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ISOTOPE EXCHANGE STUDIES ON RABBIT LIVER *N*-ACETYLTRANSFERASE

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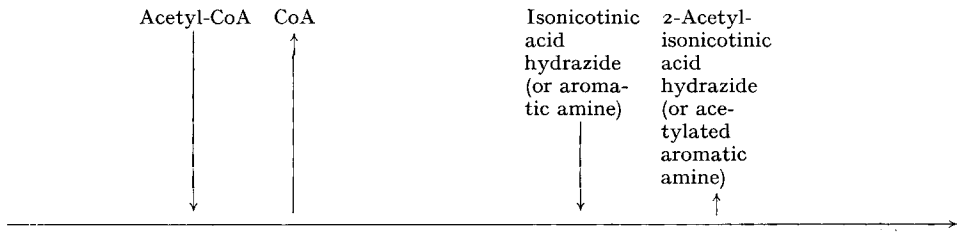
SUMMARY

N-Acetyltransferase from mammalian liver catalyzes the acetylation of isonicotinic acid hydrazide, various sulfonamides and other aromatic amines. Initial velocity and product inhibition studies reported previously indicate that this reaction proceeds according to a simple ping pong Bi–Bi mechanism. It follows that enzymatic acetylation of a drug should consist of two consecutive reactions, the first of which leads to formation of an acetyl–enzyme intermediate and the second to acetylation of the drug with regeneration of the enzyme. In the present study, the existence of these two reactions has been demonstrated by isotope exchange techniques and an acetyl–*N*-acetyltransferase intermediate capable of transferring acetyl groups to isonicotinic acid hydrazide has been isolated.

INTRODUCTION

We have been investigating the kinetic properties of an *N*-acetyltransferase (EC 2.3.1.5) from mammalian liver which catalyzes the acetylation of isonicotinic acid hydrazide (isoniazid), sulfamethazine, sulfadiazine and various structurally similar drugs. This *N*-acetyltransferase and its characteristics are of particular interest because variation in the level of its activity is responsible for the genetically determined variation in the rate of acetylation of these drugs in man[1] and in rabbit[2]. This *N*-acetyltransferase has been purified in our laboratory from liver of man, rabbit, monkey, and rat. Initial velocity and product inhibition studies with rabbit liver *N*-acetyltransferase which we have reported previously led us to the tentative proposal that the acetylation reaction proceeds according to a simple ping pong Bi–Bi mechanism[3]. Detailed studies of the kinetic properties of *N*-acetyltransferase from several other species of mammals including man, monkey and rat indicate that the acetylation of these aromatic amine and hydrazine compounds is probably catalyzed by the same mechanism in all of these species[4]. According to this mechanism, the complete reaction for the acetylation of isonicotinic acid hydrazide, or of an alternate amine substrate may be written as follows:



$$N\text{-Acetyltransferase} + \text{Acetyl-CoA} \rightleftarrows \text{Acetyl–}N\text{-acetyltransferase} + \text{CoA} \quad (1$$

$$\text{Acetyl–}N\text{-acetyltransferase} + \text{Isonicotinic acid hydrazide (or aromatic amine)} \rightleftarrows \text{2-Acetylisonicotinic acid hydrazide (or acetylated amine)} + N\text{-Acetyltransferase} \quad (2$$

where acetyl-*N*-acetyltransferase represents an acetyl–enzyme intermediate formed in the first reaction and broken down in the second.

In this report, each of these reactions is demonstrated to occur and evidence is presented for the formation of a catalytically active acetyl–enzyme intermediate. These findings together with the results of initial velocity and product inhibition studies reported previously are compatible with a ping pong Bi–Bi reaction mechanism and appear to establish this as the mechanism of acetylation by mammalian liver *N*-acetyltransferase.

MATERIALS AND METHODS

Reagents

The Acetyl-CoA, CoA (96% lithium salt), isonicotinic acid hydrazide and DEAE-cellulose were obtained from Nutritional Biochemicals Corp., Cleveland, Ohio. The dithiothreitol and 5,5′-dithiobis-(2-nitrobenzoic acid) were obtained from CalBiochem, Los Angeles, Calif. Acetanilide (zone refined) was obtained from Aldrich Chemical Company Inc., Milwaukee, Wisc. The [1-^{14}C]acetyl-CoA (58.5 mC/mmole), 2,5-diphenyloxazole and *p*-bis-2-(5-phenyloxazolyl)-benzene were obtained from New England Nuclear Corp., Boston, Mass. The [^{3}H]aniline (115 mC/mmole) was obtained from Amersham Searle, Chicago, Ill. Sephadex G-15 and G-50 were obtained from Pharmacia Fine Chemicals Inc., Piscataway, N.J. DEAE-cellulose and CM cellulose ion-exchange paper were obtained from Reeve Angel, Clifton, N.J. The phosphotransacetylase and acetylphosphate (potassium–lithium salt) were purchased from Boehringer Mannheim Corp., New York, N.Y.

Acetylisonicotinic acid hydrazide was synthesized from isonicotinic acid hydrazide by the procedure of YALE *et al.*[5]. Synthesis of [^{3}H]CoA and acetyl[^{3}H]CoA is described below.

Enzyme purification and assay

N-Acetyltransferase preparations used in this work were purified 300–500-fold by procedures described previously[3] which include ultracentrifugation, ammonium sulfate precipitation, gel filtration and chromatography on DEAE-cellulose. Enzyme

activity was measured with isonicotinic acid hydrazide at pH 9.0[3]. Protein concentration was determined by the method of WARBURG AND CHRISTIAN[6].

Radioactivity measurements

A Nuclear Chicago Mark I scintillation counter was used. All radioactive samples were counted in 15 ml of scintillation fluid prepared by mixing 4.0 g of 2,5-diphenyloxazole and 0.4 g of *p*-bis-2-(5-phenyloxazolyl)-benzene with 1.0 l of toluene and 0.4 l of absolute ethanol. All samples were counted for 40 min.

Preparation of [^{3}H]CoA and acetyl-[^{3}H]CoA

Oxidized CoA (CoA–S–S–CoA) was prepared by allowing 249 μmoles of CoA to react with 113 μmoles of 5,5'-dithiobis-(2-nitrobenzoic acid) at pH 8.0 in 0.01 M potassium phosphate buffer for 20 min at 27°. The reaction mixture was placed on a DEAE-cellulose column (18 cm × 2.0 cm) which had been equilibrated with 0.01 M potassium phosphate buffer, pH 8.0. Elution was carried out with a linear gradient (900 ml) from 0 to 0.4 M LiCl. The CoA–S–S–CoA was present in Tubes 55–76 (10.0 ml fractions) and unreacted CoA was found in Tubes 27–40. Fractions 55–76 were combined and concentrated to a small volume in a lyophilization apparatus (Virtis Research Equipment, Gardiner, N.Y.). This material was desalted by passage through a Sephadex G-15 column (100 cm × 2.5 cm) which had been equilibrated with water.

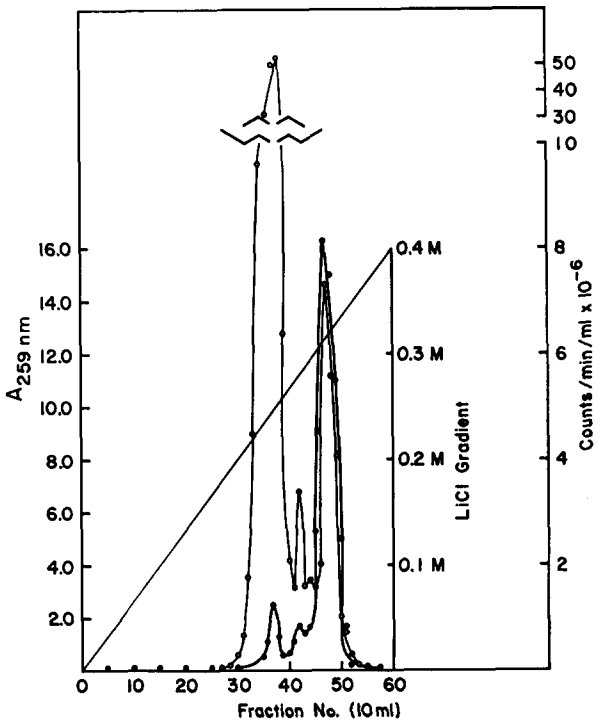


Fig. 1. DEAE-cellulose chromatography of [^{3}H]CoA–S–S–CoA. ●—●, absorbance at 259 nm; ○—○, counts/min per ml × 10^{-6}.

The fractions containing the CoA–S–S–CoA were combined and lyophilized; assay with 5,5'-dithiobis-(2-nitrobenzoic acid) in the presence of arsenite[7] showed that the final product was 97% pure (99 μmoles) CoA–S–S–CoA.

The CoA–S–S–CoA (70 mg) was labeled by exposure to $^3H_2$ at New England Nuclear Corp. The labeled material was lyophilized several times in our laboratory and chromatographed on a DEAE-cellulose column (20 cm × 2.5 cm). Linear gradient elution was performed as described in the previous paragraph and the chromatogram obtained is shown in Fig. 1. Fractions 45–52 were combined, lyophilized and desalted with acetone–methanol[8] and yielded 18 μmoles of [^{3}H]CoA–S–S–CoA. This material was reduced with an excess of dithiothreitol (180 μmoles)[7] and was placed on a DEAE-cellulose column (20 cm × 2.5 cm) previously equilibrated with 0.005 M potassium phosphate buffer, pH 7.0, in $1 \cdot 10^{-4}$ M dithiothreitol. Elution was carried out with a linear gradient (600 ml) from 0 to 0.4 M LiCl in $1 \cdot 10^{-4}$ M dithiothreitol at pH 5.0. The [^{3}H]CoA was found in Tubes 34–46 (10.0 ml fractions) as determined by the absorbance at 259 nm. Fractions 34–46 were combined, titrated to pH 7.0 with LiOH, lyophilized and chromatographed on a Sephadex G-15 column (100 cm × 2.5 cm) previously equilibrated with 0.001 M dithiothreitol at pH 5.0. Tubes 33–36 (6.0-ml fractions) contained the [^{3}H]CoA and were combined, titrated to pH 7.0 with LiOH, lyophilized and desalted with acetone–methanol. The adenine content of the final product was determined by measuring the absorption at 259 nm[7]. In the phosphotransacetylase reaction[9], 76% of the 259 nm absorbing material represented enzymatically active [^{3}H]CoA (8.2 μmoles) with a specific activity of 35.0 mC/mmole. The [^{3}H]CoA was stored at −20° with an excess of dithiothreitol (35 μmoles) at pH 5.0.

Acetyl-[^{3}H]CoA was prepared by acetylation of [^{3}H]CoA in the presence of acetylphosphate[10]. The product was purified on DEAE-cellulose ion-exchange paper by descending chromatography with an aqueous system consisting of 0.2 M LiCl in 0.01 M potassium phosphate buffer, pH 8.0. The R_F of the acetyl-[^{3}H]CoA was 0.82. The final product was assayed with phosphotransacetylase in the presence of arsenate[11] and found to be 87% acetyl-[^{3}H]CoA with a specific activity of 27.0 mC/mmole.

Separation of [^{3}H]CoA and acetyl-[^{3}H]CoA

DEAE-cellulose paper strips (57 cm × 2.0 cm) were washed in 0.01 M potassium phosphate buffer, pH 8.0, and were dried in air. After incubating a mixture of acetyl-[^{3}H]CoA and [^{3}H]CoA with excess 5,5-dithiobis-(2-nitrobenzoic acid) for 15 min, the compounds were separated by descending chromatography on the DEAE-cellulose paper strips using the aqueous LiCl system referred to in the preceeding paragraph. The R_F of the acetyl-[^{3}H]CoA was 0.82 and that of the [^{3}H]CoA, as [^{3}H]CoA 5-thio-2-nitrobenzoic acid, was 0.46.

Separation of [^{3}H]aniline and [^{3}H]acetanilide

Separation of [^{3}H]aniline and [^{3}H]acetanilide was accomplished by descending chromatography (0.01 M glycine buffer, pH 2.8) on CM-cellulose ion exchange strips (57 cm × 2.0 cm) after prewashing them with the same solution and drying in air. The [^{3}H]aniline had an R_F of 0.20 and the [^{3}H]acetanilide had an R_F of 0.74.

RESULTS

Isotope exchange studies

Acetyl-CoA and [^{3}H]CoA were incubated with *N*-acetyltransferase to determine if *N*-acetyltransferase catalyzes the first half of the ping pong Bi–Bi sequence (Eqn. 1) in the absence of the amine substrate–product pair. Aliquots removed from the incubation mixture at the intervals indicated in Fig. 2 showed that acetyl-[^{3}H]CoA was formed. After 70 min had elapsed, the reaction had almost reached equilibrium and by this time 21% (0.2 μC of the ^{3}H label initially present in [^{3}H]CoA had undergone exchange. The second partial reaction (Eqn. 2) was studied by incubating [^{3}H]-

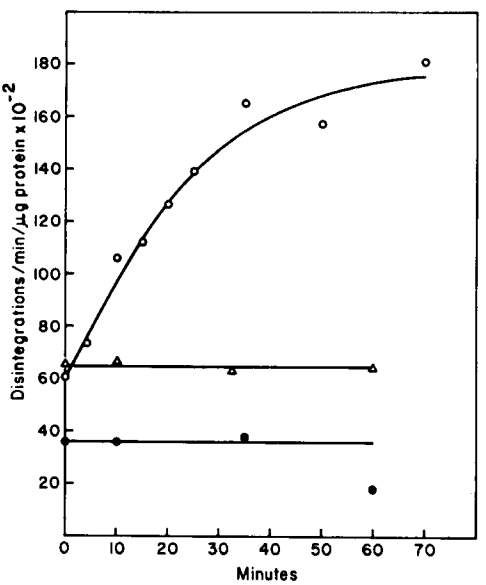


Fig. 2. Exchange reaction between acetyl-CoA and [^{3}H]CoA. The reaction mixture contained 0.027 μmole of [^{3}H]CoA (35 mC/mmole), 2.8 μmoles of acetyl-CoA, 0.10 ml of DEAE-cellulose enzyme fraction (40.6 μg protein, specific activity 0.066 μmole 2-acetylisonicotinic acid hydrazide formed per min per mg protein) and 0.8 M potassium borate buffer, pH 8.0, in a total volume of 0.27 ml of 27°. Aliquots of 0.02 ml were removed and immediately mixed with 0.15 μmole of 5,5′-dithiobis- (2-nitrobenzoic acid) (0.05 ml) to stop the reaction. Aliquots of 0.01 ml were then removed from this mixture and the acetyl-[^{3}H]CoA was separated from the [^{3}H]CoA on DEAE-cellulose ion-exchange paper as described in MATERIALS AND METHODS. The acetyl-[^{3}H]CoA area was cut out, placed in a counting vial, eluted with 0.20 ml of 0.6 M HCl and counted. ○, complete reaction mixture; ●, mixture without acetyl-CoA; △, mixture with enzyme previously inactivated by heat at 55° for 15 min.

aniline and acetanilide with *N*-acetyltransferase in the absence of acetyl-CoA and CoA. As shown in Fig. 3, labeled acetanilide was formed. By 70 min after the reaction was started, 54% (0.12 μC) of the label had undergone exchange. We would have preferred to demonstrate the second reaction with isonicotinic acid hydrazide and 2-acetylisonicotinic acid hydrazide but this was not possible since isotopically labeled isonicotinic acid hydrazide of adequate purity for this experiment was not available. It was possible, however, to show that exchange of the acetyl group between 2-

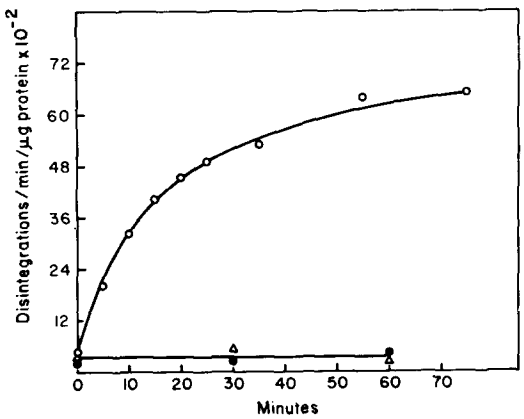


Fig. 3. Exchange reaction between [^{3}H]aniline and acetanilide. The reaction mixture contained 0.0019 μmole of [^{3}H]aniline (115 mC/mmole), 6 μmoles of acetanilide, 0.10 ml of DEAE-cellulose enzyme fraction (40.6 μg protein, specific activity 0.066 μmole 2-acetylisonicotinic acid hydrazide formed per min per mg protein) and 0.10 M sodium pyrophosphate buffer, pH 8.0, in a total volume of 0.305 ml at 27°. Aliquots of 0.01 ml were removed and were spotted onto CM-cellulose strips. The spots were treated with 0.005 ml of acetone–absolute ethanol (1:1, by vol.) to stop the reaction. The [^{3}H]acetanilide was separated from the [^{3}H]aniline as described in MATERIALS AND METHODS. The [^{3}H]acetanilide area was cut out, placed in a counting vial, eluted with 0.20 ml of 0.6 M HCl and counted. ○, complete reaction mixture; ●, mixture without acetanilide; △, mixture with enzyme previously inactivated by heat at 55° for 15 min.

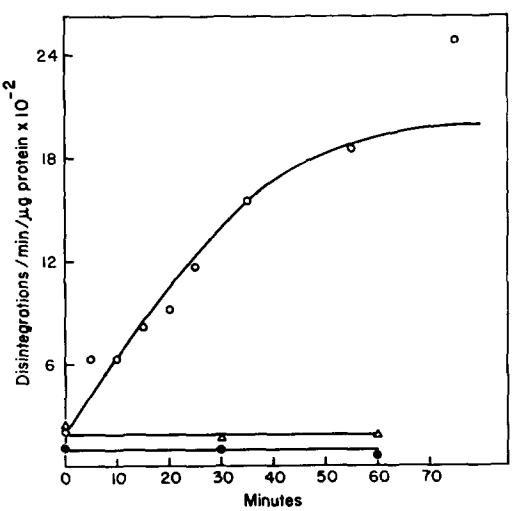


Fig. 4. Exchange reaction between [^{3}H]aniline and 2-acetylisonicotinic acid hydrazide. The reaction mixture contained 0.0029 μmole of [^{3}H]aniline (115 mC/mmole), 20 μmoles of 2-acetylisonicotinic acid hydrazide, 0.10 ml of DEAE-cellulose enzyme fraction (54.8 μg protein, specific activity 0.049 μmole 2-acetylisonicotinic acid hydrazide formed per min per mg protein) and 0.10 M sodium pyrophosphate buffer, pH 8.0, in a total volume of 0.305 ml at 27°. The remainder of the procedure was identical with that described in Fig. 3. ○, complete reaction mixture; ●, mixture without 2-acetylisonicotinic acid hydrazide; △, mixture with enzyme previously inactivated by heat at 55° for 15 min.

acetylisonicotinic acid hydrazide and [^{3}H]aniline was catalyzed by the enzyme (Fig. 4). In this reaction, by 60 min, approx. 13% (0.045 μC) of the label had undergone exchange.

Isolation of an acetyl–enzyme intermediate with catalytic activity

Labeled acetyl-CoA ([1-^{14}C]acetyl-CoA) and *N*-acetyltransferase were incubated together for 12 min at 27°. The incubation mixture was then filtered through Sephadex G-50 (Fig. 5a) and approx. 0.13 nmole of [1-^{14}C]acetyl label (0.063 nmole/mg protein) was found to be associated with protein in Fractions 10–15. The uncombined [1-^{14}C]acetyl-CoA was found in Fractions 20–30. The extent of association between the label and protein was markedly reduced after the enzyme preparation had been inactivated by heating for 15 min at 55° prior to incubation with [1-^{14}C]acetyl-CoA (Fig. 5b).

The ^{14}C label found in Fractions 10–15 could have been in the form of either

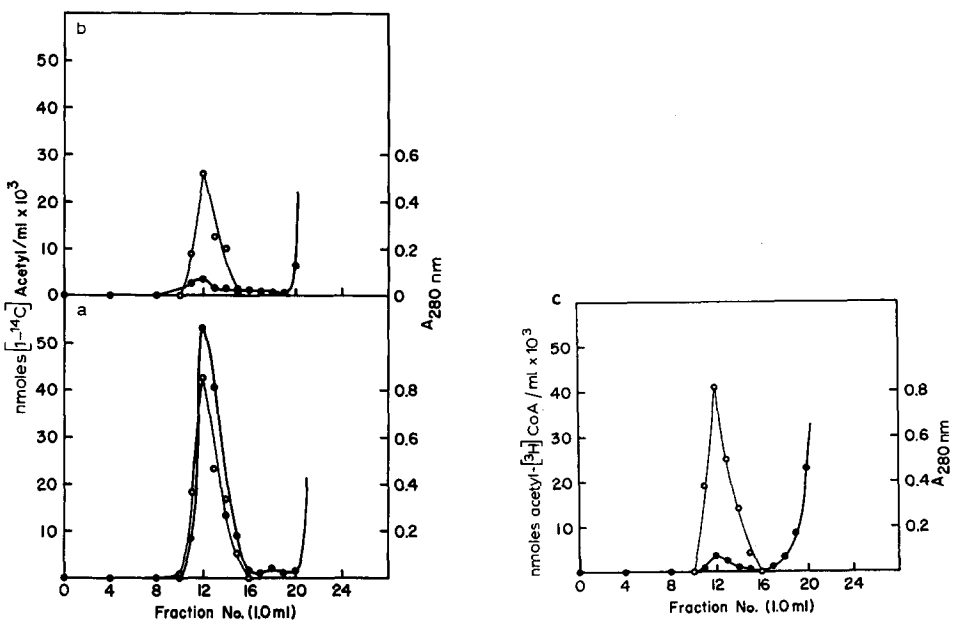


Fig. 5. Formation and isolation of a [1-^{14}C]acetyl–*N*-acetyltransferase intermediate. The DEAE-cellulose enzyme fraction (2.5 mg protein, specific activity 0.0395 μmole 2-acetylisonicotinic acid hydrazide formed per min per mg protein) was mixed with 16.4 nmoles of [1-^{14}C]acetyl-CoA (58.5 mC/mmole) and 0.01 M potassium phosphate buffer, pH 7.0, in a total volume of 0.40 ml. After 12 min of incubation at 27° the reaction mixture was cooled to 0°, placed on a Sephadex G-50 column (1.5 cm × 18.5 cm) and eluted with 0.01 M potassium phosphate buffer, pH 7.0, at 0°. A flow rate of 1.0 ml/min was maintained and fractions of 1.0 ml each were collected over a 20-min period. The fractions were analyzed for radioactivity and for absorbance at 280 nm (a). The experiment was repeated with enzyme previously inactivated by heat at 55° for 15 min (b). In c, DEAE-cellulose enzyme fraction (2.5 mg protein, specific activity 0.0395 μmole 2-acetylisonicotinic acid hydrazide formed per min per mg protein) was mixed with 43.8 nmoles of acetyl-[^{3}H]CoA (27.0 mC/mmole) and 0.01 M potassium phosphate buffer, pH 7.0, in a total volume of 0.40 ml. After 12 min of incubation at 27° the reaction mixture was cooled to 0°, filtered through the Sephadex G-50 column and the fractions collected were analyzed as described above. ○—○, absorbance at 280 nm; ●—●, nmoles of [1-^{14}C]acetyl/ml × 10^3 or acetyl-[^{3}H]CoA/ml × 10^3.

[1-^{14}C]acetyl or [1-^{14}C]acetyl-CoA attached to either *N*-acetyltransferase or to non-*N*-acetyltransferase protein. To determine whether the label was in the form of [1-^{14}C]acetyl or [1-^{14}C]acetyl-CoA, an experiment similar to that shown in Fig. 5a was performed using acetyl-[^{3}H]CoA, *i.e.* acetyl-CoA with the label in the CoA portion of the molecule, in place of [1-^{14}C]acetyl-CoA. In this experiment, the acetyl-[^{3}H]CoA was more than twice as concentrated as the [1-^{14}C]acetyl-CoA in the previous experiment. Even so the results obtained (Fig. 5c) show that only 0.004 nmole, or 0.002 nmole/mg protein, of acetyl-[^{3}H]CoA was bound to the protein in these fractions. This finding indicates that the label in Fractions 10–15 of Fig. 5a probably is in the form of [1-^{14}C]acetyl and not in the form of intact acetyl-CoA.

The *N*-acetyltransferase obtained in Fractions 10–15 is not a homogeneous preparation of the enzyme as judged by the presence of several bands of non-enzymatic protein in polyacrylamide/agarose electrophoresis patterns[12]. It was important

TABLE I

ACETYLATION OF ISONICOTINIC ACID HYDRAZIDE BY THE [1-^{14}C]ACETYL–*N*-ACETYLTRANSFERASE INTERMEDIATE

DEAE-cellulose enzyme fraction (1.6 mg protein, specific activity 0.114 μmole 2-acetylisonicotinic acid hydrazide formed per min per mg protein) was mixed with 32 nmoles of [1-^{14}C]acetyl-CoA (58.5 mC/mmole) and 0.01 M potassium phosphate buffer, pH 6.8, in a total volume of 0.40 ml. After 12 min of incubation at 27°, [1-^{14}C]acetyl–*N*-acetyltransferase was isolated as described in Fig. 5a. Then *N*-ethyl maleimide (1.0 μmole) was added and was incubated with the mixture for 1 min at 27° to inhibit the activity of any unacetylated *N*-acetyltransferase which might still be present. Isonicotinic acid hydrazide (1.0 μmole) was then added to this reaction mixture which was incubated for 30 min at the same temperature. A control without isonicotinic acid hydrazide was treated in the same manner. The experimental and control mixtures were then evaporated to dryness under a stream of N_2 at 65° and each residue was dissolved in 0.20 ml of 0.1 M HCl. The 2-[1-^{14}C]acetylisonicotinic acid hydrazide was isolated by descending chromatography on CM-cellulose ion-exchange paper with 0.01 M sodium acetate buffer, pH 5.0. The R_F of 2-[1-^{14}C]-acetylisonicotinic acid hydrazide was 0.58 and was identical with that for authentic 2-acetylisonicotinic acid hydrazide.

(1-^{14}C)Ac–NAT complex added (counts/min)	*NEM*	*INH*	*(1-^{14}C)-Acetyl-INH formed (counts/min)*	*Recovery (%)*
24 700	+	+	6500	26
24 700	+	−	440	2

Abbreviations: Ac–NAT, acetyl–*N*-acetyltransferase; NEM, *N*-ethyl maleimide; INH, isonicotinic acid hydrazide.

to determine, therefore, whether Fractions 10–15 were catalytically active; *i.e.* could transfer the acetyl group to isonicotinic acid hydrazide. In previous experiments, we had shown that *p*-chloromercuribenzoate, *N*-ethylmaleimide and various other sulfhydryl inhibitors quickly inactivated *N*-acetyltransferase[3] and that pretreatment of the enzyme with acetyl-CoA protected it against the action of these compounds. Fractions 10–15 were combined and *N*-ethylmaleimide was added to inhibit any residual unacetylated *N*-acetyltransferase which might have been present. Isonicotinic acid hydrazide was then added to this reaction mixture and incubated for 30 min. The results in Table I show that isonicotinic acid hydrazide was acetylated under these conditions.

DISCUSSION

The concept that enzymatic acetylation is a stepwise process involving the formation and breakdown of an acetylated enzyme intermediate has been suggested by several investigators. BESSMAN AND LIPMANN[13] observed that a pigeon-liver preparation reversibly catalyzed the acetyl exchange between 4-acetaminoazobenzene-4′-sulfonic acid and various arylamines including aniline, *p*-aminobenzoic acid and sulfanilamide and they deduced that an acetyl–enzyme compound was formed as an intermediate in this reaction. From additional studies, they proposed that acetyl transfer from acetyl-CoA to aromatic amines was carried out by a similar mechanism and concluded that the primary reaction involving the acetyl transfer from acetyl-coenzyme A to the enzyme was irreversible. Later, JENNE AND BOYER[14] extended this work with the pigeon liver enzyme by carrying out initial velocity studies with acetyl-CoA and isonicotinic acid hydrazide as substrates. Double reciprocal plots of initial velocity *versus* substrate concentration at various fixed concentrations of the other substrate gave sets of virtually parallel lines. They proposed a multistep reaction mechanism compatible with these findings which involved transfer of the acetyl groups from acetyl-CoA to isonicotinic acid hydrazide *via* an acetyl–enzyme intermediate. The mechanism they proposed can be included in the group of ping pong Bi–Bi mechanisms[15].

Initial velocity studies of the acetylation of isonicotinic acid hydrazide carried out in our laboratory with partially purified liver *N*-acetyltransferase from several mammalian species are compatible with this mechanism in these species as well[4]. Initial velocity data alone are consistent with either a simple or an iso-ping pong Bi–Bi mechanism[15], the only difference being isomerization of an acetyl-CoA–enzyme complex in the latter. Product inhibition studies of the reaction with rabbit-liver *N*-acetyltransferase, however, appeared to eliminate the mechanism containing an isomerization step as a reasonable alternative to the simple ping pong Bi–Bi mechanism[3].

As the number and variety of enzymes which have been studied by these kinetic techniques has increased, certain limitations have become apparent in the interpretation of such data[16]. Initial velocity patterns of the same type as those characteristic of a ping pong Bi–Bi mechanism have been observed for reactions which proceed by other mechanisms[16,17]. Furthermore, the type of product inhibition pattern found may depend upon the experimental conditions of a study[16]. It should be pointed out in this connection that our product inhibition studies referred to above could not be conducted over a wide range of concentration of the product inhibitor owing to experimental limitations.

In view of possible ambiguities in the interpretation of the initial velocity and product inhibition patterns, the studies on the mechanism of enzymatic drug N-acetylation described in this report were carried out. The data presented in Figs. 2 and 3 show that partially purified *N*-acetyltransferase from rabbit liver catalyzes the exchange between acetyl-CoA and [^{3}H]CoA in the absence of the drug substrate (Eqn. 1) and the exchange between [^{3}H]aniline and acetanilide in the absence of acetyl-CoA and CoA, (Eqn. 2). Furthermore, as shown in Fig. 5, we obtained evidence for the existence of an acetyl–*N*-acetyltransferase intermediate.

The results in Fig. 5a indicate that approx. 0.13 nmole of [1-^{14}C]acetyl residues

were bound to protein in fractions containing the enzyme. This would be equivalent to approx. 0.015 mole of acetyl residue per mole of *N*-acetyltransferase if 37 000 is taken as the molecular weight for the enzyme (R. BAUMGARTNER AND S. N. COHEN, unpublished results) and if it is assumed that the protein in these fractions was homogeneous *N*-acetyltransferase. These fractions are known, however, to be inhomogeneous preparations of *N*-acetyltransferase as noted above. The low apparent value obtained for the number of acetyl residues bound per mole of *N*-acetyltransferase is probably attributable in part at least to the presence of other protein impurities in these fractions. It is also possible that the acetyl–*N*-acetyltransferase intermediate has a very short life under the conditions of isolation and may have undergone decomposition during this procedure. The isolation of the intermediate was performed at pH 7.0 which is within the optimal pH range for *N*-acetyltransferase activity[3] and the intermediate might therefore be expected to be relatively unstable at this pH. Indeed, it was noted during preliminary attempts to isolate the intermediate that yields of catalytically active intermediate were lower than the result given in Table I whenever the time for isolation exceeded 30 min.

ACKNOWLEDGMENTS

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