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Research Article

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Monogenic Control of Variations in Antipyrine Metabolite Formation

NEW POLYMORPHISM OF HEPATIC DRUG OXIDATION

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ABSTRACT To investigate mechanisms that control large variations among normal uninduced subjects in the elimination of the model compound antipyrine (AP) and other drugs, AP was administered to 144 subjects (83 unrelated adults and 61 members of 13 families). Thereafter, at regular intervals for 72 h, the urine of each subject was collected and concentrations of AP and its three main metabolites measured. From these urinary concentrations, rate constants for formation of each AP metabolite were calculated. Trimodal curves were observed when values for each AP rate constant were plotted in 83 unrelated subjects; probit plots of these values showed inflections at the two antimodes of each trimodal distribution. All members of our 13 families were assigned one of three phenotypes determined by where their AP metabolite rate constant placed them in the trimodal distributions derived from the 83 unrelated subjects. In each family, pedigree analysis to identify the mode of transmission of these three phenotypes was consistent with their monogenic control. These results provide evidence for a new polymorphism of drug oxidation in man.

INTRODUCTION

Since the therapeutic success or failure of numerous drugs can be influenced by large interindividual variations in their metabolism, it is not surprising that causes of such differences among patients have been intensively investigated. Efforts to elucidate sources of these interindividual variations have utilized different methods and approaches. The results reflected not only the design of the particular study but also the criteria used to select subjects (1). The conclusion that genetic factors are primarily responsible for such variations

among normal subjects under carefully controlled, relatively noninducing environmental conditions derived from studies using antipyrine (AP) (2-4),¹ amobarbital (5), dicoumarol (6), ethanol (7), halothane (8), phenylbutazone (9), phenytoin (10), and salicylate (11). Nevertheless, it is also well recognized that numerous other host factors, including many disease states, can alter the genetically controlled capacity of a subject to eliminate certain drugs (1).

The present study measuring AP metabolite concentrations in carefully selected families and unrelated subjects represents an attempt to investigate variations in the primary gene products more directly than in the afore-mentioned twin studies on parent drugs. Rate constants for AP metabolite formation are as close as it is ethically possible to achieve in human studies to the primary gene products involved in AP metabolism. These primary gene products are different molecular forms of hepatic cytochrome P-450.

The three AP metabolites investigated were 4-hydroxyantipyrine (4-OH-AP), 3-hydroxymethylantipyrine (3-OHM-AP), and *N*-demethylantipyrine (NDM-AP) (3, 12-15). Evidence obtained in rats suggests that each AP metabolite is formed by a separate combination of hepatic cytochrome P-450 mediated monooxygenases (16, 17). It was concluded that variations in each separate monooxygenase are controlled by a different genetic locus (16, 17).

After AP metabolites are formed, conjugation with glucuronide occurs, rendering the oxidative products even more water soluble and hence more easily excreted in urine where the AP metabolites were identified (12-18). Thus far, AP metabolites have not been

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¹ *Abbreviations used in this paper:* AP, antipyrine; 4-OH-AP, 4-hydroxyantipyrine; k_{el} , elimination rate constant; k_i , rate constant of metabolite formation; NDM-AP, *N*-demethylantipyrine; 3-OHM-AP, 3-hydroxymethylantipyrine.

detected in human plasma. From measurements made in urine over a 72-h period after AP administration, we calculated rate constants for AP metabolite formation in normal human twins (3). In the present study we applied this method and experimental design to normal unrelated subjects and also to members of 13 families. Our main objective was to investigate mechanisms that control large variations in AP metabolite rate constants among normal uninduced subjects.

The rationale for the approach used in the present study has several sources. Firstly, conventional methodology in human genetics has repeatedly and successfully relied on testing a genetic hypothesis by analyses of pedigrees in which the phenotypes of the family members were assigned according to their places within distribution curves of data from unrelated subjects. This approach helped to elucidate several pharmacogenetic conditions, including atypical plasma cholinesterase, the acetylation polymorphism, and glucose-6-phosphate dehydrogenase deficiency. Secondly, the results of a study on normal uninduced twins suggested that genetic factors were mainly responsible for large interindividual variations in rate constants for formation of the three main AP metabolites (3). Since a twin study can implicate genetic factors but cannot establish their Mendelian mode of transmission, the present investigation utilizing pedigree analysis was indicated to test whether monogenic or polygenic mechanisms were involved and if monogenic mechanisms were operative, to determine their mode of transmission. To answer these questions, phenotypes were necessary for each of the 61 members of our 13 families. Assignment of these phenotypes was made according to conventional methods in human genetics. Thus, to permit pedigree analysis the phenotype of each parent or child was assigned according to his or her position within the trimodal curves generated in our 83 unrelated subjects.

The present study depended heavily on two additional considerations: (a) recent development of sensitive, reliable methods to measure labile AP metabolites in urine; (b) participation only of uninduced subjects selected according to rigid criteria that assured uniformity of critical environmental factors (1).

METHODS

Selection of subjects. Normal, uninduced subjects between the ages of 18 and 55 (61 from 13 two-generation families and another 83 who were unrelated) participated. They came from Hershey and vicinity and were recruited through notices placed in our medical school and in local newspapers. Subjects were carefully screened; none had a history of serious illness, or consumed any medication or ethanol regularly. None smoked tobacco or were chronically exposed to inducing or inhibiting chemicals. Subjects had an average daily caloric intake of 2,300 to 2,400 cal with low

methylxanthine consumption and the dietary ratio of protein to carbohydrate to fat was ~15:50:35%.

Sample collection. At 0900, after a 12-h fast, all subjects ingested AP (18 mg/kg) dissolved in 150 ml of water. Saliva samples (2 ml each), stimulated by chewing parafilm, were collected 3, 6, 9, 12, and 24 h after AP administration. Total urinary output was collected for 3 full days after AP administration through timed specimens at the following intervals: 0-12, 12-24, 24-36, 36-48, 48-64, and 64-72 h. Sodium metabisulfite (4 mg/ml) was added as an antioxidant to each urine sample. Aliquots of urine were frozen at -70°C. Analysis was performed within 2 wk of AP administration. All subjects abstained from caffeine, methylxanthines, alcohol, and acute medications during the 3-d study.

Assay of AP in saliva and AP metabolites in urine. Antipyrine was extracted from 1 ml saliva using the method of Prescott et al. (19). According to the procedure of Danhof et al. (18), 3-OHM-AP, 4-OH-AP, and NDM-AP were extracted from 1 ml urine. A minor modification of this procedure was a longer extraction time (60 s).

Chromatography and column preparation. AP and metabolites were separated by high performance liquid chromatography (Waters 6000 A, Waters Associates Inc., Milford, MA) and quantitated by an integrating recorder (Hewlett-Packard 3380-S Hewlett Packard Co., Palo Alto, CA). Columns (10 cm × 3.2 mm) were prepared in our laboratory as follows: Lichrosorb RP-2 (0.8 g) with a mean particle size of 5 μm (E. Merck, Darmstadt, Federal Republic of Germany) was suspended in 30 ml carbon tetrachloride in a commercially available slurry column packer (Micromeritics Instrument Corp., Norcross, GA). The suspension was stirred slowly for 5 min using a rheostat-controlled automatic stirring plate. The packer lid was then tightened; hexanes (Fisher H-291, Fisher Scientific Co., Pittsburgh, PA) were pumped slowly (0.5 ml/min) through the system. When the first drops appeared from the column outlet tubing, flow was increased to 9.9 ml/min. Pressure rose to 6,000 psi and was maintained there until flow from the column outlet tubing was drastically reduced. Pressure was then allowed to return to zero, the column removed from the packing apparatus, and a frit (2-μm pore size) placed over the column inlet. Columns were equilibrated according to the method of Danhof et al. (18). The mobile phase (0.05 M sodium phosphate buffer, pH 6.5, with 5% acetonitrile) ran through the column (0.8 ml/min) continuously throughout the entire experiment when samples were not being injected. This practice eliminated column stress from turning flow on and off daily.

Standard curves. Linear standard curves were generated by using commercially available AP (Matheson, Coleman, and Bell, Rutherford, NJ) and 4-OH-AP and NDM-AP (Aldrich Chemical Co., Milwaukee, WI). Standards of 3-OHM-AP were a generous gift of D. D. Breimer, University of Leiden, The Netherlands. To prevent loss of NDM-AP through adhesion to glass, all glassware in contact with AP metabolites was coated with a silicone preparation (Prosil-28).

Pharmacokinetic analysis. Development of a useful measurement or index of each hepatic cytochrome P-450 that participates in producing each AP metabolite requires a parameter that reflects the formation rate of each metabolite. The rate constant of metabolite formation, k_i , seemed most appropriate for this purpose. In a twin study on rate constants for AP metabolite formation (3) the utility of another measurement, the amount of each AP metabolite in timed urine specimens, was examined 4, 8, 12, and 72 h after AP administration. While amounts of each AP metabolite in urine reflected the genetic factors observed in that study, they did

so less efficiently and consistently than the rate constant for AP metabolite formation.

The disposition of AP and its metabolites can be described by a series of monoexponential equations (13, 20) and conforms to a one-compartment model in which the three main AP metabolites are produced by parallel simultaneously occurring, first order reactions. In addition, AP metabolite excretion is very much faster than AP metabolite formation (3, 13, 20). Thus:

$$\log(\text{rate of excretion})_i = \log(k_i \cdot \text{dose}) - \frac{k_{e1}t}{2.303} \quad (1)$$

in which k_i is the rate constant for formation of the i^{th} metabolite and k_{e1} is the overall rate constant describing AP elimination. In these studies, k_{e1} was calculated using the following relationship:

$$k_{e1} = 0.693/t_{1/2}, \quad (2)$$

where $t_{1/2}$ is AP half-life calculated by least squares regression analysis of the curve relating log saliva AP concentration to time after AP administration.

The first order rate constant for metabolite formation (k_i) was obtained from urinary excretion data by extrapolating to 0 the linear portion of the curve generated from equation 1, that yields:

$$k_i = \text{extrapolated time 0 intercept/dose}. \quad (3)$$

Probit analysis. The probit, y , is defined as the normal equivalent deviation, $x - \mu/\sigma$, increased by 5, where x is the log of each data point, and μ and σ are the population mean and standard deviation, respectively. Thus:

$$y = 5 + x - \mu/\sigma \quad (4)$$

Regression lines formed when probits are plotted against log drug concentrations, or in the present case log rate constants, show inflection points where antimodes occur in a distribution. For that reason, probit analysis provides an objective means of identifying modes and antimodes.

Pedigree analysis. Pedigree analysis was performed on each of 13 families investigated. The phenotype of the 61 members of these 13 families was determined by each subject's position on the distribution curves constructed in 83 unrelated subjects (Figs. 1-4).

Correlations between relatives. Parent-child and mid-parent-midchild interclass correlation coefficients were calculated according to the following formula:

$$r = \sum(xy) / [\sum(x^2) \sum(y^2)]^{1/2}, \quad (5)$$

where $\sum(xy)$ is the covariance of x (group 1) and y (group 2), respectively. Intraclass correlations were calculated for sibs as follows:

$$r = MS_b - MS_w / MS_b - (\bar{k} - 1)MS_w, \quad (6)$$

where MS_b and MS_w are mean squares between and within sibships, respectively, and \bar{k} is the geometric mean number of sibs per family. If a trait is controlled entirely by genetic factors, these values approximate the percentage of genes that relatives share. Under such conditions, correlations between parent and offspring and between sibs are expected to be 0.5. Factors that can cause these correlations to be <0.5 include effects of dominance, differential action of environmental factors on family members, nonrandom mating, and effects of statistical error due to small numbers (21).

Correlations of rate constants within subjects. To help elucidate potential relationships among factors that control each pathway for formation of the three main AP metabolites, we determined correlations within each subject for these different rate constants.

RESULTS

Four measurements based on values for rate constants of AP metabolite formation (pedigree analysis, intraclass and interclass correlations, distribution curves and probits) and also Hardy-Weinberg analysis provided evidence to indicate monogenic control of interindividual variations in rate constants for formation of each AP metabolite. Two alleles at a single locus appeared to regulate these variations.

Table I shows, in 61 members of 13 families, AP elimination rate constants, k_{e1} , and rate constants for formation of each of the three main AP metabolites, k_i . All subjects were under the carefully controlled environmental conditions described in Methods. Thus, none could be considered to exhibit markedly induced or inhibited hepatic AP-metabolizing activity. The extent of interindividual variation among these 61 family members was four-, seven-, four-, and fivefold for k_{e1} , $k_{4\text{-OH-AP}}$, $k_{3\text{-OHM-AP}}$, and $k_{\text{NDM-AP}}$, respectively. The magnitude of intraindividual variations was very much smaller than that of interindividual variations as indicated by a reproducibility within 20% of the initial value in each subject when the same AP dose was readministered 4 wk after the initial dose. Table I shows initial values.

In 83 unrelated subjects comprising a separate group from the 61 family members shown in Table I, distribution curves (Figs. 1-4) were constructed from values for k_{e1} , $k_{4\text{-OH-AP}}$, $k_{3\text{-OHM-AP}}$, and $k_{\text{NDM-AP}}$. Each of these curves was trimodal. Phenotypes of the 61 family members were determined depending on the position of each subject's value on these curves. Phenotype assignments were reproducible for values within most modes, but as values approached the antimodes, phenotype assignment became more difficult. For such subjects with values close to the antimodes, three or four repeat AP administrations were necessary to determine this assignment. For $k_{\text{NDM-AP}}$ (Fig. 4), overlap between the low and intermediate modes occurred for several of the 83 unrelated subjects; thus, phenotyping would have been uncertain for subjects in this area of overlap. Fortunately, no such problem arose in phenotyping the 61 subjects from the 13 families for 4-OH-AP or 3-OHM-AP because no values occurred in this range of overlap, but values of NDM-AP did overlap for 9 of the 61 subjects. Phenotypes for these subjects were assigned by their position in relationship to the two inflection points of the probit regression line (Fig. 4).

TABLE I
AP k_{el} and k_1 in 61 Subjects from 13 Families

Family member	$h^{-1} \times 10^{-2}$				Family member	$h^{-1} \times 10^{-2}$					
	k_{el}	$k_{4-OH-AP}$	$k_{3-OHM-AP}$	k_{NDA}		k_{el}	$k_{4-OH-AP}$	$k_{3-OHM-AP}$	k_{NDA}		
Family 1	I ₁	6.24	3.07	1.99	0.77	Family 7	I ₁	5.34	1.79	0.79	0.76
	I ₂	7.70	2.86	1.17	1.17		I ₂	11.00	1.57	0.76	0.62
	II ₁	7.53	2.13	1.54	1.17		II ₁	12.83	1.93	0.91	0.83
	II ₂	7.00	2.66	1.91	1.17	II ₂	8.09	1.94	0.72	0.50	
	II ₃	6.03	2.85	1.79	1.00	Family 8	I ₁	7.57	1.63	0.67	0.70
	II ₄	9.42	5.90	2.19	1.26		I ₂	9.40	1.52	0.73	0.73
II ₅	7.33	2.09	1.25	0.83	II ₁		7.10	1.30	0.62	0.52	
Family 2	I ₁	5.63	2.53	1.05	0.83	II ₂	6.36	1.57	0.85	0.68	
	I ₂	12.37	2.84	1.11	1.10	II ₃	10.52	2.06	0.99	0.83	
	II ₁	6.79	1.41	0.91	0.61	Family 9	I ₁	5.68	2.29	0.72	0.50
	II ₂	5.10	1.14	0.61	0.68		I ₂	8.35	1.88	0.99	0.75
	II ₃	9.76	2.49	1.09	0.73		II ₁	9.49	1.58	1.11	0.53
Family 3	I ₁	4.65	1.55	0.45	0.76	II ₂	7.77	2.65	1.33	0.93	
	I ₂	4.80	3.43	1.59	1.42	Family 10	I ₁	6.60	2.27	0.68	0.85
	II ₁	3.30	1.07	0.61	0.50		I ₂	8.06	1.74	0.56	0.49
	II ₂	4.23	1.53	1.11	0.89		II ₁	6.42	1.76	0.77	0.53
Family 4	I ₁	4.39	2.70	1.17	0.77	II ₂	7.96	2.02	0.86	0.63	
	I ₂	7.37	2.07	0.72	0.72	Family 11	I ₁	8.45	3.55	2.20	1.26
	II ₁	8.35	1.22	0.63	0.49		I ₂	4.65	3.00	2.00	1.15
	II ₂	9.12	1.25	0.59	0.39		II ₁	6.73	1.39	0.77	0.56
Family 5	I ₁	5.81	1.16	0.84	0.48	II ₂	5.73	1.41	1.17	1.04	
	I ₂	7.46	1.73	0.64	0.68	Family 12	I ₁	3.48	2.47	1.15	1.01
	II ₁	7.46	0.78	0.96	0.42		I ₂	14.43	2.26	0.91	0.95
	II ₂	5.31	1.39	0.75	0.67		II ₁	4.59	1.67	0.92	0.51
	Family 6	I ₁	5.03	1.10	0.70	0.33	II ₂	5.37	2.69	1.26	1.39
I ₂		11.88	3.11	1.00	0.67	Family 13	I ₁	5.02	2.97	0.99	1.00
II ₁		9.35	1.94	1.17	0.79		I ₂	7.00	3.46	0.42	1.30
II ₂		5.54	0.96	0.64	0.83		II ₁	7.70	3.09	0.95	2.08
II ₃		5.17	1.33	0.75	0.42		II ₂	6.86	2.90	1.87	1.06
					II ₃		4.12	0.61	0.30	0.26	
					II ₄	6.86	3.23	0.37	1.70		
					II ₅	4.85	3.49	0.38	1.92		

Probit regressions, shown in Figs. 1-4, confirmed the trimodality observed in the distribution curves. The position of the two inflection points in each probit regression shown in Figs. 1-4 was determined as follows: an arrow identifies the last subject on the slope of a curve such that the next subject clearly belongs on a curve with a different slope. This change of slope is expressed either by being marked, as in the case of the final point on each curve, or by virtue of all subjects being on a curve of perceptively different slope, as applies to the middle portion of the probit curve lying between the two arrows. By contrast, in the first portion of the probit curves shown in Figs. 1-4, the portion that contains the majority of subjects and occurs before the first arrow, some subjects lie slightly off the line. However, these subjects clearly do not start a new line

of different slope because subsequent subjects return to the same line. Such departures and subsequent returns without a change in slope appear frequently in probit analyses of drug metabolism in human subjects, as do small breaks in the curves at both ends where the number of subjects decreases (22).

Inflection points in the k_{el} regression occurred at -1.05 and -0.90 . These log values were at the antimodes (arithmetic values of 9×10^{-2} and 12.5×10^{-2}) of the k_{el} distribution curve (Fig. 1). Inflections in the regression for $k_{4-OH-AP}$ (-1.55 and -1.40), $k_{3-OHM-AP}$ (-1.80 and -1.66), and k_{NDM-AP} (-2.00 and -1.85), also occurred at antimodes (2.8×10^{-2} and 4.0×10^{-2} , 1.6×10^{-2} and 2.2×10^{-2} , and 1×10^{-2} and 1.4×10^{-2} , respectively) of their distribution curves (Figs. 2-4).

Subjects whose phenotypes were in the low rate con-

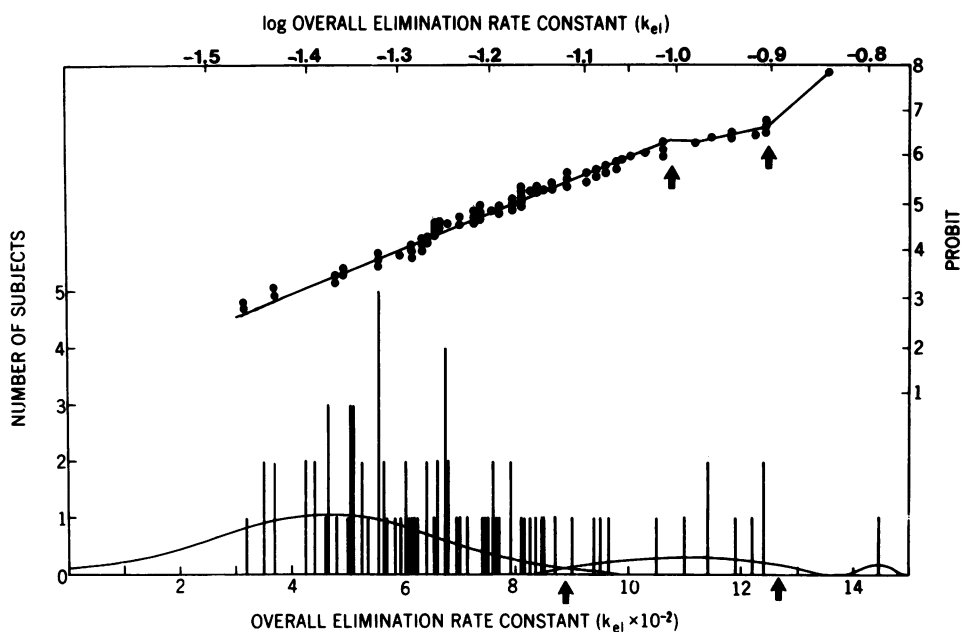


FIGURE 1 Frequency distribution of k_{el} in 83 unrelated subjects. Computerized drawings of the three modes are superimposed. Probits are plotted above the frequency distributions. Arrows indicate inflection points. Note that arrows on the probit curve point to log values, whereas on the distribution curve, corresponding arrows are on an arithmetic scale. Also note that the values at corresponding arrows are identical.

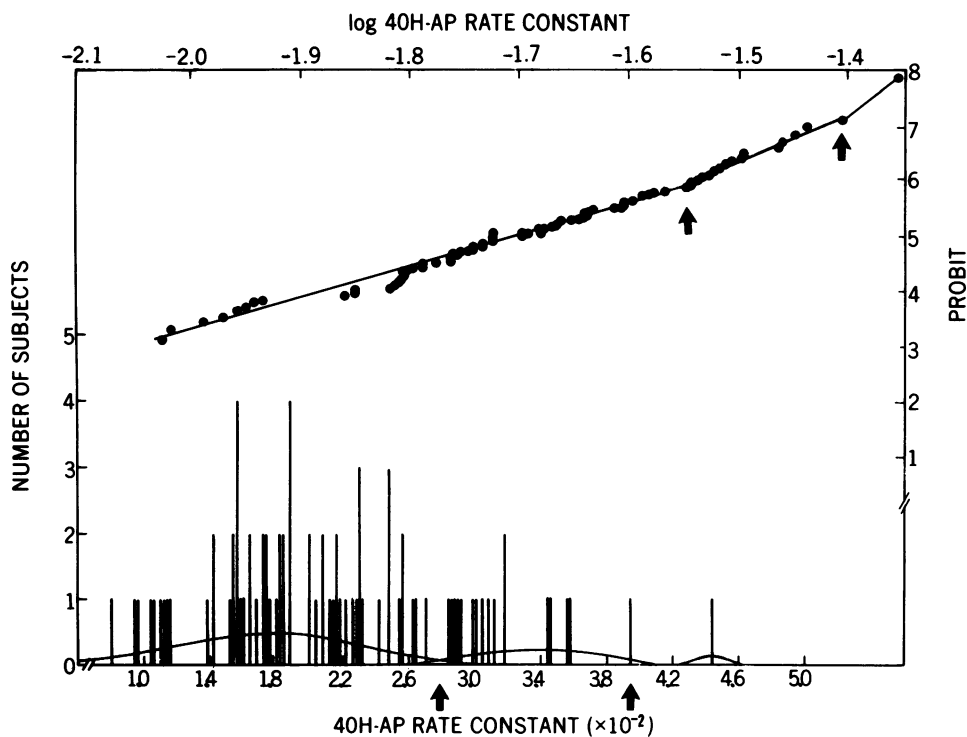


FIGURE 2 Frequency distribution of rate constants for formation of $k_{4-OH-AP}$ in 83 unrelated subjects. Computerized drawings of the three modes are superimposed. Probits are plotted above the frequency distributions. Arrows indicate inflection points. Note that arrows on the probit curve point to log values, whereas on the distribution curve, corresponding arrows are on an arithmetic scale. Also note that the values at corresponding arrows are identical.

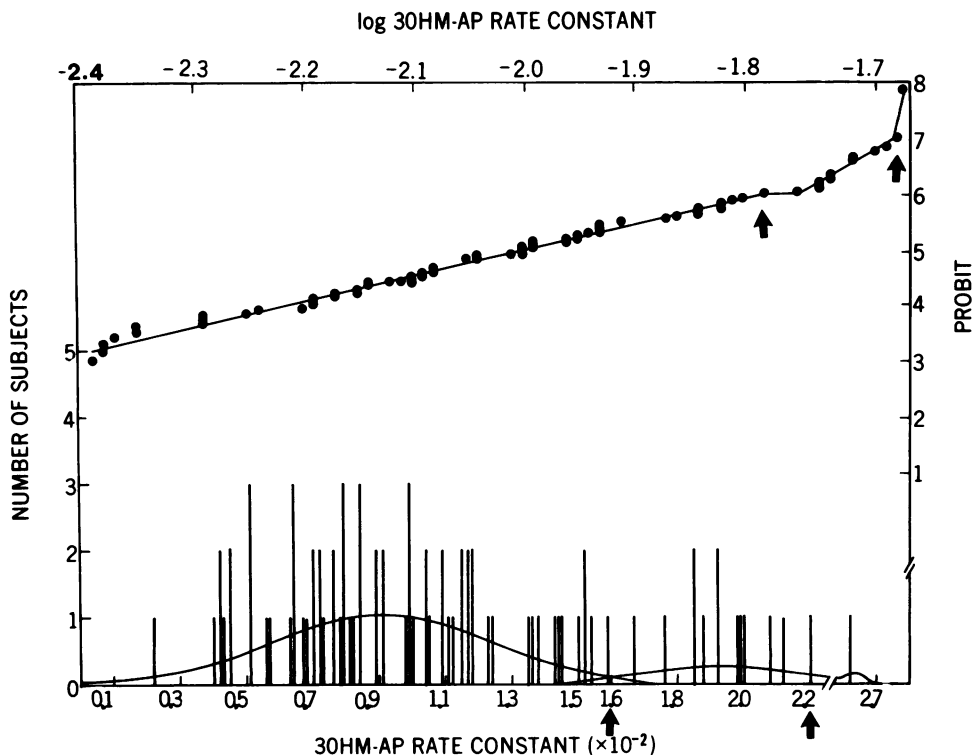


FIGURE 3 Frequency distribution of rate constants for formation of $K_{3\text{-OHM-AP}}$ in 83 unrelated subjects. Computerized drawings of the three modes are superimposed. Probits are plotted above the frequency distributions. Arrows indicate inflection points. Note that arrows on the probit curve point to log values, whereas on the distribution curve, corresponding arrows are on an arithmetic scale. Also note that the values at corresponding arrows are identical.

stant range were assigned genotypes $aa(k_{el})$, $ff(k_{4\text{-OH-AP}})$, $tt(k_{3\text{-OHM-AP}})$, and $nn(k_{\text{NDM-AP}})$. Subjects whose phenotypes fell in the intermediate range were assigned genotypes of Aa , Ff , Tt , and Nn , whereas those in the high range were given genotypes of AA , FF , TT , and NN . Alleles that determine low and high rate constants were considered codominant since identification of heterozygotes was made possible by the existence of a middle mode in each distribution curve.

Hardy-Weinberg analysis of genotype frequencies for the 83 unrelated subjects is shown in Table II. Gene frequencies were $P = 0.92$, $q = 0.08$; $P = 0.89$, $q = 0.11$; $P = 0.91$, $q = 0.09$; and $P = 0.80$, $q = 0.20$, for k_{el} , $k_{4\text{-OH-AP}}$, $k_{3\text{-OHM-AP}}$, and $k_{\text{NDM-AP}}$, respectively. Observed genotypes did not differ in frequency from Hardy-Weinberg expectations by chi-square analysis (Table II).

For each rate constant, correlations between first degree relatives (Table III) are expected to be close to 0.5. In our study midparent-midchild values were 0.40 for $k_{4\text{-OH-AP}}$, 0.48 for $k_{3\text{-OHM-AP}}$, 0.52 for $k_{\text{NDM-AP}}$, and 0.31 for k_{el} . Parent-child and sib-sib correlations were slightly lower (Table III) than the expected value

of 0.5. Large standard errors of these correlations (Table III), probably due to the relatively small number of family members studied, may explain why these correlations failed to reach statistical significance.

Correlations of 0.7, 0.4, and 0.4 ($P < 0.01$) occurred between rate constants for formation of different AP metabolites within the same subject (Table IV). For Table IV, all 144 related and unrelated subjects were used.

To attempt further separation of phenotypes than was achieved in Figs. 1-4 where some overlap occurred, we plotted values for the following ratios of rate constants: 3-OHM-AP/NDM-AP, 4-OH-AP/NDM-AP, 4-OH-AP/3-OHM-AP. Trimodality also occurred in each of these three plots, and, as in Figs. 2-4, these distributions conformed to Hardy-Weinberg expectations with gene frequencies for P and q of 0.91 and 0.09 for each ratio. However, no further separation of the three modes was achieved beyond that shown in Figs. 1-4.

Figs. 5-8 show the 13 pedigrees. Each pedigree involving rate constants for AP metabolite formation (Figs. 5-7) was consistent with a monogenic hypoth-

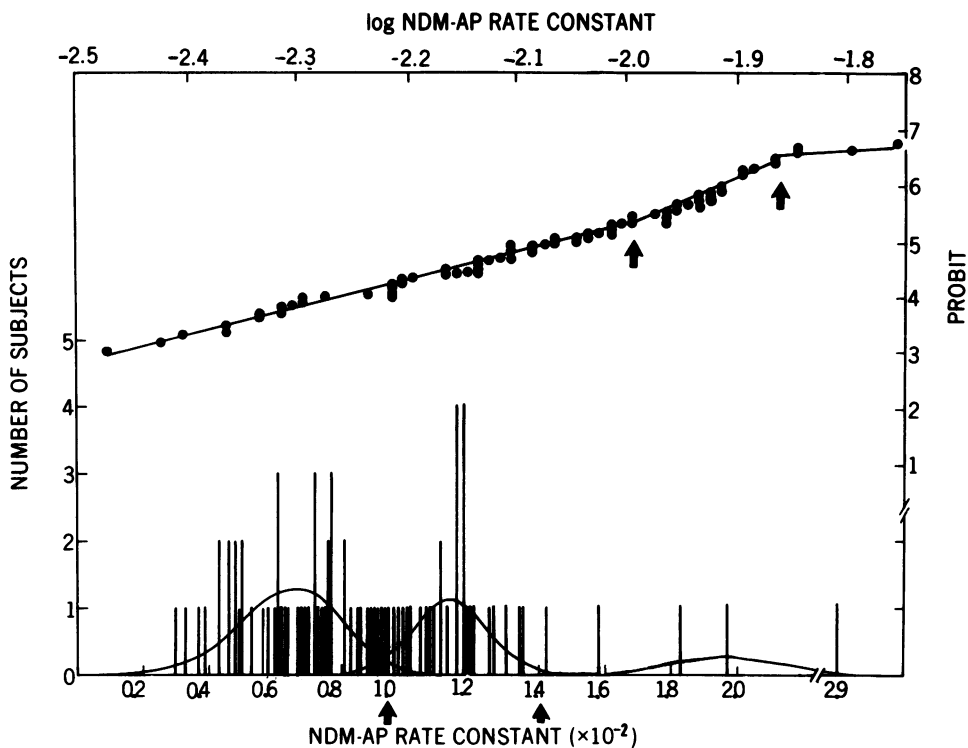


FIGURE 4 Frequency distribution of rate constants for formation of $k_{\text{NDM-AP}}$ in 83 unrelated subjects. Computerized drawings of the three modes are superimposed. Probits are plotted above the frequency distributions. Arrows indicate inflection points. Note that arrows on the probit curve point to log values, whereas on the distribution curve, corresponding arrows are on an arithmetic scale. Also note that the values at corresponding arrows are identical.

TABLE II
Hardy-Weinberg Analysis of Genotype Frequencies for k_i and AP k_{el} in 83 Unrelated Subjects

Rate constant	Genotype	Observed (O)	Expected (E)	$\frac{(O-E)^2}{E}$	Chi-square $df = 1,$ $n = 83$
$k_{4\text{-OH-AP}}$	ff	65	65.7	0.008	0.112 NS
	Ff	17	16.3	0.104	
	FF	1	1.0	0.000	
	$P = 0.89$	$q = 0.11$			
$k_{3\text{-OHM-AP}}$	tt	69	68.7	0.042	0.197 NS
	Tt	13	13.6	0.026	
	TT	1	0.7	0.129	
	$P = 0.91$	$q = 0.09$			
$k_{\text{NDM-AP}}$	nn	53	53.1	0.000	0.033 NS
	Nn	27	26.6	0.006	
	NN	3	3.3	0.027	
	$P = 0.80$	$q = 0.20$			
k_{el}	aa	70	70.3	0.001	0.504 NS
	Aa	12	12.2	0.003	
	AA	1	0.5	0.500	
	$P = 0.92$	$q = 0.08$			

esis. However, in Fig. 8, showing AP k_{el} , four pedigrees indicated by arrows are incompatible with a monogenic hypothesis, thereby requiring rejection of that hypothesis for AP k_{el} .

DISCUSSION

Our results in families on the transmission of large variations in the rate constants for formation of AP metabolites provide evidence for a new genetic polymorphism of drug oxidation in man. Previous work in

TABLE III
Correlations Between Parent and Child, Midparent and Midchild, and Between Sibs for Rate Constants for Formation of AP Metabolites, k_i , and AP Elimination Rate Constants, k_{el} , in 61 Members of 13 Families

	Interclass correlation \pm SE		Intraclass correlations \pm SE
	Parent:child	Midparent:midchild	Sib-sib
$k_{4\text{-OH-AP}}$	0.31 \pm 0.19	0.40 \pm 0.25	0.19 \pm 0.17
$k_{3\text{-OHM-AP}}$	0.41 \pm 0.18	0.48 \pm 0.24	0.38 \pm 0.16
$k_{\text{NDM-AP}}$	0.39 \pm 0.18	0.52 \pm 0.24	0.24 \pm 0.17
k_{el}	0.12 \pm 0.19	0.31 \pm 0.26	0.35 \pm 0.16

TABLE IV
Correlations Between Rate Constants for Formation of AP
Metabolites in 144 Related and Unrelated Subjects

Rate constants compared	Correlation±SE	t value	P value
$k_{4-OH-AP}-k_{3-OHM-AP}$	0.35±0.079	4.43	<0.001
$k_{4-OH-AP}-k_{NDM-AP}$	0.66±0.064	10.31	<0.001
$k_{3-OHM-AP}-k_{NDM-AP}$	0.44±0.076	5.79	<0.001
$k_{el}-k_{4-OH-AP}$	0.23±0.083	2.77	<0.01
$k_{el}-k_{3-OHM-AP}$	0.15±0.084	1.79	<0.10 (NS)
$k_{el}-k_{NDM-AP}$	0.15±0.084	1.79	<0.10 (NS)

twins indicating genetic control of interindividual variations in AP half-lives (2) and in rate constants for formation of each AP metabolite (3) formed the basis for the present investigation. Since the three principal metabolites of AP are considered to arise from several distinct molecular forms or isozymes of cytochrome P-450 (16, 17), this new polymorphism affecting AP oxidation would be expected to affect the metabolism of several other drugs biotransformed by the same isozymes as AP. Eventual isolation and characterization of these isozymic forms of cytochrome P-450 in livers from normal adults would be desirable; initial steps in this direction have been taken for the debrisoquine polymorphism (23). The new polymorphism of AP disposition differs from classical pharmacogenetic conditions in that the rarer allele of the former confers increased, rather than diminished, metabolic activity.

It is uncertain whether more than one genetic polymorphism controls the interindividual variations we observed in the rate constants for formation of the three main AP metabolites. Within a given subject, statistically significant, but low, correlations among these AP metabolite rate constants (Table IV) raise the pos-

sibility that a common genetic locus may control, or at least participate in the regulation of, all three oxidation reactions. Besides these statistically significant correlations among AP metabolite rate constants of a given subject, there was additional evidence for their regulation by a single genetic locus: similar gene frequencies for formation of each AP metabolite (Table II) and the trimodal distribution and probit curves observed (Fig. 1) for AP k_{el} . However, other evidence fails to support this hypothesis. For example, pedigree analysis of the values of AP k_{el} , a more distant reflection of the gene than the rate constant for formation of each AP metabolite, revealed a much less clear cut definition of the monogenic effect: in 4 of 13 families investigated, AP k_{el} values incompatible with a monogenic hypothesis were observed. Furthermore, lower correlations for AP k_{el} than for AP metabolites occurred between relatives. Since pedigree analysis is the most reliable and rigorous of the four parameters used to test our genetic hypothesis, identification of four families incompatible with a monogenic hypothesis necessitated its rejection for AP k_{el} and, furthermore, suggested that the parameter selected for phenotyping (AP k_{el}) was deficient and inappropriate, probably because it reflects contributions from rate constants for each AP metabolite.

Why did interindividual variations in our families for AP k_{el} fail to conform to a monogenic hypothesis (Fig. 8), despite the statistically significant correlations within a subject for rate constants for AP metabolite formation (Table IV)? These statistically significant intraindividual correlations, together with closely similar frequencies for the genes that control each AP rate constant (Table II), suggested that a single genetic locus might control variations in all three AP rate constants. Though highly statistically significant (<0.001) because of the large number of subjects investigated

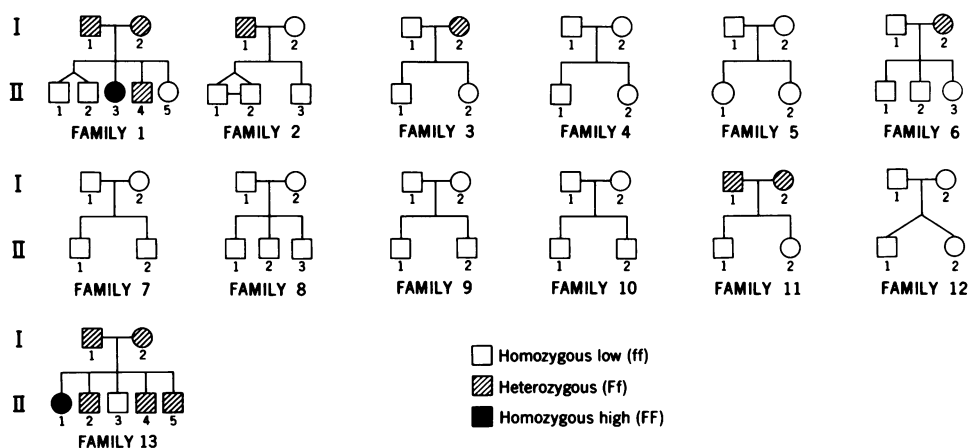


FIGURE 5 13 pedigrees comprising 61 family members phenotyped for rate constants for formation of $k_{4-OH-AP}$. All pedigrees are consistent with a monogenic hypothesis.

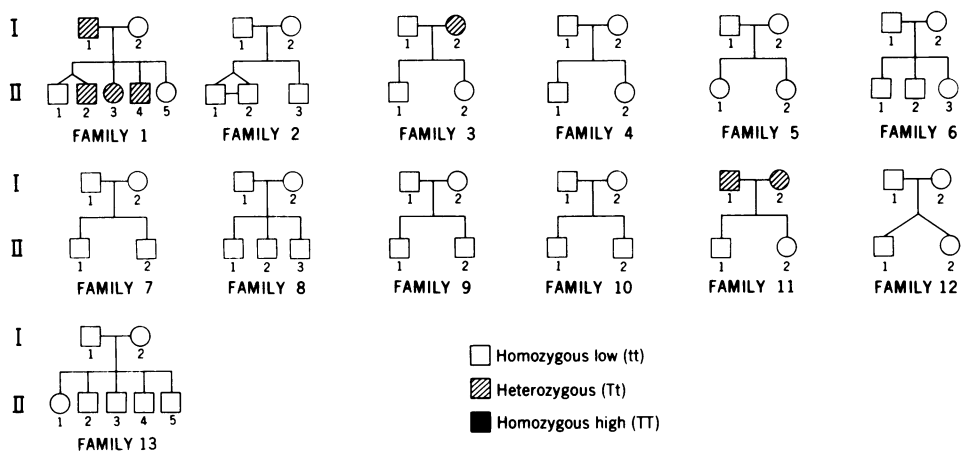


FIGURE 6 13 pedigrees comprising 61 family members phenotyped for rate constants for formation of $k_{3\text{-OHM-AP}}$. All pedigrees are consistent with a monogenic hypothesis.

(144), the correlation coefficients themselves were relatively low (0.35, 0.44, and 0.66). Furthermore, the numerical value of a correlation and the predictability of that correlation are often confused. They are not synonymous: the predictability of one variable based on its correlation to another is not determined by r but by r^2 (24), a much lower number than r . The widespread practice of using correlations as proof of a pharmacological relationship, rather than as a clue to perform a well-controlled experiment that adequately tests a hypothesis, should be discouraged (1).

These considerations suggest that while a common regulatory gene or a common rate-limiting metabolic step may be involved in controlling the three AP metabolites, this hypothesis has not yet been adequately tested. Even if such a common regulatory gene or rate-

limiting metabolic step exists, it may control only a relatively small portion of the total interindividual variation observed. Different genetic factors, independent for each of the three separate pathways of AP metabolite formation, could also play a major role. This possibility is suggested not only by the low values of r^2 (predictability) and the failure of pedigree analysis of AP k_{e1} to conform to a monogenic hypothesis (Fig. 8), but by the unimodal distribution and probit plot of AP half-life shown in Fig. 9. If only a single genetic locus controlled variations in the overall metabolism of AP, a trimodal distribution should occur for values of AP half-life rather than the clearly unimodal distribution and probit curve shown in Fig. 9.

If environmentally perturbed subjects had participated in this study, even the sensitive probe of rate

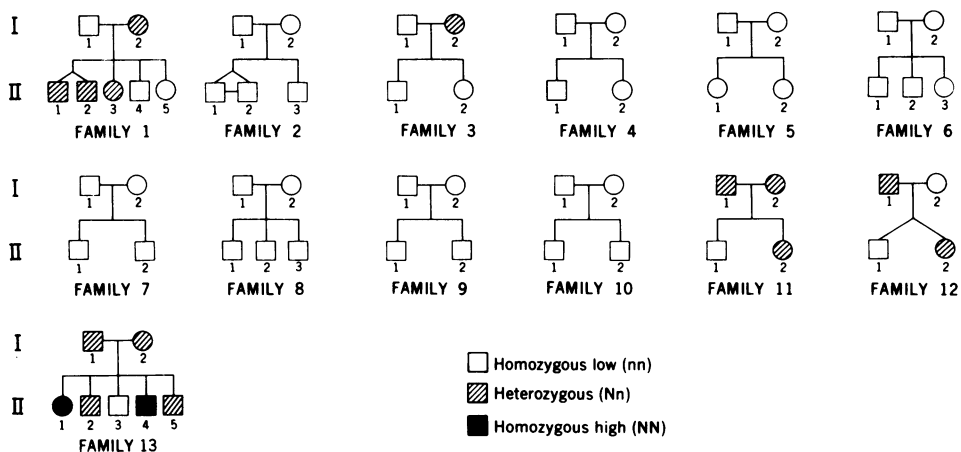


FIGURE 7 13 pedigrees comprising 61 family members phenotyped for rate constants for formation of NDM-AP, $k_{\text{NDM-AP}}$. All pedigrees are consistent with a monogenic hypothesis.

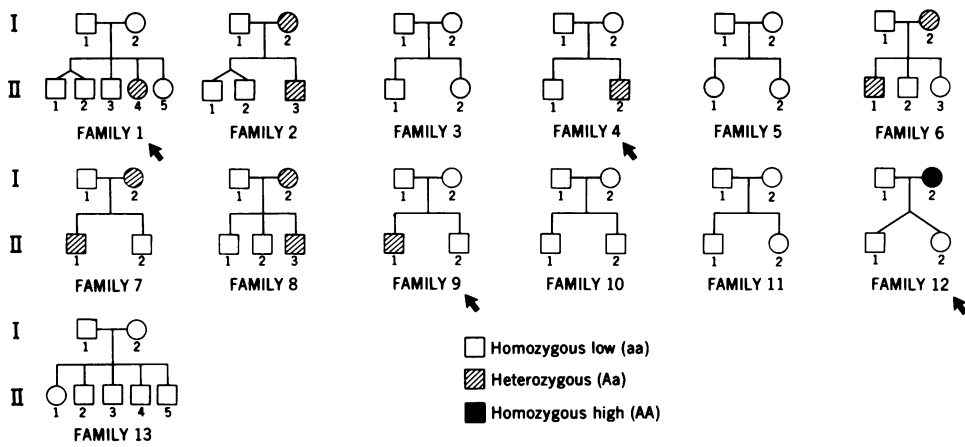


FIGURE 8 13 pedigrees comprising 61 family members phenotyped for k_{e1} . Four of these pedigrees (indicated by arrows) are incompatible with a monogenic hypothesis.

constants for AP metabolite formation might have failed to detect the hereditary factors that were discovered using uninduced subjects. Differential effects of environmental factors would have induced certain subjects and thereby concealed the transmission of these genes, as they did in a recent family study (25), in which only AP concentrations were measured. However, in other studies (2-4), where subjects were care-

fully selected to be under uniform environmental conditions, that is, uninduced, clear indications of the control exerted by these genetic factors over interindividual variations in AP metabolism could be detected. This identification of genetic factors occurred even though a relatively distant reflection of gene action, AP disappearance, was used to phenotype subjects (2-4).

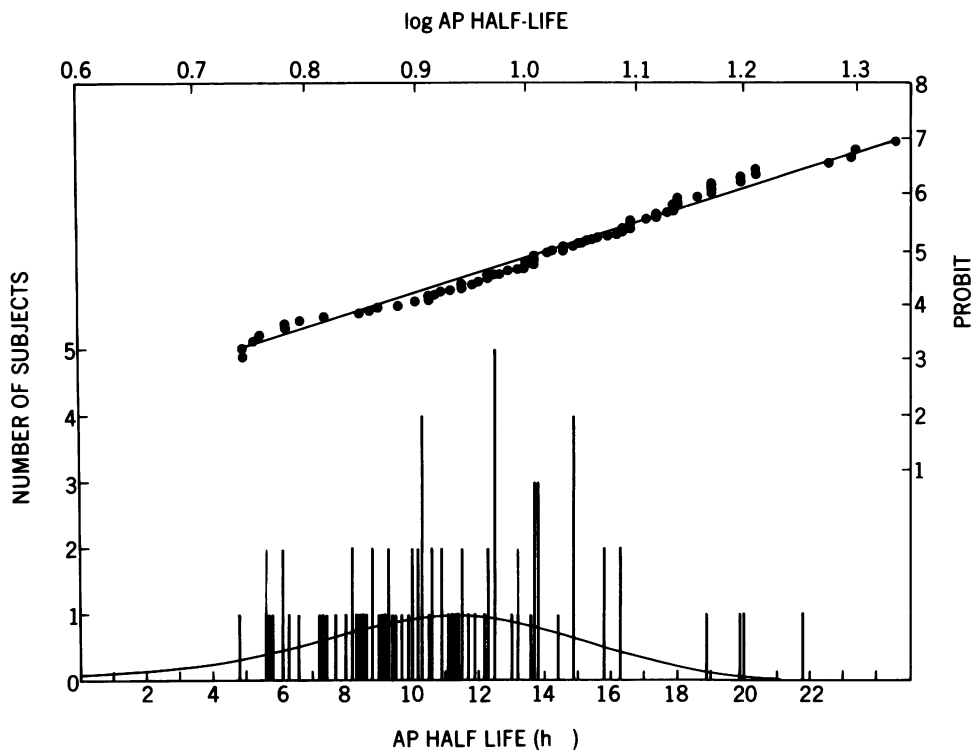


FIGURE 9 Distribution of AP $t_{1/2}$ in 83 unrelated subjects. Computerized drawing of the single mode is superimposed. A linear probit regression appears above.

The polymorphism in drug oxidation revealed using AP as substrate differs from the polymorphism of debrisoquine oxidation (26) since the debrisoquine phenotype of a subject appears unrelated to that subject's capacity to biotransform AP (27, 28). Furthermore, the gene frequency of the debrisoquine polymorphism ($P = 0.72$ and $q = 0.28$ in a Caucasian population from the United Kingdom [29]) differs from the gene frequencies we observed in the AP polymorphism (Table II).

Compared to the polymorphism discovered by measuring rate constants for production of AP metabolites, the debrisoquine polymorphism is much easier to use since subjects can be phenotyped for debrisoquine biotransforming capacity simply by measuring metabolite and parent drug concentrations in an 8-h urine specimen. Rate constants for metabolite formation need not be calculated, as they must for the AP polymorphism. Another advantage of the debrisoquine polymorphism over the AP polymorphism is further separation between the three phenotypes, which permits more accurate assignment of genotypes.

In pharmacogenetics much attention has focused on the modality of distribution curves, which in some cases can be deceptive, particularly when used by themselves. However, they can be helpful when they can be checked through use in conjunction with other methods, as in the present study. It is not surprising in light of these problems that previous AP distribution curves used alone have been conflicting; while unimodal for AP half-life (24, 30), they have deviated from unimodality for AP clearance (31), and one report even claimed trimodality for AP half-life (4).

The present study illustrates agreement among results obtained from four related pharmacogenetic parameters measured simultaneously. When all results agree, as in our present family study on rate constants for AP metabolite formation, a genetic hypothesis can be drawn more safely. If all subjects are under uniform, carefully controlled environmental conditions, then failure of the most critical parameter necessary to sustain a genetic hypothesis, consistency of the pedigrees examined with a Mendelian mechanism, is sufficient to reject a monogenic hypothesis, as in the case of our results on AP k_{el} . Since independent evidence from induction studies in rats (16, 17) suggests that three separate genetic loci contribute to AP elimination rates (k_{el}), polygenic, rather than monogenic, factors would be expected to control interindividual variations in AP k_{el} .

Much more than twins, family members representing different generations may have transmission of their genetic factors masked during pedigree analysis by environmentally or developmentally produced changes, including those from differences in age, diet, smoking, ethanol ingestion, and exposure to other in-

ducing or inhibiting agents. We attempted to select our families to minimize as much as possible effects from all such identified factors. As an example, well recognized effects of age on AP metabolism (32, 33) limited our genetic study to subjects between ages 18 and 55, which restricted it to families of only two generations.

Collectively, these considerations led to the suggestion that pharmacogenetic studies might use a tier system that begins with screening in twins to determine whether a genetic factor exists and that concludes with investigations in carefully selected and controlled families drawn from propositi with unusual values whose clearcut segregation would help to identify their Mendelian mode of transmission (34). Accordingly, the present family study was performed to define the precise mode of transmission of the genetic factors previously implicated by our twin studies (2, 3). Twin studies can strongly suggest that genetic factors control phenotypic variations, but twin studies cannot disclose the precise mode of genetic transmission, for which pedigree analysis is required.

Discovery of a new polymorphism in drug oxidation in man extends the scope of pharmacogenetic investigation. Similar family studies should be performed on rate constants for formation of the principal metabolites of amobarbital (5), dicoumarol (6), ethanol (7), halothane (8), phenylbutazone (9), phenytoin (10), and salicylate (11), since twin studies on these drugs indicated that in uninduced subjects genetic factors determined large interindividual variations in the k_{el} of the parent drug. The time is now ripe to perform family studies on the metabolites of these drugs. Sufficiently sensitive analytical methods for the principal metabolites are currently available to measure them in human blood and urine. Thus, rapid advances can now be made in pharmacogenetics to provide better understanding of why individuals vary so widely in their responses to numerous drugs.

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