

Salicylate Metabolism in Twins

EVIDENCE SUGGESTING A GENETIC INFLUENCE AND INDUCTION OF SALICYLURATE FORMATION

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ABSTRACT To evaluate the contribution of genetic influences on the individual variation in plateau serum salicylate levels, salicylate metabolism was studied in seven pairs of identical and six pairs of fraternal twins.

Under the conditions of this study, after a single i.v. dose (40 mg/kg) of sodium salicylate, the serum salicylate concentration versus time curve approximated a straight line on linear coordinates (appeared approximately zero order). The slopes of the decay curves ranged between 0.64 and 1.02. The intrapair variation for identical twin pairs was significantly less than for fraternal twin pairs ($P = 0.044$). Likewise plateau serum salicylic acid concentrations (milligrams/deciliter) and total salicylic acid excretion rate after multiple doses demonstrated significantly less intrapair variation for identical twins than for fraternal twins ($P = 0.043$ and 0.006). Plateau salicylurate excretion (milligram/kilogram per hour) differences after multiple dosing had a $P = 0.067$. Michaelis-Menton constant for salicylurate formation and hours to 50% excretion after the i.v. dose were not different when comparing identical and nonidentical twins.

Salicylurate formation rates were increased after 3 days of oral therapy, and this induction phenomenon may account for much of the apparent discrepancy between genetic influences on salicylurate formation rates observed after single and multiple dose salicylate administration.

This study suggests that the plateau concentration of serum salicylate varies among individuals given the same weight-adjusted dose in part because of geneti-

cally determined variations in their metabolism of salicylate.

INTRODUCTION

There are large individual differences in the aspirin dosage required to achieve adequate serum salicylate levels for the treatment of rheumatoid arthritis, rheumatic fever, and similar diseases. This is of special clinical importance because intensive salicylate therapy for these disorders requires the use of doses large enough to maintain salicylate concentrations just below the toxic range. Indeed, serious intoxication can result from salicylate administration in the therapeutic-dose range, especially in children (1, 2). On the other hand, an inadequate concentration may be the cause of therapeutic failure.

Published reports demonstrate significant individual differences in salicylate elimination rates as well as serum salicylate levels at constant doses (3-7). However, no satisfactory explanation of this individual variability is documented.

Levy and his associates (8-11) and others (6, 12) have demonstrated that aspirin metabolism is governed by parallel first-order and Michaelis-Menton kinetics. The excretion of two major metabolites of salicylic acid, viz, salicyluric acid and salicyl phenolic glucuronide is capacity limited in the usual therapeutic-dosage range. Thus, the excretion rates of salicyluric acid and of salicyl phenolic glucuronide are limited by the body's capacity to produce these metabolites. It is conceivable that individual differences in the maximal capacities of these pathways of salicylate metabolism could be responsible for intersubject variation of salicylate levels. This study was designed to evaluate the contribution of genetic factors in determining the individual variations in salicylate metabolism.

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METHODS

Seven pairs of identical twins (four male and three female sets) and seven pairs of fraternal twins (four male-female, two male, and one female set), with ages between 16 and 27 yr, volunteered for this study. Twins were accepted sequentially into the study as they volunteered (given that they were normal, see below, and of acceptable age). Informed consent was obtained from all individuals and from their parents where appropriate. Typing of the identical twin volunteers for 26 erythrocyte groups determined that they were identical. In the cases of the fraternal twins, sex and phenotypic differences demonstrated nonidentity; if fraternal twins appeared physically similar, they were also typed for 26 erythrocyte groups to document nonidentity. Copies of the birth certificates were obtained from the fraternal twins as proof of sibship. All were normal healthy individuals as determined by history, complete physical examination, and routine laboratory tests (including serum creatinine, alkaline phosphatase, serum glutamic oxaloacetic-acid-transaminase, serum glutamic pyruvic transaminase, total protein, albumin, and urinalysis) and had normal kidney and liver function. None had received any medication for at least 6 wk before the study except one pair of identical females (S. M. and K. M.) who continued taking birth control pills, containing 1 mg norethindrone and 0.05 mg estrogen. Before the study, four pairs of identical twins and three pairs of fraternal twins lived in the same households, whereas the members of the remaining three pairs of identical and four pairs of nonidentical twins were living in separate homes.

For acute studies, the volunteers were hospitalized in the Clinical Research Center of the University of California at Los Angeles School of Medicine, Los Angeles, Calif., and maintained on a regular diet and water ad lib. Ammonium chloride, in doses of 9–12 g/day, was given in divided doses every 4 h, skipping the night dose, to maintain the urine pH in the range of 5.0 or below. Acidification of urine minimizes the pH-dependent excretion of free salicylic acid (13), a variable which we desired to minimize for this study of individual metabolic differences. The subjects received ammonium chloride for 48 h before hospitalization and for 24 h of equilibration in the hospital. After this, a single dose of sodium salicylate, 40 mg/kg, was given i.v., followed by frequent timed collections of blood for 48 h and urine samples for at least 48 and up to 72 h after the injections. Blood was allowed to clot and serum was separated and immediately frozen for subsequent analysis. The total volume of each urine specimen was measured, and the pH was determined immediately after voiding; an aliquot was then frozen.

Serum salicylate was assayed by the single extraction method of Stevenson (14). According to this method, 0.5 ml of serum is extracted with a solution of malonic acid in 5 ml butyl ether. The ultraviolet absorption peak at 307 nm is used for identification and determination of salicylic acid. The absorbance readings obtained in repeated determinations, when standard solutions containing 10 or 20 mg/dl were treated in the same manner as serum, were within 5% of the mean value.

Free salicylic acid (FSA),¹ salicyluric acid (SU), salicylic

¹Abbreviations used in this paper: FSA, free salicylic acid; H, heritability; K_m , Michaelis constant; r , intraclass correlation coefficient; r_{DZ} , intraclass correlation coefficient, fraternal twins; r_{MZ} , intraclass correlation coefficient, identical twins; SA, salicylic acid; SAG, salicylic acid glucuronides; SU, salicyluric acid; TSA, total salicylic acid; V_m , Michaelis-Menton constant.

acid glucuronides (SAG), as well as total salicylic acid (TSA) in urine, were estimated by the colorimetric method of Levy and Procknal (15). The method consists of: (a) determination of FSA by extraction of the sample with carbon tetrachloride and re-extraction into ferric nitrate reagent solution; (b) determination of absorbance due to both FSA and SU by extraction of the sample with ethylene dichloride and re-extraction into ferric nitrate solution; and (c) determination of TSA (i.e. FSA, SU, and SAG) after complete acid hydrolysis of these metabolites to salicylic acid (SA) followed by step (a). SU content is calculated by the difference between (a) and (b) and SAG content is determined by subtracting FSA and SU values from (c). Absorbance peak was read at 530 nm using a Beckman DU spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.). Repeated determinations of standard solutions containing 10 mg of salicylic acid/100 ml and 50 mg of salicyluric acid/100 ml, treated in the same manner as urine, showed a coefficient of variation of 4%. Gentisic acid excretion was not determined.

After the acute study, and after at least 3 wk without medication, the same pairs of twins were treated as outpatients with 65 mg/kg per day of buffered aspirin (Ascriptin, William H. Rorer, Inc., Fort Washington, Pa.) given orally in five divided doses at 4-h intervals, skipping the night dose, concomitantly with ammonium chloride (6–8 g per day) to acidify the urine (kept at a pH of 5.0). After 3 days of aspirin therapy, the plateau serum salicylate levels and urinary FSA, SU, SAG, and TSA were determined during the second dosing interval on the fourth day as follows: blood and urine samples were collected immediately before and 1, 2, and 3 h after the last dose and the salicylate contents were determined as in the acute studies.

Data treatment. The maximum velocity of salicylurate formation was determined by plotting the following linearized form of the Michaelis-Menton equation:

$$\frac{S}{\nu} = \frac{K_m}{V_m} + \left(\frac{1}{V_m}\right)S_1, \quad (1)$$

where S is the amount of salicylate in the body, ν is the excretion rate of salicylurate, V_m is the theoretical maximum rate of metabolite formation (amount per unit time), and K_m is the apparent in vivo Michaelis constant (here the amount in the body, not concentration) (16). Plots were done using linear regression by the least mean-squares method. The contribution of genetic factors in determining the individual differences was estimated by comparing the variances of the differences between the members of the identical pairs with those of the fraternal pairs. The intrapair variance was determined by the following formula:

$$V = \frac{\sum_{i=1}^N [\Delta(X_{i1} - X_{i2})]^2}{2N}, \quad (2)$$

where X_{i1} represents one member of a twin pair, X_{i2} represents the other member, and N is the number of pairs (17). The heritability factor was determined according to the formula:

$$H = \frac{V_{DZ} - V_{MZ}}{V_{DZ}}, \quad (3)$$

where V_{DZ} is the variance between members of a dizygotic twin pair and V_{MZ} is the variance between members of a monozygotic twin pair (18). The intraclass correlation co-

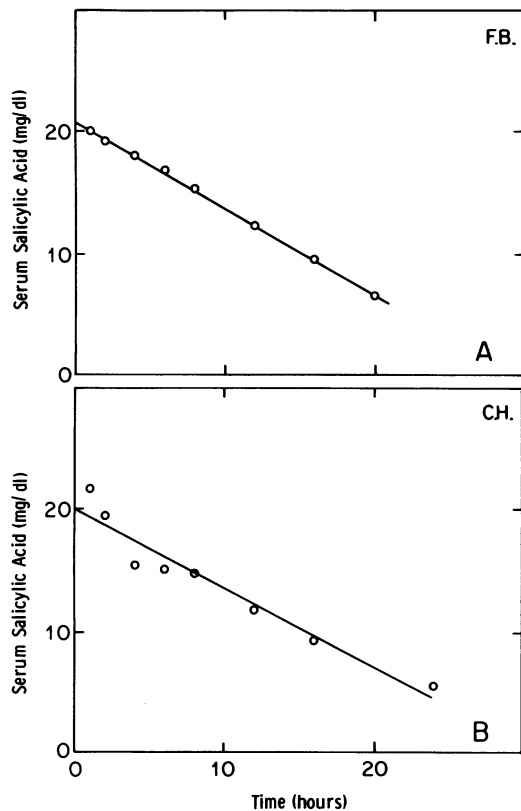


FIGURE 1 Pseudozero order plots of serum salicylate concentration vs. time after 40 mg/kg of sodium salicylate i.v. (A) represents the best, and (B) represents the poorest fitting lines for the 26 subjects.

efficient (r) was ascertained by the formula:

$$r = \frac{\sum_{i=1}^n (X_{1i} - a)(X_{2i} - a)}{nS^2}, \quad (4)$$

where X_{1i} and X_{2i} are as above, n equals the number of pairs of measurements, a is the mean for the twin measurements of all the twins in one group, and S^2 is the variance about the mean. Statistical significance was determined by the use of the Mann-Whitney U test or Student's t test. Although twin data are not normally distributed, severely and adversely affecting the usefulness of the F test, it too was calculated, for comparison with the Mann-Whitney U test. The P values were corrected for repeated determinations using the formula $P_{\text{corrected}} = nP(1-P)^{-1}$, where n is the number of variables tested (19). For the i.v. data analysis, $n = 4$, whereas $n = 3$ for the outpatient data. All values listed in this paper are means \pm SEM unless otherwise noted.

RESULTS

Acute single-dose (i.v.) study. Plots, using Cartesian coordinates, of the serum concentration versus time data (after a single i.v. dose of 40 mg/kg sodium salicylate) are shown in Figs. 1A and B. Fig. 1A represents the best fit and Fig. 1B represents the worst

fit among the subjects studied. The statistical correlation coefficient for the individual lines, r , was greater than 0.990 for 22 of the 26 subjects tested (Table I). The slopes of the linear regression lines ranged from 0.64 to 1.02 mg/dl/per h (Table I). The summary of the statistical analysis of these data is reported in Table V. The intrapair difference between the slopes was significantly smaller among the identical twins than among the fraternal twins ($P = 0.044$, Mann-Whitney U test). Heritability was 0.86; the intraclass correlation coefficient among the monozygotic twins (r_{MZ}) was 0.64; among the dizygotic twins, the intraclass correlation coefficient (r_{DZ}) was 0.32.

TABLE I
Slope of Serum Salicylate Decay Curve (Linear Regression by Least-Square Method) after i.v. Dose of Sodium Salicylate (40 mg/kg), Starting at 2 h

Subject	Sex	Age	Wt	Decay curve	r	
				slope \pm SE of estimate		
		yr	kg	mg/dl/h		
Identical twins						
Ma. B.	M	19	69.6	0.64 \pm 0.28	0.989	
Mi. B.	M	19	70.0	0.65 \pm 0.24	0.992	
Le. L.	F	17	45	0.99 \pm 0.09	0.999	
Li. L.	F	17	45.5	0.99 \pm 0.28	0.992	
L. McC.	F	19	52.5	0.82 \pm 0.21	0.994	
M. McC.	F	19	52.5	0.71 \pm 0.24	0.992	
S. M.	F	25	50.4	0.77 \pm 0.23	0.993	
K. M.	F	25	48.8	0.73 \pm 0.10	0.999	
R. P.	M	17	68.8	0.73 \pm 0.24	0.992	
H. P.	M	17	65.0	0.74 \pm 0.22	0.993	
F. B.	M	23	67.5	0.70 \pm 0.06	0.999	
A. B.	M	23	67.0	0.77 \pm 0.12	0.998	
R. C.	M	19	97.0	0.61 \pm 0.13	0.997	
D. C.	M	19	92.6	0.67 \pm 0.21	0.995	
Mean \pm SE				0.751 \pm 0.03		
Fraternal twins						
M. B.	M	21	56.5	0.66 \pm 0.39	0.987	
S. B.	F	21	51	0.73 \pm 0.36	0.991	
W. B.	M	20	77	1.02 \pm 0.17	0.997	
B. B.	F	20	68	0.73 \pm 0.46	0.978	
E. H.	F	27	47	0.73 \pm 0.27	0.992	
C. H.	F	27	52	0.64 \pm 0.49	0.969	
D. L.	M	22	92	—	—	
J. L.	M	22	79	—	—	
S. McG.	M	16	65	0.86 \pm 0.34	0.991	
P. McG.	F	16	75	0.83 \pm 0.42	0.990	
Ja. P.	M	17	68	0.85 \pm 0.19	0.995	
Ji. P.	F	17	57	0.99 \pm 0.11	0.999	
K. W.	M	23	65	0.88 \pm 0.36	0.991	
M. W.	M	23	76	0.98 \pm 0.36	0.993	
Mean \pm SE				0.825 \pm 0.04		
Overall mean	—	20.4	65.0	0.785	—	
SE	—	0.8	3.73	0.02	—	
P (corrected)	—	>0.5	>0.5	0.044	—	

Table II shows the urinary excretion of salicylic acid and its metabolites during the 72 h after the i.v. injection of sodium salicylate. The mean amount of the total salicylate excreted for all the subjects during this period was $86.3 \pm 2.1\%$ of the injected dose (range: 62.1–103.3%). Of this, $67.5 \pm 1.5\%$ was excreted as SU, $30.3 \pm 1.3\%$ as SAG, and the rest as FSA ($3.4 \pm 0.42\%$). Ranges for each of the above, as well as the percent excreted by each member of the two groups of twins, may be found in Table II. The SU excretion rate after the single i.v. dose is more or less constant during the first 16–20 h and then declines in an exponential fashion. This mean maximum observed, sustained SU excretion rate (Table III) was 0.92 ± 0.02 mg/kg per h

for the identical twins and 0.91 ± 0.03 mg/kg per h for the fraternal twins ($P > 0.5$, Student's *t* test). Estimations of the Michaelis-Menton constant, V_m , were calculated from Eq. 1. Plots of the best and worst fit to this equation are shown in Fig. 2. The mean V_m for SU was 66.3 ± 4.1 mg/h for the identical twins and 64.7 ± 5.5 mg/h for the fraternal twins ($P > 0.5$). The SE of the estimates for the individual V_m 's, as well as correlation coefficients, may be found in Table III. The time required to excrete 50% of the total injected dose of 40 mg/kg, determined from cumulative excretion curves, was 15.2 ± 0.7 h for the monozygotic twins and 13.9 ± 0.6 h for the dizygotic twins ($P > 0.5$). Ranges are seen in Table II.

TABLE II
Urinary Excretion of Salicylates during 72 h after Sodium Salicylate (40 mg/kg) Given i.v.

Name	Amount excreted		Total salicylate excreted as			Hours to 50% excretion of total dose*
	SA	Injected dose	FSA	SU	SAG	
	mg eq	%		%		
Identical twins						
Ma. B.	2,208	92.2	1.9	62.7	35.4	15.6
Mi. B.	2,273	94.4	1.6	61.4	37.0	16.0
Le. L.	1,392	77.3	1.2	69.3	29.5	10.8
Li. L.	1,251	69.5	1.1	80.4	18.5	9.9
L. McC.	1,582	87.6	4.6	62.6	32.8	13.8
M. McC.	1,334	73.9	2.4	60.0	37.8	18.0
S. M.	1,512	87.2	2.1	60.4	37.5	15.0
K. M.	1,492	88.9	1.6	60.5	37.9	14.8
R. P.	2,175	91.9	3.2	62.6	34.2	14.4
H. P.	2,014	90.1	3.6	64.9	31.5	14.9
F. B.	1,935	83.4	2.2	66.4	31.4	16.6
A. B.	1,978	85.8	3.2	61.7	35.1	15.4
R. C.	2,546	80.3	5.2	61.1	33.7	19.2
D. C.	2,787	83.5	7.0	58.8	34.2	19.0
Mean \pm SE		84.7 ± 1.9	2.9 ± 0.45	63.8 ± 1.5	33.3 ± 1.3	15.2 ± 0.7
Fraternal twins						
M. B.	2,428	103.3	5.9	75.5	18.6	15.8
S. B.	1,884	89.7	2.9	70.9	26.2	17.8
W. B.	2,467	82.2	9.2	68.5	22.3	12.3
B. B.	2,252	83.4	2.9	78.9	19.2	12.8
E. H.	1,492	79.4	1.6	67.8	30.6	13.3
C. H.	1,716	82.5	5.0	82.0	13.0	13.4
S. McG.	2,742	97.7	4.3	68.4	27.3	13.2
P. McG.	3,075	102.5	9.3	65.7	25.0	15.8
Ja. P.	1,689	62.1	3.0	74.5	22.5	12.5
Ji. P.	1,814	79.6	2.8	57.5	39.7	10.8
K. W.	3,072	102.4	2.7	71.6	26.7	13.0
M. W.	3,200	103.2	1.8	67.9	31.3	15.6
Mean \pm SE		89.0 ± 3.8	4.3 ± 0.76	72 ± 2.1	25.2 ± 2	13.9 ± 0.6
Overall mean		86.3	3.4	67.0	30.0	14.6
SE		2.1	0.42	1.5	1.4	0.47
<i>P</i> (corrected)		>0.5	>0.5	>0.5	>0.5	>0.5

* Computed from total cumulative excretion vs. time curve.

TABLE III
Urinary Excretion of Salicylurate during 72 h after Sodium Salicylate (40 mg/kg) i.v.

Name	Maximum observed sustained SU excretion rate		V_m SU mg/h	SE of estimate	<i>r</i>
	mg/h±SE	mg/kg/h			
Identical twins					
Ma. B.	63±0.21	0.905	64.5	±1.98	0.998
Mi. B.	64±2.2	0.911	82.0	±4.86	0.980
Le. L.	45±2.0	0.999	44.2	±2.95	0.994
Li. L.	50±2.5	1.11	62.5	±3.09	0.988
L. McC.	51±1.9	0.973	59.7	±2.35	0.996
M. McC.	40±1.8	0.762	48.3	±2.56	0.994
S. M.	44±0.6	0.867	50.4	±2.88	0.994
K. M.	43±0.6	0.887	45.8	±4.34	0.990
R. P.	67±1.4	0.966	78.7	±2.97	0.992
H. P.	62±1.0	0.957	68.5	±2.38	0.996
F. B.	65±5.2	0.963	75.3	±3.09	0.991
A. B.	63±0.9	0.937	72.1	±2.93	0.994
R. C.	76±1.8	0.820	93	±4.34	0.987
D. C.	76±2.7	0.788	84	±9.15	0.960
Mean±SE	58±3.2	0.918±0.02	66.3±4.1		
Fraternal twins					
M. B.	56±2.5	0.988	56.7	±4.08	0.994
S. B.	40±4.0	0.782	41.9	±12.4	0.976
W. B.	84±3.7	1.09	92.2	±5.41	0.988
B. B.	67±3.1	0.980	80.1	±2.14	0.997
E. H.	40±1.2	0.852	45.0	±5.61	0.992
C. H.	46±2.0	0.885	60.2	±3.86	0.990
S. McG.	65±3.6	0.992	63.2	±5.85	0.992
P. McG.	60±1.6	0.797	67.3	±4.75	0.989
Ja. P.	50±1.5	0.731	49.8	±8.52	0.990
Ji. P.	51±5.7	0.889	41.8	±4.05	0.990
K. W.	74±4.2	0.989	85.7	±5.68	0.981
M. W.	76±4.9	0.997	92.5	±10.7	0.963
Mean±SE	58±4.1	0.914±0.03	64.7±3.5		

Table V shows the results of the Mann-Whitney U test, testing intrapair differences among monozygotic twins as compared to intrapair differences among dizygotic twins. It also shows heritability (calculated according to equations 2 and 3) for SU and total salicylate excretion and appropriate intraclass correlation coefficients (Eq. 4). After the i.v. dose, the intrapair differences for monozygotic twins were not significantly smaller than for the dizygotic twins with respect to the maximum observed sustained SU excretion rate ($P = 0.53$). The P value for the V_m of SU was greater than 0.5, and the P value for the estimation of the hours to 50% excretion of the total salicylate dose was 0.29.

Oral multiple-dose treatment. Table IV shows serum salicylate levels and the urinary excretion rate of SU and of TSA after the twins were treated with 72 h of oral aspirin therapy (65 mg/kg per day).

Although we realize that these subjects were not yet at steady state, they were approaching plateau and, as can be seen from the data in Table IV, serum levels were relatively constant over the period of outpatient sampling (27.7±1.0 mg/dl for the identical twins; 22.9±1.8 mg/dl for the fraternal twins). The mean serum salicylate levels, SU excretion rates, and TSA excretion after multiple doses of aspirin were not statistically different when comparing the identical and fraternal twins ($P > 0.1, 0.5, \text{ and } 0.5$, respectively) (Table IV).

The intrapair differences between the multiple-dose serum salicylate levels for the identical twins were significantly smaller than for the dizygotic twins ($P = 0.04$). For SU excretion, the identical vs. nonidentical twin differences had a probability of 0.067. The intrapair differences between the identical twins were significantly smaller than those between the fraternal twins for TSA excretion ($P = 0.006$). The heritability

and intraclass correlation coefficients for all of the above substances are listed in Table V.

It is of interest that the SU excretion rate in milligrams/kilogram per hour in the oral multiple-dose treatment increased by a mean of $50.5 \pm 5.1\%$ (range: 9–96%) when compared to the single i.v. dose SU excretion rate in milligrams/kilogram per hour (Tables III and IV).

Although there were seven pairs of identical and seven pairs of fraternal twins entered in the study, one pair of fraternal twins was given an incorrect dose of salicylate i.v. A different set of fraternal twins apparently misunderstood the instructions for the multiple-dose study, and took only 300 mg/day, thus invalidating their results for this portion of the study. Therefore, only six sets of fraternal twins were analyzed in each portion of the study.

Since there were four male-female sets of dizygotic twins, it was possible that sex differences might have led to some systematic bias in the results. This possibility was tested by looking at all the males versus all the females and ascertaining if any statistically significant differences occurred between these two groups. When all the parameters tested in Table V (Statistical Summary) were compared in this way, there were no significant differences found between males and females except in the V_m . Here there was no normalization of weights, since V_m is in mg/h, and it would not be surprising to find a difference. Even here, when looking at the actual population where bias might occur (the fraternal twins), there was no significant difference found ($P = 0.12$).

DISCUSSION

This study in identical and fraternal twins suggests that some aspects of salicylate metabolism are genetically determined. Other drugs have set some precedent for this finding. Significant interindividual differences in steady-state plasma levels and biological half-lives of antipyrine (20, 21), alcohol (22), phenylbutazone (23), dicumarol (24), nortriptyline (25), and isoniazid (26) have been demonstrated and attributed to genetically determined differences in drug metabolism.

A commonly used twin method for determining the contribution of genetic versus environmental factors employs a comparison of mean monozygotic and mean dizygotic variance observed in two groups of twins (18, 27). This method was used here to compute heritability and intraclass correlation coefficients. Although heritability (H) may appear to quantitate genetic influence, used alone it may be misleading, and it is useful to look at the intraclass correlation coefficient (r) at the same time. According to Neel and Schull, r is a “. . . linear function of the ratio of two variances—the variance between members of a twin-

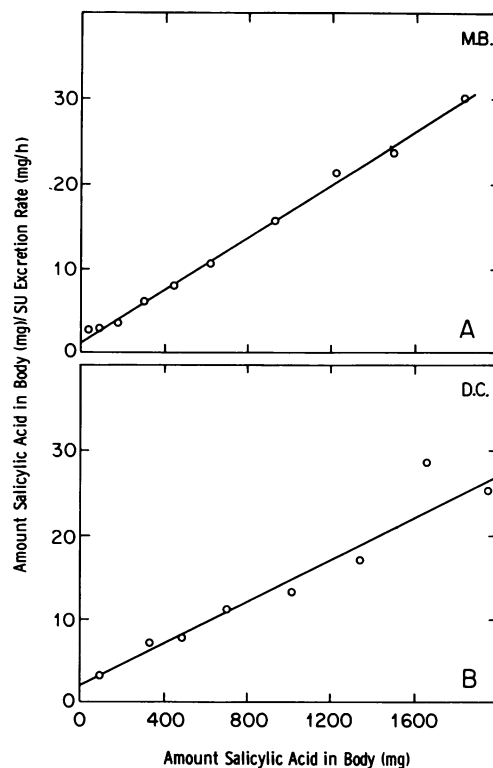


FIGURE 2 Plot of best (A) and poorest (B) fitting lines depicting data used to calculate V_m . (Amount of salicylic acid in body/excretion rate of SU vs. amount of salicylic acid in body).

pair and the variance over the whole set of observations . . .” (27). They suggest that comparison of r_{MZ} to r_{DZ} is the best comparison to appraise the relative contribution of genetic and environmental effects. Thus, the higher the r , the less difference exists between twin pairs, and the lower the r , the more the difference. Similar r 's indicate lack of large differences between sets of twins despite apparently large H .

As groups, the identical and fraternal twins were not significantly different with respect to age, weight, domicile, TSA, FSA, SU, and SAG excretion, or serum salicylate concentrations, indicating that both groups of twins are members of the same population with respect to these factors.

Serum salicylate concentration was plotted versus time. From Fig. 1, one can see that a linear relationship seems to exist, although Levy (10) showed that the decline of serum sodium salicylate over the range of this study (approximately 30–5 mg/dl) is described by parallel first-order and Michaelis-Menton kinetics, which results in a curvilinear plot. FSA, salicyl acyl glucuronide, and gentisic acid excretion follow first-order kinetics, whereas, salicylurate and salicyl phenol glucuronide excretion follow Michaelis-Menton kinetics (10). In the present study, FSA was kept to a

TABLE IV

Plateau Serum Salicylate Levels and Urinary Excretion Rates of SU and TSA after 72 h of Oral Aspirin (65 mg/kg/day)

Name	Serum levels, mean±SE	SU excretion rate			TSA excretion rate	
		mg/h, mean±SE	mg/kg/h	Increase*	mg/h, mean±SE	mg/kg/h
	<i>mg/dl</i>			<i>%</i>		
Identical twins						
Ma. B.	25.1±0.5	97.3±3.5	1.40	54.5	104.5±6.3	1.50
Mi. B.	27.8±0.6	99.3±2.4	1.42	55.7	109.7±3.3	1.57
Le. L.	24.5±0.2	67.4±1.6	1.49	49.1	89.9±10.1	1.99
Li. L.	26.0±0.3	70.4±3.7	1.54	38.7	86.4±2.9	1.89
L. McC.	25.7±0.5	78.7±4.1	1.50	54.1	110.4±2.5	2.10
M. McC.	25.8±0.8	78.2±5.3	1.49	96.6	107.2±4.1	2.04
S. M.	26.2±0.2	72.4±0.4	1.44	65.6	83.6±5.5	1.65
K. M.	26.6±0.2	71.1±2.0	1.46	64.2	85.0±5.4	1.71
R. P.	31.1±1.0	81.4±2.6	1.18	22.3	105.8±3.9	1.54
H. P.	29.7±0.8	78.1±4.0	1.20	26.6	116.7±3.7	1.80
F. B.	25.4±0.6	105.3±1.2	1.56	62.1	123.9±2.8	1.75
A. B.	25.2±0.2	108.9±2.2	1.63	73.3	124.1±2.6	1.72
R. C.	36.4±0.4	102.9±8	1.11	34.6	137.2±0.7	1.48
D. C.	35.1±0.5	99.3±6.4	1.02	30.8	145±11.1	1.50
Mean±SE	27.7±1.0	86.5±4.0	1.39±0.05	52.0±5.4	109.2±5.1	1.73±0.6
Fraternal twins						
M. B.	17.3±0.9	78.1±7.0	1.43	44.7	82.7±3.0	1.51
S. B.	16.9±1.7	76.7±7.6	1.48	90.5	93.3±7.4	1.80
W. B.	27.0±0.88	147.7±7.9	1.91	75.2	133.1±4.2	1.72
B. B.	31.4±0.71	121.1±11.2	1.78	81.6	135.9±4.1	2.00
E. H.	34.3±0.15	64.6±8.8	1.38	62.0	81±10.2	1.73
C. H.	22.0±0.05	76.6±4.5	1.47	66.1	73.4±6.6	1.41
D. L.	19.4±0.40	77.5±7.4	0.84	—	110.5±6.7	1.20
J. L.	22.8±0.47	91.3±9.9	1.15	—	115.4±11.8	1.46
S. McG.	23.9±0.24	73.6±10.6	1.13	13.9	100.6±10.5	1.54
P. McG.	25.8±0.31	68.4±7.9	0.91	14.2	89.9±9.7	1.19
K. W.	21.6±0.41	92.5±6.3	1.26	27.4	113.2±5.9	1.55
M. W.	11.9±0.48	82.4±17.4	1.09	9.3	83±17.6	1.10
Mean±SE	22.9±1.8	87.5±6.9	1.32±0.09	48.5±9.7	101±6.0	1.52±0.8

* Compared to single i.v. dose (Table III).

minimum by ammonium chloride administration (Table II). Gentistic acid is generally only a few percent of the total, leaving only one significant first-order process (salicyl acyl glucuronide formation) (10). Thus, making a linear plot on Cartesian coordinates would be a reasonable first approximation. When plotting serum salicylate concentration versus time after a single i.v. dose of sodium salicylate, the intrapair differences in slopes of the serum salicylate decay curves were significantly smaller between identical twins than between nonidentical twins ($P < 0.04$, Table V). Further, after 3 days of oral multiple-dose aspirin therapy, the intrapair variations in salicylate levels and total salicylate excretion rates were also significantly smaller between identical twins than between fraternal twins (Table V). These observations indicate an important genetic influence in determining individual variations in salicylate metabolism.

It is realized that the statistical analysis of twin studies often uses the F test. However, the F test is a parametric test and requires a normally distributed population for correct and optimum usage; Zar states that the F test “. . . is severely and adversely affected by sampling non-normal populations” (28). There is no assurance that twins are normally distributed and, in fact, Kempthorne and Osborne give a number of reasons that twins are not a normally distributed population (17). For this reason, the non-parametric Mann-Whitney U Test was used for most of the analysis in this paper. Nevertheless, for comparison, the F test also was calculated. F values, and the within-dizygotic twin mean squares and within-monozygotic twin mean squares are shown in Table VI. Only two of the seven variables tested were significantly different using the F test (and uncorrected P values). After correction of P values, only one variable remained

significantly different, illustrating the decrease in sensitivity and power when the *F* test is used to measure non-normally distributed populations.

Since salicylurate comprised the majority of the salicylate metabolites excreted in this study (67.5 ± 1.5%), it was expected that it, too, would demonstrate a genetic influence. During the acute study, we could demonstrate no such influence when examining the *V_m* (*H* = 0.27), or the maximum observed salicylurate excretion rate (*H* = 0.03) (Table V). It should be noted at this point that the *V_m*'s for SU in our 26 subjects (65.6 ± 3.3 mg/h) agreed with the values obtained by Levy et al. (11) in his four male normals (60.3 ± 3.1 mg/h). Evaluation of the *K_m* requires practically complete recovery of the dose for accurate determination, and since our recovery averaged 86% we did not include the *K_m*'s in our analysis.

Upon measuring the SU excretion rate after 3 days of oral therapy, we found smaller differences between identical than fraternal twins with a *P* = 0.067 (Table V). *H* appeared to be significant (0.89) whereas the *r*'s were only 0.18 apart (*r_{MZ}* = 0.94; *r_{DZ}* = 0.76). Thus, there were indications of genetic influence during the 3-day oral study but not during acute i.v. dose study. Part of the explanation for this apparent inconsistency may lie in another surprising finding; the maximum observed sustained SU excretion rates after 3 days of aspirin therapy were 50.5 ± 5.1% greater than those observed during the initial single i.v. dose of sodium salicylate. Even though SU excretion is capacity limited, one could object that the dose of salicylate given was larger during the outpatient portion of the study, so one would expect SU excretion to rise. It is, in fact, true that on a mole for mole basis, there was a 44.4% increase in dose during the outpatient study. However, this would only be expected to cause a change in SU excretion from the mean observed maximum SU excretion of 58.0 mg/h to one no higher than 59.7 mg/h (see Appendix A); instead the observed plateau SU excretion rate was 87.0 mg/h (Table IV). Since SU excretion is capacity limited, the only reasonable explanation is an increase in the capacity for SU formation, i.e., induction of glycine synthetase, the enzyme responsible for SU formation. Induction of SU production has not been previously demonstrated. It appeared to occur quickly (over a 3-day period) compared to meprobamate and phenylbutazone which takes weeks to months, but induction of enzymes metabolizing polycyclic hydrocarbons has been documented within 3–6 h in rats (21, 29, 30). Further support for this finding comes from Muller et al. who showed falling serum levels of salicylate over time while his subjects were taking a constant dose of aspirin (31). It should be noted that all subjects were specifically asked about recent ingestion of aspirin or salicylate-containing drugs, and denied

TABLE V
Statistical Summary

	Mann-Whitney 2-tailed U test		<i>H</i>	<i>r_{MZ}</i>	<i>r_{DZ}</i>
	Uncorrected	Corrected			
	<i>P</i>				
Slope of serum salicylurate decay curve, i.v. dose (40 mg/kg) mg/dl	0.011	0.044	0.86	0.64	0.32
Maximum salicylurate excretion rate, mg/kg/h	0.144	0.534	0.03	—	—
<i>V_m</i> , mg/h	0.157	0.628	0.27	—	—
Hours to 50% excretion of total dose	0.074	0.294	0.38	—	—
Plateau salicylic acid (mg/dl) in serum after 72 h oral dosing (65 mg/kg/day)	0.014	0.043	0.98	0.90	0.33
Salicylurate excretion rate (plateau) in mg/kg/h after 72 h oral dosing	0.022	0.067	0.89	0.94	0.76
TSA excretion rate in mg/kg/h after 72 h oral dosing	0.002	0.006	0.83	0.70	0.10

their use. Since, however, salicylates are so widely used and frequently are not identified as such, it is possible that some unintentional exposure to them had occurred in some of the volunteers. Therefore, if partial enzyme induction had occurred in some of them before the i.v. study, intrapair differences might have been magnified for the acute i.v. study, making it difficult to separate identical and fraternal twins. After uniform induction with high-dose oral therapy, however, these genetic differences might again have manifested themselves, hence no statistically significant differences, low *H*, and *r*'s during the i.v. study, but statistically significant differences and higher *H* during the longer study (Table V).

Besides the previous exposure to salicylates, the metabolism of drugs may be influenced by other environmental factors (32). For salicylates, the excretion of one of the important metabolites, FSA, is known to be dependent on urinary pH (13). In the present study, FSA excretion was almost completely prevented by the administration of ammonium chloride to acidify the urine. Other factors which might help explain

TABLE VI
Analysis of Results Using the F Test for Comparison to the Mann-Whitney U Test

	Within DZ* mean square	Within MZ† mean square	F‡	P	
				Uncorrected	Corrected
Slope of serum salicylate decay curve, i.v. dose (40 mg/kg)/mg/dl	0.0184	0.0019	9.8085	<0.05	<0.20
Maximum observed SU excretion rate (mg/kg/h)	0.0206	0.0096	0.7207	>0.50	>0.5
V _m (mg/h)	19.0	33.167	0.5723	>0.50	>0.5
Hours to 50% excretion of total dose	1.14	2.993	0.3813	>0.50	>0.5
Plateau salicylic acid (mg/dl) in serum after 72 h oral dosing (65 mg/kg/day)	21.682	0.3295	65.7987	<0.001	<0.003
SU excretion rate (plateau) in mg/kg/h after 72 h oral dosing	0.0088	0.0024	3.7262	>0.20	>0.5
TSA excretion rate in mg/kg/h after 72 h oral dosing	0.03126	0.0071	4.4109	>0.20	>0.5

$F_{0.05,(2),6,5} = 6.98^{\parallel}$

* n_{DZ}, six pairs of fraternal twins.

† n_{MZ}, seven pairs of identical twins.

‡ F = within DZ mean square/within MZ mean square.

∥ For P = 0.05, the initial value of F is 6.98, comparing seven pairs of identical twins with six pairs of fraternal twins, using a 2-tailed analysis.

the differences between the acute and plateau SU excretion rates may relate to variations in absorption kinetics, since the acute study involved an i.v. dose and the multiple-dose study involved oral doses.

When examining the serum salicylate levels achieved in these young healthy subjects, it was found that the mean serum salicylates ranged from 11.9 to 36.4 mg/dl after 65-mg/kg per day dosing for 3 days. This range is appreciably less than the range previously reported in rheumatoid arthritis patients (7). The difference between that study and the present one suggests the possibility that disease state and/or

age may also play a role in determining individual salicylate levels, in addition to the hereditary factors seen in these young, healthy individuals.

The individual twins of three identical pairs and four fraternal pairs lived apart, in different households. Environmental influences were, therefore, similarly randomized for the sets of identical and fraternal twins. Although not rigidly controlled, we feel the equal distribution of environmental differences minimizes bias contributed by this factor.

This study documents a genetic influence on the control of salicylate metabolism and suggests that salicylate formation is inducible.

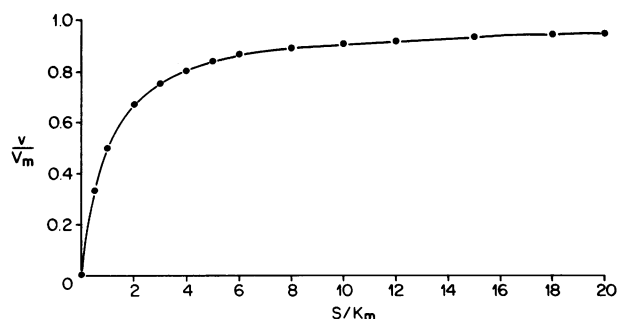


FIGURE 3 Mathematically derived plot of v/V_m vs. S/K_m .

APPENDIX A

The Michaelis-Menton equation is:

$$v = \frac{V_m S}{K_m + S},$$

where v = observed velocity; V_m = maximum velocity; K_m = Michaelis-Menton constant; and S = amount in body (as in ref. 10). Divide by V_m :

$$\frac{v}{V_m} = \frac{S}{K_m + S},$$

multiply the right side by K_m/K_m :

$$\frac{\nu}{V_m} = \frac{S/K_m}{1 + S/K_m}$$

A graph of ν/V_m vs. S/K_m from the above equation is seen in Fig. 3.

From Table III: ν = mean observed maximum SU excretion rate: 58 mg/h; V_m = mean V_m : 65.6 mg/h:

$$\frac{\nu}{V_m} = 0.884.$$

From Fig. 3 it is seen that this value is on the flat part of the curve (i.e. approaching V_m) where changes in S using a $K_m = 338$ (from ref. 10), we find a mean $S = 2,231$ mg.

If the dose given during the second portion of this study is 44.4% higher than the i.v. dose, then

$$S' = 1.444 \times 2,231 = 3,221 \text{ mg.}$$

Since:

$$K_m = 338,$$

then

$$\frac{S'}{K_m} = 9.53.$$

Again returning to Fig. 3,

$$\text{for } \frac{S'}{K_m} = 9.53,$$

$$\frac{V'}{V_m} = 0.91.$$

Since $V_m = 65.6$, $\nu' = 65.6 \times 0.91 = 59.7$ mg/h (predicted).

This is the predicted SU excretion rate, at steady state, for a 44.4% increase in dose from 2,231 mg. In our study we were not at steady state yet, so our predicted change would be less than this. Nevertheless we used the steady-state predicted value described above to be conservative in our interpretation.

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