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Drug Absorption, Metabolism, and Excretion. I. Some Pharmacokinetic Aspects of Metabolism of Acetanilide and 4-Hydroxyacetanilide[1]

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Hydroxylation of acetanilide and excretion of 4-hydroxyacetanilide in rabbits was investigated kinetically. Formation of 4-hydroxyacetanilide from acetanilide was found to be not a simple first order process but it could be well expressed by Michaelis Menten equation.

Metabolic pathways of antipyretic aniline derivatives, namely acetanilide, phenacetin and 4-hydroxyacetanilide, have been well established (Chart 1). Some other compounds such as aniline and *o*- or *p*-aminophenol are also reported as the metabolites of acetanilide,[3] and 2-hydroxyacetophenetidine[4] and S-(1-acetamido-4-hydroxyphenyl)cysteine[5] for phenacetin. However these are all minor products.

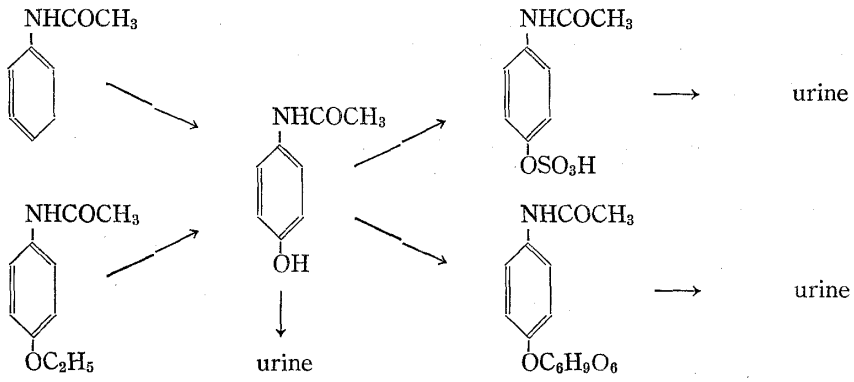

Chart 1. Metabolic Pathway of Acetanilide and Its Derivatives

In man and in rabbits acetanilide and phenacetin are mainly converted to 4-hydroxyacetanilide which is excreted as conjugates with glucuronic acid and sulfuric acid. The analgesic and antipyretic action of acetanilide and phenacetin is exerted mainly through this phenol which is an active analgesic and antipyretic. This, however, is not free from criticism. Conney and coworkers[6] have demonstrated that phenacetin itself has antipyretic activity.

If the mixture of 4-hydroxyacetanilide and its precursor, acetanilide or phenacetin, is given to a subject, formation of 4-hydroxyacetanilide by the metabolism of the precursor will fill and compensate the decrease of coadministered 4-hydroxyacetanilide due to excretion.

1) Presented in part to the 87th Annual Meeting of Pharmaceutical Society of Japan, Kyoto, April 1967.
2) Location: *4-23 Bunkyo-cho, Nagasaki.*
3) R.T. Williams, "Detoxication Mechanism," John Wiley and Sons Co., New York, 1959, p. 432, 443.
4) A. Klutch, M. Harfenist, and A.H. Conney, *J. Med. Chem.*, **9**, 63 (1966).
5) O.R. Jagenburg and K. Toczko, *Biochem. J.*, **92**, 639 (1964).
6) A.H. Conney, M. Sansur, F. Soroko, R. Koster, and J.J. Burns, *J. Pharmacol. Exptl. Therap.*, **151**, 133 (1966).

It seems possible to maintain the constant plasma level of the active antipyretic 4–hydroxyacetanilide by the simultaneous administration of these drugs.

To make up the dosage schedule from the point of view described above, it is necessary to know the kinetic behavior of individual drug, namely all the rate constants such as hydroxylation of acetanilide, excretion of unchanged acetanilide, glucuronic acid conjugation of 4–hydroxyacetanilide, and so forth. In order to get all these informations, kinetic studies are necessary.

In 1952 kinetics of acetanilide metabolism and excretion in rabbits was reported by Bray.[7] Two hundred and fifty miligrams per kilogram body weight of acetanilide was administered to rabbits and excreted conjugates were analysed. According to Bray the hydroxylation of acetanilide and glucuronic acid conjugation obey the first order kinetics and the sulfuric acid conjugation of 4–hydroxy derivative is a process which is saturated with the substrate. The rate constants reported by Bray are shown in Table I.

Nelson[8] studied the metabolism of 4–hydroxyacetanilide in man and demonstrated that 3% of the dose was excreted unchanged, and most of the drug was excreted as conjugated forms both with glucuronic acid and sulfuric acid, the former being predominant. Excretion rate of conjugates was very large and conjugation was rate–limiting step. Rate constants reported by Nelson are also given in Table I.

Brodie and Axelrod[9] performed a quantitative study of acetanilide metabolism in man. Kinetic interpretation of Brodie's data[10] has been attempted in a similar way to the present work. The results of which satisfactorily support our conclusion described below.

Very recently Cummings and coworkers[11] separated thin–layer chromatographically 4–hydroxyacetanilide, 4–hydroxyacetanilide sulfate and 4–hydroxyacetanilide glucuronide excreted in the urine after the administration of 4–hydroxyacetanilide to man, and estimated spectrophotometrically. In Table I, rate constants reported by Cummings are given.

Table I. Survey of Rate Constants reported for 4–Hydroxyacetanilide Metabolism and Excretion

	Species	Dose	Rate constant (hr^{-1}) for			
			Glucur. acid conj.	Sulfuric acid conj.	Excret. unchang. drug.	Eliminat. unchang. drug.
Bray[a]	rabbit	250 mg/kg per os	0.50	—	—	—
Nelson[b]	man	1000 mg per os	—	—	0.0083—0.0185	0.245—0.427
Cummings	man	12 mg/kg ?	0.13[c]—0.16	0.07[c]—0.09	0.01—0.014	0.27—0.35

a) Hydroxylation rate constant of acetanilide was reported to be 0.43 hr^{-1}, following oral administration of acetanilide 250 mg/kg body weight.
b) Nelson assumed that excretion of the metabolites of 4–hydroxyacetanilide would be rate–limited by the conjugation step.
c) Excretion rate constants of the conjugates are also reported by Cummings which are 0.47—1.01 hr^{-1} for glucuronide and 0.80—1.53 hr^{-1} for sulfate.

The present report describes the results of kinetic study of acetanilide metabolism and 4–hydroxyacetanilide excretion in rabbits, emphasis being placed mainly on the conversion of acetanilide to 4–hydroxyacetanilide.

7) H.G. Bray, B.G. Humphris, W.V. Thorpe, K. White, and P.B. Woods, *Biochem. J.*, **52**, 412, 416, 419, 423 (1952).
8) E. Nelson and T. Morioka, *J. Pharm. Sci.*, **52**, 864 (1963).
9) B.B. Brodie and J. Axelrod, *J. Pharmacol. Exptl. Therap.*, **94**, 29 (1948).
10) To be published.
11) A.J. Cummings, M.L. King, and B.K. Martin, *Brit. J. Pharmacol.*, **29**, 150 (1967).

Methods

Animal——Unanesthetized male white rabbits weighing 2 to 3.5 kg were used. Acetanilide was dissolved in hot water (40°) to make 300 mg/50 ml solution, which was administered either orally or intravenously. As for 4–hydroxyacetanilide, 300 mg/10 ml solution was administered. Blood specimens were taken (0.9 ml each) with one ml syringe containing 0.1 ml of 3.8% sodium citrate solution from ear vein or directly from the rabbit heart. Urine collections were made through Nelaton's catheter inserted to the bladdar. Food was withheld during the experiment, although water was given through the catheter passed to the stomach frequently to keep constant urine flow.

Drug——Acetanilide and 4–hydroxyacetanilide used were J.P. grade.

Analytical Methods——Acetanilide, 4–hydroxyacetanilide, and total conjugated 4–hydroxyacetanilide were estimated by the method described by Brodie and Axelrod[12] with a slight modification.

***β*-Glucuronidase Treatment of Urine**——To one ml of the urine 8.5 ml of 1/15 N acetate buffer (pH 5.5) and 0.5 ml of *β*–glucuronidase solution (13000 unit/ml Tokyo–Zoki Chemical Co.) were added. The mixture was incubated for 18 hours at 37°. Determination of free 4–hydroxyacetanilide mentioned above was carried out with the incubated solution. The results gave the sum of the concentration of free and glucuronic acid conjugated 4–hydroxyacetanilide.

The concentration of sulfuric acid conjugated drug was obtained by calculation: total 4–hydroxyacetanilide minus free and glucuronic acid conjugated drug. This procedure of calculation is not entirely free from criticism, for the assurance has not been given that the *β*–glucuronidase used by the authors was free from sulfatase, and for all the experimental errors are accumulated on the sulfate concentration.

Results and Discussion

Conjugation and Excretion of 4-Hydroxyacetanilide in Rabbits

The amounts of 4–hydroxyacetanilide, 4–hydroxyacetanilide glucuronide, and 4–hydroxyacetanilide sulfate excreted in the urine up to 10 hours after dosage of 300 mg of 4–hydroxyacetanilide are given in Table II. These results show that the glucuronide is the major metabolite accounting for an average of 92% of the dose administered, the corresponding values for the sulfate and unchanged 4–hydroxyacetanilide are 4—7% and 1%, respectively. These results are qualitatively consistent with those of Nelson[8] and of Cummings.[11]

Excreted amounts shown in Table II are the experimental values. Although the administration of the drug was performed through intravenous route, still the period of time required for distribution has to be taken into account, for which 30 minutes will be sufficient.

Table II. The Cumulative Amount (mg Equivalent of 4–Hydroxyacetanilide) of 4–Hydroxyacetanilide (Be), 4–Hydroxyacetanilide Glucuronide (Ge), and 4–Hydroxyacetanilide Sulfate (Se) Excreted in the Urine of Rabbits after Intravenous Administration of 4–Hydroxyacetanilide 300 mg

Rabbit	B	I			P				X		
Weight kg	3.1	3.2			2.3				3.1		
Time hr	Total	Be+Ge	Se	Total	Be	Ge	Se	Total	Be+Ge	Se	Total
1	152.3	96.0	9.7	105.7	0.5	66.2	11.4	78.1	145.4	12.4	157.8
2	223.0	183.9	15.2	199.1	0.9	172.9	13.1	186.9	214.2	11.7	225.9
3	267.0	237.9	18.9	256.8	1.4	227.9	14.8	244.1	244.0	15.6	259.6
4	285.7	256.0	31.3	287.3	1.6	248.9	16.1	266.6	258.8	17.7	276.5
5	293.8	268.8	33.1	301.9	2.0	264.4	12.2	278.6	265.4	17.8	283.2
6	298.5	275.3	34.8	310.1	2.1	273.1	10.3	285.5	268.7	17.5	286.2
7	301.6	278.4	36.1	314.5	2.3	276.3	10.8	289.4	270.6	17.5	288.1
8	303.0	280.6	36.9	317.5	2.8	278.0	11.5	292.3	271.9	17.9	289.8
9	303.8	282.5	37.0	319.5	3.1	279.5	12.0	294.7	272.3	18.8	291.1
10	304.3	283.9	37.1	321.0	3.3	280.4	12.5	296.2	272.6	19.1	291.7

12) B.B. Brodie and J. Axelrod, *J. Pharmacol. Exptl. Therap.*, **94**, 22 (1948).

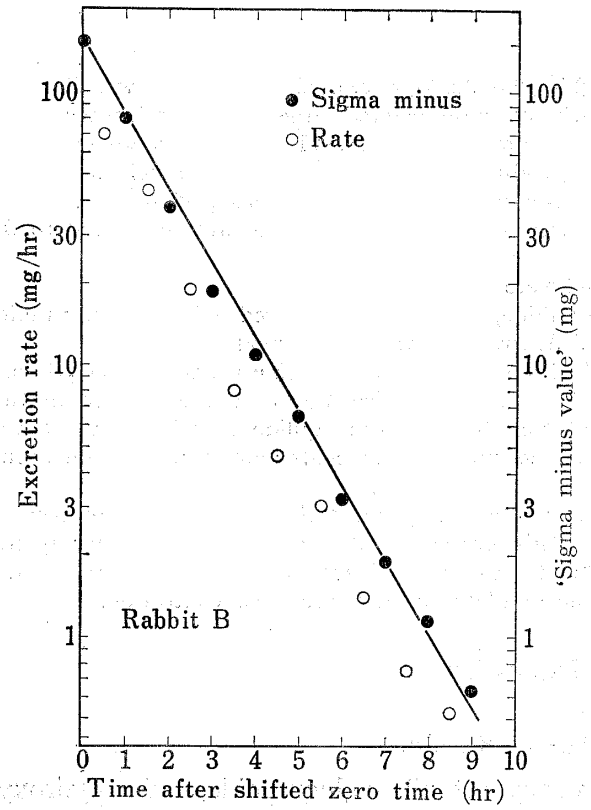

Fig. 1. Log 'Rate' Plot (○) and Log 'Sigma Minus' Plot (●) relating to the Excretion of Total 4–Hydroxyacetanilide (Free and Conjugated) in the Urine of Rabbit B after Dosage of 4–Hydroxyacetanilide 300 mg

Gilman and coworkers[13] allowed 20 minutes for equilibration of injected thiosulfate. When the excretion in one hour[14] is subtracted from each value in the Table II, the values with the shifted zero time are obtained.

The amount of total 4–hydroxyacetanilide excreted during each consequtive one hour (in other words, the difference of the adjacent values in Table II) was plotted in logarithmic scale against the mid point of each time interval. The graph for rabbit B is shown in Fig. 1. This is the logarithmic plot of excretion rate against time after shifted zero time. The authors will refer to this kind of plot after Martin[15] as log 'rate' plot.

The plot could be interpreted as linear, therefore;

$$\log \frac{dC}{dt} = a - bt \qquad (1)$$

where a is the interecept of the straight line at $t=0$, b the slope of the line and C the amount of total 4–hydroxyacetanilide excreted in the urine. Multiplication of the both sides of equation (1) with 2.303 gives equation (2).

$$\ln \frac{dC}{dt} = 2.303a - 2.303bt \qquad (2)$$

Antilogarithm of equation (2) is,

$$\frac{dC}{dt} = a' \exp(-b't) \qquad (3)$$

where $a' = \exp(2.303\ a)$, and $b' = 2.303\ b$.

If B is the total amount of unchanged and conjugated 4–hydroxyacetanilide in the body after shifted zero time and B_0 is B at shifted zero time, the balance of drug amount gives equation (4).

$$B_0 = B + C \qquad (4)$$

Differentiation of the both sides of equation (4) gives equation (5).

$$0 = \frac{dB}{dt} + \frac{dC}{dt} \qquad (5)$$

Rearrangement of equation (5) and substitution by equation (3) gives equation (6).

13) A. Gilman, F.S. Philips, and E.S. Koelle, *Am. J. Physiol.*, **146**, 348 (1946).
14) In the experiment of Table II urine collections were made every one hour, and 30 minute excretion data were not available.
15) B.K. Martin, *Brit. J. Pharmacol.*, **29**, 181 (1967).

$$\frac{dB}{dt}=-\frac{dC}{dt}=-a'\exp(-b't) \tag{6}$$

Equation (7) is obtained by integration of equation (6) with the condition $B=0$ at $t=\infty$.

$$B=\frac{a'}{b'}\exp(-b't) \tag{7}$$

Equation (8) is obtained by comparing equation (6) with equation (7).

$$\frac{dB}{dt}=-b'B \tag{8}$$

Equation (8) indicates that the elimination process of total 4-hydroxyacetanilide, free and conjugated, is first order.

Hereafter total elimination rate constant, K, is used instead of b', therefore,

$$\frac{dB}{dt}=-KB \tag{9}$$

Applying equation (6), equation (10) is obtained.

$$\frac{dC}{dt}=KB \tag{10}$$

Therefore K is also interpreted as total excretion rate constant of 4-hydroxyacetanilide.

C_{rest}, the amount of 4-hydroxyacetanilide which was to be excreted after 10 hours (9 hours after shifted zero time), when the experiment was terminated, could be calculated by equation (11), assuming that the excretion of the drug continues to be first order.

$$C_{\text{rest}}=\left(\frac{dC}{dt}\right)_{t=10}\Big/K \tag{11}$$

Sum of $C_{t=10}$, shown in Table II, and C_{rest}, calculated by equation (11), gives the total amount of drug excreted after dosage. The theoretical total amount of the drug excreted after shifted zero time is obtained by subtracting the excretion of one hour from the sum. The calculated total amount excreted minus the cummulative amount excreted at each time, was plotted on a logarithmic scale against time after shifted zero time. The graph for rabbit B is shown in Fig. 1. The authors will refer to this kind of plot, after Martin,[15] as log 'sigma minus' plot.

The total excretion rate constant of 4-hydroxyacetanilide, K, was obtained by calculation of the slope of the log 'sigma minus' plot. Because of less scattering of the plot due to averaging effect of integration, log 'sigma minus' plot is preferable to log 'rate' plot for the estimation of the rate constant.

When the excretion rate constant of conjugated 4-hydroxyacetanilide is much greater than the formation rate constant of the conjugates, or, in other words, conjugation is the rate-limiting step of 4-hydroxyacetanilide excretion, as assumed by Nelson, *et al.*[8] and generally believed, then the total excretion rate constant, K, is the sum of the rate constants which govern the excretion of unchanged 4-hydroxyacetanilide, and formation of 4-hydroxyacetanilide glucuronide and sulfate, and the individual rate constants are estimated from the relative amount of unchanged drug and each metabolites ultimately excreted.

There have been presented, however, some facts that do not necessarily support the assumption mentioned above.

According to Cummings[11] excretion rate constant of 4-hydroxyacetanilide glucuronide, 0.47 to 1.01 hr^{-1}, is not so much greater than the formation rate constant of the glucuronide, 0.13 to 0.16 hr^{-1}, in man.

Relatively high values have been reported by Brodie, *et al.*[9] for the plasma concentration of conjugated 4–hydroxyacetanilide after the oral administration of acetanilide to man. The accrual of the blood concentration of the conjugate indicates that the excretion rate constant of the conjugate is not so mcuh greater than its formation rate constant.

Taking these facts into consideration, the authors of this paper refrain from the evaluation of the formation rate constant of each conjugates, 4–hydroxyacetanilide glucuronide and sulfate.

Total excretion, rate constant of 4–hydroxyacetanilide, K, obtained from the slope of log 'sigma minus' plot is shown in Table III.

Table III. Total Excretion Rate Constant of 4–Hydroxyacetanilide calculated from the Slope of Log 'Sigma Minus' Plot

Rabbits	K (hr^{-1})
B	0.667
I	0.594
P	0.640
X	0.735
Average	0.659

Hydroxylation of Acetanilide in Rabbits

The total amount of 4–hydroxyacetanilide excreted in the urine up to 10 to 11 hours after the dosage of 300 mg of acetanilide[16] are given in Table IV.

Table IV. The Cumulative Amount of Total 4–Hydroxyacetanilide (Free and Conjugated) excreted in the Urine of Rabbits after Dosage of Acetanilide 300 mg

Time (hr)	Rabbits					
	D[a] 2.75 kg	G 3.7 kg	J 2.75 kg	N[a] 2.8 kg	Q 2.5 kg	W 3.3 kg
0.5	3.5	7.1	5.5	0.9	—	0.3
1.0	20.6	13.3	20.5	6.5	23.0	0.9
1.5	53.9	43.2	—	24.2	—	—
2.0	92.1	75.1	63.3	59.1	65.9	52.8
2.5	116.2	112.8	—	95.6	—	—
3.0	143.1	153.2	127.2	129.6	127.8	101.9
3.5	176.9	—	—	153.7	—	—
4.0	201.9	208.0	184.4	188.5	185.0	149.4
5.0	236.6	252.4	231.8	232.5	223.0	199.7
6.0	259.9	273.5	275.3	253.6	254.3	229.0
7.0	270.8	284.9	294.4	269.9	275.9	249.2
8.0	275.6	290.2	301.5	279.2	286.7	258.4
9.0	278.3	293.5	305.6	285.0	290.5	261.0
10.0	280.7	295.5	308.3	290.2	293.3	262.2
11.0	282.4	296.8	—	—	294.7	263.0

a) Drug was administered orally.

The excreted amounts shown in Table IV are the experimental values. In order to allow necessary period of time for distribution of the injected drug to the body fluid, the excretion in 30 minutes was subtracted from the each value in Table IV and the values with shifted

16) 300 mg dose of acetanilide to a rabbit is, on a body weight basis, ten times greater than the ordinary dose of acetanilide in man described in J.P. VII.

zero time were obtained. In case of oral administration, 2 hours excretion was subtracted to get the values with shifted zero time.

Log 'rate' plot and log 'sigma minus' plot were made for rabbit G as described in the previous section, and are shown in Fig. 2.

It has been shown in the previous section that the total excretion process of 4–hydroxyacetanilide is first order. The following model could be assumed to govern the formation of 4–hydroxyacetanilide and its excretion.

Acetanilide in the body $\xrightarrow{?}$

4–Hydroxyacetanilide total, in the body $\xrightarrow{K}$

4–Hydroxyacetanilide total excreted

In the above scheme K is as defined in the previous section. The excretion of unchanged acetanilide has been estimated and found not more than a few percent of the dose, therefore the process has been neglected.

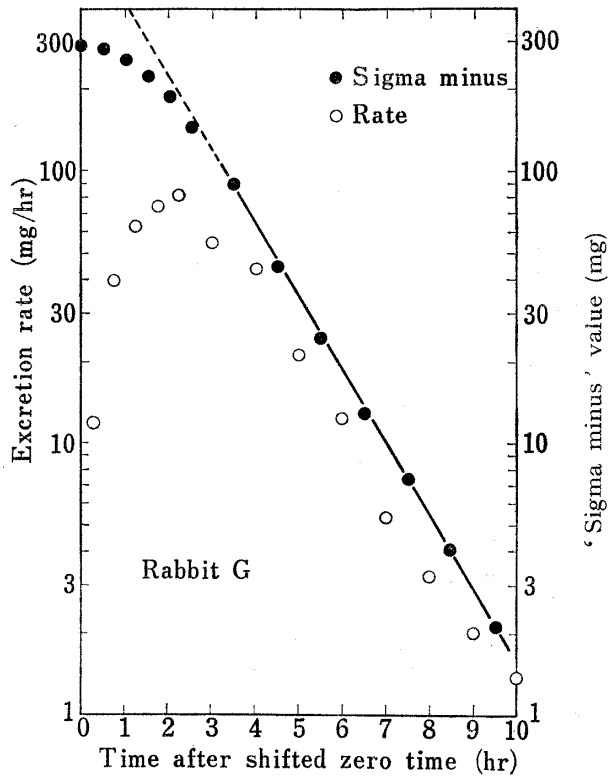

Fig. 2. Log 'Rate' Plot (○) and Log 'Sigma Minus' Plot (●), relating to the Excretion of Total 4–Hydroxyacetanilide (Free and Conjugated) in the Urine of Rabbit G after Dosage of Acetanilide 300 mg

From equation (10), the excretion rate of total 4–hydroxyacetanilide is directly proportional to the amount of total 4–hydroxyacetanilide in the body. Therefore, the log 'rate' plot shown in Fig. 2 is also the measure of relative amount of total 4–hydroxyacetanilide in the body respect to the time course. The amount of total 4–hydroxyacetanilide in the body at any time may be obtained by equation (12).

$$B=\frac{dC}{dt}\Big/K \qquad (12)$$

After attaining the maximum value, the excretion rate of total 4–hydroxyacetanilide decreases and log 'rate' plot eventually appear to become linear. The rate constant calculated from the slope of the terminal linear portion of the log 'rate' plot was essentially the same as the value of total excretion rate constant of 4–hydroxyacetanilide shown in Table III. This fact indicates that the process of acetanilide hydroxylation had become negligible and excretion of total 4–hydroxyacetanilide become predominant. The total excretion rate constant of 4–hydroxyacetanilide, K, was obtained by calculation of the slope of the linear part of log 'sigma minus' plot, which was 0.63 hr^{-1} for rabbit G.

The amount of total 4–hydroxyacetanilide, free and conjugated, which were excreted in the urine after shifted zero time was calculated as the sum of C_{rest} and $C_{t=10}$ as described in the previous section. The calculated total amount excreted minus the cumulative amount excreted at each time and minus the amount of total 4–hydroxyacetanilide in the body calculated by equation (12) at each time was plotted on a logarithmic scale against time after shifted zero time. The graph for rabbit G is shown in Fig. 3. This is the calculated amount of unchanged acetanilide in the body.

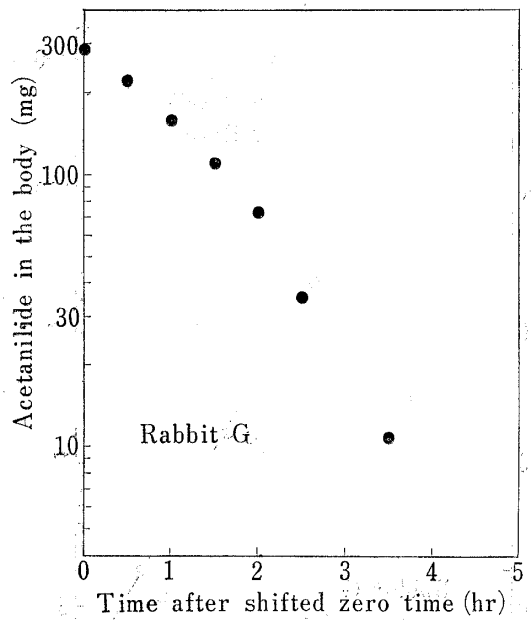

Fig. 3. Calculated Amount of Unchanged Acetanilide in the Body after Dosage of 300 mg Acetanilide to Rabbit G

A period of time which is necessary for equilibration of the drug between blood and distributing fluid after administration of the drug have been allowed sufficiently. If the process of acetanilide elimination, *i.e.* the formation of 4-hydroxyacetanilide, excretion of unchanged acetanilide being neglected, is first order, then log-plot of the amount of acetanilide in the body against time course should be linear from $t=0$.

This does not seem to be the case with the hydroxylation of acetanilide. The decline of log-amount plot of Fig. 3 continues to increase and eventually become linear, which indicates that the process of hydroxylation of acetanilide is not a simple first order but the one that is saturated. In order to examine this behavior of acetanilide elimination from the body, various amount of acetanilide was administered to rabbits, and the determination of acetanilide concentration in blood was carried out periodically. The results are shown in Table V.

Table V. The Blood Concentration (mg%) of Unchanged Acetanilide after Dosage of Various Amount of Acetanilide to Rabbits

Rabbit		I	K	S	Z	A	D[a]	G	J	N[a]	Q	W
Wt. (kg)		3.2	3.7	3.2	3.0	2.8	2.75	3.7	2.75	2.8	2.5	3.2
Dose (mg)		30	30	30	100	200	300	300	300	300	300	300
Time (hr)	0.25	3.06	2.54	—	—	—	—	—	—	—	—	—
	0.5	1.72	2.02	1.82	6.18	12.29	12.03	23.98	21.31	10.03	13.76	22.05
	0.75	—	1.20	1.04	5.47	10.01	17.52	20.06	16.30	13.48	—	19.81
	1.0	0.84	0.90	0.70	4.49	8.30	17.79	16.68	14.98	11.68	14.83	17.89
	1.5	0.34	0.44	0.30	2.93	6.51	15.96	12.64	13.45	8.44	—	14.43
	2.0	—	0.16	0.16	1.78	3.95	9.90	8.32	10.86	6.36	10.61	12.17
	2.5	—	—	—	1.11	2.81	7.44	7.28	8.87	4.89	—	—
	3.0	—	—	—	0.33	1.79	5.04	4.04	7.34	3.39	5.93	5.72
	3.5	—	—	—	—	—	2.19	2.50	—	2.45	—	—
	4.0	—	—	—	0.20	0.36	1.11	1.22	4.13	1.41	3.30	2.20
	4.5	—	—	—	—	—	0.36	—	—	0.80	—	—
	5.0	—	—	—	—	0.09	0.09	0.42	1.90	0.31	2.14	0.28
Rate const.[b]		1.77	1.70	1.74	—	1.51	2.41	—	—	1.84	—	—

a) Drug was administered orally.
b) Rate constant was calculated from the slope of linear portion of the plot. (hr^{-1})

The concentrations have been plotted on a logarithmic scale against time after shifted zero time to make certain for establishing equilibrium of distribution. The graph is shown in Fig. 4. It is clear from Fig. 4 that when the acetanilide concentration in blood is less than 1 mg%, the elimination of acetanilide is log-linear indicating that the process of hydroxylation is apparent first order, of which the rate constant is about 1.7 hr^{-1} as shown in Table V, but that when the concentration is more than 2 mg%, the elimination is no more first order process. The slope of the tangent line to the curve of Fig. 4, which is the measure of rate constant, decrease with the increase of the concentration of the drug.

It is easily considered that the hydroxylation of acetanilide to 4–hydroxyacetanilide is carried out through a series of complicated enzymatic reactions. The rate–limiting step, however, may be a simple reaction which obeys Michaelis Menten equation.

As far as the conjugation is concerned, some non–first order processes were reported. According to Bray,[7] sulfuric acid conjugation of phenols in rabbits was saturated with the substrate. Recently Levy has reported that the sulfuric acid conjugation of salicylamide[17] and glycine conjugation of salicylate[18,19] in man are also saturated, and that the Michaelis Menten equation is applicable to express these processes.

Hydroxylation of acetanilide is not a conjugation process but it is a production process of a 'hand grip' for glucuronic acid or sulfuric acid to conjugate with. Although no saturated process has yet been reported with this sort of reaction, it is not unreasonable to assume that the process can be expressed by Michaelis Menten equation.

The authors presumed that Michaelis Menten equation could be applied to express the process of 4–hydroxyacetanilide formation from acetanilide. The differential equation expressing this process is,

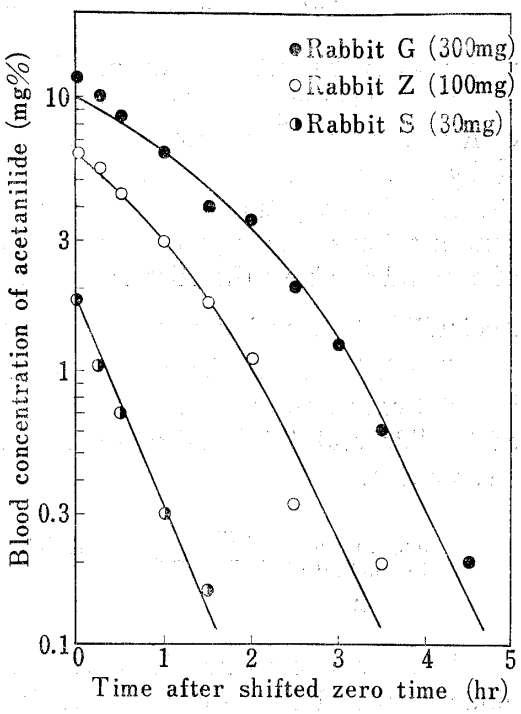

Fig. 4. Logarithmic Plot of Experimentally Obtained Blood Concentration of Unchanged Acetanilide after Dosage of Various Amount of Acetanilide

Plot was made after shifted zero time. The solid lines were drawn by equation (16), where $\bar{A}_0$=20.0 mg%, $\bar{V}m$=9.806 mg%/hr, and $\bar{K}m$=5.768 mg% for rabbit G. For rabbit Z, parameters were $\bar{A}_0$=6.18 mg%, $\bar{V}m$=5.374 mg%/hr, and $\bar{K}m$=3.161 mg%. And for rabbit S, $\bar{A}_0$=1.82 mg% and $\bar{V}m/\bar{K}m$=1.74 hr^{-1}

$$\frac{dA}{dt}=-\frac{VmA}{Km+A} \tag{13}$$

where A is the amount of acetanilide in the body, Vm and Km are constants. If A becomes negligiblly small compared with Km, equation (13) can be rewritten as equation (14),

$$\frac{dA}{dt}=-\frac{Vm}{Km}A \tag{14}$$

and the process is apparent first order, of which the rate constant is Vm/Km.

When the volume of distribution of acetanilide is Vd and the blood concentration of acetanilide is $\bar{A}$, equation (14) becomes equation (15).

$$\frac{d\bar{A}}{dt}=-\frac{\bar{V}m\bar{A}}{\bar{K}m+\bar{A}} \tag{15}$$

Where $\bar{V}m=Vm/Vd$, and $\bar{K}m=Km/Vd$.

Integration of equation (15) gives equation (16),

17) G. Levy and T. Matsuzawa, *J. Pharm. Sci.*, **55**, 222 (1966).
18) G. Levy, *J. Pharm. Sci.*, **54**, 496 (1965).
19) G. Levy, *J. Pharm. Sci.*, **54**, 959 (1965).

$$\bar{K}m(\ln \bar{A}_0 - \ln \bar{A}) + \bar{A}_0 - \bar{A} = \bar{V}mt \qquad (16)$$

where $\bar{A}_0$ is the initial condition of $\bar{A}$.

If 5.768 mg%, 9.806 mg%/hr and 20.0 mg% are chosen,[20] for example, as the values of $\bar{K}m$, $\bar{V}m$ and $\bar{A}_0$ respectively, for rabbit G, t can be calculated for the various values of $\bar{A}$ by equation (16). Results of calculation are shown as a solid curve of Fig. 4. The theoretical curves thus calculated pass almost all spots of the experimentally obtained blood concentrations. This fact confirms the validity of the assumption that the hydroxylation of acetanilide obeys the Michaelis Menten equation.

Acetanilide Hydroxylation and Total Excretion of 4-Hydroxyacetanilide in Rabbits

The following model was assumed to govern the formation of 4–hydroxyacetanilide and its total excretion after administration of acetanilide and attainment of equilibrium between acetanilide and total 4–hydroxyacetanilide in blood with these materials in the fluid of distribution.

acetanilide in the body (A) $\xrightarrow{Vm, Km}$ total 4–hydroxyacetanilide in the body (B) $\xrightarrow{K}$ total 4–hydroxyacetanilide excreted in the urine (C)

In the above scheme K is first order rate constant of total excretion of 4–hydroxyacetanilide, Vm and Km are constants for Michaelis Menten kinetics for 4–hydroxyacetanilide formation.

If at any time t, A is the amount of acetanilide in the body in mg, B the amount of total 4–hydroxyacetanilide in the body as mg acetanilide, C the excreted total 4–hydroxyacetanilide in the same unit, the differential equations expressing the scheme above are,

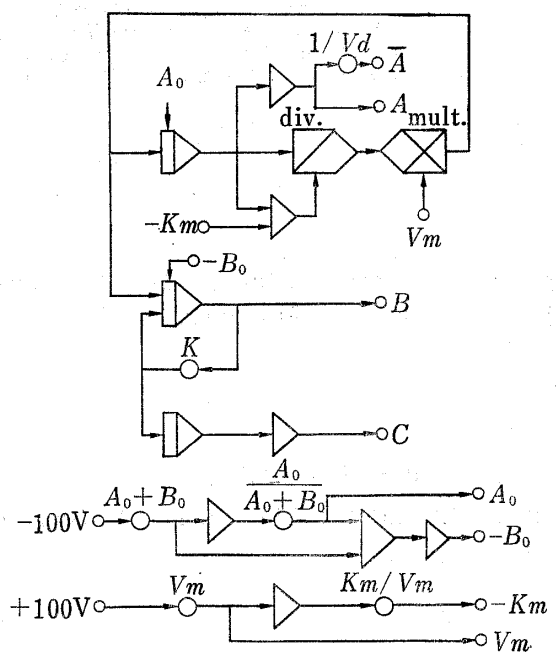

Fig. 5. Analog Computer Program used for Investigation of Acetanilide Metabolism Model proposed

$$\frac{dA}{dt} = -\frac{VmA}{Km+A} \qquad (17)$$

$$\frac{dB}{dt} = \frac{VmA}{Km+A} - KB \qquad (18)$$

$$\frac{dC}{dt} = KB \qquad (19)$$

And the initial conditions are $A=A_0$ and $B=B_0$, when $t=0$.

Instead of performing numerical integration of these differential equations, rate constants and calculated curves were obtained by an analog computer. The program used by the authors is shown in Fig. 5.

Experimental data with shifted zero time were first plotted on the recording chart. Various values were tried for the each rate constant on the computer setting and looked for the best fit of the theoretical curves. In the program shown in Fig. 5, it was intended that the ratio of Vm/Km was constant and that the sum of A_0 and B_0 was constant and equal to the total amount of recovered drug. Therefore, during the course of searching the

20) See appendix for the detail of obtaining these numerical values.

best rate constant that fit the experimental data, the ratio of Vm/Km, and the sum of A_0 and B_0 were kept constant automatically. The curves obtained by the analog computer are shown in Fig. 6, and 7, and the rate constants thus obtained are listed in Table VI.

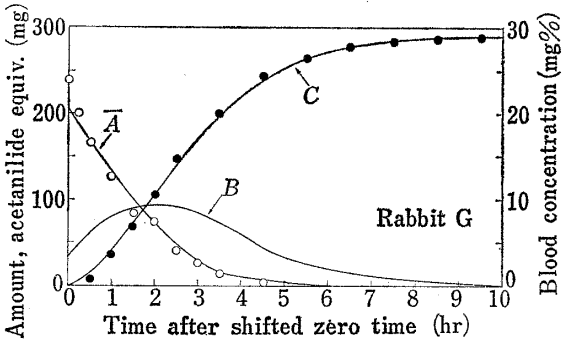

Fig. 6. Plots of Unchanged Acetanilide Elimination from the Blood (○) and Total 4–Hydroxyacetanilide Excretion (●) for Rabbit G

The solid lines were drawn by an analog computer programmed as shown in Fig. 5, where A_0=266.5 mg, B_0=23.5 mg, Vm=120.4 mg/hr, Km=71.0 mg, K=0.73 hr,$^{-1}$ and Vd=1276 ml.

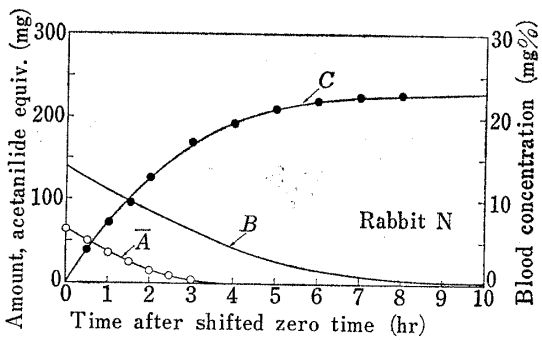

Fig. 7. Plots of Unchanged Acetanilide Elimination from the Blood (○) and Total 4–Hydroxyacetanilide Excretion (●) for Rabbit N

The solid lines were drawn by an analog computer programmed as shown in Fig. 5, where A_0=93.4 mg, B_0=140.2 mg, Vm=60.0 mg/hr, Km=35.4 mg, K=0.56 hr^{-1}, and Vd=1464 ml.

Table VI. Rate and Other Constants involved in Acetanilide Metabolism and Excretion obtained by the Analog Computer

Constants	Rabbits						
	A	D	G	J	Q	N	W
Vm (mg/hr)	108.0	119.6	120.4	88.0	76.0	60.0	78.0
Km (mg)	63.7	70.6	71.0	51.9	44.8	35.4	46.0
K (hr^{-1})	0.68	0.72	0.73	0.80	0.69	0.56	0.74
Vd (ml)	1433	1361	1276	1618	1446	1464	1172

The good agreement of calculated cruves and experimental data shows the validity of the model proposed for the metabolism and excretion of acetanilide.

In the most recent report, Wagner[21] has pointed out the fallacy in concluding there are zero order kinetics. The authors of present paper have been very cautious not to misinterprete the experimental data. The authors are fully confident about the existence of Michaelis Menten type process in hydroxylation of acetanilide by the following reason. Acetanilide was administered to rabbits *via* intravenous route and furthermore thirty minutes time shift was made to make sure of completion of the drug distribution in blood and attainment of equilibrium between the drug in blood with that in the fluid of distribution. Even under these strict conditions curvature of semi–logarithmic plots of the drug blood level was evidently noticed.

Appendix

Determination of Michaelis Menten Parameters from the Experimental Data of Acetanilide Hydroxylation

Differential equation that express Michaelis Menten kinetics is equation (15),

21) J.G. Wagner, *J. Pharm. Sci.*, **56**, 586 (1967).

$$\frac{d\bar{A}}{dt}=-\frac{\bar{V}m\bar{A}}{\bar{K}m+\bar{A}} \tag{15}$$

where $\bar{A}$ is the blood concentration of acetanilide, $\bar{V}m$ and $\bar{K}m$ are Michaelis Menten parameters. From equation (15), it is clear that when $\bar{A}$ is negligiblly small compared with $\bar{K}m$, equation (15) could be expressed as equation (20).

$$\frac{d\bar{A}}{dt}=-\frac{\bar{V}m}{\bar{K}m}\bar{A} \tag{20}$$

Equation (20) is nothing but the first order rate equation. And $\bar{V}m/\bar{K}m$ is the rate constant of this apparent first order process. Therefore, when the experimental blood concentration is plotted on a logarithmic scale, $\bar{V}m/\bar{K}m$ may be calculated from the slope of the terminal straight line portion of the curve.

Integration of equation (15) gives equation (16),

$$\bar{V}mt=\bar{K}m(\ln\bar{A}_0-\ln\bar{A})+(\bar{A}_0-\bar{A}) \tag{16}$$

where $\bar{A}_0$ is $\bar{A}$ at $t=0$.

Rearrangement of equation (16) gives equation (21).

$$\bar{K}m=\frac{\dfrac{(\bar{A}_0-\bar{A})}{t}}{\dfrac{\bar{V}m}{\bar{K}m}-\dfrac{(\ln\bar{A}_0-\ln\bar{A})}{t}} \tag{21}$$

If $\bar{V}m/\bar{K}m$ is known, $\bar{K}m$ can be calculated by equation (21), because $\bar{A}_0$, $\bar{A}$, and t are all known as experimental data.

The determination of the parameters for equation (16) are illustrated with the data of rabbit G in Table V.

Logarithm of the blood concentration was plotted against 0.5 hours shifted time scale (solid circle plot of Fig. 4). Thirty minutes time shift was made to allow sufficient period of time necessary for equilibrium of distribution after intravenous administration of the drug.

Where A is less than 1 mg%, the plot is linear. From this straight line portion, $\bar{V}m/\bar{K}m$ was calculated by equation (22), which was 1.7 hr^{-1}.

$$\bar{V}m/\bar{K}=\text{slope of linear portion}\times 2.303 \tag{22}$$

Since $\bar{V}m/\bar{K}m$ was obtained, $\bar{K}m$ was calculated with the experimental data by equation (21) as shown in Table VII.

Table VII. $\bar{K}m$ Values calculated by Equation (21) from Experimentally obtained $\bar{A}$ and t with Various $\bar{A}_0$ Values ($\bar{V}m/\bar{K}m=1.70$ hr^{-1}) (Rabbit G)

t (hr)	$\bar{A}$ (mg%)	$\bar{K}m$ (mg%)			
		$\bar{A}_0=23.98$ mg%	$\bar{A}_0=22.0$ mg%	$\bar{A}_0=20.0$ mg%	$\bar{A}_0=18.0$ mg%
0.25	20.06	15.904	5.832	—	—
0.5	16.68	14.992	9.282	4.966	1.706
1.0	12.64	10.704	8.170	5.930	3.399
1.5	8.32	10.502	8.672	6.982	5.444
2.0	7.28	7.564	6.418	5.324	4.297
2.5	4.04	8.078	7.028	6.022	5.066
3.0	2.50	7.566	6.668	5.794	4.959
3.5	1.22	7.660	6.798	5.958	5.150
4.5	0.42	6.536	5.846	5.172	4.518

Unfortunately, when 23.98 mg% (experimentally obtained $\bar{A}_0$ value) was used as $\bar{A}_0$ value, calculated $\bar{K}m$ varied from one $\bar{A}$ to another.

This happened because experimentally obtained $\bar{A}_0$ was extraordinarily large due to analytical error. Therefore, this value was discarded, and looked for appropriate value for $\bar{A}_0$. When 20.0 mg% was used

for $\bar{A}_0$, deviation of $\bar{K}m$'s became smallest. Average value of $\bar{K}m$ was 5.768 mg% and consequently $\bar{V}m$ was 9.806 mg%/hour.

When the value of the volume of distribution of acetanilide, Vd, is known, Vm and Km are calculated by,

$$Vm=\bar{V}m\cdot Vd/100, \qquad Km=\bar{K}m\cdot Vd/100$$

The volume of distribution of acetanilide, Vd, of rabbit G was 1276 ml from Table VI. Therefore Vm and Km were 125.1 mg/hour and 73.6 mg respectively. These values show a very good agreement with the constants shown in Table VI, which were obtained by the analog computation.

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