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Research Article

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Partial Purification and Properties of the Isoniazid Transacetylase in Human Liver. Its Relationship to the Acetylation of *p*-Aminosalicylic Acid *

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The heritable trait of rapid and slow inactivation of isoniazid (isonicotinic acid hydrazide) in man is a topic of considerable interest in the developing field of pharmacogenetics (1). Although differences in acetylation appear to be the principal reason for inactivation differences, knowledge of the specific enzymic basis as well as of the relationship of this trait to the acetylation of chemically related compounds is scant.

Some *in vitro* information has been obtained. Localization of the variability in acetylation to the transacetylase has been reported by two laboratories. Evans and White, using homogenate of wedge biopsies of human liver, successfully correlated isoniazid inactivation phenotype and the disappearance rate of either isoniazid or sulfamethazine [N'-(4,6-dimethyl-2-pyrimidinyl)sulfanilamide] in the presence of both generated and fixed concentrations of acetyl-CoA (2, 3). A suggestive correlation was found with the rate of hydralazine (1-hydrazinophthalazine) disappearance as well. *p*-Aminobenzoic acid (PABA) and sulfanilamide were not metabolized.

While using different methods, we reported in a preliminary communication that, in the presence of constant acetyl-CoA, wide variations in isoniazid acetylation activity were found in the soluble fraction of postmortem liver and intestinal mucosa (4). The activity could be concentrated by 50% saturation with ammonium sulfate. Hydralazine was a very strong inhibitor of the reaction, but *p*-aminosalicylic acid (PAS) inhibited only weakly. Acetylation of *p*-nitroaniline (PNA) and, at that time, PAS, was not detected.

Remarkably, a similar trait exists in the rabbit. Frymoyer and Jacox found a bimodal distribution of sulfadiazine (2-sulfanilamidopyrimidine) half-lives that correlated with the half-life of isoniazid but not PAS (5). Furthermore, *in vitro* measurements of sulfadiazine acetylation activity showed a trimodal distribution suggestive of the presence of a heterozygous state (6).

Whether the variation in isoniazid acetylation activity in man represents a difference in enzyme-substrate affinity, differences in the amount of enzyme synthesized, or various other possibilities (7) remains to be shown and is the principal subject of this report.

Human *in vivo* work has shown that the excretion of acetylated sulfamethazine is bimodal, with rapid inactivators of isoniazid acetylating more sulfamethazine as well (3, 8). However, as in the rabbit, a different principal pathway for PAS acetylation in man has been suggested by our failure to find any correlation between the iv half-life for isoniazid and PAS (7). Furthermore, Evans found a unimodal distribution for the ratio of total PAS metabolites excreted to the free PAS fraction (9). Such a dual arylamine acetylation pathway in the individual human and, perhaps, the rabbit, contrasts with previous concepts based on work in pigeon liver (10, 11). However, the present report, containing both *in vitro* kinetic data and excretion data for PAS together with recent comparative data for isoniazid and sulfanilamide excretion obtained by Peters, Gordon, and Brown (8), adds to a growing body of evidence that, in man, simple aromatic amines are principally acetylated independently of isoniazid, in con-

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trast to the polycyclic amine mentioned above and perhaps others.

Methods

Identification of isoniazid inactivator phenotype. With the iv fall-off technique (12) patients were classified as rapid ($t_{\frac{1}{2}}$ 40 to 80 minutes) or slow ($t_{\frac{1}{2}}$ 140 to 200+ minutes) inactivators. The majority of patients were of two varieties. One group was under treatment for tuberculosis but not acutely ill, and the other consisted of patients with terminal diseases, mostly malignancies, with no apparent involvement of liver or kidney. A toast and coffee breakfast was not specified for the ill patients.

Collection of tissue specimens. Only those tissues obtained between 3 and 5 hours post-mortem were used for quantitative comparisons of enzyme activity, although tissues of patients classified before death were assayed up to 21 hours post-mortem. Tissues were frozen at − 20° C, and usually processed within a few days but never more than 2 months later. Numerous assays failed to disclose any detectable loss of activity during this 2-month period.

In the morgue cooler at 8° C, rectal temperatures fell about 1° C per hour. At 37° C, excised liver lost about 40% of its activity in 4 hours. Samplings of excised liver and liver left *in situ* revealed no significant loss of activity while cooling from 35° to 30° C. At 25° C, excised liver was quite stable in this regard for at least 18 hours. In summary, it was estimated that liver lost about 25% of its activity by 3 to 5 hours post-mortem and much less thereafter, up to 18 hours.

Special chemicals. The sources and purity of isoniazid, PAS, and acetyl-CoA have been described previously (11). PNA was recrystallized from ethanol-water and had a mp of 152 to 153° C. Hydralazine was obtained as Apresoline HCl [1] of highest purity and it decomposed at 273° C.

Enzyme preparations. All procedures were carried out at 4° C. Fifteen to one hundred g of liver or intestinal mucosa was optimally extracted by homogenizing for 60 seconds in 4 vol of water in a Waring blendor and centrifuging at 78,000 × *g* (average) for 90 minutes in a Spinco model L preparative centrifuge. The supernatant was brought to 50% saturation with ammonium sulfate (final pH 6.1 to 6.3) and, after 30 minutes, centrifuged at 8,000 × *g* (average) for 15 minutes. The sediment was resuspended in a small volume (3 to 15 ml) of 0.01 M K phosphate buffer at pH 7.0, recentrifuged, and the clear supernatant used for routine assays as the "ammonium sulfate" fraction, or further processed. A 45-minute delay was required after preparation of the enzyme solution for maximal uniformity of activity, since activity dropped initially about 40% before stabilizing. Neither 5 mM cysteine nor adjustment of pH to 7.0 prevented this drop. When added to the postmortem loss, this loss brought the final activity measurement to an estimated 50% of the original *in vivo* activity.

[1] Ciba Pharmaceutical Co., Summit, N. J.

For kinetic studies, the enzyme was further purified through Sephadex G-100. The gel was equilibrated with 0.01 M K phosphate buffer, pH 7.0, and poured into either a 4- × 60-cm column to a final depth of 45 cm, or a 2- × 40-cm column to a depth of 30 cm. The larger column was used for large scale purification, starting with the ammonium sulfate fraction from 30 to 100 g of liver dissolved in 10 ml of buffer. The small column was used for preparation of highly concentrated enzyme solutions, starting with the ammonium sulfate fraction from 10 to 30 g of liver dissolved in 2 to 5 ml of buffer. The effluents were collected in 10- or 3-ml fractions, respectively. Enzyme activity emerged well behind the main protein peak and slightly behind hemoglobin. Active fractions could be kept at 4° C for 24 hours or stored at − 20° C for at least 10 days, either intact or lyophilized, without loss of activity.

Further purification was obtained by passage through a 1- × 3-cm column of DEAE equilibrated to the same buffer. Serial elutions were made with 6-ml batches of successively increasing molarity (0.016 to 0.2 M phosphate, pH 7.0). The most active fractions were subjected to polyacrylamide gel electrophoresis in 0.1 M Tris-borate-EDTA buffer, pH 9.2. They were first concentrated by suction in a collodion bag,[2] washed several times with the electrophoresis buffer, and brought to a volume of 0.3 ml before application to the gel.

Protein was determined by the method of Lowry, Rosebrough, Farr, and Randall (13).

Assay of enzyme activity. The spectral assays for isoniazid and PAS acetylation activity were first described with pigeon liver enzyme (11). They depend upon an increase in absorbancy as the acetylated compounds are formed. The isoniazid assay is especially sensitive and was performed at 303 mμ at pH 9.0 ± 0.02 and 25° C in a Beckman model DU spectrophotometer. Sample and blank usually contained 0.3 ml of 0.01 M isoniazid, 0.3 to 2.0 ml of enzyme solution, and 0.1 M sodium pyrophosphate buffer, pH 9.6, to a total volume of 3.0 ml. Addition of EDTA or a reducing substance was not helpful. When Sephadex fractions were used, enzyme was omitted from the blank. The reaction was usually started by the addition of 0.3 ml of 3.0 mM acetyl-CoA to the sample. The pH was adjusted, the contents thoroughly mixed, and, after equilibrating for 3 minutes, absorbancy measured at 1-minute intervals for 20 to 30 minutes, depending on the activity. The reaction was linear for the first 0.2 OD unit increase, then it slowly fell off due to depletion of acetyl-CoA. Suitable controls ruled out interfering side reactions, and velocity was proportional to enzyme concentration.

Repeated assays on the same ammonium sulfate preparation revealed a standard deviation of 19% (14 determinations) in the range of activity of 60 mμmoles per L per minute per mg, and 14% (8 determinations) at 260 mμmoles per L per minute per mg. Thirteen repeated extractions and assays from these two livers had a standard

[2] Carl Schleicher and Schuell Co., Keene, N. H.

deviation of 21%. The standard deviation of eight assays on a single "rapid" Sephadex preparation was only 3%.

PAS acetylation activity was measured at 320 mμ and usually at a pH of 9.0. After adding acetyl-CoA, a 10-minute equilibration period was required because of a slight increase in optical density at this wave length due to a reaction between acetyl-CoA and the enzyme solution.

In all studies, constancy of acetyl-CoA concentration was maintained, since it had been found with the pigeon liver transacetylase that the apparent Michaelis constant (K_M) for either isoniazid or acetyl-CoA increased with an increase in concentration of the other reactant (11). In that case, kinetic considerations suggested the formation of an acetyl-enzyme intermediate complex, and a similarity of reaction mechanism might be anticipated.

Inhibition of isoniazid acetylation by PAS was measured at 340 mμ and a pH of 9.0. At this wave length, the density increase due to simultaneous acetylation of PAS itself is small. It was separately determined and an appropriate correction made. However, at higher isoniazid concentrations, this acetylation was also inhibited so that some inaccuracy was inevitable in assigning the proper correction.

Hydralazine inhibition of isoniazid acetylation was also measured at 340 mμ and a pH of 9.0. Alkaline decomposition of hydralazine (14) determined by the Ninhydrin reaction (15) was only 6% per 20 minutes at pH 9.0 and at the lowest hydralazine concentration used, and so it was ignored. Density changes associated with the acetylation of hydralazine itself did not interfere with initial velocity determinations.

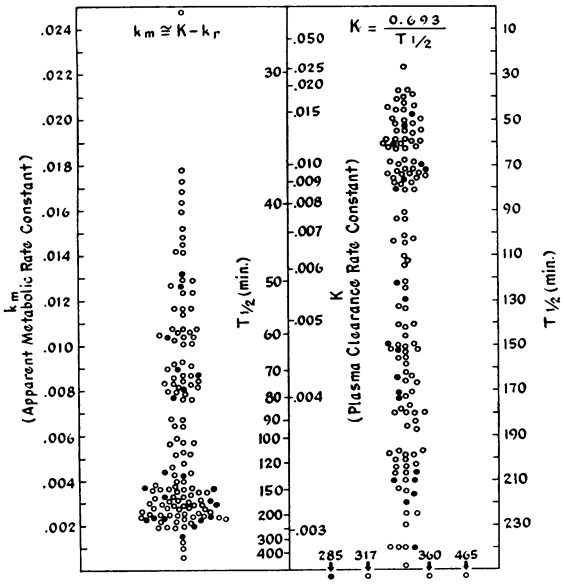

FIG. 1. CUMULATIVE EXPERIENCE WITH ISONIAZID HALF-LIFE DETERMINATIONS. On the right, plasma clearance rate constants are compared with a linear half-life scale. On the left, the presumed metabolic rate constants are plotted on a linear scale and compared with the half-lives. k_r is the rate constant for renal clearance. Solid circles (●) designate patients with terminal malignancies in Figures 1 and 2.

Paper chromatographic methods. For confirmation of isoniazid acetylation, concentrated alcoholic extracts of the assay solutions were subjected to ascending chromatography with methanol-*n*-butanol-benzene-water in a proportion of 2:1:1:1, stained with 1% benzidine in 10% acetic acid and exposed to cyanogen bromide vapor. Isoniazid appeared as a blue fluorescent spot at an R_f of 0.77 and acetylated isoniazid as a purple spot at an R_f of 0.85. Ascending chromatography of PAS was performed with *n*-butanol-ethanol-3% ammonia in proportions of 4:1:5 and stained with 1% ferric nitrate in 0.07 M nitric acid (16). PAS appeared as a dark blue spot at an R_f of 0.37 and acetylated PAS as a purple spot at an R_f of 0.50.

Urine excretion studies of PAS acetylation. Patients under treatment for tuberculosis were given a packet of crystalline PAS equivalent to 3.0 g of the acid [3] the evening after a morning isoniazid half-life determination. All other drug intake had been suspended for 24 hours. Urine was collected overnight on ice for a 12-hour period. Determinations of PAS and acetyl-PAS were performed in duplicate by a chemical and by a chromatographic method. Free and total amines were measured with the Bratton and Marshall method as modified for PAS (17), with an 18-hour hydrolysis at 38° C in a final concentration of a 1.2 N HCl as advocated by Way and associates for total amines (18). Under these conditions, slight decomposition of PAS occurred requiring a correction with appropriate standards. Since this method also determines the glycine conjugate, more specificity was realized with the use of paper chromatography. Urine was usually diluted 1:100 and 0.2 ml spotted on paired strips. After development, one strip of each pair was stained with the ferric nitrate reagent and the areas corresponding to PAS and acetyl-PAS on the other strip were excised, eluted into 4 ml of water, and treated with the ferric nitrate reagent. Duplicate samples and unchromatographed PAS standards were read at 500 mμ. The acetyl-PAS readings were increased 6% to correct for the slightly different absorption spectrum of ferric nitrate with this substance. Recoveries were essentially quantitative, and duplicates showed a mean variation of 10%. A second group of four rapid and four slow inactivators was given 300 mg of PAS 60 hours or more after the last drug intake and the urine analyzed with the Bratton and Marshall method only.

Results

Identification of enzyme. Evidence that the enzyme under study is responsible for or representative of differences in inactivation is given in Figures 1 and 2. Figure 1 shows our cumulative isoniazid half-life determinations. The majority of

[3] Hellwig, Inc., Chicago, Ill.

patients were under treatment for tuberculosis but essentially in a normal physiological state. When their half-lives are transposed into rate constants by the relationship

$$t_{\frac{1}{2}} = \frac{0.693}{K},$$

where K is the plasma disappearance rate, the rapid inactivators become spread out, and the reverse applies to the slow inactivators. This effect is even more pronounced when, in the left half of Figure 1, the presumed metabolic rate constant, k_m, is calculated by subtracting the rate constant for renal clearance ($k_r = 0.00095$) (7) from the over-all clearance constant. Thus,

$$K = k_m + k_r.$$

The shape of the k_m distribution is similar to the shape of the distribution of measured liver enzyme activities in the left half of Figure 2. These activities were determined on the ammonium sulfate fractions of 54 livers obtained at random and processed from 3 to 5 hours post-mortem, and they are expressed as millimicromoles per liter isoniazid acetylated per minute per milligram protein of the fraction. It seems most likely that we have two groups of activities, divided at about 75 mμmoles per L per minute per mg with 25 or 46% of the livers in the rapid group. However, the rather large variance in the assay does not definitely preclude a skewed unimodal distribution, or even more than two groups.

The right half of Figure 2 shows the correlation between *in vivo* phenotype and *in vitro* activity. These patients were all very ill, and most of them were poorly nourished. The patient with a half-life of 100 minutes had some of his isoniazid injected subcutaneously, possibly prolonging his half-life. The patient with a half-life of 39 minutes, a patient with meningitis, went from a state of good nutrition to emaciation between the *in vivo* and *in vitro* determinations, with a possible secondary effect on his enzyme activity. An attempt to evaluate the effect of nutrition is made in Figures 1 and 2 by designating those patients with metastatic cancer. No effect is evident. In spite of these slight discrepancies in Figure 2, there is no question that inactivation phenotype correlates with liver activity, and it seems reasonable to conclude

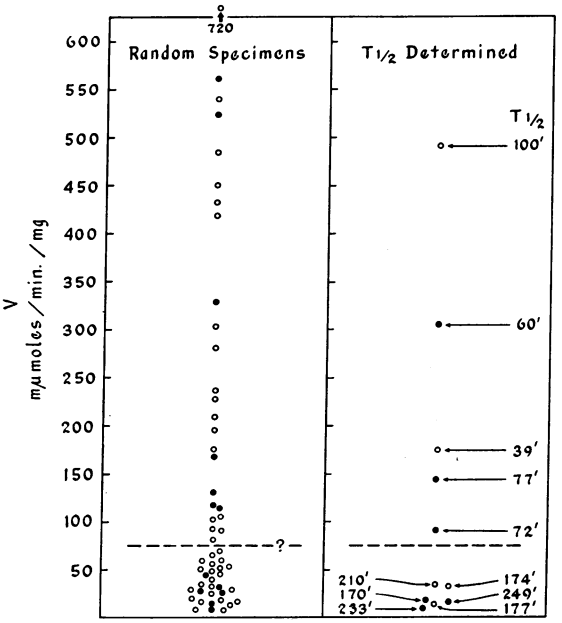

FIG. 2. ISONIAZID ACETYLATION ACTIVITIES OF HUMAN LIVER. Specific activities are expressed as millimicromoles isoniazid acetylated per minute per milligram protein by the ammonium sulfate fraction. V = initial enzyme velocity. Values on the left are from livers obtained 3 to 5 hours post-mortem. The right half shows the correlation of activity with *in vivo* isoniazid half-life determinations. These livers were obtained 8 to 21 hours post-mortem.

that the enzyme in question is responsible for acetylation of isoniazid.

Enzyme purification. Figure 3 demonstrates the purification beyond the ammonium sulfate step. The Sephadex G-100 step was particularly useful since, despite considerable purification, there was practically complete recovery of activity. The DEAE elution activity profile was not entirely uniform, with the peak sometimes at 0.05 or 0.06 M phosphate. Activity for acetylation of PAS paralleled that for isoniazid, but was much less.

Table I tabulates the stepwise purification data in this instance. All fractions were assayed together. Pooling only the most active fractions of DEAE, a purification of 255-fold was obtained with a recovery of 15% of the activity units originally present. Another 15% was obtained with additional fractions added at an over-all purification of 175-fold. The final product was not yet pure, since polyacylamide gel electrophoresis of the 0.28 to 0.30 DEAE eluates indicated three minor and one major bands of proteins.

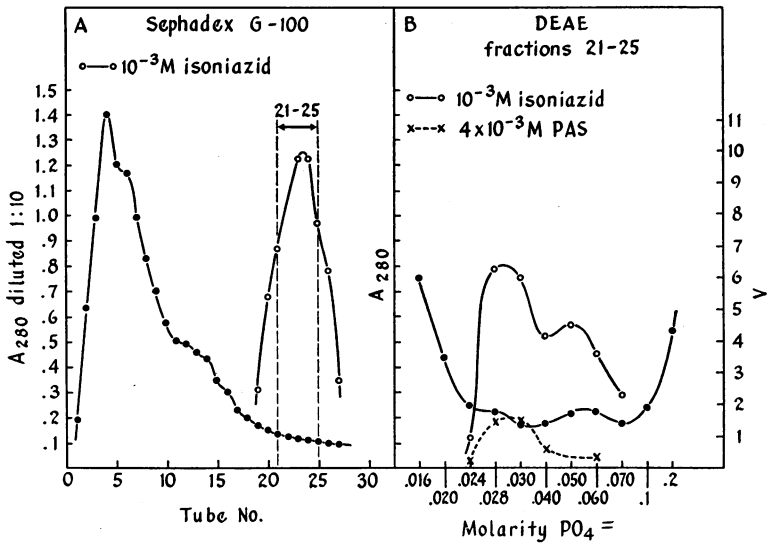

FIG. 3. FURTHER PURIFICATION OF THE AMMONIUM SULFATE FRACTION. Fifty g of a "rapid" liver was processed. Section A is an elution profile from a 4- × 45-cm Sephadex G-100 column, and section B is a batch elution from a 1- × 3-cm DEAE column at pH 7.0. The closed circles represent absorbancy of the fractions at 280 mμ (A_{280}), and the open circles represent the initial velocities expressed as micromoles isoniazid acetylated per minute per milliliter of enzyme solution. Measurements were at 303 mμ (isoniazid) and 320 mμ [*p*-aminosalicylic acid (PAS)], with acetyl-CoA 0.3 mmole and 0.5 ml enzyme solution in a total volume of 3.0 ml at pH 9.0 and 25° C.

In other runs, purification up to 328-fold was obtained, and mean purification was about 300-fold.

Marked concentration of enzyme activity by passage through a 2-cm Sephadex column allowed positive identification of the correct activity peak from livers giving practically no indication of activity upon routine assay of the ammonium sulfate fraction. For study of the properties of the "slow" enzyme, a liver was used for two of the three K_M and hydralazine inhibition studies having only $\frac{1}{70}$ of the activity of the rapid liver with which it was compared. The remaining comparisons were in livers differing ten- to twentyfold.

Properties of the rapid liver transacetylase. At a pH of 6.1 to 6.3, the rapid transacetylase precipitated rather sharply from the soluble fraction between 40 and 50% saturation by ammonium sulfate. It was inactivated by pH below 5.6 and by 40% cold acetone. It withstood freezing and lyophilization, but dialysis of the ammonium sulfate fraction in 0.02 M phosphate buffer at pH 7.0 hastened loss of activity for reasons not known.

When 1 mM isoniazid was used, enzyme activity was unaffected by 10 mM EDTA, 1 mM *p*-chloromercuribenzoate, 2 mM cysteine, or 0.33 mM concentrations of Ca^{++}, Mg^{++}, Mn^{+}, Fe^{++}, and Zn^{++}. Inhibition did occur with Cu^{++}, however, increasing

TABLE I

Purification of isoniazid transacetylase from 50 g of liver

	Total activity	Total protein	Specific activity	Purification
	μmoles/minute	*mg*	*μmoles/minute/mg*	
Soluble fraction* (160 ml)	680	3,360	0.20	1
50% Ammonium sulfate (17 ml)	427	1,240	0.34	1.7
Sephadex fractions 21–25 (50 ml)	370	61	6.1	30
DEAE peak fractions (12 ml)	100	1.96	51.0	255

* Soluble fraction after tissue homogenization.

from a slight effect of 0.1 mmole per L to strong inhibition by 0.33 mM concentrations, introducing the possibility of complex formation with isoniazid (19). One-tenth M cysteine completely inactivated the enzyme, and 0.5 NaCl was strongly inhibitory, but the effect of ionic strength was not explored.

The pH activity profiles for the rapid enzyme in Figure 4 indicate a drop in activity with rising pH from 7.0 to 9.5. However, an activity plateau

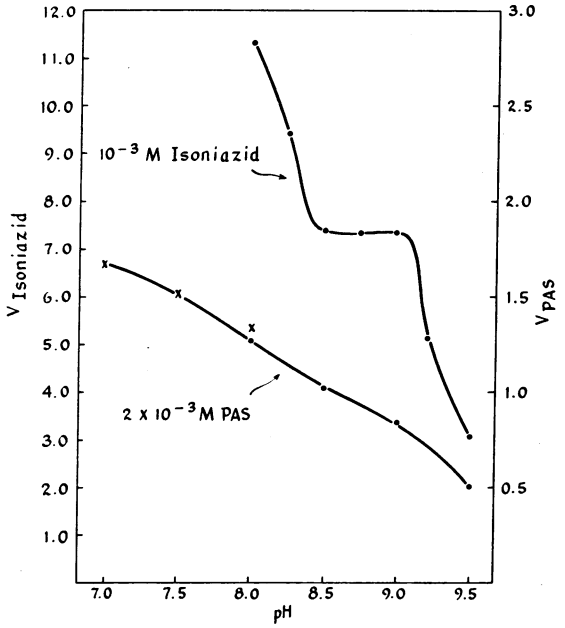

FIG. 4. pH ACTIVITY PROFILES. Sephadex preparation from a rapid liver. Velocities and conditions are as in Figure 3. Buffers are 0.05 M Na pyrophosphate (dots) and 0.05 M K phosphate (x's).

was found at 8.5 to 9.0 for isoniazid, but not PAS, underlining the need for careful control of pH in the assays.

The rapid enzyme displayed marked temperature instability with rapid loss of activity of the Sephadex fractions after 10 minutes of exposure to temperatures over 45° C (Figure 5).

The apparent Michaelis constant for acetylation of isoniazid averaged 0.22 mmole per L (0.19, 0.21, 0.22, 0.25 mmole per L) and 2.5 mmoles per L for acetylation of PAS (2.2, 2.4, 2.7 mmoles per L). With both isoniazid and PAS there occurred substrate inhibition evident at and above 5 mM isoniazid (Figure 6) and 8 mM PAS. The inhibition constant (K_I) for hydralazine inhibition of isoniazid acetylation was 0.025 mmole per L (0.019, 0.024, 0.025, 0.026, 0.029 mmole per L), and for PAS inhibition was 3.1 mmoles per L (2.4, 2.9, 3.1, 3.2, 3.3, 3.7 mmoles per L). Both substances inhibited competitively (Figures 7, 8). The similarity of the K_I for PAS and its apparent acetylation K_M suggests that PAS inhibits by virtue of its competition as substrate for the enzyme.

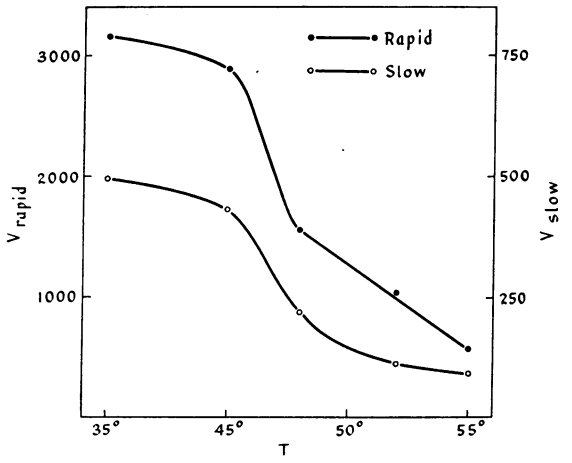

FIG. 5. HEAT INACTIVATION OF A RAPID AND A SLOW LIVER. Sephadex fractions were heated for 10 minutes, cooled, and their activities expressed as millimicromoles isoniazid acetylated per minute per milligram protein.

No special attempt was made to explore substrate specificities at this time. However, with the sensitive spectral assay for acetylation of PNA consisting of a drop in optical density at 420 mμ (20), the results were entirely negative. This contrasted sharply with our results and those of

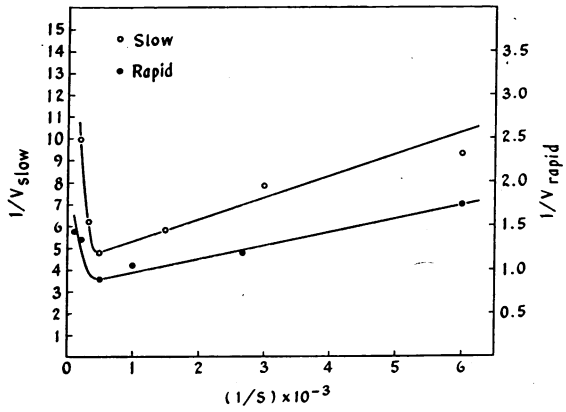

FIG. 6. COMPARATIVE AFFINITY OF A RAPID AND A SLOW LIVER FOR ISONIAZID. Reciprocal plots were made using Sephadex preparation. Conditions and velocities are as in Figure 3. S = substrate concentration.

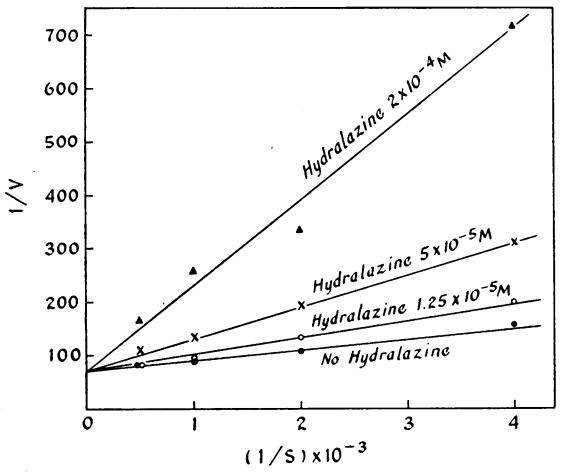

FIG. 7. HYDRALAZINE INHIBITION OF ISONIAZID ACETYLATION. Reciprocal plots were made using Sephadex preparations from a rapid liver. Conditions are as in Figure 3, except measurements at 340 mμ.

others (20) with pigeon liver transacetylase. In summary, the affinity of the enzyme for isoniazid was about ten times that for PAS, and hydralazine was at least 100 times as potent an inhibitor of isoniazid acetylation as PAS. The enzyme had only low affinity for the simple aromatic amines.

Properties of the slow liver transacetylase. The slow enzyme was found to have a number of properties in common with the rapid enzyme. Its ammonium sulfate precipitation requirements and Sephadex behavior were identical. It had similar temperature instability (Figure 5). Its apparent Michaelis constant for isoniazid was the same, averaging 0.22 mmole per L (0.20, 0.22, 0.24 mmole per L), and it showed substrate inhibition at the same isoniazid concentration (Figure 6). Two-tenths mM hydralazine inhibited acetylation of 2 mM isoniazid approximately 50%, concordant with the rapid enzyme. Acetylation activity toward PAS was correspondingly less, as shown by paper chromatography. Further comparisons were not feasible with the limited activity available.

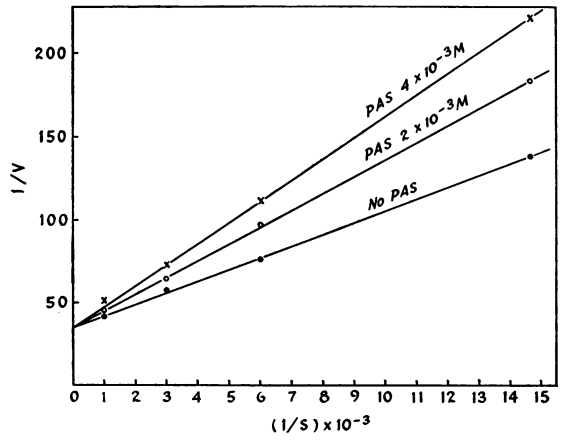

FIG. 8. PAS INHIBITION OF ISONIAZID ACETYLATION. Reciprocal plots were made using Sephadex preparation from a rapid liver. Conditions are as in Figure 3, except measurements at 340 mμ.

The presence of an enzyme inhibitor accounting for the low activity in the slow enzyme preparations was felt to be unlikely, since mixing of the rapid and slow Sephadex preparations with a 30-minute incubation at room temperature produced no inhibition of the rapid enzyme. Mixing of the crude soluble preparations was likewise negative. Twenty-four–hour dialysis of the slow enzyme did not increase its activity.

TABLE II

Isoniazid acetylation activity of matched specimens of liver and jejunal mucosa

"Slow"		"Rapid"	
Liver	Intestine	Liver	Intestine
	mμmoles/minute/mg		
66	46	560	553
53	38	540	570
49	<10	482	82
40	<10	305	214
16	<10	132	100
<10	<10	97	95
<10	<10		
<10	<10		

With acetyl-isoniazid as the substrate and omitting acetyl-CoA, no evidence of deacetylation by the soluble and ammonium sulfate fractions of the slow liver was found. A decreasing optical density at 303 mμ would have been observed in this event.

Extrahepatic acetylation of isoniazid. The transacetylase for isoniazid in small intestinal mucosa was not studied in detail. The specific activities in 14 cases were found to correlate rather well with liver activity, as seen in Table II. The enzyme was similar to the liver enzyme in its ammonium sulfate precipitation requirements and its Sephadex filtration behavior. The enzyme was equally present (specific activity) in ileum and jejunum. The spleen had some enzyme activity, but this was not quantitated. The kidney was always negative.

TABLE III

*12-hour urinary excretion of PAS and AcPAS by rapid and slow inactivators receiving 3.0 g PAS by mouth**

		Bratton-Marshall method			Chromatography		
	t½ INH†	"Free" PAS	AcPAS	AcPAS / Free PAS	PAS	AcPAS	AcPAS / PAS
	minutes	*mg*			*mg*		
				Rapid inactivators			
	45	1,650	1,750	1.06	1,140	1,980	1.74
	50	670	1,590	2.38	480	1,720	3.58
	63	940	1,290	1.37	960	1,400	1.46
	69	1,120	1,640	1.47	1,150	1,850	1.61
	94	1,500	1,360	0.91	1,130	1,400	1.07
Mean	64	1,180	1,530	1.44	1,000	1,670	1.89
				Slow inactivators			
	153	1,300	1,150	0.88	910	1,230	1.35
	193	1,600	1,770	1.11	1,350	1,750	1.30
	204	610	1,990	3.26	650	2,020	3.11
	210	1,870	1,590	0.85	1,450	1,580	1.09
	225	1,500	1,680	1.12	1,100	1,590	1.45
	240	1,540	1,470	0.96	1,400	1,470	1.05
Mean	204	1,400	1,610	1.36	1,140	1,610	1.56

* PAS = *p*-aminosalicylic acid. AcPAS = acetylated PAS.
† INH = isonicotinic acid hydrazide (isoniazid).

Urine excretion studies of PAS acetylation. Table III lists the 12-hour levels of "free" and acetylated PAS in the urine of five rapid and six slow inactivators of isoniazid who were given 3.0-g acid equivalents of PAS by mouth. Both the Bratton-Marshall determination and paper chromatographic quantitation showed no detectable difference in the excretion of acetylated PAS in the two groups or in the ratio of acetylated to free PAS. In the subjects given only 300 mg of PAS, there was a surprisingly sharp increase in the ratio of acetylated to free drug (mean ratio 10.4 ± 2.8), but the two groups again showed no differences.

Discussion

A physiological function for this enzyme has not been elucidated apart from its role in drug detoxification. In view of the mode of its detection and study, it seems appropriate to refer to it for the time being as "isoniazid transacetylase," anticipating a change in nomenclature if a natural substrate is found. Because of its reduced affinity for PNA, PAS, and sulfanilamide, it cannot be thought of as the primary transacetylase in man for aromatic amines.

Recent work by Peters, Miller, and Brown (21) on the urine metabolites of isoniazid shows conclusively that the acetylation step is solely responsible for the differences in isoniazid metabolism between rapid and slow inactivators. Hydrazones of isoniazid appear simply to reflect the amount of isoniazid available for their formation, and differences in isonicotinic acid formation reflect differences in the amount of their principal precursor, acetyl-isoniazid.

There is abundant evidence that the acetylation differences are genetically determined (22–25). Sunahara, Urina, and Ogawa have adequately identified the heterozygote from serum levels in Japanese populations (25). Whether the heterozygote can be identified in Caucasians from serum levels is not yet clear. When the genotype of the offspring was deduced from study of the parents, Evans, Manley, and McKusick found a slightly higher serum level in the heterozygous rapid inactivator than in the homozygous, but levels in a large population still gave only a bimodal distribution (24). Dufour, Knight, and Harris have claimed a trimodal distribution of serum levels (26). These authors grouped a small number of patients (6%) whose serum levels at 6 hours were 0.11 μg per ml or below into a homozygous rapid

category. This could easily have created an artificial third mode from what might simply have been a tailing off of values from the larger intermediate mode (0.13 to 0.8 μg per ml). The hazards of assigning modes from a group of single serum levels after ingestion of a drug have been recently emphasized by Nelson (27).

Examination of our data in this regard is of no help. The half-life data hint of an intermediate mode between 68 and 81 minutes, but the number of individuals comprising this group, 16%, is not in accord with the Hardy-Weinberg binomial distribution. The *in vitro* data indicate a wide range of activities, the majority grouped at a low level, but no clear group separation. Perhaps this is due to the disadvantages of working with postmortem tissue, as well as an assay that lacks precision. The commencement of *in vitro* acetylation rates at relatively lower values than the calculated *in vivo* metabolic rates suggests that the latter actually include other processes contributing to drug disappearance, such as hydrazone formation or continuing diffusion into tissue.

It is desirable to relate these *in vitro* enzyme activities to the amounts of acetylated isoniazid found in urine in rapid and slow inactivators. Peters and co-workers found mean excretions of this metabolite to be 33.8% (32.0 to 35.6) and 17.8% (9.5 to 24.6) of the total in five rapid and three slow inactivators, respectively, during the 24-hour period after ingestion of 10 mg per kg isoniazid. Unchanged isoniazid constituted 3.9% (3.0 to 4.5) and 11.9% (10.5 to 13.7), respectively (21).

Isoniazid circulates until it is either metabolized or excreted as the free drug or its hydrazones. With a renal clearance for the free form of only 41 ml per minute, the kidneys account for only about one-fourth of the plasma clearance in slow inactivators, and one-twelfth in rapid ones (7, 28). The concentrations of isoniazid in plasma at a dose of 10 mg per kg would peak at about 0.1 mmole per L and would average far less. With an apparent K_M of 0.22 mmole per L in each group, the enzyme is quite unsaturated. From Figure 2, typical enzyme activities are 40 mμmoles per L per minute per mg in the slow group and 300 mμmoles per L per minute per mg in the rapid, or a ratio of about one to seven. Since the area under the plasma isoniazid curve after an oral dose is about three times as great in a slow as in a rapid inactivator (29), the slow enzyme is exposed to three times the time-concentration product and works almost three times as hard. Consequently, net production of acetylated isoniazid is almost one-half as great as in the rapid inactivator. The low renal clearance enables isoniazid to recirculate until it is finally metabolized, compensating to a considerable extent for the smaller enzyme activity.

Failure to find a difference in apparent Michaelis constant for isoniazid acetylation in the two groups eliminates the factor of enzyme substrate affinity as an explanation of the gross activity differences observed. It suggests, rather, that we are dealing with differences in concentration of an identical or closely similar enzyme molecule as further strengthened by the similarity in other characteristics of the enzyme in the two groups. However, only complete characterization and quantitation of the purified enzyme molecule can absolutely establish this point.

Other possible mechanisms seem to be eliminated. No deacetylation activity has been demonstrated *in vitro* or *in vivo* (21, 30). An additional acetylating enzyme in the rapid group is no longer likely, since it probably would have been eliminated in the purification procedure leaving similar activities in the two groups. Finally, no evidence can be found by the usual tests for the presence of an inhibitor in the slow preparation.

The mechanism of acetylation of simple aromatic amines in the two groups requires further *in vitro* study. With PAS, failure of the excretion studies to show group differences in acetylation argues strongly against a major role for the isoniazid transacetylase. Since the large 3.0-g dose used might have obscured differences in enzyme activity if production of acetyl-CoA is limiting, the 300-mg dose was also tried and, despite more complete acetylation, it also failed to reveal differences. In the studies of Peters and associates, although the proportions of acetylated isoniazid in the two groups were not greatly different, the ratios of acetylated to unchanged isoniazid differed markedly. The same was true for sulfamethazine administered in comparable doses (8). This contrasted sharply with their results for sulfanilamide and with our results for PAS. Finally, preliminary observations of Evans and White suggest that the capacity of jejunal mucosa to acetylate PABA is monomorphically distributed (31).

Thus, the simple aromatic amines appear not to be significantly acetylated by the isoniazid-sulfamethazine pathway. Some acetylation of PAS by the enzyme is evident from the *in vitro* data and must account for the rather weak inhibition of isoniazid acetylation by PAS *in vivo* when given in massive doses as a companion drug in the treatment of tuberculosis (*7, 32, 33*).

Summary

A soluble enzyme is described in human postmortem liver and small intestinal mucosa that is believed responsible for the heritable differences in acetylation of isoniazid. The enzyme has been partially purified 300-fold. Its affinity for isoniazid is approximately ten times that for *p*-aminosalicylic acid (PAS). PAS and hydralazine inhibit isoniazid acetylation competitively, the latter over 100 times as strongly as PAS. No acetylation of *p*-nitroaniline can be detected. From livers representative of the "rapid" and "slow" groups, concentrated preparations of the enzyme showed no difference in apparent Michaelis constant for isoniazid, and, in several other respects, the enzymes behaved identically. The differences in activity of these groups do not appear to be on the basis of differences in affinity of the enzyme for isoniazid, but may be due to differences in amount of an identical enzyme molecule, representing a mutation in the genic mechanism controlling rate of enzyme synthesis. Characterization of a completely purified enzyme is necessary to establish this fact.

Excretion of free and acetylated PAS in the urine of rapid and slow inactivators of isoniazid does not show acetylation differences. A study of sulfanilamide excretion by others shows a similar lack of differences. Although some overlap probably exists, there appear to be two acetylation pathways available in man: one with widely differing acetylation capacities utilized by isoniazid and certain polycyclic amines, and another with uniform capacity utilized by simple aromatic amines.

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References

1. Kalow, W. Pharmacogenetics. Philadelphia, Saunders, 1962.
2. Evans, D. A. P. Pharmacogénétique. Méd. et Hyg. (Genève) 1962, **20**, 905.
3. Evans, D. A. P., and T. A. White. Human acetylation polymorphism. J. Lab. clin. Med. 1964, **63**, 394.
4. Jenne, J. W. Isoniazid acetylation by human liver and intestinal mucosa. Fed. Proc. 1963, **22**, 540.
5. Frymoyer, J. W., and R. F. Jacox. Investigation of the genetic control of sulfadiazine and isoniazid metabolism in the rabbit. J. Lab. clin. Med. 1963, **62**, 891.
6. Frymoyer, J. W., and R. F. Jacox. Studies of genetically controlled sulfadiazine acetylation in rabbit livers: possible identification of the heterozygous trait. J. Lab. clin. Med. 1963, **62**, 905.
7. Jenne, J. W., F. M. MacDonald, and E. Mendoza. A study of the renal clearances, metabolic inactivation rates, and serum fall-off interaction of isoniazid and para-aminosalicylic acid in man. Amer. Rev. resp. Dis. 1961, **84**, 371.
8. Peters, J. H., G. R. Gordon, and P. Brown. The relationship between the capacities of human subjects to acetylate isoniazid, sulfanilamide and sulfamethazine. Life Sci. (Oxford) 1965, **4**, 99.
9. Evans, D. A. P. Pharmacogenetics. Amer. J. Med. 1963, **34**, 639.
10. Johnson, W. J. The inhibition of sulfanilamide acetylation by aromatic and heterocylic carboxamides and carboxhydrazides. Canad. J. Biochem. 1955, **33**, 107.
11. Jenne, J. W., and P. D. Boyer. Kinetic characteristics of the acetylation of isoniazid and *p*-aminosalicylic acid by a liver-enzyme preparation. Biochim. biophys. Acta (Amst.) 1962, **65**, 121.
12. Jenne, J. W. Studies of human patterns of isoniazid metabolism using an intravenous fall-off technique with a chemical method. Amer. Rev. resp. Dis. 1960, **81**, 1.
13. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. Protein measurement with the Folin phenol reagent. J. biol. Chem. 1951, **193**, 265.
14. Schulert, A. R. Physiological disposition of hydralazine (1-hydrazinophthalazine) and a method for its determination in biological fluids. Arch. int. Pharmacodyn. 1961, **132**, 1.
15. Perry, H. M., Jr. A method of quantitating 1-hydrazinophthalazine in body fluids. J. Lab. clin. Med. 1953, **41**, 566.

16. Way, E. L., C. T. Peng, N. Allawada, and T. C. Daniels. The metabolism of p-aminosalicylic acid in man. J. Amer. pharm. Ass., Sci. Ed. 1955, **44**, 65.
17. Marshall, E. K., Jr. Determination of para-aminosalicylic acid in blood. Proc. Soc. exp. Biol. (N. Y.) 1948, **68**, 471.
18. Way, E. L., P. K. Smith, D. L. Howie, R. Weiss, and R. Swanson. The absorption, distribution, excretion and fate of para-aminosalicylic acid. J. Pharmacol. exp. Ther. 1948, **93**, 368.
19. Albert, A. The affinity of isonicotinic hydrazide for metals. Experientia (Basal) 1953, **9**, 370.
20. Tabor, H., A. H. Mehler, and E. R. Stadtman. The enzymatic acetylation of amines. J. biol. Chem. 1953, **204**, 127.
21. Peters, J. H., K. S. Miller, and P. Brown. Studies on the metabolic basis for the genetically-determined capacities for isoniazid inactivation in man. J. Pharmacol. exp. Ther. In press.
22. Bönicke, R., and B. P. Lisboa. Uber die Erbbedingtheit der intraindividuellen Konstanz der Isoniazidausscheidung beim Menschen. Naturwissenschaften 1957, **44**, 314.
23. Knight, R. A., M. J. Selin, and H. W. Harris. Genetic factors influencing isoniazid blood levels in humans. Transactions of the 18th Conference on Chemotherapy of Tuberculosis, Veterans Administration Armed Forces 1959, 52.
24. Evans, D. A. P., K. A. Manley, and V. A. McKusick. Genetic control of isoniazid metabolism in man. Brit. med. J. 1960, **2**, 485.
25. Sunahara, S., M. Urina, and M. Ogawa. Genetical and geographic studies on isoniazid inactivation. Science 1961, **134**, 1530.
26. Dufour, A. P., R. A. Knight, and H. W. Harris. Genetics of isoniazid metabolism in Caucasians, Negro, and Japanese populations. Science 1964, **145**, 391.
27. Nelson, E. Kinetic considerations in pharmacogenetic studies of drug metabolism. Life Sci. (Oxford) 1965, **4**, 949.
28. Jenne, J. W. Pharmacokinetics and the dose of isoniazid and p-aminosalicylic acid in the treatment of tuberculosis. Antibiot. et Chemother. (Basel) 1964, **12**, 407.
29. Bartmann, K., and W. Massmann. Der Blutspiegel des INH bei Erwachsanen und Kindern. Beitr. klin. Tuberk. 1960, **122**, 239.
30. Yard, A. S., and H. McKennis, Jr. Aspects of the metabolism of isoniazid and acetylisoniazid in the human and the dog. J. med. pharm. Chem. 1962, **5**, 196.
31. Evans, D. A. P. Acetylation polymorphisms. Proc. roy. Soc. Med. 1964, **57**, 508.
32. Peters, J. H. Studies on the metabolism of isoniazid. II. The influence of para-aminosalicylic acid on the metabolism of isoniazid by man. Amer. Rev. resp. Dis. 1960, **82**, 153.
33. Lauener, H., and G. Favez. The inhibition of isoniazid inactivation by means of PAS and benzoyl-PAS in man. Amer. Rev. resp. Dis. 1959, **80**, 26.