Effects of Alkyl Chain Length on Biological Activity: Alkyl *p*-Aminobenzoate-Induced Narcosis in Goldfish

S. H. YALKOWSKY [x], T. G. SLUNICK, and G. L. FLYNN

Abstract □ On the basis of a diffusional model and certain well-known physical-chemical relationships, equations were derived which describe the relative biological activities of alkyl homologs. These equations were applied to the goldfish turnover time produced by both saturated solutions and equimolar solutions of eight *n*-alkyl esters of *p*-aminobenzoic acid. The results show that the apparant parabolic structure–activity curve can be described quite well by a simple solubility-limited diffusional model.

Keyphrases □ *p*-Aminobenzoic acid ester-induced narcosis in goldfish—chain length effect, solubility-limited diffusional model, equations □ Chain length effect—alkyl *p*-aminobenzoate-induced narcosis in goldfish, solubility-limited diffusional model, equations □ Goldfish turnover—chain-length effect of alkyl *p*-aminobenzoates, diffusion model, equations

The role of the lipid–water partition coefficients of a series of compounds in determining their relative biological activities has long been recognized (1–5). It is also well known that linear relationships between the logarithms of the partition coefficient and biological activity break down at high partition coefficients (6). In certain instances (7–9), this is due to the fact that the solubilities of highly lipoidal substances are not sufficient to elicit the desired responses. However, there are many instances of deviations from linearity occurring at concentrations that are well below saturation. One of the first theoretical models capable of explaining these deviations was presented by Zwolinski *et al.* (10), who pointed out that diffusion layers can play an important role in the penetration of biological barriers. These diffusion layers have been repeatedly demonstrated on *in vitro* systems (11–13) and recently were shown to be present *in situ* (14) and *in vivo* (15, 16).

Equations accounting for diffusion layers have been built upon (13, 17) to describe the transport properties of *n*-alkyl *p*-aminobenzoates across silicone rubber membranes. It was shown that certain chain length–activity relationships that were predicted and observed in synthetic membrane systems are also observed in biological systems. Several specific examples of the various expected relationships were described in the literature, but no single example of all of the predicted dependencies could be found.

In this report, an attempt is made to verify all of the authors' earlier predictions on a single biological system. As in previous studies, the approach here involves the incremental changing of physical–chemical properties of the permeating species. This approach, which is implemented by restricting the study to the members of a homologous series, greatly simplifies the analysis and interpretation of the data. A literature analysis (13) strongly suggested that the relative degrees of narcosis produced by alkyl *p*-aminobenzoates in goldfish were in accord with the predictions of a diffusion layer membrane model. Thus, this system was chosen as a model system to demonstrate the hypotheses. Some additional practical reasons for choosing the goldfish system are the relative simplicity of experimental design, the low cost of the fish, the clearly defined end-point, and the ease of controlling experimental variables.

THEORETICAL

In the field of structure–activity relationships, it is generally true that the complete description of a model consists of: *(a)* the statement of the underlying assumptions (in mathematical form, if possible), and *(b)* the arithmetic manipulation of the postulated equations to give new equations which can be used to explain and hopefully to predict biological results. The model advanced in this report, to explain and to predict the relative narcotic activity of alkyl homologs in goldfish, is based on the following five postulates:

1. The differences in the observed biological activities of each member of the series, α_n, is directly related to that compound's ability to permeate biological barriers and to reach the receptor site, *i.e.*:

$$\alpha_n = \gamma F_n \qquad \text{(Eq. 1)}$$

where γ is a constant relating the rate of transport of the homolog to the site or flux, F_n, to its activity. The constancy of γ precludes the situation where there are differences in the metabolism or binding of the homologs. In other words, since the intrinsic activity, γ, is a constant, the observed differences in activity are due solely to differences in flux. Although this supposition is somewhat speculative, it is intuitively reasonable and is generally accepted. The four remaining criteria have been demonstrated numerous times by the authors and others in both *in vivo* and *in vitro* studies.

2. The flux, F, of a substance across a region consisting of aqueous and lipid barriers is given by (12):

$$\log F = \log C - \log (R_{aq} + R_m/PC) \qquad \text{(Eq. 2)}$$

where C is the concentration differential across the region; R_{aq} and R_m are the sums of the resistances of the aqueous and lipid regions, respectively; and PC is the lipid–water partition coefficient of the substance. All lipid regions involved are regarded as identical in polarity, and all aqueous regions are assumed to be of the same polarity. Factors such as thickness, porosity, tortuosity, diffusivity, and cross-sectional area are included in the overall resistance and need not be considered separately.

3. The maximum flux of a particular series of barriers is obtained when the donor region is saturated and the receptor region is at a negligible concentration, *i.e.*, when the concentration differential, C, is replaced by the solubility, S. Thus:

$$\log F^{max} = \log S - \log (R_{aq} + R_m/PC) \qquad \text{(Eq. 3)}$$

4. The partition coefficients of the members of a homologous

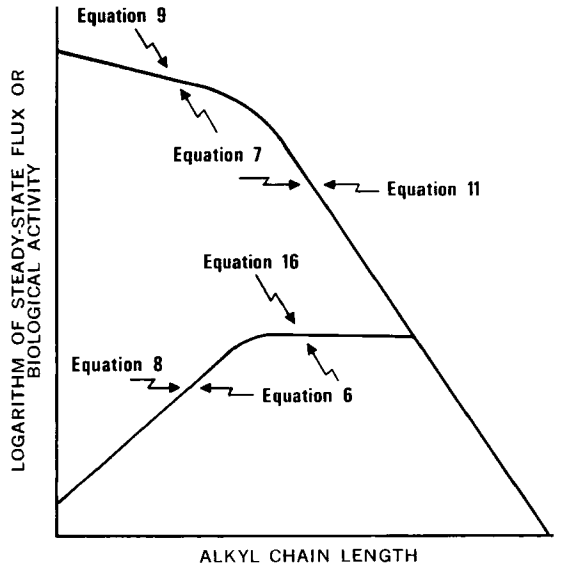

Figure 1—*Hypothetical chain length–activity relationships.*

series are described by:

$$\log PC_n = \log PC_0 + \pi n \qquad \text{(Eq. 4)}$$

where PC_n is the lipid–water partition coefficient of the homolog having chain length n, PC_0 is the extrapolated partition coefficient of the reference compound of the series, and π is a constant dependent on the polarity of the lipid.

5. The aqueous solubility of the homologs can be represented by:

$$\log S_n = \log S_0 - \delta n \qquad \text{(Eq. 5)}$$

where S_n and S_0 are the solubilities of the nth member of the series and the reference compound, respectively, and δ is a constant which is independent of the biological system.

On the basis of these equations, it is possible to derive equations that describe the observed biological activity in terms of alkyl chain length. Substituting Eqs. 4 and 5 into Eqs. 2 and 3 and the resulting equations into Eq. 1 gives[1]:

$$\log \alpha^c = \log \gamma + \log C + \log PC_0 + \pi n - \log (R_m + R_{aq}PC_0 10^{\pi n}) \qquad \text{(Eq. 6)}$$

and:

$$\log \alpha^s = \log \gamma + \log S_0 + \log PC_0 + (\pi - \delta)n - \log (R_m + R_{aq}PC_0 10^{\pi n}) \qquad \text{(Eq. 7)}$$

which describe the relative activities of the members of the series studied at a particular concentration, α^c, or the relative activities of saturated solutions of the homologs, α^s. These equations are depicted in Fig. 1 for arbitrary values of the constants. It can be seen that both curves undergo a change in slope at the same value of n. This change is due to the fact that when n is small, R_m can dominate the last term of Eqs. 6 and 7 so that they are approximated by:

$$\log \alpha^c = \log \gamma + \log C + \log PC_0 + \pi n - \log R_m \qquad \text{(Eq. 8)}$$

and:

$$\log \alpha^s = \log \gamma + \log S_0 + PC_0 + (\pi - \delta)n - \log R_m \qquad \text{(Eq. 9)}$$

But when n is large, $-\log (R_m + R_{aq}PC_0 10^{\pi n})$ approaches $-\log R_{aq} - \log PC_0 - \pi n$, and Eqs. 6 and 7 become, respectively:

$$\log \alpha^c = \log \gamma + \log C - \log R_{aq} \qquad \text{(Eq. 10)}$$

and:

$$\log \alpha^s = \log \gamma + \log S_0 - \delta n - \log R_{aq} \qquad \text{(Eq. 11)}$$

[1] A more complete derivation of Eqs. 6–11 can be found in Ref. 17.

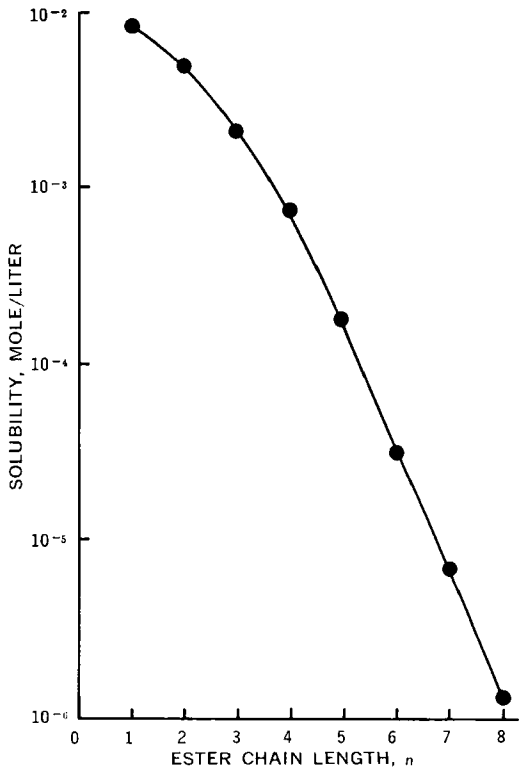

Figure 2—*Solubilities of* n*-alkyl* p*-aminobenzoates in water at 24 ± 1°.*

Equations 8 and 9, in which the only resistance appearing is the lipid or membrane resistance, describe the conditions most commonly encountered. This condition is referred to as membrane or lipid control of activity. The last two equations contain only the aqueous resistance and describe the condition of diffusion layer or aqueous control of activity. The meanings of these equations[2] in terms of chain length–activity relationships are evident from Fig. 1.

Basically, only four distinct conditions are described by the equations. Properties dependent upon flux from solutions of uniform concentration are characterized by Eqs. 6, 8, and 10. Equation 8 applies only to the situation in which there is membrane control of flux, while Eq. 10 applies when the diffusion layers offer the major resistance to transport. The special case in which the applied solutions are saturated is described by Eq. 7. Equations 9 and 11 delineate the subcases of membrane and diffusion layer control, respectively.

Linearity of the logarithms of both PC_n and S_n with chain length is not vital to this treatment. Although this simple case was used to facilitate deriving the necessary equations, any single-valued function that expresses log S_n or log PC_n as a function of n could have been used. In fact, as pointed out later, solubility is not a simple exponential function of chain length for the alkyl *p*-aminobenzoates.

EXPERIMENTAL

Materials—The alkyl *p*-aminobenzoates used in this study were either purchased or synthesized from *p*-nitrobenzoyl chloride and the appropiate alcohol[3]. Deionized water was used to prepare all solutions.

Goldfish—Common variety goldfish *(Carassius auratus)* weighing between 2 and 4 g were purchased locally.

[2] They and their implications are described extensively in Refs. 13 and 17.

[3] The sources were: *p*-nitrobenzoyl chloride, Matheson, Coleman and Bell; methyl *p*-aminobenzoate, Eastman; ethyl *p*-aminobenzoate, Aldrich; *n*-propanol, Eastman; *n*-butanol, source unknown; *n*-pentanol, Aldrich; *n*-hexanol, Aldrich; *n*-heptanol, Aldrich; *n*-octanol, Eastman; *n*-nonanol, Aldrich; *n*-dodecanol, Aldrich; *n*-hexadecanol, Eastman; and *p*-aminobenzoic acid, Eastman.

Table I—Average Turnover Times Produced by Various Concentrations of Alkyl *p*-Aminobenzoates

Ester	Fractional Saturation	Concentration, mole/liter	Number of Fish	Mean Turnover Time, min	Standard Error of Mean	Mean Reciprocal Turnover Time
Methyl	Saturated	8.3×10^{-3}	5	0.320	0.051	3.15
	2/3	5.52×10^{-3}	10	0.410	0.030	2.44
	1/2	4.15×10^{-3}	10	0.640	0.098	1.52
	1/3	2.76×10^{-3}	10	0.933	0.088	1.07
	1/4	2.07×10^{-3}	10	1.230	0.076	0.813
	1/6	1.40×10^{-3}	10	2.270	0.183	0.44
	1/10	8.3×10^{-4}	10	4.390	0.189	0.23
	1/20	4.15×10^{-4}	10	45.0	15.7	0.022
	1/40	2.07×10^{-4}	10	—[a]	—	—
	1/100	8.3×10^{-5}	10	—[a]	—	—
Ethyl	Saturated	—	5	0.340	0.062	2.95
	2/3	4.83×10^{-3}	10	0.270	0.015	3.70
	1/2	2.37×10^{-3}	10	0.460	0.040	2.17
	1/3	1.58×10^{-3}	10	0.550	0.045	1.82
	1/4	1.18×10^{-3}	10	0.990	0.095	1.01
	1/6	7.8×10^{-4}	10	1.210	0.159	0.83
	1/10	4.83×10^{-4}	10	2.800	0.226	0.36
	1/20	2.37×10^{-4}	10	6.50	0.64	0.15
	1/40	1.18×10^{-4}	10	47	9.3	0.021
	1/100	4.83×10^{-5}	10	—[a]	—	—
Propyl	Saturated	2.03×10^{-3}	6	0.333	0.042	3.0
	2/3	1.3×10^{-3}	10	0.450	0.037	2.22
	1/2	9.8×10^{-4}	10	0.510	0.038	1.96
	1/3	6.5×10^{-4}	10	0.680	0.039	1.47
	1/4	4.9×10^{-4}	10	0.900	0.097	1.11
	1/6	3.3×10^{-4}	10	1.150	0.097	0.87
	1/10	2.03×10^{-4}	10	3.150	0.442	0.32
	1/20	9.8×10^{-5}	10	8.250	0.720	0.12
	1/40	4.9×10^{-5}	10	30.50	3.06	0.03
	1/100	2.03×10^{-5}	10	—[a]	—	—
Butyl	Saturated	7.35×10^{-4}	5	0.340	0.025	2.94
	2/3	4.7×10^{-4}	10	0.640	0.072	1.56
	1/2	3.6×10^{-4}	10	0.730	0.070	1.37
	1/3	2.4×10^{-4}	10	1.110	0.094	0.90
	1/4	1.8×10^{-4}	10	1.510	0.157	0.66
	1/6	1.2×10^{-4}	10	2.600	0.256	0.39
	1/10	7.35×10^{-5}	10	4.650	0.289	0.22
	1/20	3.6×10^{-5}	10	9.950	0.664	0.10
	1/40	1.8×10^{-5}	10	70.60	18.44	0.014
	1/100	7.35×10^{-6}	10	—[a]	—	—
Pentyl	Saturated	1.79×10^{-4}	10	1.370	0.104	0.73
	3/4	1.34×10^{-4}	10	1.830	0.146	0.55
	2/3	1.29×10^{-4}	10	2.380	0.133	0.42
	1/2	9.0×10^{-5}	10	3.310	0.293	0.30
	1/3	5.95×10^{-5}	10	4.500	0.632	0.22
	1/4	4.5×10^{-5}	10	6.380	0.495	0.16
	1/6	2.98×10^{-5}	10	8.850	0.311	0.11
	1/10	1.79×10^{-5}	10	15.50	1.06	0.065
	1/20	9.0×10^{-5}	10	43.33	3.67	0.023
	1/40	4.5×10^{-5}	6	63.60	5.05	0.016
	1/100	1.79×11^{-6}	5	—[a]	—	—
Hexyl	Saturated	3.1×10^{-5}	10	6.70	0.26	0.15
	3/4	2.4×10^{-5}	10	9.20	0.41	0.11
	2/3	2.0×10^{-5}	10	13.00	0.45	0.078
	1/2	1.5×10^{-5}	10	14.30	1.68	0.070
	1/3	1.0×10^{-5}	10	25.00	2.17	0.040
	1/4	7.5×10^{-6}	10	31.20	5.88	0.032
	1/6	5.0×10^{-6}	10	—[a]	—	—
Heptyl	Saturated	7.2×10^{-6}	6	30.00	3.72	0.033
	1/2	3.6×10^{-6}				
Octyl	Saturated	—	2	—[a]	—	—

[a] Inactive.

Solubility Determination—The solubilities of the esters studied were determined by equilibrating excess drug with deionized water for at least 48 hr at 24°. The solutions were then filtered, diluted, and read at 285 nm on a spectrophotometer[4]. All esters had molar absorptivities of 20,700.

Turnover Time Measurements—The time required for a goldfish to lose its righting reflex after being placed in a specific solution at 24° was taken as the turnover time. Most values reported are the average of 10 individual experiments. The experimental details were described previously (18).

Extrapolation of Data—As explained previously (18), it is experimentally impossible to measure accurately turnover times of less than about 20 sec. Because the observed turnover times produced by saturated solutions of the lower esters were in this range, it was felt that they might be artifactual. Furthermore, these values were not in agreement with the rest of the concentration–turnover time data for these esters. More meaningful, al-

[4] Zeiss.

Table II—Dose–Response Equations

Ester	Equation
Methyl	$1/t = -0.164 + 4.76 \times 10^{-2} \times C$
Ethyl	$1/t = -0.105 + 1.11 \times 10^{-3} \times C$
Propyl	$1/t = -0.742 + 2.17 \times 10^{-3} \times C$
Butyl	$1/t = -0.040 + 3.76 \times 10^{-3} \times C$
Pentyl	$1/t = -0.003 + 3.78 \times 10^{-3} \times C$
Hexyl	$1/t = -0.0006 + 4.35 \times 10^{-3} \times C$

though hypothetical, indications of the activities of these esters were obtained by extrapolating the dose-response data for the remaining concentrations to saturation.

Extrapolations of the equations:

$$\frac{1}{t} = A\frac{C}{S} + B \qquad \text{(Eq. 12)}$$

and:

$$\log t = A' \log \frac{C}{S} + B' \qquad \text{(Eq. 13)}$$

where A, A', B, and B' are constants, to $C/S = 1$ give similar values for turnover time, t, at saturation. The averages of the extrapolated values determined by each equation for each lower ester are used in the remainder of this report in lieu of experimental values.

RESULTS

Solubility—The solubilities of the esters at 24° are plotted semilogarithmically *versus* chain length in Fig. 2. Above $C = 4$, log S is a linear function of n and there is significant deviation from this linearity for the lower esters. An explanation for this deviation in terms of changes in crystal packing with increasing chain length was already presented (19).

Turnover Time—The reciprocal mean turnover times produced by various concentrations of the esters studied are listed in Table I. An interpretation of these data in terms of absorption kinetics was recently given (18).

The data in Table I can be described satisfactorily by the equations listed in Table II. With the exception of the above-mentioned extrapolated points, all data discussed in this paper were obtained by interpolation of these equations. Figure 3 shows the results of calculations of the minimum concentration of each ester required to produce turnover in the following specified periods: 1, 3, 10, and 30 min. The vertical bars at the end of each line indicate that the next homolog is incapable of producing turnover in the designated period. The fact that there is no single, most active homolog is readily apparent from the figure. The lower the degree of activity measured, the higher is the chain length of the most active member of the series. The role of solubility in producing this relationship was first noted by Ferguson in 1939 (8) but has been largely ignored by many workers currently involved in structure-activity theory.

As stated by Ferguson (7, 8) and by the authors (9, 17), it is generally better to measure the biological end-point produced by a given concentration of drug than to measure the amount of drug required to produce a given end-point. This fact will become apparent in the discussion.

DISCUSSION

Equimolar Studies—To begin testing the model and evaluating the important parameters, one can consider the equimolar data at various concentrations. These data (Fig. 4) were obtained from the equations of Table II.

The value of the constant, π, can be determined from the initial slopes of the lines of either Fig. 3 or 4 or from the data in the last column of Table II. The best value of π appears to be 0.40, which is in good agreement with the value of 0.413 determined from the data of Adams *et al.* (20) for the lower alkyl *p*-aminobenzoates. It is also in agreement with other data for goldfish (21, 22).

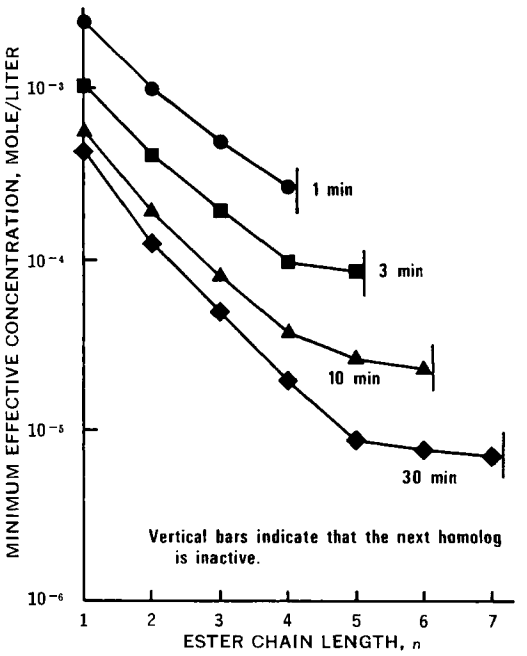

Figure 3—*Minimum concentrations of* n-*alkyl* p-*aminobenzoates required to produce goldfish turnover in specified time periods. Data were interpolated from the equations of Table II.*

According to Eqs. 8 and 10, the intercept at $n = 0$ divided by the concentration can be used to calculate log $[\gamma(PC_0/R_m)]$, and the plateau value divided by the concentration can be used to determine log (γ/R_{aq}).

The curves in Fig. 4 that fit the equimolar data quite well were generated from Eq. 2 with $R_{aq}/\gamma = 2 \times 10^{-3}$, $R_m/\gamma PC_0 = 6 \times 10^{-4}$, and $\pi = 0.40$. The factor γ is not important since it would appear in each term. In all cases, the data (not shown) for the lower concentrations, where the bulk concentration of drug approached the threshold concentration required for activity, fell considerably below the calculated lines.

Saturated Solutions—As mentioned, the logarithms of the solubilities of the homologs are not a linear function of chain length. The solubility data can, however, be adequately described by:

$$\log S_n = -2.042 + 0.342n - 0.837n^2 + 0.000303n^4 \qquad \text{(Eq. 14)}$$

To make Eqs. 7, 9, and 11 applicable to the alkyl *p*-aminobenzoates, log S must be replaced by Eq. 14, which approximates all of the data with a standard error of 9%. The incorporation of the higher order terms presents no difficulties other than the elimination of the expected linearity of log α with n for saturated solutions. The function does, however, approach linearity for $n > 4$.

Although log S is not a linear function of n, it is assumed that log PC increases linearly with alkyl chain length. The nonlinearity of the solubility data is due to alterations in crystal packing with chain length, which were shown to be inapplicable to partitioning (19). The log PC's of the esters of this series were shown to be linear functions of n in four different partitioning systems (19, 23).

To test the model further, Eq. 7 can be solved with Eq. 14 and the determined values of π, $R_m/\gamma PC_0$, and R_{aq}/γ. As shown in Fig. 4, the data for the longer chain lengths and Eq. 7 both have a limiting slope of about −0.6. This slope would be expected most frequently because the value of δ is usually about −0.6 for most homologous series in water.

All data points of Fig. 4 are in excellent agreement with the theoretical lines. These lines are based on only three biological parameters (π, $R_m/\gamma PC_0$, and R_{aq}/γ) and the aqueous concentration or solubility. In changing to another series, the only parameter (other than solubility which can easily be measured) that will change is PC_0. Therefore, if a solvent can be found that truely mimics the partitioning behavior of the diffusion-limiting membrane of the goldfish, it would be possible to characterize

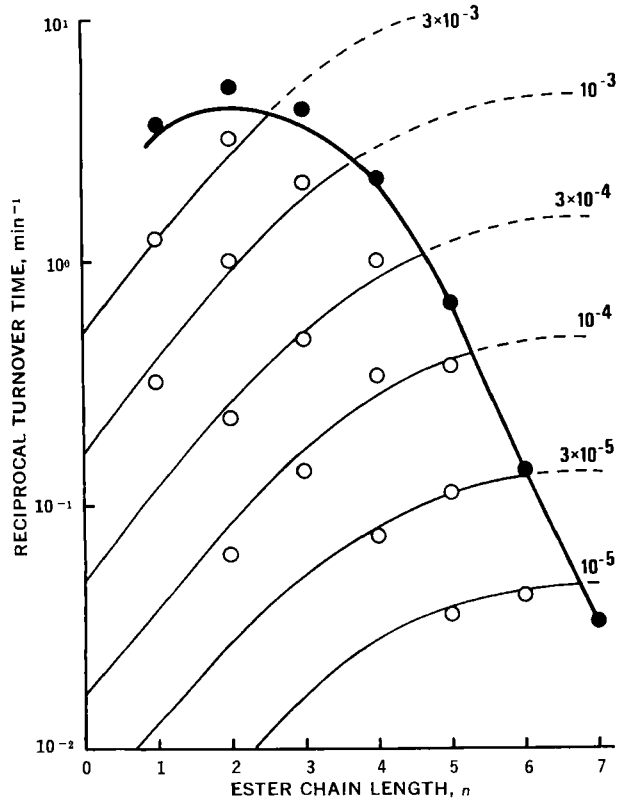

Figure 4—*Turnover times produced by various concentrations and saturated solutions of* n-*alkyl* p-*aminobenzoates. Points are experimental* (○, *unsaturated; and* ●, *saturated*), *and lines are theoretical* (——, *unsaturated; and* ——, *saturated*).

physically all important parameters. Then, since π and γ are constant and R_m and R_{aq} only change slightly, it should be possible to predict completely the relative activities of the members of any group of structurally related, nonspecifically acting compounds.

It is also clear from Fig. 4 that when the compounds are studied on an equimolar basis, rather than on an equitoxic basis as in Fig. 3, there is a definite descending portion of the curve. The term equimolar as used here refers to equivalent amounts of the homolog being present, even if the amounts in solution are not equivalent. The term is used in this manner because frequently a series of drugs is tested by noting the effect of the addition of equal amounts of each member. In many instances, it is not known whether all of the added drug is dissolved or not. The dosing of lower animals with very high milligram per kilogram levels of drug dissolved in an oil or surfactant can easily result in precipitation and diminished activity, which would not occur if the dose were comparable on a milligram per kilogram basis to that given in humans.

The effect of the choice of concentration studied is illustrated by the curves of Fig. 4. The most dilute concentration, 10^{-5} *M*, showed an optimum activity for the hexyl ester. The same compounds tested on the same biological system at 10^{-3} *M* showed maximal activity for the butyl ester while saturated solutions of the ethyl ester were the most active. These curves can be regarded as indicating the expectable error in determining the most active member of a series in humans from rat data when significantly different milligram per kilogram doses are given.

In this particular study, the decrease in activity with increasing chain length results only from the diminished solubility of the higher esters. In no way do the authors intend to suggest that the only reason for decreasing activity with chain length is solubility. Numerous phenomena can be activity limiting (17, 24). Most of these phenomena show chain-length dependencies similar to those described by Eq. 5 or 14 and can be treated in the same manner as solubility has been treated here. In fact, Higuchi and Davis (25) showed that partitioning into a hydrocarbon-like lipid phase also results in an ultimate slope of −0.6 for log α *versus n*.

The equations presented here were derived on the basis of Eq. 2, which describes the transport of a substance across a series of aqueous and lipid barriers. Other models have the same mathematical form as Eq. 2, such as the partitioning model of Wagner and Sedman (26). All of the presented equations could have been derived equally well on the basis of Wagner and Sedman's (26) equations. However, the diffusional model as used here offers a greater degree of simplicity and intuitive satisfaction.

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