Acrylate and methacrylate esters: Relationship of hemolytic activity and *in vivo* toxicity

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Quantitative hemolysis assays of acrylate and methacrylate esters provided estimates of the intrinsic hemolytic activity (H_i, the slope of the concentration–response curve) and the concentrations effecting 5% (H_5) and 50% (H_{50}) hemolysis. The dependence of hemolytic activity and LD_{50} (mice) on physical properties (lipophilicity, molar refraction, and molecular volume) of the esters was determined by multiple regression analysis. The observed correlations were: H_i, $R^2 = 0.94$; H_5, $R^2 = 0.95$; H_{50}, $R^2 = 0.94$; and LD_{50}, R^2 (all compounds) = 0.80, R^2 (all compounds less the methyl esters) = 0.94. The difference of the methyl esters was associated with the smaller steric volume of the methyl ester substituent and the presence (methacrylates) or absence (acrylates) of the branched methyl group. Associative steric contributions of the branched methyl group and the ester substituents were probably responsible for greater variability in the methyacrylate series. The results were consistent with the conclusion that the mechanism of the action of the esters is membrane mediated and relatively nonspecific and that *in vivo* biotransformation was not a significant factor. Also, long-term toxic liability of the esters may be more closely related to intrinsic toxicity than acute toxicity.

INTRODUCTION

Acrylate and methacrylate esters are used widely in the formulation of polymeric materials for medical, dental, orthopedic, and industrial applications. The volatility of the esters make them a potential hazard in the work environment, and residual unpolymerized esters or esters released on degradation of acrylic or methacrylic polymers pose a toxic liability in the biological application of such materials. Although direct contact or inhalation poses the most serious hazard, more subtile questions of biological compatibility and potential toxic liability are present in *in situ* polymerization procedures contacting soft and hard tissues. In those procedures, tissues are exposed to significant concentrations of free monomer and the toxic liability of the esters is a primary concern.

Diechmann[1] and Spealman et al.[2] tested a series of methacrylate esters in small animals and found the methyl ester to be the most toxic, followed by the ethyl and *n*-butyl esters. Pozzani, Weil, and Carpenter[3] and Treon et al.[4] reported that acute toxicity was correlated with water solubility; *in vivo* meth-

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ylacrylate was more toxic than ethylacrylate and both were more toxic than the corresponding methacrylate esters. Those conclusions were confirmed by Lawrence et al.[5] and Bass et al.[6] who examined the structure activity relationship of 18 acrylic and methacrylic compounds. Singh, Lawrence, and Autian[7] reported presumptive evidence of teratogenicity of acrylates and methacrylates. The observed activity appeared to be directly correlated with water solubility. Mir, Lawrence, and Autian,[8,9] using isolated organ parameters, found no direct correlation of toxicity with water solubility except for the rate of contraction of rabbit heart.

Since *in vivo* biological activity depends simultaneously upon secondary time-dependent mechanisms (e.g., transport, biotransformation, and excretion) and intrinsic toxicity (the time-independent toxicant- receptor interaction), both of which are modulated by the physicochemical properties of the toxicant, the object of this investigation was to examine the relationship among those parameters directed toward rationalization of *in vitro* intrinsic toxicity with *in vivo* toxicity of acrylate and methacrylate esters.

HEMOLYSIS ASSAY SYSTEM

Evaluation of intrinsic biological activity requires an equilibrium system such as that described by Higuchi and Davis.[10] Dillingham et al.[11] demonstrated that a monolayer tissue culture assay meets the constraints described, and a high degree of correlation ($r = 0.98$) between tissue culture and hemolysis assays was reported for methyl- and halogen-substituted alcohols. The hemolysis assay system was selected over the tissue culture system based on the following considerations: (1) the opportunity for secondary hydrophobic partitioning to added serum is 60% lower, (2) oxidative metabolism in erythrocytes is low, (3) metabolic activity associated with growth and reproduction is absent, and (4) the assay time is short, 1 h as opposed to 72 h for the tissue culture system. The hemolysis assay system, therefore, more closely approximated an equilibrium system relatively unaffected by secondary time-dependent processes.

EXPERIMENTAL

Materials

The acrylate and methacrylate esters (Table I) were used as received: compounds 1, 2, 7, 8, and 11;* compounds 5 and 6;† and compounds 3, 4, 9, and 10.‡ The monoethylether of hydroquinone was present in concentrations of 5–15 ppm. Gas chromatography revealed no other significant quantities of organic compound. Additions in the hemolysis assay system of hydroquinone or the monoethylether of hydroquinone at concentrations two times higher did not change the apparent estimates of intrinsic hemolytic activity.

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Hemolysis assay

Fresh oxalated rabbit blood (2% potassium oxalate in saline; 1 mL/20 mL of rabbit blood) was diluted with normal saline such that 0.2 mL of diluted blood hemolyzed in 10 mL of distilled water gave a spectrophotometric reading at 545 nm of 0.9–1.0 absorbance (positive control, 100% lysis).

Arbitrary concentrations near saturation of the esters in saline were employed as stock solutions. Preliminary range finding assays were carried out to establish the hemolysis concentration-response range and ten-step dilution series were prepared yielding hemolysis values between 2 and 95%. The assays were carried out in test tubes with 10 mL in each tube and threefold replication at each concentration, including a negative control, normal saline. The tubes containing toxicant and control solutions were equilibrated for 30 min at 37°C, and 0.20 mL of diluted rabbit blood was added to each tube with gentle mixing. After 60 min incubation following the addition of blood, all tubes were centrifuged for 5 min (500 × g), the optical density (OD) of each supernatant was determined at 545 nm, and replicate values averaged. The percent hemolysis was calculated as follows:

$$\%H = \frac{\text{OD test solution} - \text{OD negative control}}{\text{OD positive control} - \text{OD negative control}} \times 100$$

Concentration-response curves were prepared and the biological response data—H_5, H_{50}, and H_i (Table I)—were determined from smooth curves showing best fit with the mean values (visual estimation) (Fig. 1). The H_5 and H_{50} values were taken from the smooth curves. The slope of the concentration-response curve, H_i, was taken as the tangent to the curve at inflection point of the curve, the curves being relatively linear in that region, between 40 and 60% hemolysis.

TABLE I
Biological Response Parameters

Compound	H_5 (mole/L)[a]	H_{50} (mole/L)[b]	H_i (%/mole)[c]	LD_{50} (mole/10^6 g)[d]
1. Methyl acrylate	2.12×10^{-1}	2.33×10^{-1}	2.32×10^{3}	2.95
2. Ethyl acrylate	1.04×10^{-1}	1.11×10^{-1}	6.69×10^{3}	5.98
3. *n*-Propyl acrylate	1.86×10^{-2}	2.59×10^{-2}	2.33×10^{4}	5.80
4. *i*-Propyl acrylate	1.38×10^{-2}	1.83×10^{-2}	2.79×10^{4}	6.42
5. *n*-Butyl acrylate	1.48×10^{-3}	2.45×10^{-3}	1.20×10^{5}	6.64
6. *i*-Butyl acrylate	9.65×10^{-4}	1.44×10^{-3}	3.46×10^{5}	5.92
7. Methyl methacrylate	7.15×10^{-2}	8.84×10^{-2}	8.87×10^{3}	11.29
8. Ethyl methacrylate	2.58×10^{-2}	3.60×10^{-2}	1.82×10^{4}	10.88
9. *n*-Propyl methacrylate	4.52×10^{-3}	6.83×10^{-3}	9.11×10^{4}	7.89
10. *i*-Propyl methacrylate	2.47×10^{-3}	3.16×10^{-3}	2.28×10^{5}	11.63
11. *n*-Butyl methacrylate	2.77×10^{-4}	3.83×10^{-4}	8.76×10^{5}	10.47

[a] H_5 = concentration effecting 5% hemolysis.
[b] H_{50} = concentration effecting 50% hemolysis.
[c] H_i = slope of the dose-response curve between 40 and 60% hemolysis.
[d] Male ICR mice.

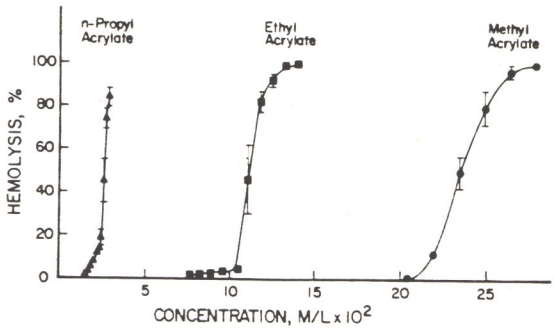


Figure 1. Hemolytic activity: typical concentration-response curves.

In vivo toxicity

The 7-day LD_{50} values (male ICR mice) used in this study were obtained from Lawrence et al.[5] and Bass et al.[6] (Table I).

Physical parameters

Physical parameters selected for correlation analysis with biological response included lipophilicity, molar refraction, and molecular volume (Table II). The log *P* values and the fragment constant values for calculating the lipophilicity of the R_1 and R_2 substituents of the acrylate and methacrylate esters were provided by Dr. Albert Leo, Pomona College, Claremont, California. Molar refraction and molecular volume values were obtained from the literature by Hansch and Leo[12] and by Bondi,[13] respectively.

Correlation analysis

Linear regression of biological parameters was carried out on all possible combinations of 1–5 physical parameters. The statistical program BMD P9R (ref. 14) was employed. The regression equation in each subset comprised of 1–4 independent variables (physical parameters) having the highest multiple regression coefficient (R) was determined for the dependent variables H_i, H_5, H_{50}, and LD_{50}. The addition of a fifth independent variable either did not improve or decreased R.

The correlation among physical parameters was examined by univariate correlation analysis.

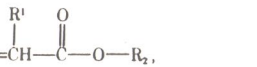
RESULTS AND DISCUSSION

Biological response parameters

The biological response data are presented in Table I. Typical concentration-response curves are shown in Figure 1. The hemolytic activity of the acrylate and methacrylate esters increased with respect to the chain length

TABLE II
Physical Parameters

Compound[a]	P	Log P	πR_1	πR_2	MR	MR(R_1)	MR(R_2)	V_ω	$V_\omega R_1$	$V_\omega R_2$
1. Methyl acrylate	4.217	0.625	0	0.89	21.85	0	5.65	49.02	3.44	13.67
2. Ethyl acrylate	14.622	1.165	0	1.55	26.03	0	10.30	59.25	3.44	23.90
3. *n*-Propyl acrylate	50.699	1.705	0	1.97	26.50	0	14.96	69.47	3.44	34.12
4. *i*-Propyl acrylate	30.549	1.485	0	1.97	26.50	0	14.96	69.47	3.44	34.12
5. *n*-Butyl acrylate	175.792	2.245	0	2.51	31.15	0	19.61	79.70	3.44	44.35
6. *i*-Butyl acrylate	272.270	2.435	0	2.51	31.15	0	19.61	79.70	3.44	44.35
7. Methyl methacrylate	8.810	0.945	0.89	0.89	27.50	5.65	5.65	59.25	13.67	13.67
8. Ethyl methacrylate	30.549	1.485	0.89	1.55	31.68	5.65	10.30	69.48	13.67	23.90
9. *n*-Propyl methacrylate	105.925	2.025	0.89	1.97	32.15	5.65	14.96	79.70	13.67	34.12
10. *i*-Propyl methacrylate	63.826	1.805	0.89	1.97	32.15	5.65	14.96	79.70	13.67	34.12
11. *n*-Butyl methacrylate	367.282	2.565	0.89	2.51	36.80	5.65	19.61	89.94	13.67	44.36

[a] General structure: 
where R_1 = hydrogen or methyl and R_2 = methyl, ethyl, propyl, isopropyl, butyl, or isobutyl.

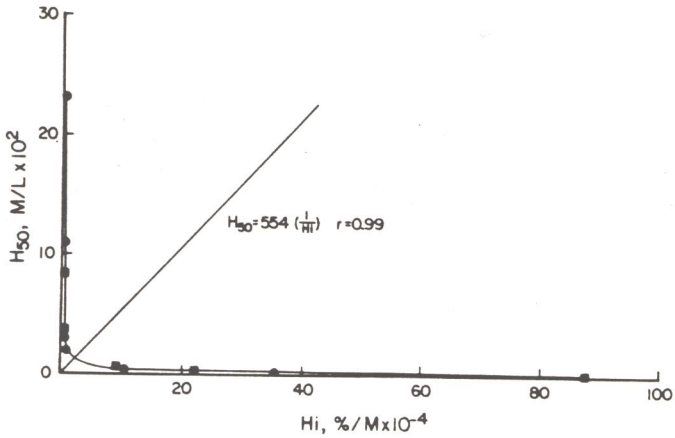


Figure 2. Correlation of H_{50} and H_i. ●, Acrylates; ■, methacrylates.

of the ester substituent, with the methacrylate esters showing uniformly higher activity. All isocompounds had slightly lower activity compared to the corresponding straight chain compounds. The observed intrinsic hemolytic activity (H_i) was inversely proportional to water solubility, agreeing more closely with isolated organ response than *in vivo* toxicity.[1-9]

The relationship between H_i and H_{50} (Fig. 2) is hyperbolic ($r = 0.99$), which is consistent with the conclusion that the hemolysis assay system is a pseudoequilibrium system appropriate for the estimation of intrinsic biological activity,[10] in this case, intrinsic hemolytic activity. The same basic hyperbolic relationship was observed between H_i and H_5. The slope of the concentration–response curve, therefore, was considered to be a valid estimate of intrinsic hemolytic activity (H_i). The relationship between primary toxicant–membrane interaction, as expressed by hemolytic activity, and *in vivo* toxicity was examined through correlation analysis. Although correlation per se does not establish mechanism, it is influenced by mechanism and is relevant to the assessment of *in vitro* assay systems for prediction of *in vivo* toxicity.

TABLE III
Squared Correlation Matrix: Physical Parameters[a]

	Log *P*	πR_1	πR_2	MR	MR(R_1)	MR(R_2)	V_ω	$V_\omega R_1$	$V_\omega R_2$
Log P	1.00[b]								
πR_1	0.02	1.00							
πR_2	0.91	0.01	1.00						
MR	0.70	0.38	0.48	1.00					
MR(R_1)	0.02	1.00	0.01	0.38	1.00				
MR(R_2)	0.91	0.01	0.99	0.46	0.01	1.00			
V_ω	0.92	0.12	0.80	0.84	0.12	0.80	1.00		
$V\omega R_1$	0.02	1.00	0.01	0.38	1.00	0.01	0.12	1.00	
$V\omega R_2$	0.91	0.01	0.99	0.46	0.01	1.00	0.80	0.01	1.00

[a] See Table II.
[b] Matrix values are univariate correlation coefficients squared.

TABLE IV
Linear Regression Analysis: Biological Response on Physical Parameters

Log biological response	Best one- through four-parameter equations	Statistics $n = 11$				
		R^2	df[a]	S[b]	F	Equation number
H_i	$= 1.23 \log P + 2.59$	0.900	1,9	0.265	81.1	1
	$= 0.11\ MR(R_1) + 0.07\ V_\omega R_2 + 2.31$	0.944	2,8	0.210	67.7	2
	$= 0.241 \log P + 0.09\ MR + 0.03\ V_\omega R_2 + 0.76$	0.933	3,7	0.246	32.4	3
	$= 0.371 \log P - 0.008\ MR + 0.10\ MR\ (R_1) + 0.05\ V_\omega R_2 + 2.5$	0.946	4,6	0.238	26.4	4
H_5	$= 1.44 \log P - 0.39$	0.931	1,9	0.254	122.2	5
	$= 0.10\ MR + 0.05\ V_\omega R_2 - 2.50$	0.949	2,8	0.232	74.8	6
	$= 0.05\ MR + 0.06\ MR(R_1) + 0.06\ V_\omega R_2 - 1.54$	0.952	3,7	0.239	47.1	7
	$= 0.42 \log P + 0.02\ MR + 0.06\ MR(R_1) + 0.05\ V_\omega R_2 - 1.00$	0.954	4,6	0.253	31.5	8
H_{50}	$= 1.36 \log P - 1.39$	0.920	1,9	0.261	103.6	9
	$= 0.10\ MR + 0.05\ V_\omega R_2 - 2.40$	0.941	2,8	0.235	64.9	10
	$= 0.05\ MR + 0.05\ MR(R_1) + 0.06\ V_\omega R_2 - 1.52$	0.945	3,7	0.244	40.3	11
	$= 0.27 \log P + 0.03\ MR + 0.06\ MR(R_1) + 0.05\ V_\omega R_2 - 1.17$	0.946	4,6	0.262	26.3_ω	12
LD_{50}	$= -0.05\ MR(R_1) + 2.26$	0.666	1,9	0.108	17.9	13
	$= 0.25 \log P - 0.06\ MR + 3.57$	0.756	2,8	0.098	12.4	14
	$= 0.30 \log P - 0.40\ \pi R_2 - 0.07\ MR(R_1) + 2.54$	0.783	3,7	0.098	8.46	15
	$= 0.391 \log P - 0.33\ \pi R_2 - 0.03\ MR - 0.04\ MR(R_1) + 3.07$	0.800	4,6	0.102	6.01	16

[a] Degrees of freedom: numerator and denominator.

[b] Standard error of estimates.

Physical parameters

Three basic physical parameters were used in the analysis: P (octanol/water partition coefficient), $V\omega$ (molecular volume, cm^3/mole), and MR (molar refraction). The first two have been widely used in correlation analysis, while MR has been used relatively recently.[12] Values for the parent compound and for the R_1 and R_2 substituents (Table II) were employed in the analysis.

The cross-correlation of physical parameters was examined by univariate correlation analysis (Table III). Where significant cross-correlations were present, the parameter giving the highest multiple correlation with biological response was selected.

Correlation analysis

Regression of biological response (H_i, H_5, H_{50}, and LD_{50}) on all possible subsets of physical parameters (Table II) was executed using statistical program BMD P9R.[14] The best one- through four-parameter equations are found in Table IV. The four-parameter equations uniformly gave the highest multiple correlation (R^2) with biological response [eqs. (4), (8), (12), and (16)]. However, the two-parameter equations [eqs. (2), (6), and (10)] with independent variables MR or MR(R_1) and $V_\omega R_2$ provide almost as good correlation as the four-parameter equations. The improvement of R^2 on the addition of $V_\omega R_2$ is statistically significant but small in magnitude compared to the correlation with the best single parameter, log P [eqs. (1), (5), and (9)]. The relationship of H_{50} and log P is shown in Figure 3. The relationship is very similar for H_5 and H_i except that isobutyl acrylate showed slightly lower activity falling off the trend for the corresponding straight chain compounds.

Although the equations generated are the best selected by the statistical program employed, they are not necessarily better than equations that substitute highly cross-correlated parameters for the parameter appearing in the selected equation (Table IV). The R_1 parameters only reflect the presence or absence of the methyl group and any one functions equally well as an indicator

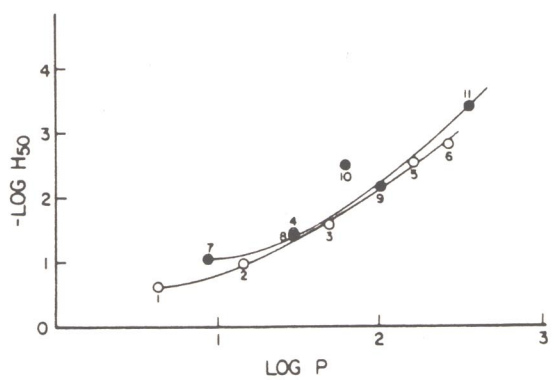


Figure 3. Relationship of H_{50} and log P. See Table I for compound identification. O, Acrylates; ●, methacrylates.

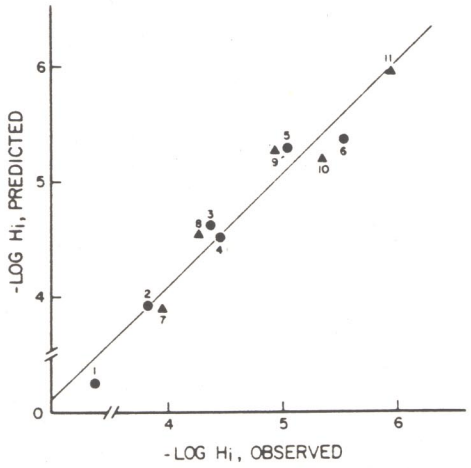


Figure 4. H_i observed (Table I) and predicted [eq. (4), Table IV]. See Table I for compound identification. ●, Methacrylates; ▲, acrylates.

variable in the analysis. This is particularly apparent in the equations for LD_{50} [eqs. (13)–(16)]. Any of the R_1 parameters discriminate LD_{50} equally well in the one-parameter equation, a reflection of the fact that LD_{50} values for the acrylate and methacrylate esters are significantly different in magnitude and show no well-defined trends with respect to the ester substituents. The overall correlation of LD_{50} with physical parameters is lower than similar correlations with hemolytic activity. A comparison of observed and predicted values for hemolysis and LD_{50} data are found in Figures 4–7. For the LD_{50}, it is apparent that the methyl esters are outliers that contribute significantly to the reduction of the overall correlation (univariate correlation of observed and predicted values) r (overall) = 0.89 and r (all compounds less the methyl esters) = 0.97,

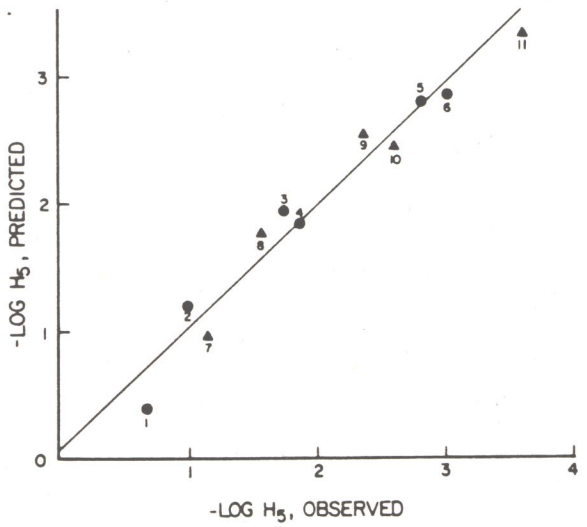


Figure 5. H_5 observed (Table I) and predicted [eq. (8), Table IV]. See Table I for compound identification. ●, Acrylates; ▲, methacrylates.

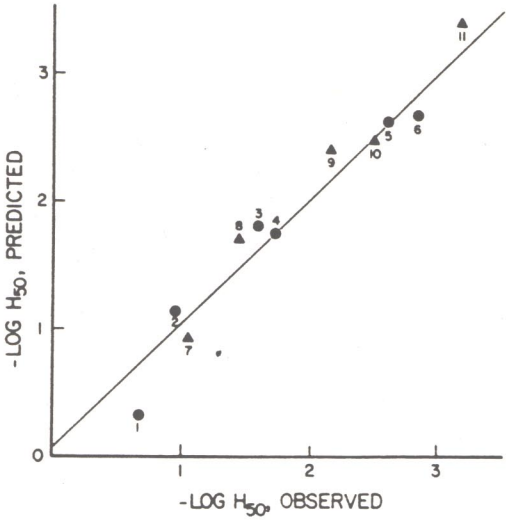


Figure 6. H_{50} observed (Table I) and predicted [eq. (12), Table IV]. See Table I for compound identification. ●, Acrylates; ▲, methacrylates.

the latter equal to the correlation with *in vitro* data. Since the predicted value for methyl methacrylate is high and for methyl acrylate is low, the steric parameters of both the R_1 and R_2 substituents are probably important to the differential expression of toxicity of those compounds *in vivo*. The relatively good correlations for all compounds where R_2 is ethyl or larger suggests that R_2 is a primary factor in the expression of toxicity. The associative contributions of R_1 and R_2, however, are probably responsible for the greater variability within the methacrylate esters.

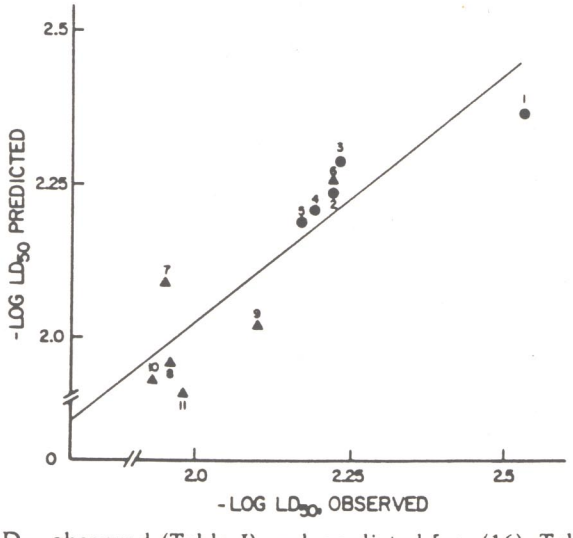


Figure 7. LD_{50} observed (Table I) and predicted [eq. (16), Table IV]. See Table I for compound identification. ●, Acrylates; ▲, methacrylates.

Relationship of *in vitro* and *in vivo* response

The excellent correlation of biological response with physical parameters and the uniformity of physical parameters in the four-parameter equations, πR_2 being highly cross-correlated with $V_\omega R_2$, suggests a fundamental relationship between the *in vitro* and *in vivo* biological response. The direct correlations (ratios) of *in vitro* response (H_i) to *in vivo* response (LD_{50}) (A, Table V) show a very large (106-fold) variation. The correlation analysis indicates log P to be a dominant factor modulating biological response. To the degree that the reciprocal of the octanol/water partition coefficient ($1/P$) is a valid estimate of the effective aqueous concentration of low molecular weight toxicants *in vivo*, and to the degree that the intrinsic biological activity of toxicants is the same *in vitro* and *in vivo*, the product of A and $1/P$ (B, Table V) should be a constant if biotransformation is not a significant factor. The overall variation of B is 5.6-fold compared to 106-fold for A. Since the largest variation was in the methacrylate group, those values apply also to that group. For the acrylate group, the value of A was 78-fold and the value of B was 2.8-fold. The differential response (B) of methyl acrylate and methyl methacrylate is apparent as is the greater variability of B for the members of the methacrylate group, probably modulated by the associative steric relationships of R_1 and R_2. Since it can reasonably be assumed that intrinsic biological activity (H_i) can be free of secondary processes of biotransformation, transport and excretion, the residual variation in B may be related to those *in vivo* processes. It is not possible, however, to discriminate among those factors and statistical error in the present analysis. The overall variation of B (5.6-fold) is approximately the same as observed previously for methyl- and halogen-substituted alcohols[11] where similar relationships with lipophilicity and steric parameters were demonstrated. Biotransformation of a toxicant to significantly higher or lower toxicity metabolites can be expected to significantly affect the LD_{50} and the value of B, as demonstrated in the alcohol study.[11] Since the overall

TABLE V
Relationship of P to *in vitro-in vivo* Response

Compound	$A = H_i/LD_{50} \times 10^{-5}$	$B = H_i/LD_{50}\,P \times 10^{-5}$
1. Methyl acrylate	7.9	1.87
2. Ethyl acrylate	11.2	0.77
3. *n*-Propyl acrylate	40.2	0.79
4. *i*-Propyl acrylate	43.4	1.42
5. *n*-Butyl acrylate	180.6	1.03
6. *i*-Butyl acrylate	584.0	2.15
7. Methyl methacrylate	7.9	0.89
8. Ethyl methacrylate	16.73	0.55
9. *n*-Propyl methacrylate	115.5	1.09
10. *i*-Propyl methacrylate	196.0	3.07
11. *n*-Butyl methacrylate	837.0	2.28
Variation:	106-fold	5.58-fold

variability of the acrylate and methacrylate esters with respect to B was of the same order of magnitude as that of the alcohols, the observed variability is consistent with the conclusion that the metabolites of those compounds in the course of a 7-day LD_{50} assay, where there is ample time for biotransformation, do not significantly affect the LD_{50}, i.e. the compounds act by the same basic mechanism modulated by the physicochemical properties of the parent compound.

That *in vivo* toxicity in mice can be rationalized to a significant degree through consideration of *in vitro* intrinsic hemolytic activity supports the conclusion that the mechanism of action is membrane mediated and relatively nonspecific for acrylate and methacrylate esters. Intrinsic toxicity is related to lipophilicity (inversely related to water solubility), which suggests that the toxic liability of long-term exposure *in vivo* may be more closely related to intrinsic toxicity than acute toxicity which is consistent with observed differences in acute and chronic LD_{50} values for the phthalate esters.[15] The value of *in vitro* assay systems for the estimation of intrinsic biological activity and the relevance of intrinsic activity to higher order biological response were demonstrated.[16]

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