Kinetics of Pharmacologic Response II: Equation for Turnover Time of Goldfish as a Function of Concentration of Ethanol and a Theoretical Derivation Based on a Combination of Occupation and Rate Receptor Theories

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Abstract □ Turnover time (T) of goldfish in 2–8% v/v ethanol–water (C) was determined in two crossover studies: (*a*) three body weight groups of six fish each, and (*b*) six larger fish. Each fish had a different treatment schedule. Analyses of variance of log T showed: (*a*) no residual effects of a treatment, and (*b*) no significant differences among fish. Log T was linearly related to log C and the variances were homogeneous. For individual fish: $T = (a/C)^b$, where a and b are apparently normally distributed with 95% C.I. of 9.31 ± 3.8 and 2.28 ± 1.17, respectively. For all 24 fish, $a.b = D$, where D is essentially a constant but has a narrow apparently normal distribution with a coefficient of variation of 11.5%; hence, $T = (D/bC)^b$. This equation was also derived by marrying occupation and rate receptor theories. Basic assumptions are: $dT/T = -b(dv/v)$ and $v \ll Vm$, where v is the overall reaction rate of the receptor-ethanol reaction and Vm is the maximum rate. Also note that $dv/v = dn/n$, where n is the fraction of receptors occupied. Literature data are shown to obey the derived equations. Hence $1/T \propto C$ only in those rare instances when $b = 1$. The theory assumes only a partitioning across the absorbing membranes of the fish. It differs from a previous theory which assumes a first order rate constant for absorption is involved.

Keyphrases □ Pharmacologic response—kinetics □ Ethanol effect—goldfish turnover time □ Kinetic equations—goldfish response, ethanol □ Partitioning theory—ethanol transport □ Theoretical equations—pharmacologic response

When a goldfish is allowed to swim in an alcohol-water solution it will eventually turn on its side. The time interval between the time the fish was placed in the solution and the time of turnover has been called the turnover (or overturn) time. If the fish is taken out of the solution immediately after it turns over and is placed in freshly aerated water it will recover. However, if the fish is left in the solution after it turns over it will eventually die. The time interval between the time the fish was placed in the solution and the time of death has been called the lethal (or survival) time. Over the past 70 years there have been numerous reports (1–9) giving the turnover and/or lethal times of both goldfish and guppies exposed not only to different concentrations of alcohol but also to different concentrations of a wide variety of drugs and chemicals. These reports have not answered many obvious questions such as the following. (*a*) What functional relationship exists between turnover or lethal time and the concentration of the drug? (*b*) How well does the appropriate equation apply to data collected on individual fish as well as averaged data? (*c*) Could the appropriate functional relationship be derived "from scratch" on the basis of theory alone? (*d*) How much was day-to-day variation influencing the results? (*e*) Are there any residual effects? That is, if a fish was exposed to a certain concentration of alcohol on Day 1 did this exposure affect the turnover time observed in a different concentration of alcohol on Day 2? (*f*) What is the quantitative effect of changing the size of the fish? This report provides answers to most, if not all, of these questions.

EXPERIMENTAL

Goldfish, *Carassius auratus*, common variety, were used. Between tests the fish were maintained in a large aquarium which had a charcoal and glass-wool filter and was well aerated. For test runs 0.05 M phosphate buffer, adjusted to pH 6.1, was used to prepare solutions containing 2, 3, 4, 5, 6, and 8% v/v ethanol. During the tests the solutions were maintained at 24 ± 1°.

Evaluation of Pharmacologic Response—The determination of turnover time was carried out with individual fish in 200 ml. of ethanol solution contained in 800-ml. beakers. The turnover time was taken as the time required for the fish to lose the ability to maintain itself upright after immersion in the alcohol solution. When the first signs of turnover were observed the end point was tested by attempting to turn the fish on its side with a stirring rod.

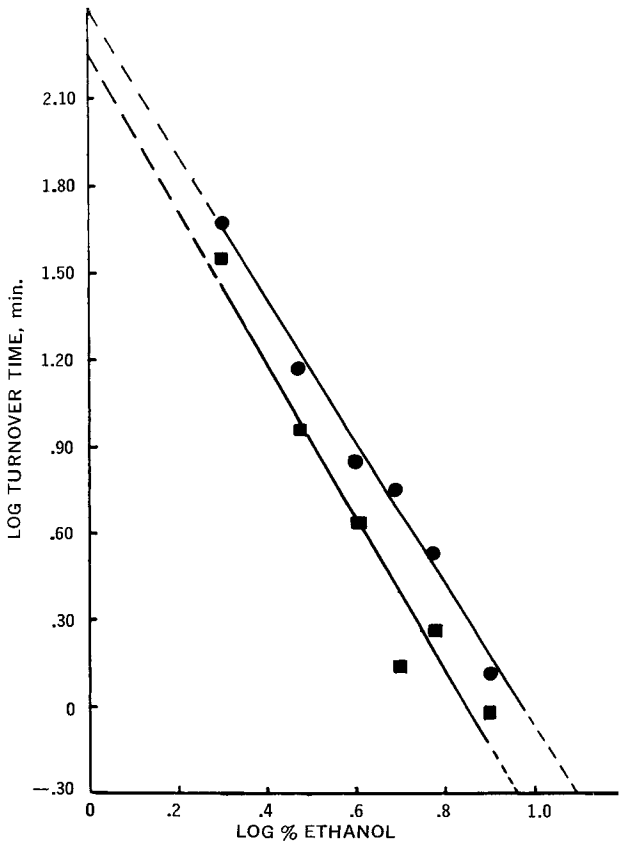

Figure 1—*Representative plots of logarithm of turnover time against logarithm of alcohol concentration for individual fish. Key:* ■, *fish No. 6 of Group I;* ●, *fish No. 8 of Group II.*

Table I—Treatment Schedules and Turnover Times in Minutes of Individual Fish Observed in the Two Crossover Studies

Group	Fish	Day 1	2	3	4	5	6	Fish Av.
				Crossover Study No. 1				
I	1	(2) 13.54[a]	(3) 12.80	(4) 8.95	(5) 7.42	(6) 2.00	(8) 1.15	7.64
	2	(3) 24.25	(8) 0.86	(2) 50.92	(4) 10.30	(5) 5.24	(6) 2.28	15.65
	3	(4) 2.94	(5) 3.56	(8) 2.41	(6) 4.90	(3) 7.29	(2) 25.82	7.82
	4	(5) 3.32	(6) 1.51	(3) 19.77	(2) 17.64	(8) 0.55	(4) 5.73	8.09
	5	(6) 3.96	(4) 6.57	(5) 4.39	(8) 1.18	(2) 27.25	(3) 10.71	9.01
	6	(8) 0.95	(2) 36.17	(6) 1.83	(3) 9.10	(4) 4.00	(5) 1.30	8.89
II	7	(3) 10.32	(2) 32.00	(6) 2.65	(5) 2.85	(4) 2.73	(8) 1.20	8.63
	8	(2) 48.29	(4) 7.29	(8) 1.28[b]	(3) 14.80	(6) 3.43	(5) 5.65	13.46
	9	(4) 7.05	(8) 1.58	(5) 10.86	(2) 41.85	(3) 14.36	(6) 2.50	13.03
	10	(6) 2.46	(3) 17.68	(4) 9.67	(8) 1.09	(5) 3.00	(2) 32.56	11.08
	11	(8) 2.04	(5) 4.91	(3) 10.21	(6) 1.67	(2) 27.05	(4) 2.98	8.14
	12	(5) 10.3[c]	(6) 4.72	(2) 24.47	(4) 5.88	(8) 1.80	(5) 7.08	9.04
III	13	(4) 13.09	(8) 0.78	(2) 59.00	(6) 1.33	(5) 1.54	(3) 11.23	14.50
	14	(3) 16.03	(2) 70.12	(4) 4.00	(8) 1.05	(6) 0.83	(5) 3.07	15.85
	15	(6) 8.50	(5) 3.33	(3) 19.97	(2) 25.17	(4) 2.22	(8) 0.86	10.01
	16	(8) 1.08	(4) 1.18	(6) 3.47	(5) 2.18	(3) 4.39	(2) 23.00	5.88
	17	(2) 163.24	(6) 3.48	(5) 1.61	(3) 6.19	(8) 0.71	(4) 3.65	29.81
	18	(5) 7.43	(3) 12.17	(8) 2.03	(4) 7.30	(2) 22.72	(6) 1.78	8.91
Day averages		18.82	12.26	13.19	8.99	7.28	7.92	
		Day 7	8	9	10	11	12	
				Crossover Study No. 2				
IV	19	(2) 32.33	(4) 7.25	(3) 10.45	(6) 2.50	(8) 2.43	(5) 2.32	9.55
	20	(3) 20.55	(5) 6.18	(4) 6.41	(8) 2.30	(2) 14.78	(6) 1.92	8.64
	21	(4) 14.06	(6) 3.16	(5) 2.29	(2) 25.4	(3) 11.70	(8) 1.35	9.66
	22	(5) 4.74	(8) 2.43	(6) 2.13	(3) 5.90	(4) 3.30	(2) 23.38	6.98
	23	(6) 5.44	(2) 18.24	(8) 1.4	(4) 3.70	(5) 4.72	(3) 6.10	6.60
	24	(8) 0.70	(3) 14.31	(2) 20.4	(5) 3.46	(6) 3.12	(4) 3.96	7.66
Day averages		12.92	8.60	7.18	7.21	6.68	6.51	

[a] Bracketed numbers show the alcohol concentration (% v/v). [b] Fish looked ill and was replaced on Day 4. [c] Fish died and was replaced on Day 2.

Unaffected fish will either not allow themselves to be placed on their side or will immediately right themselves. The end point was judged as the time when a fish placed on its side did not right itself immediately. This was the same procedure as used by Gibaldi and Nightingale (8). Time was measured with a clock reading to 0.01 min. Immediately after the end point was reached the fish was removed from the alcohol solution and placed in a recovery tank containing about 5 gal. of distilled water. After recovery fish were placed in the large aquarium.

Crossover Study 1—Three groups of six fish each were chosen on the basis of body weight. The group number, average body weight, and range of body weights were as follows: I, 6.2 g., 5–7 g.; II, 8 g., all 8 g.; III, 10.5 g., 10–12 g. Since there were six different treatments there were 6! or 720 possible different treatment schedules. Eighteen of these different possible treatment schedules were chosen by a random process and one of the schedules was assigned to each of the 18 fish. Each fish was exposed to a different concentration of alcohol on each of six consecutive days. The experimental design is indicated by Table I.

Crossover Study 2—Six fish with average body weight of 13.8 g. (range 11–17 g.) were used. Each fish had a different treatment schedule. In this study the design was balanced in that each treatment followed another treatment an equal number of times. The experimental design is indicated by Table I.

RESULTS

The individual turnover times, the fish averages, and the day averages are given in Table I. The treatment averages with their coefficients of variation are given in Table II. Also given in Table II are the averages of the logarithms (base 10) of the turnover times and their corresponding variances. Bartlett's test indicated their variances were homogeneous. Hence all analyses of variance were performed on the logarithms of the turnover times.

Table III is an analysis of variance table based on the data collected in Crossover Study 1. There were no significant differences among groups of fish or among fish per group. Most of the variance was associated with treatments (different alcohol concentrations) but the mean square for periods or days was highly significant ($p < 0.001$). There was no evidence of residual effects of a treatment by inspection of the data collected in Study 1.

Table IV is an analysis of variance table resulting from the usual analysis of variance for crossover design applied to the logarithms of the turnover times observed in Crossover Study 2. Again, there were no significant differences among fish. In this study there were no significant differences among periods or days. As before, most of the variance was associated with treatments. Table V is an analysis of variance table showing a test for residual effects in Crossover Study 2. This was feasible because of the balanced design. There were no significant residual effects of one treatment on another treatment when the treatments were separated by 24 hr. Almost all the variance was associated with direct effects of a treatment.

When the logarithm of turnover time was plotted against the logarithm of the alcohol concentration for individual fish in the concentration range studied (2–8% v/v alcohol) linear plots were obtained. Typical plots are shown in Fig. 1. The intercept (corresponding to $C = 1$) and the slope of the straight lines for each fish were calculated by the method of least squares and are shown in Table VI. The coefficients of determination listed in Table VI are a measure of the fit of the points to each line, if all the points were exactly on the line the coefficient of determination would equal unity. The coefficient of determination is equivalent to the fraction of the variance of the log T values which is accounted for by differences in the log C values. If the intercept is $\log(a^b) = b \log a$, where a and b are constants for each fish, then division of the intercept by the absolute value of the slope, b, will yield the parameter a. The values of a so calculated are listed in Table VI. Hence, for individual fish, the relationship between turnover time and ethanol concentration is given by:

$$T = \left(\frac{a}{C}\right)^b \qquad \text{(Eq. 1)}$$

The parameters a and b appeared to be normally distributed. The 95% C.I. of a was 9.31 ± 3.8 for individual fish. The 95% C.I. of b was 2.28 ± 1.17 for individual fish. The 95% C.I. of the average

Table II—Average Turnover Times and Average of the Logarithms of Turnover Times with Measures of Dispersion

Alcohol Concentration, % v/v	Turnover time, min. Av.	Turnover time, min. *CV* %[a]	Logarithm of Turnover Time Av.	Logarithm of Turnover Time Variance[b]	Estimated[c] Av. Turnover Time, min.	Error,[d] min.
2	36.47	85.4	1.4778	0.05660	33.3	−3.2
3	13.51	42.4	1.0582	0.03801	13.2	−0.3
4	6.02	55.5	0.7111	0.6763	6.88	+0.86
5	4.40	57.5	0.5775	0.06060	4.13	−0.27
6	2.97	55.9	0.4185	0.04760	2.73	−0.24
8	1.38	43.5	0.1022	0.03566	1.41	+0.03

[a] *CV* (%) = standard deviation/average × 100. [b] Bartlett's test (14) indicated that the variances were homogeneous since $\chi^2 = 3.75$ with 5 degrees of freedom, which is not significant ($0.7 > p > 0.5$). [c] Estimated average turnover time = $(9.31/C)^{2.28}$, where C is the alcohol concentration in % v/v and the numbers 9.31 and 2.28 are the average values of a and b, respectively (see Table VI). [d] Error is the difference between the estimated and observed average turnover times.

Table III—Analysis of Variance Table Resulting from the Usual Analysis of Variance for Crossover Design. Data were Logarithms of Turnover Times for Fish of Groups I, II and III

Source of Variation	*df*	*SS*	*MS*	*F*	Significance Level
Total	107	29.4694	—	—	—
Fish	17	1.0064	0.05920	1.36	N.S. ($0.25 > p > 0.10$)
Groups	2	0.1742	0.08712	2.00	N.S. ($0.25 > p > 0.10$)
Fish/Group	15	0.8322	0.05548	1.27	N.S. ($p > 0.25$)
Periods, days	5	1.1753	0.2351	5.39	Sig. ($p < 0.0001$)
Treatments	5	23.8003	4.7601	109	Sig. ($p \ll 0.001$)
Residual	80	3.4875	0.04359	—	—

values of a and b were 9.31 ± 0.77 and 2.28 ± 0.24, respectively.

Empirically it was found that the parameters a and b for individual fish were related in that their product, $a \cdot b$, was essentially a constant, D, for all fish. These values are also listed in Table VI. Values of D appeared to be normally distributed with a very narrow distribution. The coefficient of variation was only 11.5% and the 95% C.I. of the individual D value was 20.3 ± 4.8. Support for this relationship between the parameters a and b is given in Figs. 2 and 3. Figure 2 is a plot of the intercepts against the slopes of the linear regressions of log T on log C for the 24 individual fish studied. This plot illustrates that the intercepts of the log T *versus* log C plots are a power function of the slopes, b; that is: intercept = log $(a^b) = b \log a$. The equation of the least-squares line drawn through the points in Fig. 2 is:

$$b \widehat{\log} a = 0.7147 + 0.6310\, b \qquad \text{(Eq. 2)}$$

whence

$$a = \text{antilog}\left\{\frac{0.7147}{b} + 0.6310\right\} \qquad \text{(Eq. 3)}$$

Using the average value of b, namely 2.276, for the 24 fish (see Table VI) to substitute into Eq. 3 one finds $a = 8.804$ which is very similar to the average value of a, namely 9.306, calculated from the 24 individual a values (see Table VI). Figure 3 is a plot of a *versus* $1/b$ for the 24 individual fish. The line drawn through the points is $a = 20.33/b$ where the slope of 20.33 is the average value of D (*i.e.*, the average value of the products of a and b for the 24 fish). One can see that the line represents the individual points very well with the possible exception of Fish No. 16 which is an outlier. Hence for all 24 fish one can write the equation:

$$T = \left(\frac{D}{bC}\right)^b \qquad \text{(Eq. 4)}$$

Literature Data—Previous authors have only reported average turnover times or just the slopes and intercepts of plots $1/T$ *versus* C; hence much useful data concerning the relationship between T and C for individual fish is unavailable. Hall and Hayton (7) studied both goldfish and guppies in various concentrations of ethanol using both turnover time and lethal time end points. These authors kindly supplied the authors the average turnover times and corresponding alcohol concentrations which they used to calculate the intercepts and slopes of their $1/T$ *versus* C plots. Gibaldi and Nightingale (8) reported two sets of average turnover times as a function of alcohol concentration. Log T was plotted *versus* log C for all of these sets of data with the results shown in Table VII. Since average turnover or lethal times were employed we have included results obtained with our four groups of fish at the bottom of Table VII for comparison purposes. Inspection of Table VII indicates that the log-log plot adequately describes all literature data including the data of Gibaldi and Nightingale (8) where the corresponding $1/T$ *versus* C plot was interpreted as three linear segments. When average turnover times are employed the coefficients of determination of the regressions of log T on log C are all very high indicating little scatter of the points about the regression lines. When the end point was turnover time, the slopes (b) ranged from 1.722 to 2.256 for literature data and from 1.856 to 2.716 for data from this study. A plot of $1/T$ *versus* C will be linear and pass through the origin only when $b = 1$; this accounts for the high negative intercepts reported by Hall and Hayton (7) and the S-shaped plot which was resolved as three linear components by Gibaldi and Nightingale (8). This suggests that a plot of $1/T$ *versus* C is an inappropriate function *for these*

Table IV—Analysis of Variance Table Resulting from the Usual Analysis of Variance for Crossover Design.[a]

Source of Variation	*df*	*SS*	*MS*	*F*	Significance Level
Total	35	6.049	0.1680	—	—
Sequences (fish)	5	0.09174	0.01835	0.56	N.S. ($p > 0.25$)
Periods, days	5	0.3337	0.06673	2.02	N.S. ($0.25 > p > 0.10$)
Treatments	5	4.963	0.9926	30.0	Sig. ($p \ll 0.001$)
Residual	20	0.6606	0.03303	—	—

[a] Data were logarithms of turnover times for fish of Group IV.

Table V—Analysis of Variance Table Showing Test for Residual Effects.[a]

Source of Variation	*df*	*SS*	*MS*	*F*	Significance Level
Sequences (fish)	5	0.09174*	0.01835	0.49	N.S. ($p > 0.25$)
Periods, days	5	0.3337*	0.06673	1.79	N.S. ($0.25 > p > 0.10$)
Direct effects (unadj.)	5	17.71	3.541		
Residual effects (adj.)	5	0.1011	0.02023	0.54	N.S. ($p > 0.25$)
Residual effects (unadj.)	5	0.1831*	0.03662		
Direct effects (adj.)	5	4.881*	0.9763	26.2	Sig. ($p \ll 0.001$)
Error	15	0.5594[b]	0.03730		
Total	35	6.049			

[a] Data were logarithms of turnover times for fish of Group IV. [b] The error sum of squares is the total sum of squares less the sums of squares marked with an asterisk.

Table VI—Parameters Calculated from the Logarithms of Turnover Time (*T*) and the Logarithms of the Ethanol Concentrations (*C*) for Individual Fish

Group	Fish	Weight, g.	Intercept, log (*a*)[b]	Slope (*b*)	Coefficient of Determination[a]	*a*	$D = a \cdot b$
			From Regression of log T on log C (% v/v)				
I	1	5	2.4697	2.618	0.891	8.778	22.98
	2	6	2.7308	2.997	0.979	8.151	24.43
	3	6	1.6683	1.501	0.746	12.93	19.41
	4	7	2.1746	2.507	0.861	7.369	18.47
	5	6	2.0669	2.076	0.963	9.900	20.55
	6	7	2.2506	2.666	0.935	6.985	18.62
II	7	8	2.0842	2.285	0.921	8.168	18.66
	8	8	2.4083	2.483	0.987	9.330	23.17
	9	8	2.2865	2.268	0.801	10.19	23.11
	10	8	2.3840	2.567	0.967	8.486	21.78
	11	8	1.9241	1.969	0.853	9.488	18.68
	12[b]	8	1.7811	1.623	0.905	10.97	17.80
III	13	10	2.7265	3.249	0.917	6.905	22.43
	14	11	2.7392	3.286	0.937	6.817	22.40
	15	10	2.0779	2.136	0.657	9.393	20.06
	16	10	1.6404	1.822	0.651	7.949	14.48
	17	12	2.8330	3.412	0.823	6.766	23.09
	18	10	1.9686	1.882	0.877	11.12	20.93
IV	19	14	2.0227	2.021	0.896	10.02	20.25
	20	16	1.8487	1.697	0.790	12.28	20.84
	21	12	2.1353	2.176	0.853	9.579	20.84
	22	13	1.6644	1.579	0.810	11.32	17.87
	23	17	1.6200	1.485	0.797	12.33	18.31
	24	11	2.1143	2.325	0.917	8.117	18.87
Av.			2.1508	2.276		9.306	20.33
SD			0.3659	0.563		1.834	2.33
Coefficient of variation, %			—	24.7		19.7	11.5
95% C.I. of individual values			2.15 ± 0.76	2.28 ± 1.17		9.31 ± 3.8	20.3 ± 4.8
95% C.I. of average			2.15 ± 0.15	2.28 ± 0.24		9.31 ± 0.77	20.3 ± 1.0

[a] Coefficient of determination = $(\Sigma_{obs.}^2 - \Sigma_{dev.}^2)/\Sigma_{obs.}^2$. It is equivalent to the fraction of variance of the log *T* values which is accounted for by differences in the log *C* values. [b] Parameters calculated from data collected with 2,3,4,6, and 8% v/v alcohol on Days 2 through 6 of the study on a second fish since the first fish died on Day 1 after exposure to 5% v/v alcohol.

data. When the end point was lethal time, the slopes (*b*) ranged from 1.006 to 1.918; hence these were generally lower (but still greater than unity) than those obtained with the turnover end point. The values of the parameters *a* and *D* were correspondingly higher when the end point was lethal time compared with the values when the end point was turnover time.

Correlation of Parameters with Body Weight of Fish—The parameters *a* and *b* of Eq. 1 are not related to the body weight, and hence the size, of the fish. For the 24 goldfish employed in these studies the correlation coefficient for parameter *a* with body weight of fish was 0.308 ($p > 0.10$). The correlation coefficient for parameter *b* with body weight of fish was −0.251 ($p > 0.10$).[1] Also, as stated formerly, analysis of variance of log *T* in both crossover studies, indicated no significant differences among fish. These results strongly suggest that the constants relating turnover time of goldfish to concentrations of ethanol do not involve the surface area of the absorbing fish membrane(s).

Value of Constant D if Alcohol Concentration is Expressed in Moles/Liter—When the alcohol concentration is expressed as % v/v the average value of *D* for the 24 fish was 20.33. Since the density of ethanol is 0.78522 g./ml. at 25° the corresponding average value of *D* would be 52.4 if the concentration of ethanol in the bath fluid was expressed in moles/liter.

THEORETICAL

A feasible approach to the empirical Eqs. 1 and 4 is by combining both occupation (10, 11) and rate (12) receptor theories. In occupation theory the pharmacologic response is assumed to be related to the fraction of the receptors occupied or its equivalent, the rate of formation of products or overall reaction rate. In rate theory the pharmacologic response is assumed to be related to the rate of receptor combination.

[1] Logarithmic transforms gave similarly low correlation coefficients implying lack of correlation of *a* and *b* with surface area of the fish.

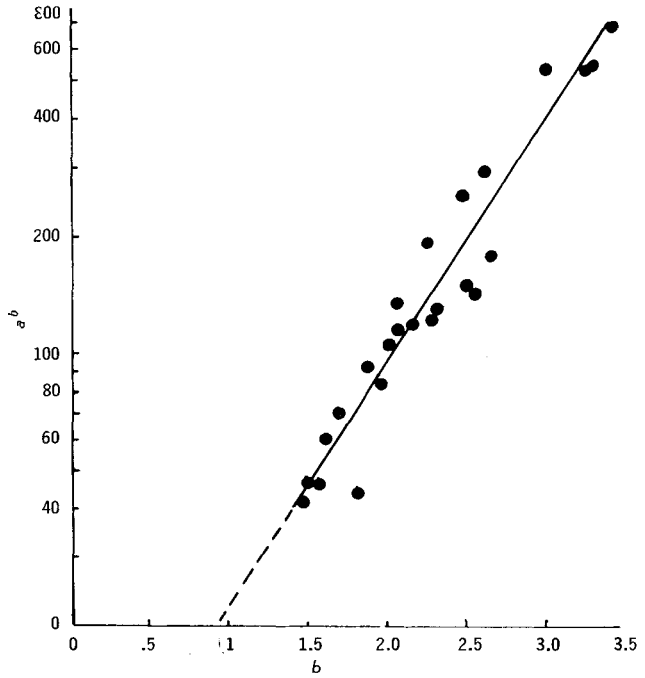

Figure 2—*Plot of the intercepts* [*log* a^b = b *log* a] *corresponding to* C = *1 against the slopes* (b) *for 24 individual goldfish. The intercepts and slopes were obtained by the method of least squares using the logarithm of the turnover time* (*min.*) *as ordinate and the logarithm of the alcohol concentration* (%*v*/*v*) *as abscissa. The equation of the line drawn through the points is* b $\widehat{log}$ a = *0.7147* + *0.6310* b.

Symbolism—Let:

- Ci = the concentration of ethanol in the biophase next to the receptor inside the fish;
- Co = the concentration of ethanol in the bath fluid in which the fish is swimming;
- Kp = Ci/Co = the partition coefficient for ethanol across the absorbing fish membrane(s);
- n_T = the total number of receptors available for complexing with ethanol;
- n = the number of receptors complexed with ethanol;
- p = the concentration of products resulting from breakdown of the alcohol-receptor complex;
- (dP/dt) = $k_3n = v$ = overall reaction rate;
- T = turnover time of goldfish when the ethanol concentration is Co;
- K = $(k_2 + k_3)/k_1$, where K is analogous to the extended Michaelis constant;
- Vm = k_3n_T, hence Vm corresponds to v when all receptors are occupied by ethanol.

Scheme—Consider the following reaction scheme:

$$\underset{(Ci)}{\text{ethanol}} + \underset{(n_T - n)}{\text{receptor}} \underset{k_2}{\overset{k_1}{\rightleftarrows}} \underset{(n)}{\text{drug-receptor complex}} \xrightarrow{k_3} \underset{(P)}{\text{products}}$$

Scheme I

Derivation—Based on Scheme I:

$$\frac{dn}{dt} = k_1Ci(n_T - n) - (k_2 + k_3)n \qquad \text{(Eq. 5)}$$

$$(dP/dt) = k_3n = v \qquad \text{(Eq. 6)}$$

At the steady state, $dn/dt = 0$, hence from Eq. 5

$$k_1Ci(n_T - n) = (k_2 + k_3)n \qquad \text{(Eq. 7)}$$

whence,

$$n = \frac{k_1}{k_2 + k_3}(n_T - n)Ci = \frac{(n_T - n)Ci}{K} \qquad \text{(Eq. 8)}$$

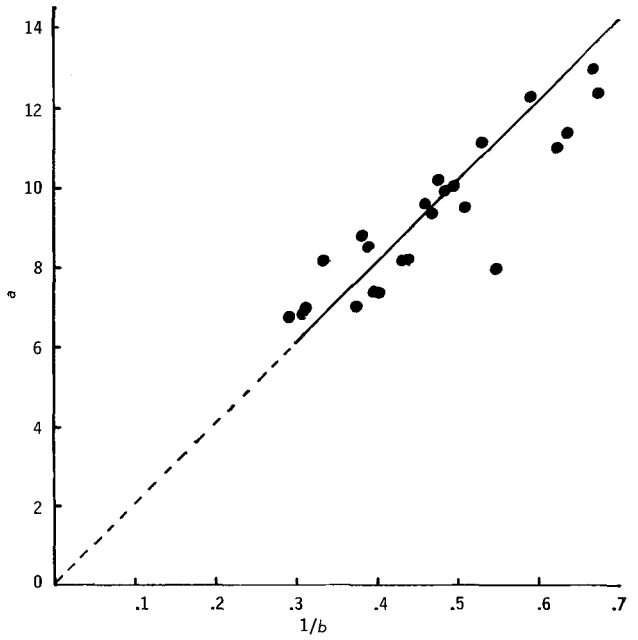

Figure 3—*Plot of the parameter* a versus *the reciprocal of the parameter* b. *The line drawn through the points has the equation:* â = *20.33*/b *where the slope of 20.33 is the average value of the products,* a·b, *for the 24 individual goldfish.*

Substituting for n in Eq. 6 from Eq. 8 yields

$$v = k_3\frac{(n_T - n)}{K}Ci = \frac{(Vm - v)Ci}{K} \qquad \text{(Eq. 9)}$$

Eq. 9 may be rearranged to yield the well-known result as follows:

$$v = \frac{VmCi}{K + Ci} \qquad \text{(Eq. 10)}$$

Substituting $KpCo$ for Ci in Eq. 10 and taking the reciprocal of both sides gives

$$\frac{1}{v} = \frac{K + KpCo}{VmKpCo} \qquad \text{(Eq. 11)}$$

Assume that for any given individual fish,

$$\frac{dT}{T} = -b\frac{dv}{v} \qquad \text{(Eq. 12)}$$

or its equivalent, namely

$$\frac{dT}{dv} = -b\frac{T}{v} \qquad \text{(Eq. 13)}$$

where b is a dimensionless proportionality constant. Now, since

$$\frac{dv}{v} = \frac{dn}{n} \qquad \text{(Eq. 14)}$$

the assumption stated in Eq. 12 is also equivalent to

$$\frac{dT}{T} = -b\frac{dn}{n} \qquad \text{(Eq. 15)}$$

From Eq. 12 we may obtain:

$$\int_{T^1}^{T}\frac{dT}{T} = -b\int_{v^1}^{v}\frac{dv}{v} \qquad \text{(Eq. 16)}$$

Performing the integration:

$$\ln\frac{T}{T^1} = -b[\ln v - \ln v^1] = \ln\left(\frac{v^1}{v}\right)^b \qquad \text{(Eq. 17)}$$

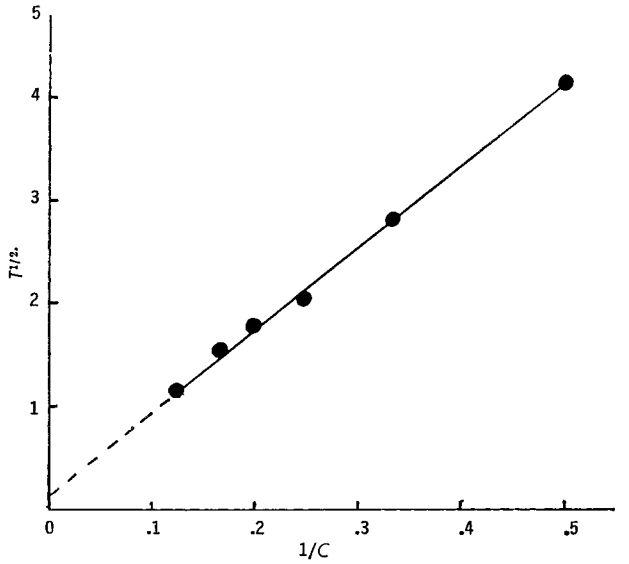

Figure 4—*Plot of* $T^{1/b}$ versus 1/C *in conformity with Eq. 21. Average turnover times of 24 fish were used for* T *and alcohol concentrations (%v/v) for* C. *The equation of the line drawn through the points is* $T^{1/2.5} = 0.137 + 8.107/C$ *and represents an approximate but not a least-squares fit. Using Eq. 21 one finds* $a_1 = 0.137$ *and* $a_2 = 59.2$.

whence

$$T = \left(\frac{G}{v}\right)^b, \text{ where } G = (T^1)^{1/b} \cdot V^1 \qquad \text{(Eq. 18)}$$

and

$$\frac{1}{v} = \frac{T^{1/b}}{G} \qquad \text{(Eq. 19)}$$

Substituting for $1/v$ from Eq. 19 into Eq. 11:

$$T^{1/b} = G\left(\frac{K + KpCo}{VmKpCo}\right) = \frac{G}{Vm} + \frac{GK}{VmKpCo} \qquad \text{(Eq. 20)}$$

If $G/Vm = a_1$ and $K/Kp = a_2$, then Eq. 20 becomes

$$T^{1/b} = a_1 + \frac{a_1 a_2}{Co} \qquad \text{(Eq. 21)}$$

An approximate fit, using the average turnover times of the 24 fish, in conformity with Eq. 21, is shown in Fig. 4. One could also fit turnover times, *T*, corresponding to different ethanol concentrations, *Co*, with Eq. 21 by an iterative method using a suitable nonlinear estimation program and a high speed digital computer. The three parameters, a_1, a_2, and *b* would be estimated. Such a process would require preliminary estimates of the parameters; although *b* may be guessed quite accurately, this is not the case with parameters a_1 and a_2. If this process were carried out for data derived from individual fish little would be gained since the parameters a_1 and a_2 are ratios of two other fundamental constants of the hypothesized model.

If the data are collected in a range where $v \ll Vm$ then an approximation is feasible. Equation 11 leads to

$$\frac{1}{v} - \frac{1}{Vm} = \frac{K}{KpCoVm} \qquad \text{(Eq. 22)}$$

When $v \ll Vm$ Eq. 22 is approximated by Eq. 23.

$$\frac{1}{v} = \frac{K}{KpCoVm} \qquad \text{(Eq. 23)}$$

Substituting for $1/v$ in Eq. 23 from Eq. 19 and simplifying gives

$$T^{1/b} = \frac{GK}{VmKpCo} \qquad \text{(Eq. 24)}$$

If:

$$a = \frac{GK}{VmKp}$$

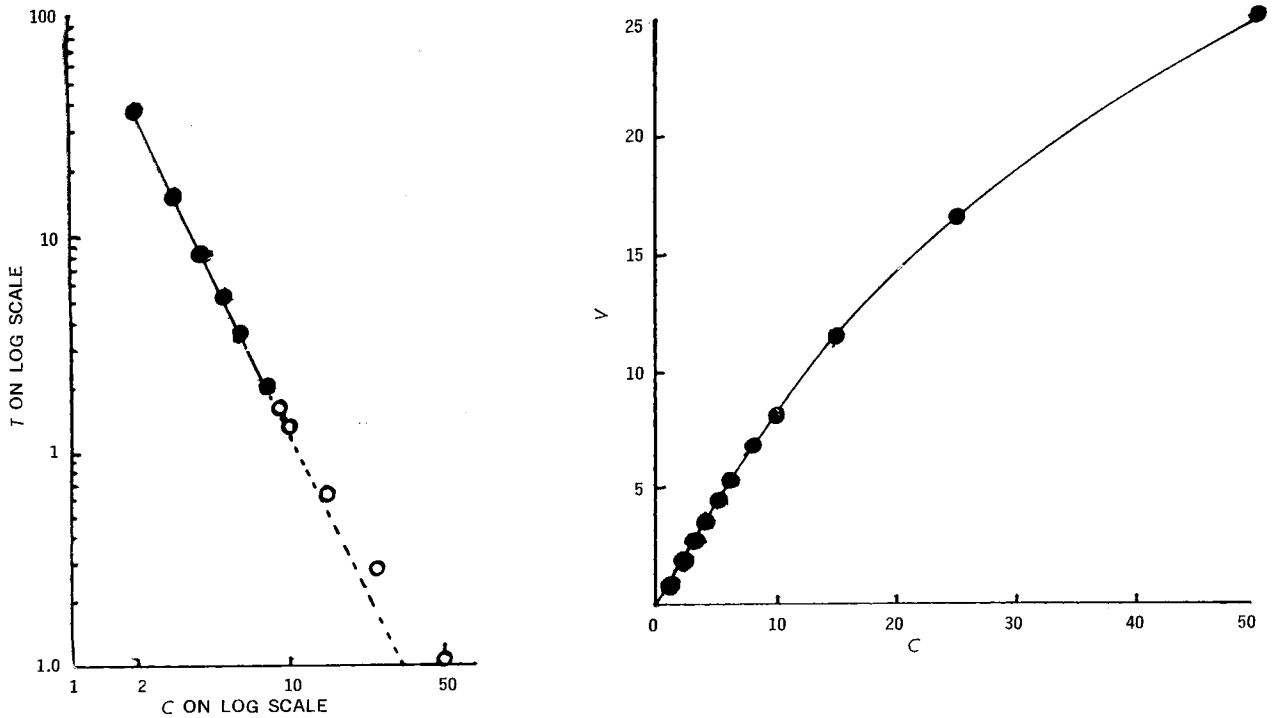

Figure 5—T versus C *on log-log plot derived from synthetic data. Least-squares line based on solid points (corresponding to* C = 2, 3, 4, 5, 6, *and 8) is log* T = 2.1846 − 2.100 log C. *The estimated values of the parameters* b, a, *and* D *are 2.100, 10.97 and 23.04, corresponding to the actual values of 2.276, 9.34, and 21.26, respectively. Insert: plot of* v versus C *based on the equation* v = 50 C/(50 + C). *Synthetic data was derived from latter equation and* $T = (9.34/v)^{2.276}$. *Note that when there is marked curvature in the* v versus C *plot (corresponding to* C > 8 *and* v/Vm > 0.14) *the points of the log* T versus C *plot deviate from the line established in the concentration range of 2 to 8 when* v/Vm < 0.14.

Table VII—Parameters of the Equations $T = (a/b)^b$ and $T = (n/bC)^b$ Calculated from Average Turnover Times and Average Lethal Times Reported in the Literature and a Comparison of the Slopes and Intercepts of Reciprocal Plots as Reported by the Original Authors[a]

Species	Code: Original Author	Code: This Report	From Regression of log T on log C (% v/v): Intercept log (a^b)	Slope (b)	Coefficient of Determination	a	$D=a.b$	From Reciprocal Plot of $1/T$ *vs.* C as Interpreted by Original Authors: Slope	Intercept
Data from Literature: end point was turnover of the fish, hence T is average turnover time (min.)									
Goldfish	IF (7)	1A	2.0216	1.829	0.983	12.75	23.32	7.65	−17.25
Goldfish	IF(7)	2A	2.3630	1.933	0.976	16.69	32.26	4.82	−12.68
Goldfish	Table III and Fig. 2 (8)	7A	2.0713	1.944	0.992	11.62	22.59	Not reported	
Goldfish	Table IV and Fig. 3 (8)	8A	1.7304	1.889	0.978	8.654	16.35	Interpreted as three linear segments	
Guppie	IIG (7)	4A	2.3309	1.908	0.985	16.65	31.77	5.74	−13.45
Guppie	IVG (7)	5A	2.1154	1.722	0.987	16.92	29.14	5.87	−14.70
Guppie	VIIG (7)	6A	2.4992	2.256	0.996	12.81	28.90	5.70	−15.07
Data from Literature: end point was death of fish, hence T is average lethal time (min.)									
Goldfish	IF (7)	1B	2.4170	1.346	0.971	62.47	84.08	0.89	− 0.93
Goldfish	IF (7)	2B	2.3160	1.006	0.998	20.05	20.17	0.48	− 0.36
Guppie	IG (7)	3B	2.1829	1.365	0.990	39.74	54.25	1.81	− 1.91
Guppie	IVG (7)	5B	2.1836	1.458	0.956	31.46	45.87	2.26	− 3.50
Guppie	VIIG (7)	6B	2.5838	1.918	0.989	22.24	42.66	2.04	− 4.12
Data from These Studies: end point was turnover of the fish and T is average turnover time (min.)									
Goldfish	—	Fish of Group I	2.1895	2.285	0.993	9.082	20.75		
Goldfish	—	Fish of Group II	2.1679	2.188	0.985	9.790	21.42		
Goldfish	—	Fish of Group III	2.4716	2.716	0.961	9.590	26.05		
Goldfish	—	Fish of Group IV	1.9209	1.856	0.998	10.84	20.12		

[a] As an additional comparison, parameters for the author's equation were also calculated from these average turnover times.

then Eq. 24 becomes

$$T = \left(\frac{a}{Co}\right)^b \qquad \text{(Eq. 25)}$$

which is the same as Eq. 1. Taking logarithms of both sides of Eq. 25 gives

$$\log T = \log (a^b) - b \log C \qquad \text{(Eq. 26)}$$

Hence a plot of log T *versus* log C should yield a straight line with slope equal to $-b$ and intercept equal to log (a^b) or b log a.

From the empirical relationship found, namely $a \cdot b = D$ is essentially a constant for all fish, we obtain

$$a = \frac{D}{b} \qquad \text{(Eq. 27)}$$

Substitution of Eq. 27 into Eq. 25 gives

$$T = \left(\frac{D}{bCo}\right)^b \qquad \text{(Eq. 28)}$$

which is the same as Eq. 4.

Also, substituting Eq. 27 into the expression for a gives

$$D = \frac{bGK}{VmKp} \qquad \text{(Eq. 29)}$$

If it is assumed that the small variability in D from fish to fish is due to small variability in $K/VmKp$, then for D tc remain essentially constant, b and G must be inversely proportional. This inverse relationship between b and G is evident from Eq. 19. When b increases, G must decrease, and conversely, when b decreases, G must increase proportionately when v and T are fixed.

It should be noted that, assuming the theory applies, the tangent to the v *versus* Ci line through the origin is the apparent first-order rate constant Gb/DKp. For an individual fish this tangent is simply G/aKp which is equivalent to Vm/K.

It should also be noted that Eqs. 24 through 29 are valid only for low concentrations when $n \ll n_T$ and $v \ll Vm$. The expectation of the theory is that when the concentration is raised higher (corresponding to v/Vm values greater than about 0.15) the points will be *above* the straight line established for log T *versus* log C at low concentrations. The lethal time data of Powers (2) and the synthetic example discussed later both show this type of deviation.

DISCUSSION

Literature—Powers (2) showed that plots of the reciprocal of survival (lethal) time *versus* concentration of various chemicals were not linear but only apparently linear in a certain concentration range in some cases. These cases coincide with b values near unity. Using Powers' survival times of goldfish in ethanol solutions in the concentration range 1.6 to 6.25% v/v we obtained the least-squares regression line:

$$\log T = 2.1738 - 1.069\ C \qquad \text{(Eq. 30)}$$

and hence

$$T = \left(\frac{149.1}{C}\right)^{1.069} \qquad \text{(Eq. 31)}$$

This b value of 1.069 agrees quite well with the b value of 1.006 determined from one set of data of Hall and Hayton but does not agree with the b value of 1.346 obtained with the other set of data from Hall and Hayton (see Table VII under *Lethal Time*).

When the end point is turnover (overturn) time the b values are much higher and averaged 2.28 in these studies and near this value when average turnover times were used from the literature (see Tables VI and VII). Hence the reciprocal plot ($1/T$ *versus* C) is not the appropriate function with ethanol–water solutions when turnover is the end point. This explains the plot of Gibaldi and Nightingale (8) shown as their Fig. 3.

Ostwald (1) studied survival of fish in various salt solutions and claimed his data were fit by the equation:

$$tc^m = K_1 \qquad \text{(Eq. 32)}$$

If we let $t = T$, $m = b$ and $K_1 = a^b$, then Ostwald's equation becomes identical to Eq. 1. Powers (2) was critical of Ostwald's equation

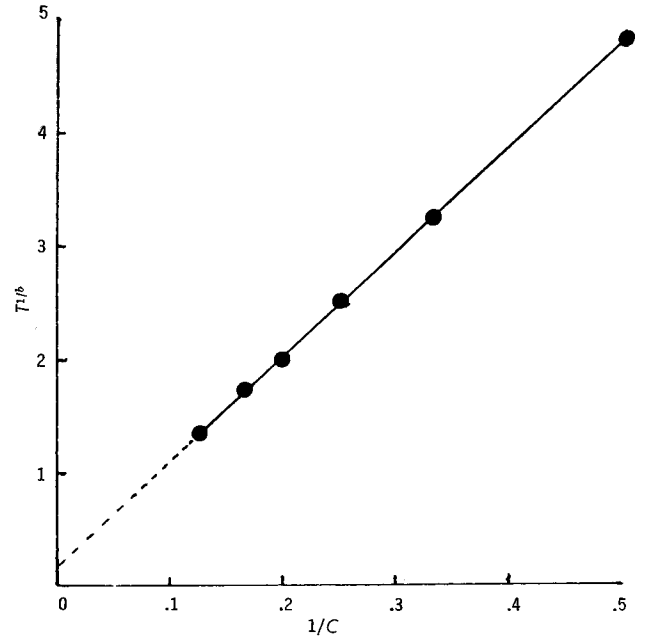

Figure 6—*Plot of* $T^{1/b}$ versus *1/C based on the complete equation:* $T^{1/b} = G/Vm + GK/VmKpC$ *using the synthetic data. The method of least squares gave the line* $T^{1/b} = 0.187 + 9.31/C$. *The intercept agrees with the known value* $G/Vm = 9.34/50 = 0.187$ *and the slope agrees with the known value* $GK/VmKp = (9.34 \times 50)/50 = 9.34$.

since he said Ostwald disregarded the extremes of his data, particularly survival times at high salt concentrations. The theoretical section of this report indicates why such deviations from the log-log will occur.

The previously published theory of Levy *et al.* (4–6) assumes that the rate-limiting step in such goldfish experiments is absorption of the ethanol or other drug being studied. They assume that the slope of the $1/T$ *versus* C plot (when the line goes through the origin) is a function of an absorption rate constant. The theory of this report assumes that ethanol partitions across the absorbing membrane(s) of the fish extremely rapidly, and that the concentration in the biophase, Ci, is related to the concentration in the bath fluid, Co, simply by the expression $Ci/Co = Kp$ where Kp is the partition coefficient. Hence the bile salt potentiation reported by Gibaldi and Nightingale (9) may be explained by a change in Kp produced by the bile salt. The theory of this report indicates that the rate-limiting step is the ethanol-receptor reaction, and that both the overall reaction rate, v, and its differential, dv, *or* the number of receptors occupied, n, and its differential, dn, are involved and related to turnover time as in Eqs. 12 and 13.

Synthetic Data—To illustrate the general applicability of Eq. 21 and the range of applicability of Eqs. 25 and 26, a set of synthetic data was generated. These data were generated by assigning values of 50,50, 9.34, 1, and 2.276 to Vm, K, G, Kp, and b, respectively. The assignments made for the first four constants should not be construed as bearing any relation to the actual values in the goldfish-ethanol case since the actual values are unknown. Substitution of these values into Eqs. 10 and 18 gave Eqs. 33 and 34 from which the synthetic data were generated.

$$v = \frac{50C}{50 + C} \qquad \text{(Eq. 33)}$$

$$T = \left(\frac{9.34}{v}\right)^{2.276} \qquad \text{(Eq. 34)}$$

The plot of log T *versus* log C for these synthetic data is shown in Fig. 5. The method of least squares applied to the solid circles (corresponding to alcohol concentrations of 2, 3, 4, 5, 6, and 8% v/v as in the goldfish studies) gave the equation:

$$\log T = 2.1846 - 2.100 \log C \qquad \text{(Eq. 35)}$$

The estimated values of the parameters b, a, and D were 2.100, 10.97, and 23.04 corresponding to the actual values of 2.276, 9.34, and 21.26, respectively. The dotted line in Fig. 5 is an extrapolation of this least square line. It may be seen that the points progressively deviate from the line but are always above the extrapolated line. Insert in Fig. 5 is the plot of v *versus* C. The linear portion of the log T *versus* log C plot (2–8% v/v) corresponds to the second through eighth points of the insert figure. When the v *versus* C plot becomes markedly curved ($v/Vm > 0.15$) the points on the log-log plot deviate from the line established at lower concentrations.

Figure 6 is a plot of $T^{1/2.276}$ *versus* $1/C$ for the synthetic data in conformity with Eq. 21. The plot is linear over the entire concentration range. The method of least squares gave the equation:

$$T^{1/2.276} = 0.187 + \frac{9.31}{C} \qquad \text{(Eq. 36)}$$

The intercept agrees with the known value of $P/Vm = 9.34/50 = 0.187$ and the slope agrees with the known value of $GK/Vm = (9.34 \times 50)/50 = 9.34$.

Other Applications—It seems reasonable to expect that the theory and equations discussed in this report may be applied to other drugs and chemicals as well as ethanol. For acidic and basic drugs one most probably would have to make appropriate changes involving the pKa and the pH.

The theory may also explain the data of Morozowich *et al.* (13). These authors reported that the logarithm of the LT_{50} (lethal time, 50% or time for 50% of the animals to die) of mice, administered lethal doses of benzphetamine and etryptamine orally by stomach tube in the form of various salts, was linearly related to the logarithm of the equivalent rate of dissolution of the salts measured *in vitro* at pH 7.2 and 37°. Their trend line, based on all points, had a slope of −0.5. However, inspection of their plot suggested that the points corresponding to the benzphetamine hydrochloride and etryptamine acetate were markedly influencing the slope of the line. These compounds had the highest rates of dissolution of all the salts. Availability of the active free bases to the receptors in the mice from these two salts may not have been rate limited by rate of dissolution. Theory discussed in this report also suggests deviations at high concentrations and hence also at high rates of dissolution. The data of Morozowich *et al.* (13) were re-evaluated by omitting the points for these two salts. The remaining seven salts of benzphetamine gave the least-squares regression line:

$$\log LT_{50} = 1.6651 - 0.6054 \log R \qquad \text{(Eq. 37)}$$

where R represents the equivalent rate of dissolution measured *in vitro*. The remaining five etryptamine compounds gave the regres-

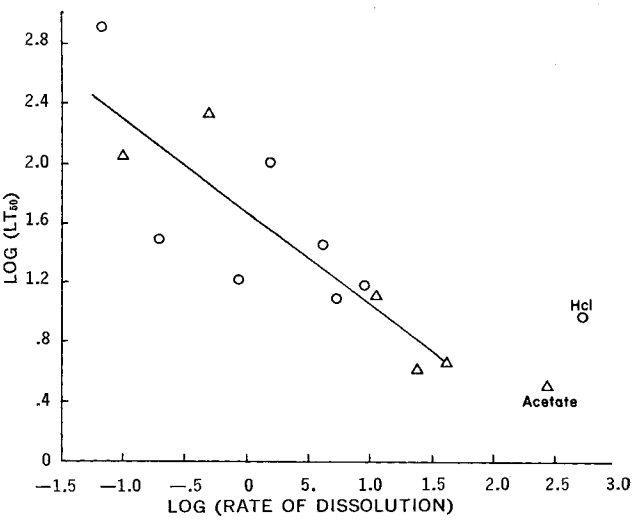

Figure 7—*Plot of the logarithm of the* LT_{50} *(min.) in mice against the logarithm of the equivalent rate of dissolution (mg. free base/cm.²/hr.) determined in an* in vitro *test for various salts of benzphetamine and etryptamine. Key:* ○, *benzphetamine salts;* △, *etryptamine free base and four of its salts. Data from Morozowich* et al. *(13).*

sion line:

$$\log LT_{50} = 1.6987 - 0.6493 \log R \qquad \text{(Eq. 38)}$$

A Student's t test indicated the two slopes were not significantly different ($t = 0.14$, $p > 0.25$), hence the data for all twelve compounds were pooled and these yielded the regression line:

$$\log LT_{50} = 1.6755 - 0.6276 \log R \qquad \text{(Eq. 39)}$$

The correlation coefficient was -0.848 ($p < 0.001$). The plot of these data with the line corresponding to Eq. 39 drawn through the points is shown in Fig. 7. If one assumes that R is a reflection of Ci the relevance these data have to the theory discussed and to the goldfish problem is evident.

The relationship between the theoretical equations derived in this report and the equation relating intensity of pharmacologic response to drug concentration reported by Wagner (15) in the first paper of this series will be discussed in a future publication.

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Effects of Some Enzymes, Surface-Active Agents, and Calcium Chloride on the Aqueous Extraction of Alkaloids from Belladonna Leaves

JOSÉ HELMAN

Abstract □ Treatment of belladonna leaves with the enzymes described, prior to extraction with surface-active agents and calcium chloride, results in higher yields. This was observed in simple aqueous extraction and also when hydrochloric acid was added.

Keyphrases □ Alkaloid extraction—belladonna leaves □ Surfactant effect—alkaloid extraction □ Enzymes effect—alkaloid extraction □ Calcium chloride effect—alkaloid extraction

Previous studies concerning the effect of various surface-active agents on the extraction of alkaloids have shown that the yield in aqueous medium varies according to the agent used. In general the yield decreases with anionic agents, is slightly increased with nonionic, and much more so with cationic agents. Nonionic agents such as polyoxyethylene sorbitan monolaurate and mono-oleate, sorbitan laurate ester, polyethylene glycols 400 and 600, propylene glycol, and glycerol esters (1–3) have been assayed in the extraction from hyoscyamus, belladonna, ipecac, cinchona, hydrastis, *etc.*

Experiments performed by Cadórniga *et al.* (4, 5) with anionic agents proved that at low concentrations the yield decreases, but increases at high concentrations. Results considerably above controls were obtained with cationic agents, especially with quaternary ammonium compounds (6, 7).

Gupta and Sen Gupta treated powdered kurchi (*Holarrhena antidysenterica*), belladonna, nux vomica, and ipecac with diastase prior to extraction (8). White *et al.* (9), in a study directed to obtain proteins and other kinds of cellular material from leaves, subjected these to the action of *Clostridium roseum* cultures, exposing them to an anaerobic fermentation.

The purpose of this work is to determine the yield of alkaloid extraction from belladonna, using aqueous media and with the aid of enzymes, surface-active agents, and calcium chloride.

EXPERIMENTAL

Materials—Powdered belladonna leaves (*Atropa belladonna*), 40 mesh (0.19 mm. sieve opening), dried at 60° were used.