N-Acetylation of Drugs: Isolation and Properties of an N-Acetyltransferase from Rabbit Liver

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(Received January 9, 1967)

SUMMARY

The characteristics and kinetic properties of an *N*-acetyltransferase have been studied in partially purified preparations from rabbit liver. The enzyme catalyzes the acetylation of isonicotinic acid hydrazide, sulfadiazine, sulfamerazine, sulfamethazine, and sulfanilamide. A "ping-pong Bi-Bi" mechanism, which involves the formation of an acetylated enzyme, is proposed for the acetylation of INH from our studies of initial velocity patterns and product inhibition. The possible limitations of the Michaelis constant (K_m) as a means of characterizing an enzyme which catalyzes "ping-pong Bi-Bi" reactions or other reactions involving two or more substrates and two or more products are noted.

INTRODUCTION

Inactivation of a drug by conjugation is complex because it involves two (or more) substrates and yields two (or more) products. Relatively few drug conjugation mechanisms have been analyzed in detail. This report contains data on the purification and characteristics of an enzyme from rabbit liver which catalyzes the conjugation of isonicotinic acid hydrazide (INH) and several sulfonamides, including sulfadiazine (SD) and sulfamethazine (SM), with the acetyl group from acetyl coenzyme A. Kinetic properties of this *N*-acetyltransferase have been determined and on the basis of initial rate data and product inhibition studies (1, 2), a mechanism is proposed for the acetylation reaction.

MATERIALS AND METHODS

Reagents. SM, INH, acetyl coenzyme A, and coenzyme A were obtained from Nutritional Biochemicals. We wish to thank Merck Sharp and Dohme Laboratories, Rahway, New Jersey, and Lederle Laboratories, Pearl River, New York, for samples of *N*-4-acetyl SM. We wish to thank Hoffmann-La Roche, Inc., Nutley, New Jersey for a sample of 2-acetyl INH.

Enzyme assays. INH and SM were used as substrates to assay for the presence of *N*-acetyltransferase activity. Activity was determined with INH by following the rate of appearance of 2-acetyl INH at 303 mμ (3) in either a Beckman DU-2 spectrophotometer or a Gilford Model 2000 automatic recording spectrophotometer. The experimental cuvette contained INH (1.0 μmole), acetyl coenzyme A (0.5 μmole), an aliquot of enzyme, and borate buffer, 0.8 M, pH 9.0 in a final volume of 1.0 ml. Acetyl coenzyme A was omitted from the control cuvette. The reaction was started by the addition of the acetyl coenzyme A to the experimental cuvette.

Activity was determined with SM by measuring the disappearance of free sulfonamide in an experimental tube compared with the concentration of free drug in a control by a modification of the Bratton-

[1] Recipient of Career Scientist Award of the Health Research Council of the City of New York under contract No. I-441.

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Marshall method (4). The experimental tube contained SM (0.03 μmole), acetyl coenzyme A (0.20 μmole), an aliquot of enzyme and phosphate buffer, 0.10 M, pH 6.8. Acetyl coenzyme A was omitted from the control tubes. The reaction was started with SM and stopped during the zero-order phase (usually after 10 min) by the addition of 2.0 ml of trichloroacetic acid to a final concentration of 3–5%.

All assays were conducted at 27°.

Activity was determined with the various sulfonamides by the method outlined above at all pH's, but it was necessary to modify the assay for activity with INH below pH 9.0. Under these conditions the amount of 2-acetyl INH formed at the lower pH's was determined spectrophotometrically by removing aliquots at intervals during the zero-order phase of the reaction and transferring them to cold borate buffer, 0.8 M, pH 9.0.

Activity was expressed as micromoles of drug acetylated per minute per milliliter of enzyme fraction. Protein concentration was determined by the method of Warburg and Christian (5) on all enzyme fractions except for determinations on tissue homogenates, for which the method of Lowry (6) was used.

Enzyme purification. *N*-Acetyltransferase was purified from rabbit liver as shown in Table 1. New Zealand White rabbits, classified as rapid inactivators of SD (genotypes RR or Rr) (7), were sacrificed by a sharp blow on the head. All steps in purification were carried out between 0 and 5°. The liver was removed immediately and homogenized with four volumes of phosphate buffer, 0.1 M, pH 7.4 in a Waring Blendor for 2 min. The homogenate was centrifuged at 10,000 *g* for 15 min and the precipitate was discarded. The supernatant fraction was then centrifuged at 100,000 *g* for 60 min in a Spinco Model "L" preparative ultracentrifuge, and the clear supernatant fraction was used for further fractionation.

Saturated ammonium sulfate, adjusted to pH 7.2 was added slowly, with stirring, to 0.45 saturation. After 1 hour, the precipitate was collected by centrifugation and discarded. The supernatant fraction was treated with additional saturated ammonium sulfate to 0.60 saturation. The precipitate was collected by centrifugation and dissolved in phosphate buffer, 0.10 M, pH 7.4 to a final protein concentration of 20–30 mg/ml. The (0.45–0.60) ammonium sulfate fraction was then dialyzed for 16 hr against phosphate buffer, 0.01 M, pH 7.4 without loss of activity.

The dialyzed (0.45–0.60) ammonium sulfate fraction was concentrated in an ultrafiltration apparatus (Schleicher and Schuell, Keene, New Hampshire) to a final protein concentration of 120–180 mg/ml prior to gel filtration on Sephadex G-100. The concentrated protein solution was used to charge a Sephadex G-100 column (2.2 × 52 cm), which was then eluted with phosphate buffer, 0.005 M, pH 7.4 over 5–8 hr. The effluent from this column was collected in 5-ml fractions; 80% of the protein was

TABLE 1
Purification of N-acetyltransferase from rabbit liver

Step	Activity[a] (units/ml)	Protein concentration (mg/ml)	Specific activity (units/mg)
Supernatant fraction (100,000 *g*)	0.014	30.6	0.00046
Ammonium sulfate fraction (0.45–0.60)	0.032	25.6	0.0012
Sephadex G-100 (peak fraction)	0.010	1.1	0.0091
DEAE-cellulose (peak fraction)	0.021	0.14	0.16

[a] The enzyme was assayed with INH at pH 9.0 as described under Materials and Methods. One unit of activity represents 1 micromole of 2-acetyl INH formed per minute per milliliter of enzyme fraction.

recovered in fractions 13–28, and activity was recovered in fractions 26–31, with a peak of activity in fractions 29 and 30.

Fractions containing most of the activity were combined, and EDTA (tetrasodium salt) was added to a final concentration of 0.001 M. A DEAE-cellulose column (0.8 × 7.5 cm), equilibrated with phosphate buffer, 0.005 M, pH 7.15 and EDTA 0.001 M, was used for further fractionation of this material. Gradient elution was performed with KCl in EDTA, 0.001 M, and phosphate buffer, pH 7.15, beginning with 0.005 M and increasing to 0.3 M. The effluent was collected in 5-ml fractions; 80% of the protein was recovered by fraction 14, and activity appeared in fractions 14–29, with most of the activity in fractions 18–22. The total activity recovered represented about 15% of the activity in the initial homogenate. The specific activity in DEAE-cellulose fractions 18–22 was within the range 0.16–0.18 μmole INH acetylated per minute per milligram of protein.

It was not possible to saturate *N*-acetyltransferase simultaneously with both acetyl coenzyme A and INH. Since the acetylation of INH proceeds by a "ping-pong" mechanism (see below), it would still be possible to relate the *N*-acetyltransferase activity to its concentration if the ratio, AcCOA:INH, initially present in the assay were constant (2, 8). However, we have found that there are other enzymes in the cruder *N*-acetyltransferase fractions which utilize acetyl coenzyme A (unpublished observations). In addition, the extent to which protein binding may alter the free substrate concentration in these fractions is unknown. Therefore, neither the specific activity of the enzyme in the early fractions nor the overall degree of purification in the DEAE-cellulose fractions can be estimated with certainty.

RESULTS

Distribution in Tissues

N-Acetyltransferase activity was found in the liver and intestine (jejunum) of rapid inactivator rabbits when either SD, SM, or INH was used as substrate. Activity was not detected with SD in the 10,000 *g* supernatant fraction of heart, kidney, skeletal muscle, spleen, brain, or adrenal. The activity at pH 6.8 with either SM or INH was 20–25-fold greater in liver than in intestine.

Stability

The 100,000 *g* supernatant fraction retained over 50% of its activity for at least 1 month when frozen and for 48 hr when stored at either 4° or 27°. Repeated freezing and thawing did not inactivate the enzyme. The (0.45–0.60) ammonium sulfate fraction (both dialyzed and undialyzed) and the Sephadex fractions all retained over 50% of their activity for at least 1 week when frozen. They were not affected by exposure to 27° for at least 8 hr. The DEAE-cellulose fractions retained at least 80% of their activity when stored at 4° for 4–5 days, but lost over 50% of their activity when frozen for the same length of time.

Substrate Specificity

INH, SD, SM, and sulfamerazine are substrates for *N*-acetyltransferase in the DEAE-cellulose fractions. The ratio of activity with SM versus INH as substrates did not change appreciably during purification. Sulfanilamide (SA) was also a substrate for *N*-acetyltransferase in the DEAE-cellulose fractions. The ratio of activity with SA versus INH as substrates also was unchanged during purification.

pH Optimum

The pH for optimum activity of *N*-acetyltransferase was determined in the presence of INH, SM, SD, and SA. The optimum pH varied according to the drug used as substrate, as shown in Fig. 1.

Inhibitors

N-Acetyltransferase purified through the Sephadex stage was used for inhibitor studies. *p*-Chloromercuribenzoate (*p*CMB) in concentrations of 5×10^{-6} M and 1×10^{-4} M produced 10 and 90% inhibition, respectively. Sodium thioglycolate (7×10^{-3} M) restored 40% of the activity in

preparations completely inhibited by *p*CMB.

Various metals, Cu^{+}, Zn^{++}, Mn^{++}, and Ni^{++} (1.4×10^{-4} M) each inhibited *N*-acetyltransferase. Copper and zinc inhibited the

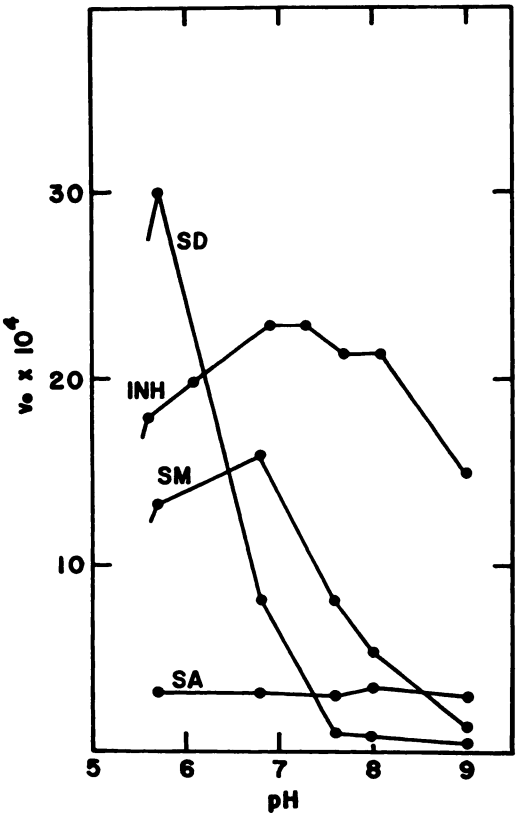


FIG. 1. *pH-activity curves for N-acetyltransferase determined in pyrophosphate buffer, 0.1 M, with four drug substrates*

Velocity units are micromoles of drug acetylated per minute per milliliter of enzyme fraction. The enzyme preparation was the (0.45–0.60) ammonium sulfate fraction. Each point represents the average of duplicate determinations. The pK_a of each drug substrate is: SD, 6.5; SM, 7.4; INH, 10.3; and SA, 10.5.

activity more than 95%; the latter two metals each produced about 80% inhibition. Preincubation of the enzyme with EDTA (2.8×10^{-4} M) for 5 min reduced the inhibition by each metal to less than 20%, but the addition of EDTA after preincubation with the metal had no protective effect. Neither iron (ferrous and ferric) nor magnesium inhibited *N*-acetyltransferase activity.

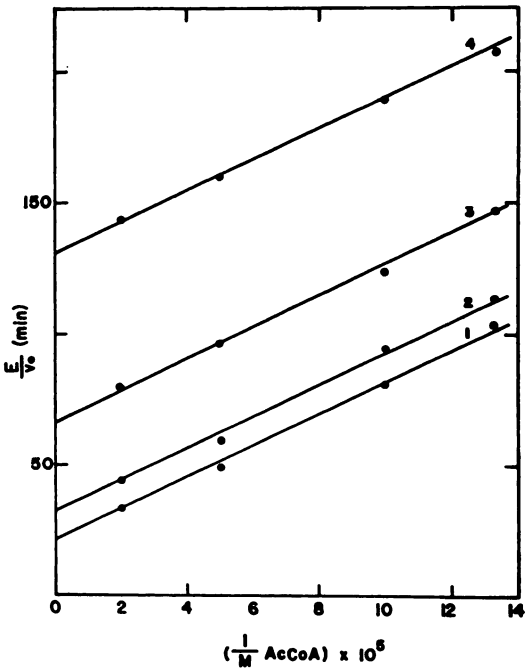


FIG. 2. *Initial velocity pattern determined with acetyl coenzyme A as the varied substrate in borate buffer, 0.8 M, pH 9.0*

INH concentration: curve *1*, 2.0 mM; curve *2*, 1.0 mM; curve *3*, 0.4 mM; and curve *4*, 0.2 mM. The enzyme preparation was the DEAE-cellulose fraction (18–22). The protein concentration in each assay was 2.26×10^{-2} mg/ml.

Kinetics

Families of parallel lines were obtained when double reciprocal plots of the initial rate data and substrate concentration were made for the acetylation of INH at pH 9 (Figs. 2 and 3). The slope and intercept of each line obtained from these plots are replotted in Fig. 4. Values for the kinetic coefficients ϕ_0, ϕ_1, ϕ_2, and ϕ_{12}, in the initial rate equation

$$\frac{E}{v_0} = \phi_0 + \frac{\phi_2}{[\text{INH}]} + \left(\phi_1 + \frac{\phi_{12}}{[\text{INH}]}\right)\frac{1}{[\text{AcCoA}]}$$

where E/v_0 is 1/(micromoles of INH acetylated per minute per milliliter of enzyme fraction), were obtained from these secondary plots (1). These constants, as well as

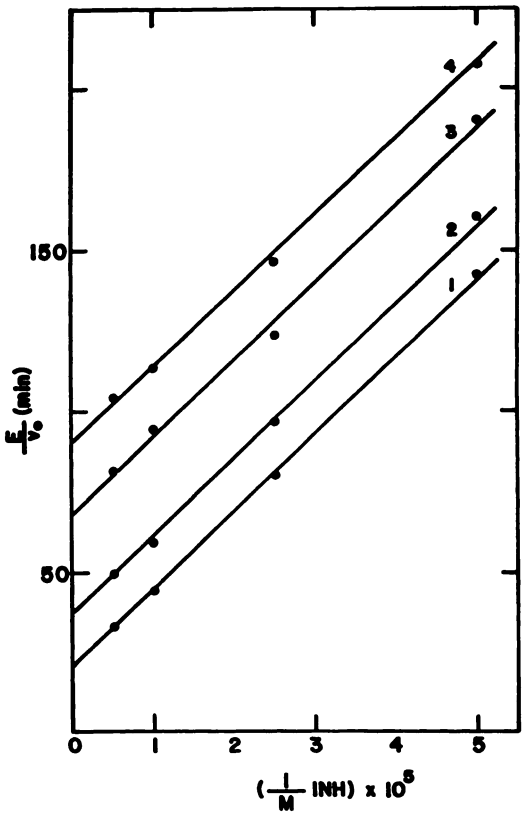


FIG. 3. *Initial velocity pattern determined with INH as the varied substrate in borate buffer, 0.8 M, pH 9.0*

Acetyl coenzyme A concentration: curve *1*, 0.5 mM; curve *2*, 0.2 mM; curve *3*, 0.1 mM; and curve *4*, 0.075 mM. The enzyme preparation was the DEAE-cellulose fraction (18–22). The protein concentration in each assay was 2.26×10^{-3} mg/ml.

K_m values calculated for each substrate in the presence of an infinite concentration of the other substrate are given in Table 2.

Product Inhibition

Coenzyme A and acetylated drug each inhibit the acetylation of INH by *N*-acetyltransferase. Coenzyme A is a noncompetitive inhibitor with respect to acetyl coenzyme A (Fig. 5A) and a competitive inhibitor with respect to INH (Fig. 5B). It was not possible to study the inhibition of INH acetylation in the presence of 2-acetyl INH due to the high blank absorption of this compound. However, an acetylated sulfonamide, *N*-4-acetyl SM, was found to be a competitive inhibitor with respect to acetyl coenzyme A when INH was the substrate (Fig. 6). *N*-4-Acetyl SM may be regarded as an alternate product (9) since the substrate specificity of *N*-acetyltransferase includes SM.

It has not been possible to demonstrate reversibility of the reaction, i.e., the deacetylation of 2-acetyl INH or *N*-4-acetyl SM, by purified *N*-acetyltransferase in the presence of coenzyme A.

TABLE 2

Kinetic coefficients and apparent Michaelis constants[a] for INH acetylation at 27° with DEAE-cellulose fractions of N-acetyltransferase[b] from rabbit liver

				K_m	
ϕ_0	ϕ_1	ϕ_2	ϕ_{12}	AcCoA (M × 10^4)	INH (M × 10^3)
5.0	0.00775	0.0382	0	15.0	7.6

[a] K_m values for each substrate, defined as that concentration which gives half the maximum rate in the presence of an infinite concentration of the other substrate, were calculated from the following relationships: for AcCoA, $K_m = \phi_1/\phi_0$ and INH, ϕ_2/ϕ_0 (1).

[b] Purification and initial rate measurements were performed as described in Materials and Methods.

DISCUSSION

Studies of the substrate specificity of partially purified *N*-acetyltransferase from rabbit have not been reported previously. We have found that DEAE-cellulose fractions of rabbit liver *N*-acetyltransferase catalyze the acetylation of INH, SM, SD, sulfamerazine, and SA, and there was a parallel increase in specific activity during purification. These results suggest that a single enzyme in rabbit liver may catalyze the acetylation of these drugs. However, it is still possible that several *N*-acetyltransferases may be present in the DEAE-cellulose fractions and that further purification will separate them.

The particular pH for optimum activity and the shape of the pH-activity curve both vary with the drug substrate used (Fig. 1).

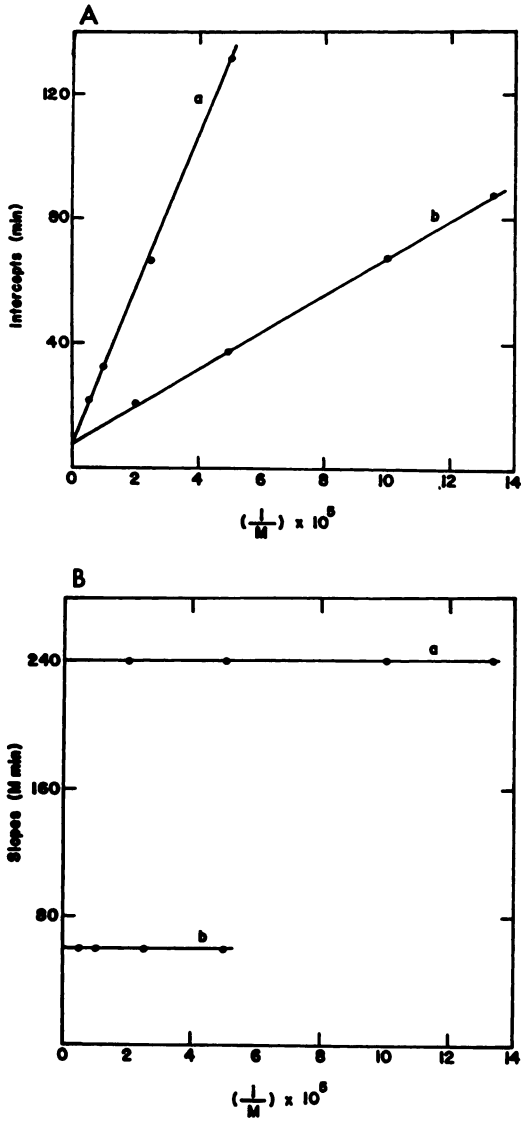


FIG. 4. *Secondary plots: Variation of intercepts (A) and slopes (B) of the primary plots of Figs. 2 and 3 with the reciprocals of the INH concentration* (○) *and acetyl coenzyme A concentration* (●), *respectively*

In (A) the common intercept on the ordinate is ϕ_0 and the slopes are ϕ_2 for curve *a* and ϕ_1 for curve *b*. In (B), the common slope for curves *a* and *b* is ϕ_{12} (1).

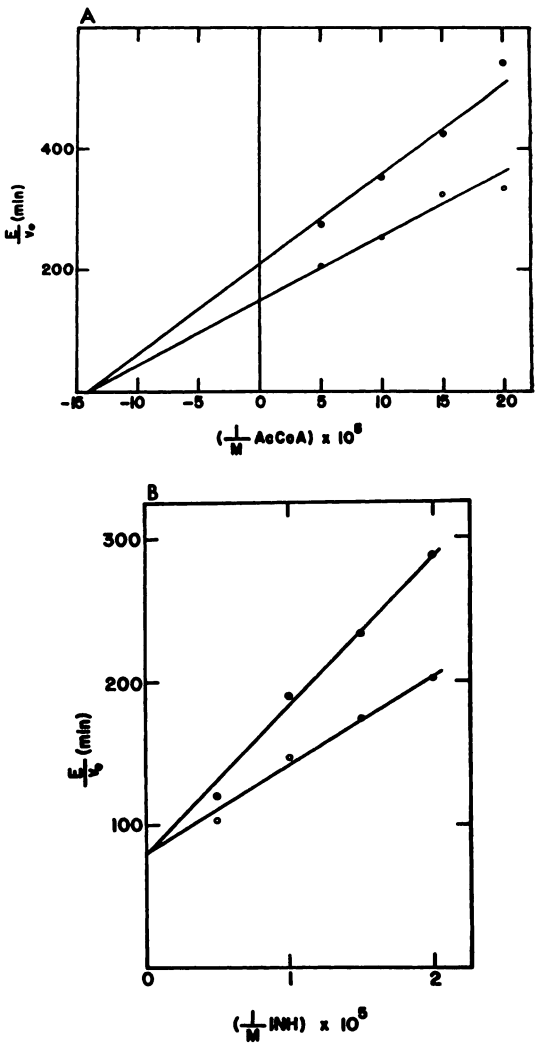


FIG. 5. *Reciprocal plots for N-acetyltransferase with coenzyme A as inhibitor* (●); *(A) with acetyl coenzyme A as the varied substrate at an INH concentration of 0.4 mM; (B) with INH as the varied substrate at an acetyl coenzyme A concentration of 0.1 mM*

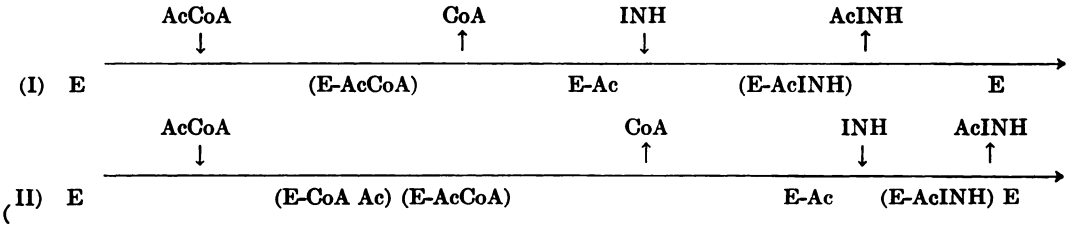
The coenzyme A concentration was 0.53 mM. The enzyme preparation was the DEAE-cellulose fraction (18–22). The protein concentration in each assay was 1.14×10^{-2} mg/ml. Assays were performed in borate buffer, 0.8 M, pH 9.0.

The activity with SD as substrate is maximal at pH 5.7 and drops rapidly on either side of this pH. In contrast, there is a broad optimal range with SM between pH 5.7 and pH 7.4. There is little variation in activity with INH between pH 5 and 9, and even less variation with SA. Since all drugs studied are weak organic acids and the activity with each is relatively greater at

pH's below the pK_a of the drug, it appears that the uncharged form of the drug interacts more strongly with the enzyme.

The initial rate data, presented in Figs. 2 and 3, indicate that INH is acetylated by a "ping-pong Bi-Bi" mechanism (2). In such a mechanism, one substrate reacts with the enzyme with the release of one product before the second substrate enters the reaction. Reaction of the enzyme with the first substrate produces a second stable form of the transferring enzyme. We have shown that coenzyme A is a noncompetitive inhibitor with respect to acetyl coenzyme A (Fig. 5A) and a competitive inhibitor with respect to INH (Fig. 5B) when INH is used as the drug substrate. From these inhibitor studies, it is concluded that acetylation of the enzyme must be the first step in the overall acetylation reaction. The existence of an acetylated form of *N*-acetyltransferase was suggested on the basis of studies of INH acetylation in a pigeon liver preparation (3), but direct evidence for this form of the enzyme is yet to be obtained.

The initial rate data, together with the coenzyme A inhibition studies, suggest that the total reaction for INH acetylation may be represented by one of the following schemes:



(I) represents an uncomplicated "ping-pong Bi-Bi" mechanism, and (II) involves isomerization of the acetyl coenzyme A–enzyme complex. Since *N*-4-acetyl SM is a competitive inhibitor with respect to acetyl coenzyme A, mechanism (I) probably represents the mechanism of INH acetylation (2).

Experimental K_m values can be calculated from each of the lines in Figs. 2 and 3. None of these apparent K_m values is characteristic of *N*-acetyltransferase, however, since each depends upon the concentration of the fixed substrate. Variation of apparent K_m values is consistently found in "ping-pong" reactions and may also be seen in two-substrate, two-product reactions which proceed by other mechanisms (2). Thus, experimental K_m values are not necessarily characteristic of an enzyme which catalyzes such a reaction. The kinetic constants in Table 2 provide a basis for characterization of such an enzyme (1, 2).

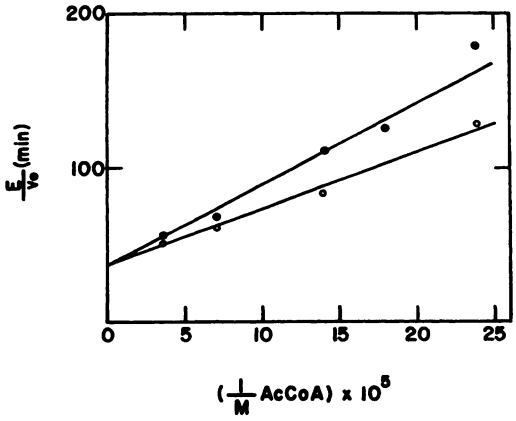


FIG. 6. *Reciprocal plot for N-acetyltransferase with acetyl coenzyme A as the varied substrate and N-4-acetyl SM (0.35 mM) as the inhibitor* (●)

The concentration of INH was 0.5 mM. The enzyme preparation was the DEAE-cellulose fraction (18–22). The protein concentration in each assay was 4.5×10^{-2} mg/ml. Assays were performed in borate buffer, 0.8 M, pH 9.0.

ACKNOWLEDGMENTS

We wish to thank Dr. B. N. La Du for his advice throughout these studies.

This investigation was supported in part by U.S. Public Health Service Grant AM 08577 from the National Institutes of Health.

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