N-Acetylation of Drugs

OBSERVATIONS ON THE PROPERTIES OF PARTIALLY PURIFIED N-ACETYLTRANSFERASE FROM PERIPHERAL BLOOD OF RABBIT

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A method is described for the partial purification of *N*-acetyltransferase (EC 2.3.1.5) from peripheral blood of rabbit. This enzyme purified from both rapid- and slow-isoniazid-acetylator phenotype rabbits was examined with regard to stability, substrate specificity, cation effect, rate of inactivation, temperature and pH optimum. Data show that in the biochemical characteristics examined the enzyme is independent of acetylator phenotype. The possible significance of these findings to the evolutionary origin of drug-acetylating enzymes in rabbit blood and liver is discussed.

Rapid and slow inactivation of isoniazid and sulphamethazine is a human genetic trait attributable to different amounts of drug-acetylating enzyme activity (*N*-acetyltransferase, EC 2.3.1.5) in the liver and duodenal mucosa of different individuals (Evans & White, 1964). A genetic polymorphism of a similar sort exists for these drugs in rabbits (Frymoyer & Jacox, 1963*a,b*). The rate of elimination of these drugs from blood of individuals of both species is highly correlated with the activity of *N*-acetyltransferase in these tissues.

Recent comparative studies of liver *N*-acetyltransferases from rapid- and slow-isoniazid-acetylator rabbits of their heat-stability characteristics and stability characteristics *in situ*, inhibition properties and immunochemical properties indicate that they are significantly different enzymes (Weber *et al.*, 1978). Previous biochemical studies had failed to produce evidence of the basis for this pharmacogenetic trait (Jenne, 1965; Weber, 1971*a*).

In addition to its occurrence in liver and duodenal mucosa, drug-acetylating *N*-acetyltransferase activity is widely distributed in other extrahepatic tissues. Studies in rabbits have shown that extrahepatic *N*-acetyltransferase activity exhibits less individual variation than hepatic activity and that it does contribute appreciably to the total drug-acetylating capacity of the individual, particularly in the

slow acetylator (Hearse & Weber, 1973). Extrahepatic *N*-acetyltransferase activity differs from hepatic *N*-acetyltransferase in substrate specificity, i.e. isoniazid is not a substrate of the former, and with respect to certain other biochemical characteristics, but also shares some properties with hepatic *N*-acetyltransferase activity. Extensive study of *N*-acetyltransferase in peripheral blood of rabbits indicates that this activity is inversely correlated with the activity of hepatic isoniazid *N*-acetyltransferase (Weber *et al.*, 1976). In addition, the reciprocal relationship between liver isoniazid *N*-acetyltransferase activity and blood *N*-acetyltransferase activity is expressed in erythrocytes and lymphocytes (Szabadi *et al.*, 1978), but not in polymorphonuclear leukocytes (C. A. McQueen, W. Brenner & W. W. Weber, unpublished work). Initial biochemical and tissue-distribution studies on the extrahepatic *N*-acetyltransferase from slow- and rapid-acetylator rabbits showed (Hearse & Weber, 1973) that: (1) extrahepatic tissues were found to contribute at least two-thirds of the total acetylating capacity to the acetylation of polymorphic substrates, e.g. sulphamethazine in the slow-acetylator rabbit; (2) multiple forms of *N*-acetyltransferase existed in different tissues; (3) certain tissues contained more than one form, e.g. liver; (4) a comparison of some of the biochemical properties of *N*-acetyltransferases isolated from the liver (hepatic) and gut mucosa, spleen, kidney and peripheral blood (extrahepatic) indicated some features in common.

The studies reported in the present paper were designed to investigate the biochemical properties of *N*-acetyltransferases isolated from the peripheral

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blood of rapid- and slow-acetylator rabbits and to compare these properties with those of the *N*-acetyltransferases isolated from the liver of rapid- and slow-isoniazid-acetylator rabbits.

Experimental

Reagents

p-Aminobenzoic acid, sulphamethazine and sulphadiazine were obtained from the Sigma Chemical Co., St. Louis, MO, U.S.A. Acetyl-coenzyme A was obtained from Nutritional Biochemical Corp., Cleveland, OH, U.S.A. Dithiothreitol from Calbiochem, Richmond, CA, U.S.A. *N*-(1-Naphthyl)ethylenediamine and routine laboratory chemicals were from Fischer Co., Springfield, NJ, U.S.A. DE-52 DEAE-cellulose (Whatman) was obtained from Reeve Angel, Clifton, NJ, U.S.A. CM-Sephadex was from Pharmacia, Piscataway, NJ, U.S.A.

Animals

Known lines of New Zealand White rabbits (5 months of age or older) of both sexes maintained in our colony on a standard diet were used throughout this study. The acetylator phenotype of the rabbits was determined by using sulphadiazine, by a modification (Weber *et al.*, 1976) of the procedure of Frymoyer & Jacox (1963a).

Enzyme purification

Animals were anaesthetized and blood was collected by cardiac puncture and immediately mixed with heparin (as an anti-coagulant) and then centrifuged at 1000 *g* and the plasma removed. All steps were carried out at 4°C. The erythrocytes were washed two times with an equal volume of 0.1 M-potassium phosphate buffer, pH 7.20, and then haemolysed with an equal volume of cold water. This solution was kept in an ice bath for 15 min. The haemolysate was centrifuged at 100 000 *g* for 90 min and the supernatant solution was retained as starting material. The 100 000 *g* supernatant was chromatographed on a CM-Sephadex column (C-50; 40 cm × 2.5 cm) equilibrated with 0.01 M-potassium phosphate buffer, pH 6.70, containing 0.001 M-di-

thiothreitol, 0.001 M-MgCl₂ and 0.001 M-EDTA. The enzymic activity, which was contained in the void volume, was rechromatographed on a DE-52 DEAE-cellulose column (20 cm × 2.5 cm) with the same buffer as in the previous column procedure and a linear KCl gradient from 0 to 0.3 M. The *N*-acetyltransferase activity was eluted at 0.2 M-KCl and the activity was concentrated under N₂ by ultrafiltration using an Amicon model 52 ultrafiltration cell and a PM-30 membrane. Enzyme at this concentration was used in all experiments.

The *N*-acetyltransferase from liver was purified as previously described (Weber, 1971b).

Enzyme assay

The enzymic activity was assayed at 27°C as previously described (Hearse & Weber, 1973). The mixture was left for 10 min and the absorbance at 540 nm was measured on a Beckman DU spectrophotometer against water as blank. The extent of acetylation was obtained by subtracting the experimental reading from the control reading. A decrease in absorbance at 540 nm of 167.5 corresponds to the acetylation of 1 μmol of substrate. The limit of detection is 0.1 μmol of acetylated substrate.

Specific activity of enzyme preparations

Activity was usually expressed as μmol of substrate acetylated/ml of enzyme per min at 27°C. Specific activity was defined as μmol of substrate acetylated/mg per min at 27°C. The protein concentration was determined by the method of Warburg & Christian (1942).

Results

Purification

A typical purification is shown in Table 1. In the CM-Sephadex step, *N*-acetyltransferase from rabbit blood was eluted in the void volume, whereas haemoglobin was retained on the gel. In the DEAE-cellulose step enzyme purified from both the rapid- and slow-acetylator phenotype was eluted at the same concentration of salt (0.2 M). This procedure is simple and a good recovery of activity was obtained, although some was lost on concentration

Table 1. *Purification of N-acetyltransferase from rabbit peripheral blood*
The enzyme assay and specific activity are defined in the Experimental section.

Purification step	Volume (ml)	Activity (units/ml)	Protein (mg/ml)	Specific activity	Purification	Recovery (%)
100 000 <i>g</i> supernatant	52	2.08	128.20	0.016	1	100
CM-Sephadex	104	1.07	17.40	0.062	3.8	103
DEAE-Cellulose	120	0.66	0.13	5.05	312	73
Amicon PM-30	11.5	5.13	1.10	4.76	288	55

by ultrafiltration. The purification described was over 300-fold, but up to 1000-fold purification has been obtained.

Stability

It can be seen from Table 2 that *N*-acetyltransferase partially purified from rabbit blood is unstable at 4°C. To purify and to study the biochemical properties of the enzyme a way had to be found to stabilize the enzyme. Previous work had shown the requirement for dithiothreitol, MgCl₂ and EDTA in stabilizing the enzyme isolated from human blood (H. C. Kelker & M. Gross, unpublished work). The stability of the partially purified enzyme could be maintained by dithiothreitol either alone or in combination with MgCl₂ and EDTA. The latter two compounds either alone or together were insufficient to maintain activity under the conditions described in Table 2.

The data in Table 2 are that for enzyme purified from the blood of rapid-acetylators rabbits. Identical results were obtained for enzyme purified from the blood of slow-acetylators rabbits.

Substrate specificity

A series of drugs was studied with respect to the substrate specificity of *N*-acetyltransferase from peripheral rabbit blood. The series comprised drugs that were either acetylated monomorphically, e.g. *p*-aminobenzoic acid, *p*-aminosalicylic acid and sulphanilamide, or the remainder, which were acetylated polymorphically, by the hepatic *N*-acetyltransferase. Table 3 shows that *p*-aminobenzoic acid was the best substrate. Of the remainder aniline was the preferred substrate, but was acetylated at only 4.9% of the rate of *p*-aminobenzoic acid acetylation. *p*-Aminosalicylic acid and sulphanilamide were not substrates for the enzyme, by comparison with

p-aminobenzoic acid, the other monomorphically-acetylated drug tested. No difference was found in substrate specificity between the enzyme purified from rapid- and slow-acetylators phenotypes.

Effect of metals

Peripheral blood *N*-acetyltransferase was tested against a series of cations to determine if there was any difference in the enzyme purified from the different acetylators phenotypes. It can be seen from Table 4 that Cu²⁺, Hg²⁺, Zn²⁺ and Cd²⁺ completely

Table 3. *Substrate specificity of rabbit blood N-acetyltransferase*

The final drug concentration was 0.1 mM, except anileridine, where the final concentration was 1 mM. The enzyme was assayed and specific activity defined as described in the Experimental section. N.D. indicates that the activity was not detectable by the assay used.

Drug	Specific activity	Activity (% of <i>p</i> -aminobenzoic acid activity)
<i>p</i> -Aminobenzoic acid*	7.70	100.0
Aniline	0.38	4.9
Sulphamethazine	0.30	3.9
Anileridine	0.13	1.7
Sulphadiazine	0.12	1.6
Procainamide	0.06	0.8
Sulphanilamide*	0.05	0.6
Sulphamerazine	N.D.	0
Sulphapyridine	N.D.	0
<i>p</i> -Aminosalicylic acid*	N.D.	0
Isoniazid	N.D.	0

* Drugs that are acetylated monomorphically by hepatic *N*-acetyltransferase.

Table 2. *Effect of dithiothreitol, MgCl₂ and EDTA on the stability of rabbit blood N-acetyltransferase*

The activity was expressed as specific activity (see the Experimental section). Final concentrations of dithiothreitol, MgCl₂ and EDTA were 1 mM. The preparation was dialysed for 16 h at 4°C against 0.01 M-phosphate, pH 6.80. Protein concentration was 9.4 µg/assay and the enzyme was assayed as described in the Experimental section. N.D. indicates that the activity was not detectable by the assay used.

Additions	Time of storage at 4°C (days) ...	Specific activity				
		0	2	4	6	8
None	—	3.32	2.63	1.49	0.21	N.D.
Dithiothreitol	—	—	3.23	3.57	2.83	2.92
MgCl ₂	—	—	0.72	0.13	0.14	N.D.
EDTA	—	—	1.06	1.19	0.42	0.21
Dithiothreitol, MgCl ₂	—	—	3.18	3.06	3.01	2.89
Dithiothreitol, EDTA	—	—	2.76	3.18	2.55	2.03
MgCl ₂ , EDTA	—	—	1.85	1.74	0.85	N.D.
Dithiothreitol, MgCl ₂ , EDTA	—	—	3.06	3.18	3.10	2.89

Table 4. *Effect of metals and inhibitors on rabbit blood N-acetyltransferase*

'Rapid' and 'slow' are enzyme preparations isolated from the blood of animals that have been characterized as rapid- and slow-isoniazid acetylators respectively. The enzyme was assayed as described in the Experimental section. Each metal salt was present as the chloride at a concentration of 0.1 mM. The enzyme was dialysed against 0.1 M-phosphate buffer, pH 6.80, before use.

Metal	Enzyme	...	Inhibition (%)	
			Rapid	Slow
Mn ²⁺			0	0
Ca ²⁺			0	0
Fe ³⁺			0	8
Mg ²⁺			13	11
Fe ²⁺			70	76
Cd ²⁺			96	100
Cu ²⁺			100	100
Hg ²⁺			100	100
Zn ²⁺			100	100
<i>p</i> -Chloromercuribenzoate				
(0.1 mM)			100	100
(5 μ M)			100	100

inhibited *N*-acetyltransferase. Ca²⁺, Mn²⁺, Mg²⁺ and Fe³⁺ produced either slight or no inhibition, whereas Fe²⁺ inhibited both by approx. 70%. With all the metals tested there was very little difference between the amount of inhibition of the enzyme purified from the different acetylator phenotypes. The difference observed was minimal and can be explained by experimental error.

p-Chloromercuribenzoate at two concentrations completely inhibited the enzyme activity. This inhibition is indicative of essential thiol groups being present.

Rate of inactivation

The rate of inactivation of peripheral blood *N*-acetyltransferase at 40 and 50°C was examined. At the lower temperature 50% of enzymic activity purified from both acetylator phenotypes was lost in 41 min compared with 6.8–8.5 min at the higher temperature. At 40°C there was no difference between the enzyme isolated from the different acetylator phenotypes, whereas at 50°C there was a slight but minimal difference in the time required for 50% of the initial activity to be lost.

Temperature optimum

N-Acetyltransferase exhibits a broad temperature optimum as is illustrated in Fig. 1. The curves show the temperature optimum for the partially purified enzyme from slow and rapid acetylators respectively. No difference in optimum was noted.

Fig. 1. *Temperature optimum of rabbit blood N-acetyltransferase*

The enzyme was dialysed against 0.01 M-phosphate, pH 6.80, for 16 h before use. The enzyme assay was carried out as described in the Experimental section after a 5 min preincubation at the appropriate temperature.

Fig. 2. *pH optimum of rabbit blood N-acetyltransferase*

The enzyme was dialysed against 0.01 M-phosphate, pH 6.80. The final concentration of the buffer in the digest was 0.2 M. Citrate/phosphate buffer was used in the pH range 4.60–6.30. Phosphate buffer was used in the pH range 6.30–7.80.

pH optimum

The pH optima of *N*-acetyltransferase purified from the different acetylator phenotypes are illustrated in Fig. 2. In both cases curves are biphasic with peaks at pH 6.6–6.7 and at pH 7.20. In both cases the specific activity is higher at the lower pH compared with the higher pH.

Discussion

The results presented above provide evidence that the partially purified *N*-acetyltransferase isolated from the peripheral blood of rabbits of different acetylator phenotypes are indistinguishable with respect to the biochemical properties examined.

The studies of Jenne (1965) and Weber (1971a) have shown that isoniazid *N*-acetyltransferase is associated with liver and duodenal mucosa in man and rabbit. Extensive study of extrahepatic *N*-acetyltransferase in rabbits has shown a direct relationship exists between the activity of this enzyme and the activity of the hepatic *N*-acetyltransferase (Weber *et al.*, 1976). How they are related is not known, but previous biochemical studies have noted possible similarities between the extrahepatic and hepatic *N*-acetyltransferase (Hearse & Weber, 1973). The evidence presented above when taken in conjunction with previous studies (Weber, 1971a; Hearse & Weber, 1973; Weber *et al.*, 1976) suggests an overlap of certain biochemical properties (Table 5), i.e. biphasic pH optimum with a common peak at pH 7.20, isoelectric points with a common point around pH 5.30, *p*-aminobenzoic acid as a common substrate, both heat labile and inhibited by *p*-chloromercuribenzoate, although the hepatic enzyme appears less susceptible to both forms of inactivation. This suggests that the enzymes are closely related, but nevertheless display differences.

The biphasic nature of the pH optimum (Fig. 2) is similar to that reported in a previous study of extrahepatic *N*-acetyltransferase (Hearse & Weber,

1973). The pH profiles of the partially purified enzyme (purified to a much higher degree than in previous studies) exhibit a much deeper trough between pH 6.60 and pH 7.20 than previously reported (Hearse & Weber, 1973). This suggests the existence of either more than one form of *N*-acetyltransferase, which have become better resolved on greater purification or the existence of multiple sites on the same enzyme molecule with a different pH optimum. Evidence obtained with the liver enzyme while studying heat stability, inhibition and immunochemical properties indicates that both the polymorphic (sulphamethazine) and monomorphic (*p*-aminobenzoic acid) *N*-acetyltransferase activity exists in the same enzyme molecule (Weber *et al.*, 1978).

The information obtained in the present study when taken in conjunction with other studies (Weber, 1971a; Hearse & Weber, 1973; Weber *et al.*, 1978) supports the concept that the drug-acetylating *N*-acetyltransferases in the rabbit may all have been derived from a single primordial gene. This would suppose that the catalytic unit of these enzymes is determined at the same genetic locus. If the primordial locus coded for an *N*-acetyltransferase with specificity for acetylation of *p*-aminobenzoic acid, this would provide a relatively simple explanation for the observation in liver of rabbits (Weber, 1971a) and man (Evans, 1965; Glowinski *et al.*, 1978) that rapid and slow acetylators alike have virtually the same capacity to acetylate *p*-aminobenzoic acid, despite large differences in isoniazid-acetylating capacity. To account for the catalytic difference in *N*-acetyltransferase, such as those in liver and blood of the same individual, the gene product could have undergone post-translation modification that is tissue-specific. Such a mechanism has been shown to be a reasonable possibility to account for the multiple forms of human deaminase present in blood and other tissues (Hirschhorn, 1975).

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Table 5. Comparison of *N*-acetyltransferases in extrahepatic and hepatic tissues of rabbit

	Tissue	Extrahepatic enzyme ... Widespread (including liver and duodenal mucosa)	Hepatic enzyme Liver, duodenal mucosa
Drug substrates	...	<i>p</i> -Aminobenzoic acid	Isoniazid, sulphamethazine, <i>p</i> -aminobenzoic acid
Thermostability ($t_{1/2}$ at 50°C)	...	7–8 min 100%	28 min 10%
Inhibition (<i>p</i> -chloromercuri- benzoate, 5 μ M)			
pH optimum		6.60, 7.20	5.60, 7.20
Isoelectric point		Slow 5.42, 6.28 Rapid 5.17, 6.44	5.30 5.31, 6.67

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