aldrich	Water
4-Methoxyaniline	Sigma	None
4-Hydroxyaniline	Aldrich	70% Ethanol
Trifluorotoluidine	Sigma	None
4-Nitroaniline	Matheson, Coleman and Bell	None
4-Methylaniline	Sigma	Petroleum ether
3-Methylaniline	Aldrich	Distilled
2-Methylaniline	Sigma	Water–ethanol
4-Ethylaniline	Aldrich	Distilled
3-Ethylaniline	Aldrich	Distilled
2-Ethylaniline	Aldrich	Distilled
4-Propylaniline	Aldrich	Distilled
2,6-Dimethylaniline	Aldrich	Distilled
2,5-Dimethylaniline	Aldrich	Distilled
2,4-Dimethylaniline	Aldrich (gold label)	None
2,3-Dimethylaniline	Aldrich	Distilled
3,5-Dimethylaniline	Aldrich	Distilled
3,4-Dimethylaniline	Aldrich	Petroleum ether
4-Aminobenzenesulfonic acid	Mallinckrodt	Water
3-Aminobenzenesulfonic acid	Pfaltz–Bauer	Water
2-Aminobenzenesulfonic acid	Pfaltz–Bauer	Water
4-Aminobenzoic acid	Sigma	None
3-Aminobenzoic acid	Calbiochem	Water
2-Aminobenzoic acid	Aldrich (gold label)	None

(Andres *et al.*, unpublished method). To phenotype rabbits from a random population 0.5 g liver was homogenized on ice with a Polytron (Kinematica GMBH, Lucerne, Switzerland) using 6 vol ice-cold, degassed buffer containing 20 mM Tris/HCl, pH 7.5, at 4°C, 1 mM DTE, 1 mM EDTA, 50 μM phenylmethylsulfonyl fluoride, and 10 μM leupeptin (buffer 1). The crude homogenate was centrifuged for 3 min in an Eppendorf/Brinkmann centrifuge, Model 3200. The supernatant was kept on ice. Activity was measured with 0.1 mM acetyl-CoA and 0.2 mM PABA. Liver tissue can be stored at −70°C without loss of activity for at least 3 months when shock frozen in liquid nitrogen. Mouse blood (50 μl) was obtained by orbital sinus puncture using heparinized glass capillaries, immediately lysed in 500 μl buffer 1, and kept on ice until assayed for NAT activity. To obtain mouse bladder NAT, whole bladders were homogenized on ice with micro tissue grinders (Konte's Glass Co., Vineland, N. J.) in 200 μl buffer 1. The crude homogenate was centrifuged for 3 min in an Eppendorf/Brinkmann centrifuge, Model 3200. The supernatant was kept on ice. Activity was measured with 0.5 mM acetyl-CoA and 0.1 mM PABA.

Standard Procedure for Spectrophotometric Assay

NAT assays were carried out in a total volume of 90 μl in capped polypropylene 1.5-ml microsample tubes. To 50 μl of appropriately diluted enzyme (buffer 1), 20 μl of a solution was added containing 225 mM Tris/HCl, pH 7.5, at 37°C, 4.5 mM DTE, 4.5 mM EDTA, 22.5 mM acetyl phosphate, 2.25 U/ml phosphotransacetylase, and 0.45–0.9 mM arylamine. After a preincubation period of 5 min at 37°C, the reaction was started by the addition of 20 μl of acetyl-CoA in concentrations which varied from 0.45 to 2.25 mM. The reaction was terminated by the addition of 50 μl of 20% (w/v) trichloracetic acid. Using whole homogenates or blood lysates, the samples were centrifuged for 1 min in an Eppendorf/Brinkmann centrifuge, Model 3200. Five hundred microliters 5% (w/v) dimethylaminobenzaldehyde in acetonitrile was added. The samples were recentrifuged and incubated for at least 10 min at room temperature. Blank values were obtained by substituting water for AcCoA in the incubation mixture. Absorbance at 450 nm was recorded. Samples were incubated in triplicate. Using these standard conditions, the units of NAT activity expressed in nanomoles amine acetylated/min was calculated from the equation

$$\frac{\text{nmol amine acetylated}}{\text{min} \times \text{ml}} = \frac{(\text{OD}_{450\ \text{nm}}\ \text{blank} - \text{OD}_{450\ \text{nm}}\ \text{sample}) \times 12.8 \times 10^{6}}{\text{incubation time (min)} \times (\text{cm}^{-1}\ \text{M}^{-1})}.$$

Note that dimethylaminobenzaldehyde is highly allergenic and that its bioavailability is increased by acetonitrile. Therefore, gloves should be worn to avoid skin contact.

General Procedure for NAT-Radiolabeled Assay

A buffer was prepared (Buffer 2) containing 450 mM Tris, pH 8.0, at 37°C, 4.50 mM DTE, 4.5 mM EDTA, 22.5 mM $MgCl_2$, 0.9 mM coenzyme A, 22.5 mM ATP, 2.25 mM [^{3}H]acetate (sp act: 2 Ci/mmol), and 1.5 U/ml acetyl-CoA synthetase (EC 6.2.1.1). For each sample, 20 μl buffer 2, 50 μl of enzyme preparation, and 10 μl of a buffer containing 100 mM Tris, 1 mM EDTA, and 2 mM DTE at pH 7.8 were preincubated at 37°C in 2-ml polypropylene microfuge tubes for 5 min. The reaction was started by the addition of a 10-μl aliquot of 4.5 mM 2-aminofluorene in 50% DMSO. Sample blanks were run simultaneously by substituting a 10-μl aliquot of 50% DMSO without AF. The reaction was stopped by the addition of 10 μl of a 5 N NaOH/100 mM sodium acetate solution. The [^{3}H]acetylaminofluorene formed was extracted by shaking the samples vigorously in a horizontal position for 10 min on a New Brunswick Scientific laboratory rotator with 1.5-ml chloroform containing 10 μmol AAF.

The two phases were separated by a 1-min centrifugation in an Eppendorf/Brinkman centrifuge, Model 3200. The organic layer was transferred to glass scintillation minivials and evaporated. To redissolve each sample, 300 μl of DMSO was added followed by the addition of 3 ml Omnifluor/toluene-based scintillation cocktail. Samples were counted on a Tracor Mark III Model 6881 liquid scintillation counter. The sample activity was calculated using the activity determined for a known amount of [^{3}H]acetate from the equation

$$\frac{\text{nmol amine acetylated}}{\text{min}} = \frac{\text{dpm sample} - \text{dpm blank}}{(\text{dpm std.} - \text{dpm blank}) \times \text{incubation time (min)}}.$$

Determination of the Solubility of Different Arylamines

To 70 μl of buffer 1, 20 μl of a solution was added containing 225 mM Tris/HCl, pH 7.5, at 37°C, up to 22.5% (v/v) DMSO, ethanol, or acetone and several increasing concentrations of the arylamine. The samples were incubated for 30 min at 37°C and centrifuged for 1 min in an Eppendorf/Brinkmann centrifuge, Model 3200. Depending on the arylamine concentration, suitable aliquots of the supernatant were withdrawn and buffer 1 was added to a final volume of 90 μl. Fifty microliters of 20% (w/v) trichloroacetic acid and 500 μl of 5% (w/v) dimethylaminobenzaldehyde in acetonitrile were added. The samples were incubated for at least 10 min and the absorbance was monitored at 450 nm against a blank in which the arylamine was omitted. To estimate the solubility limit for a particular compound, the theoretical concentration was compared with the determined concentration, calculated from the known molecular extinction coefficient.

Protein Determination

Mouse blood protein concentrations were determined with a modified biuret assay described by C. Watters using a bovine serum albumin standard (16). To obtain valid protein readings in hemoglobin-containing samples, a sample blank was monitored in addition to the reagent blank. The sample blank contained sodium tartrate and sodium hydroxide, but no copper sulfate. The sum of both blank values was subtracted from the sample reading.

RESULTS AND DISCUSSION

Development of a spectrophotometric detection system. To carry out structure activity studies with NAT for a wide variety of arylamines, a detection method applicable to any arylamine was desired. When developing such a detection method, either product formation or substrate disappearance can be measured. For routine work, derivatization of the amino group of the substrate with dimethylaminobenzaldehyde and spectrophotometrical quantification of the derivative was selected because

1. The formation of a Schiff's base is a chemically well-characterized one-step reaction (Fig. 1B).
2. The Schiff's bases for all arylamines show broad absorption optima around 450 nm.
3. The formation of Schiff's base is completed within seconds and the adduct is stable for at least 1 h.
4. Schiff's base formation is linear versus arylamine concentration up to 0.2 mM, and,
5. most arylamine derivatives have high molecular extinction coefficients in the range 50×10^3 cm^{-1} M^{-1} (Table 2).

Note that this method cannot be used with diamines like phenylendiamine, benzidine, methylene dianiline, or dapsone. With these compounds, both diamine substrate and the mono-N-acetyldiamine product react with dimethylaminobenzaldehyde forming adducts with different molecular extinction coefficients. Because the relative amount of each compound is not known, a quantitative estimate of the enzymatic acetyl transfer for each step is impossible. This is a common feature of any method that involves the de-

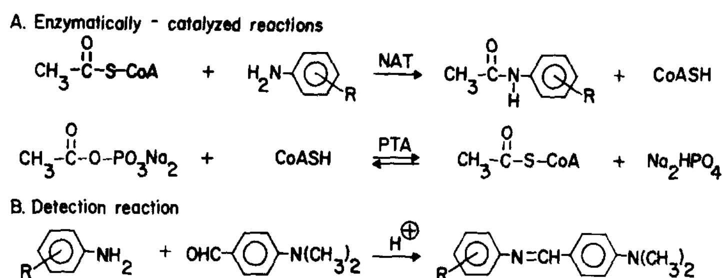


FIG. 1. Reaction scheme of the *N*-acetyltransferase assay. (A) The acceptor amine is acetylated by NAT using acetyl-CoA as acetyl donor. The product CoASH is recycled to acetyl-CoA by the acetyl phosphate/phosphotransacetylase system. (B) The enzymatic activity is measured by quantifying the disappearance of the arylamine substrate as reflected by decreasing Schiff's base formation with dimethylaminobenzaldehyde.

rivatization of amino groups of the substrate, such as the Bratton–Marshall method. Only procedures which include product separation prior to quantification will give significant results for diamines.

Optimization of the enzymatic reaction system. As discussed in the introduction, measurement of NAT activity at V_{max} requires low and constant levels of acetyl-CoA and prevention of CoASH inhibition. These two features are combined in the "recycling system" shown in Fig. 1A. CoASH produced in the NAT reaction is immediately recycled to acetyl-CoA by the acetyl phosphate/phosphotransacetylase system. Phosphotransacetylase (PTA) was chosen because of its broad pH optimum which allows adjustments of pH to optimize NAT activity without sacrificing the auxiliary enzyme activity. In addition, PTA is commercially available at high specific activity and low cost.

Using this recycling system, one should be aware that acetyl phosphate is capable of chemically acetylating amines. This reaction is favored at high concentrations of acetyl phosphate, with prolonged incubation at elevated temperature, at high pH, and with amines having high pK_a values.

Using 5 mM acetyl phosphate and aniline as the acceptor amine ($pK_a = 4.6$), no significant nonenzymatic acetylation was observed after 1 h at 37°C in buffers ranging from pH 7.0–8.5. Although nonenzymatic acetylation may occur with a more basic amine, the

TABLE 2

MOLECULAR EXTINCTION COEFFICIENTS AT 450 nm FOR THE REACTION PRODUCT OF *p*-DIMETHYLAMINOBENZALDEHYDE WITH VARIOUS ARYLAMINES

Aniline derivative	$E_{450\ nm} \times 10^3$ (cm^{-1} M^{-1})
-H	48.8 ± 0.4
2-CH_3	50.5 ± 0.8
3-CH_3	48.6 ± 0.3
4-CH_3	52.5 ± 0.1
2-C_2H_5	20.0 ± 0.2
3-C_2H_5	50.2 ± 2.1
4-C_2H_5	49.3 ± 0.3
2-SO_3H	64.1 ± 0.2
3-SO_3H	54.2 ± 0.4
4-SO_3H	60.2 ± 0.1
2-COOH	48.0 ± 0.3
3-COOH	61.9 ± 0.5
4-COOH	62.5 ± 0.3
4-F	47.7 ± 0.5
4-Cl	56.8 ± 0.6
4-Br	56.7 ± 1.6
4-I	62.1 ± 0.5
2,3-Dimethyl	20.1 ± 0.5
2,4-Dimethyl	23.3 ± 0.2
2,5-Dimethyl	21.5 ± 0.3
2,6-Dimethyl	4.5 ± 0.1
3,4-Dimethyl	51.9 ± 0.4
3,5-Dimethyl	49.0 ± 0.3
4-CN	46.5 ± 0.9
4-CF_3	52.7 ± 1.3
4-OCH_3	52.9 ± 0.2
2-C_3H_7	19.0 ± 0.3
Procainamide	62.1 ± 2.4
Sulfamethazine	63.4 ± 0.3
Sulfapyridine	59.3 ± 0.2
Sulfadiazine	56.9 ± 1.0
p-Aminosalicylic acid	52.6 ± 0.6

Note. Values are means ± SD; $n = 4$.

acetyl phosphate concentration cannot be lowered when using 0.1–0.2 mM acetyl-CoA, because at least a 25-fold excess is needed to shift the equilibrium of the PTA reaction to favor the formation of acetyl-CoA. The acetyl phosphate/PTA recycling system can be replaced by either acetyl-*O*-carnitine/acetyl-CoA:carnitine *O*-acetyltransferase (EC 2.3.1.7) or by acetate, ATP/acetyl-CoA:synthetase systems. In the former system, the acetylation reagent, acetyl-*O*-carnitine, is an oxygen ester with a lower free energy of hydrolysis than that of the mixed anhydride acetyl phosphate. This prevents nonenzymatic acetylation under assay conditions. In the latter system, the energy to synthesize acetyl-CoA is derived from the anhydride ATP. ATP is not readily saponified by aniline derivatives and even if saponification occurs, the phenylphosphoramide formed decomposes in aqueous solution, preventing a persistant chemical modification of the free amine. Unfortunately, the latter recycling system can only be used at pH values above pH 7.0 due to the pH optimum of acetyl-CoA synthetase. With 5 mM acetyl-*O*,L-carnitine and 1 U/ml acetyl-CoA:carnitine *O*-acetyltransferase identical results have been obtained compared with the acetylphosphate/PTA recycling system (data not shown).

The newly developed assay system was optimized for purified rabbit liver NAT using 4-methylaniline as the model substrate. With 0.1 mM acetyl-CoA, a K_m of 25 μM was determined for this particular acceptor amine. Enzyme activity was linear over time and with varying protein concentrations. The assay was linear until three-fourths of the acceptor amine was converted to its acetylated derivative, or at least 1 h (Fig. 2B). After an incubation of 1 h, activities as low as 0.4 nmol 4-methyl acetanilide formed/min could be detected. Because linearity with time and concentration was achieved with this assay, a single time point was sufficient to calculate the initial reaction velocity. In similar experiments with the previously used assay, which uses high concentrations of acetyl-CoA and low concentrations of acceptor amine, activity was measured at multiple time points and the initial velocity was determined from the slope of a tangent to the curve at time zero (Fig. 2A).

To ensure that the new assay was capable of measuring NAT activity in whole homogenates, we determined NAT activity in 10,000*g* supernatants of liver homogenates from 38 New Zealand White rabbits. We found that slow acetylators had a mean specific activity of 6.2 ± 3.2 (SD, n = 7), rapid acetylators 140 ± 14 (SD, n = 6), and animals with intermediate activity 53.2 ± 19.1 (SD, n = 25) nmol/min/g liver wet wt. In no case was overlap between these groups observed.

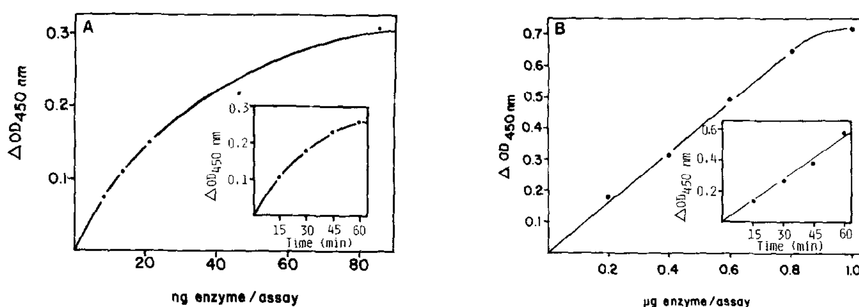


FIG. 2. Comparative *N*-acetyltransferase activity determination with the old and the improved assays using purified rabbit liver enzyme. (A) The dependence of the activity on protein is shown for the old *N*-acetyltransferase assay using 2.2 mM acetyl-CoA and 44 μM acceptor amine. In the inset, the relationship between activity and time are depicted. The results represent means from four experiments; variation coefficient was ± 5%. (B) The same plot for the improved procedure using 0.1 mM acetyl-CoA, 0.2 mM 4-methylaniline and the acetylphosphate/phosphotransacetylase recycling system. The results represent means from four experiments; variation coefficient was ± 2%.

This is in accordance with genetic analysis described before (17), in which a trimodal distribution of NAT activity was observed in the rabbit population.

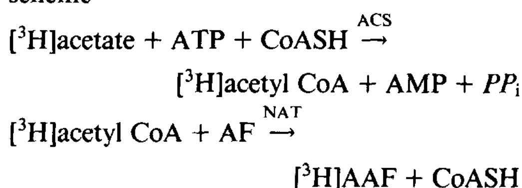
Our laboratory has a long-standing interest in the development of genetic animal models for the human acetylation polymorphism, including an inbred mouse model. We thus adapted the assay to small blood samples enabling us to determine the phenotype of live mice. Figure 3 shows the frequency distribution curve for PABA–NAT activity from C57BL/6J, A/J, and $B6.AF_1$ mice. The mean specific activity for A/J (slow acetylator) mice was 0.09 ± 0.08 (SD, $n = 9$), for $B6.AF_1$ (heterozygous) mice 1.05 ± 0.24 ($n = 14$), and for C57BL/6J 1.80 ± 0.14 ($n = 16$) nmol/min/mg protein.

The applicability of the modified assay was also examined with another tissue of interest. Homogenates of individual mouse bladders assayed for NAT activity showed 2.87 ± 0.42 (SD, $n = 12$) nmol/min/mg protein for C57BL/6J and 1.73 ± 0.14 (SD, $n = 9$) nmol/min/mg protein for A/J mice.

The spectrophotometric assay thus appears to be applicable to a wide range of acceptor amines and biological systems.

Development of a radiochemical-detection system. In some instances, measurement of product formation rather than substrate disappearance is necessary, or preferable. Problems arise with the spectrophotometric assay when acceptor amine concentrations greater than 0.3 mM are needed to saturate the enzyme. When using high concentrations of acceptor amine, large amount of Schiff's base are formed which produce intensely colored solutions preventing accurate absorbance measurements. For such cases a radiochemical assay system has been developed which measures the production of *N*-acetylarylamine rather than substrate disappearance. To optimize this assay we used the model carcinogen 2-aminofluorene (AF).

The radiochemical assay uses the reaction scheme



$$[^3H]\text{acetate} + \text{ATP} + \text{CoASH} \xrightarrow{\text{ACS}} [^3H]\text{acetyl CoA} + \text{AMP} + PP_i$$

$$[^3H]\text{acetyl CoA} + \text{AF} \xrightarrow{\text{NAT}} [^3H]\text{AAF} + \text{CoASH}$$

Acetyl-CoA synthetase and [^{3}H]acetate were used instead of the phosphotransacetylase/acetyl phosphate system because, (i) radiolabeled [^{3}H]acetyl phosphate is not readily available and (ii) using [^{3}H]acetyl-CoA and nonradiolabeled acetyl phosphate would result in a decrease in specific activity of the acetyl-CoA as the reaction proceeds. Tritiated acetate, which is readily available at high specific activity, enables a constant specific activity of [^{3}H]acetyl-CoA to be maintained throughout the reaction. Thus, the two major difficulties set forth above, i.e., AcCoA depletion and CoA inhibition, are both satisfactorily avoided. Separation of [^{3}H]AAF from [^{3}H]acetate is readily accomplished by a single extraction with chloroform. The extraction efficiencies of acetate and AAF were determined with radiolabeled standards. AAF was found to have a 95% extraction efficiency whereas less than 0.5% acetate was extracted. Measurement of [^{3}H]acetyl CoA extraction was unnecessary since the [^{3}H]AcCoA produced is hydrolyzed to [^{3}H]acetate and CoASH by the addition of NaOH to stop the reaction.

AF, like many other arylamines, is sparingly soluble in aqueous solutions, and DMSO was used to increase the solubility. Solubility was assessed by the procedure de-

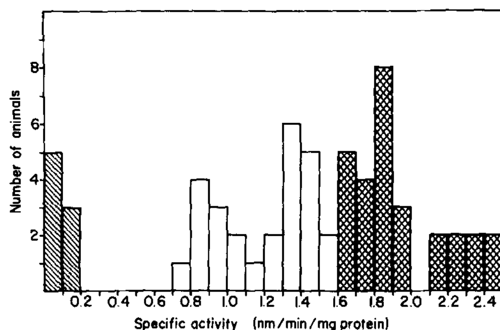


FIG. 3. Frequency distribution curve for blood PABA *N*-acetyltransferase activity from C57BL/6J (■), A/J (▨), and $B6.AF_1$ (□) mice. Activity was determined with 0.2 mM acetyl-CoA and 0.1 mM PABA.

scribed under Materials and Methods which is generally applicable to arylamines. However, NAT activity decreased as the concentration of DMSO increased in the reaction mixture. DMSO inhibition of NAT was found to be concentration dependent rather than time dependent. Other solvents such as acetonitrile, ethanol, and dimethylformamide also inhibited NAT activity. Therefore, when selecting a solvent, depression of NAT activity should be assessed and care must be taken that the concentration used is sufficient to keep the acceptor amine in solution, but not in such excess that NAT activity is greatly affected. We found that DMSO concentrations of about 5% met these criteria, allowing AF to remain soluble under the assay conditions up to a concentration of 0.5 mM.

Using purified rabbit liver NAT, [^{3}H]AAF formation in this assay depended on enzyme concentration (Fig. 4). The rate of formation of [^{3}H]AAF was linear through 30 min (Fig. 4, inset), although 10-min incubations were sufficient to produce accurate measurements of enzyme activity. The limit of detection was 0.1 nmol AAF produced/min when incubating 10 min. The K_m determined for AF using this assay was 0.5 mM at 0.2 mM acetyl CoA. This K_m value was identical to that found with the spectrophotometric procedure.

We have developed specific and sensitive methods that can be used to measure NAT activity in a variety of biological systems and with a broad range of acetyl acceptor amine substrates. The arylamine substrates or the arylamide products can be quantified quickly and accurately and NAT activity can be assayed in crude tissue homogenates and blood lysates. The assays were developed to minimize NAT inhibition by CoASH, while recognizing the problems encountered in dealing with enzymes which exhibit ping-pong kinetics.

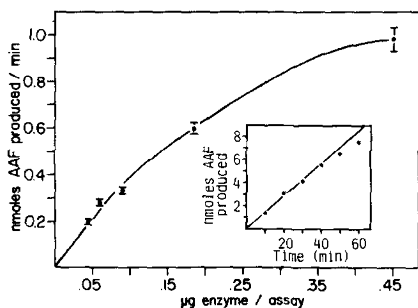


FIG. 4. N-Acetyltransferase activity with aminofluorene as acceptor amine using purified rabbit liver enzyme. Experimental details are given under Materials and Methods. The dependence of the activity on the protein is shown. In the insert, the relationship between activity and time is depicted. The results are means ± SD.

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