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RESEARCH ARTICLES

Effect of Lidocaine and Derivatives on Time of Death and Overturn in Goldfish

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Abstract □ The effect of lidocaine and five derivatives on the time of overturn and time of death was examined in goldfish. A relationship between the minimum effective concentration of drug necessary to produce the pharmacological response and the partition coefficient between *n*-heptane or *n*-octanol and pH 7.4 buffer was found. Further investigations confirmed that, in goldfish, the nonionized local anesthetic agent is solely responsible for the observed effects on overturn time and time of death. Experiments performed to determine the amount of drug in the goldfish at the time of pharmacological response indicated that, for five of the six compounds studied, the same concentration of drug was found in the fish at the time of response. This finding supports the observation that, for the compounds studied, the critical concentration of drug in the goldfish necessary to produce a given response is identical, the difference in time of response being due to differences in absorption rate. The relationship between overturn time and time of death in goldfish was examined, and linear regression of 1/time of death *versus* 1/overturn time yielded $1/T_D = 0.2860(1/T_O) + 0.0001$ ($r = 0.9648$). The relationship between overturn and death may simply be a function of the amount of drug in the fish at the time of the pharmacological response, and the relationship between the amounts of the drug in the fish at the two end-points may be constant regardless of the derivative evaluated.

Keyphrases □ Lidocaine—effect on time of death in goldfish, effect on time of overturn in goldfish, derivatives, ionization, absorption □ Goldfish—effect of lidocaine and derivatives on times of death and overturn, effect of pH on times of death and overturn □ Toxicity—lidocaine and derivatives, use of goldfish as biological models

Goldfish may be used as biological models for the assessment of absorption and toxicity of chemical compounds and for the elucidation of the physical-chemical parameters of a pharmacological response (1–6). Goldfish were used as a model for the assessment of structure–toxicity relationships between substituted phenothiazines (4); differences in toxicity may have been due to differences in absorption. Goldfish also were used to study the effects of alkyl chain length on biological activity for *n*-alkyl esters of *p*-aminobenzoic acid (5). The activity of local anesthetic agents was studied using goldfish, and a possible relationship between the minimum effective concentration (MEC) of drug in the bathing solution and the minimum blocking concentration of the same anesthetic agent on isolated nerve and muscle fibers was observed (6).

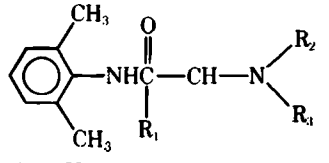
Whenever goldfish are used as biological models, the investigator has the choice of two common pharmacological end-points: the overturn time, defined as the loss of the righting reflex, or the time of death, defined as the complete cessation of gill and mouth movements. This report evaluates the activity of lidocaine and a series of lidocaine derivatives in producing overturn and death and examines the relationship between these two end-points.

EXPERIMENTAL

Goldfish of the variety *Carassius auratus*, 2–4 g, were used initially. The overturn and death times of individual goldfish in 200 ml of a solution of lidocaine and its derivatives[1] in pH 7.4 phosphate buffer (0.05 *M*) were determined. All solutions were freshly prepared before each experiment. The goldfish tank and test solutions were maintained at 22 ± 2°.

The overturn and death times of individual goldfish in 200 ml of various concentrations of lidocaine and benzocaine[2] in 0.05 *M* phosphate buffer in solutions of varying pH were also determined.

Partition coefficients of lidocaine (I) and each derivative (II–VI) were determined between *n*-octanol and pH 7.4 phosphate buffer (0.05 *M*). Various volumes of organic phase and 20 ml of aqueous phase containing



I: $R_1 = H$, $R_2 = R_3 = C_2H_5$
II: $R_1 = H$, $R_2 = R_3 = CH_3$
III: $R_1 = R_2 = R_3 = CH_3$
IV: $R_1 = H$, $R_2 = CH_3$, $R_3 = C_2H_5$
V: $R_1 = CH_3$, $R_2 = R_3 = C_2H_5$
VI: $R_1 = CH_3$, $R_2 = C_2H_5$, $R_3 = C_3H_7$

[1] Supplied by Astra Pharmaceuticals, Worcester, Mass.
[2] Eastman Organic Chemicals, Rochester, N.Y.

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Table I—Average Overturn Time of Goldfish in Solutions of Local Anesthetic Agents in pH 7.4 Buffer

Compound	Concentration, m*M*	Number of Fish	Mean Overturn Time, min	*SD*	Reciprocal of Mean Overturn Time, min^{-1}
I	0.2	5	93.11	10.21	0.0107
	0.3	5	53.36	8.26	0.0187
	0.5	13	16.26	8.19	0.0615
	0.7	5	17.33	1.51	0.0577
	0.8	3	3.34	0.77	0.2977
	0.9	5	4.26	2.09	0.2347
	1.0	26	4.16	3.62	0.2402
	1.1	6	2.62	1.40	0.3817
	1.2	6	1.56	0.54	0.6397
II[a]	0.8	3	70.86	22.69	0.0141
	1.0	3	44.69	11.26	0.0224
	1.1	3	31.98	12.38	0.0313
	1.2	3	16.22	4.66	0.0616
	1.5	3	13.22	7.44	0.0757
III[b]	0.5	5	25.46	5.79	0.0393
	0.8	5	19.02	4.62	0.0526
	1.0	8	12.48	3.57	0.0801
	1.2	6	11.43	2.96	0.0875
	1.4	3	10.80	5.59	0.0926
	1.5	3	13.53	4.31	0.0739
IV[c]	0.5	3	22.61	9.61	0.0442
	0.8	3	12.22	0.19	0.0819
	1.0	3	9.54	4.35	0.1048
	1.1	3	9.85	3.97	0.1016
	1.2	6	7.78	5.89	0.1285
V[d]	0.3	3	20.50	16.02	0.0488
	0.5	3	3.30	0.65	0.3028
	0.8	6	2.96	1.35	0.3384
	1.0	3	2.66	0.66	0.3759
	1.2	3	1.15	0.49	0.8673
VI[e]	0.1	8	9.42	5.28	0.1062
	0.3	9	3.09	1.75	0.3233
	0.5	11	1.24	0.51	0.8097
	0.8	6	1.17	0.36	0.8562
	1.0	6	0.70	0.21	1.4389

[a] W36017. [b] W36024. [c] W36004. [d] W36023. [e] W36032.

Table II—Average Death Time of Goldfish in Solutions of Local Anesthetic Agents in pH 7.4 Buffer

Compound	Concentration, m*M*	Number of Fish	Mean Death Time, min	*SD*	Reciprocal of Mean Death Time, min^{-1}
I	0.2	5	138.22	33.52	0.0072
	0.3	5	86.35	16.27	0.0115
	0.5	13	38.64	15.90	0.0259
	0.7	5	40.43	5.99	0.0247
	0.8	3	15.48	6.59	0.0646
	0.9	5	12.58	3.88	0.0795
	1.0	26	13.20	8.91	0.0758
	1.1	6	9.96	4.28	0.1004
	1.2	6	7.31	1.56	0.1369
II	0.8	3	216.77	11.82	0.0046
	1.0	3	132.55	43.29	0.0075
	1.1	3	46.68	14.47	0.0214
	1.2	3	69.07	20.71	0.0145
	1.5	3	34.85	2.86	0.0287
III	0.5	5	130.59	96.62	0.0077
	0.8	4	67.55	45.29	0.0148
	1.0	6	73.33	3.48	0.0136
	1.2	6	40.02	14.61	0.0250
	1.4	3	36.08	21.45	0.0277
	1.5	3	36.92	7.31	0.0271
IV	0.5	3	73.82	22.97	0.0136
	0.8	3	33.03	13.82	0.0302
	1.0	3	30.87	5.94	0.0324
	1.1	3	20.18	6.73	0.0496
	1.2	6	19.68	13.55	0.0508
V	0.3	3	41.33	23.46	0.0242
	0.5	3	16.55	2.88	0.0604
	0.8	6	9.91	3.82	0.1010
	1.0	3	9.68	2.52	0.1033
	1.2	3	4.85	0.33	0.2061
VI	0.1	8	40.65	7.48	0.0246
	0.3	9	10.55	5.66	0.0948
	0.5	11	6.73	1.60	0.1485
	0.8	6	3.28	1.22	0.3049
	1.0	6	2.11	0.73	0.4735

the local anesthetics were placed in glass bottles and shaken[3] for a minimum of 16 hr at 100 oscillations/min. Equilibrium was established by repetitive sampling. Assay for each drug in the aqueous phase was performed spectrophotometrically[4] at 262 nm. All determinations were performed at room temperature (22 ± 2°).

The amounts of drug in individual fish at the pharmacological endpoints (overturn and death) also were determined. Fish weighing 4–6 g were placed in 200 ml of 0.5, 1.0, or 1.5 m*M* solutions of lidocaine or 1.0 m*M* solutions of derivatives buffered to pH 7.4 with a 0.05 *M* phosphate buffer. All experiments were performed at room temperature (22 ± 2°). At the time of overturn, fish were removed from solution and washed with 100 ml of distilled water.

Whole fish were then sectioned and placed in vials, and 5 ml of distilled water and 2.0 ml of a 2 *N* NaOH solution were added; the fish were homogenized[5] and the vial was rinsed with 5 ml of distilled water, which was added to the homogenate. Samples were centrifuged[6] at 10,000 rpm for 10 min, and the supernate was added to 20 ml of carbon tetrachloride and shaken[7] for 5 min. The aqueous layer was aspirated, and the organic layer was filtered. The sample was added to 10 ml of distilled water as a wash and shaken for 5 min.

The aqueous layer was removed, and 10 ml of the organic layer was added to an equal volume of a 0.004% bromthymol blue solution buffered to pH 6.0 with a 0.05 *M* phosphate buffer. This mixture was shaken for 5 min and centrifuged[8] at 4000 rpm for 6 min. The aqueous layer was aspirated, and 5 ml of the organic layer was added to 5 ml of a 0.1 *N* NaOH solution. Then this mixture was shaken for 5 min and centrifuged[9] at 2000 rpm for 5 min. The top layer was read at 615 nm[4].

Calibration plots were obtained by adding known amounts of each drug and 5 ml of distilled water to vials containing a sectioned fish and 1 ml of 2 *N* NaOH. Blanks were run for each experiment and consisted of 5 ml of distilled water added to a sectioned fish and 1 ml of 2 *N* NaOH. Although nonlinearity was observed at some high drug concentrations, all determinations were made in the linear portion of the absorbance *versus* concentration plots.

[3] Precision water bath shaker model 25, Precision Scientific Co.
[4] Perkin-Elmer model 124.
[5] Brinkmann Polytron.
[6] Sorvall RC-5 superspeed refrigerated centrifuge.
[7] Eberbach shaker unit, model 6000.
[8] Damon/IEC Division model HN-S centrifuge.
[9] Clay Adams Dynac centrifuge.

THEORETICAL

The Levy–Gucinski (7) model assumes that the absorption rate of drug into the fish is proportional to the drug concentration in the bathing solution:

$$R = KC \qquad \text{(Eq. 1)}$$

where R is the absorption rate into the fish, K is the relative absorption rate constant, and C is the drug concentration in the bathing solution.

If the drug concentration in the aqueous medium is high enough to elicit the pharmacological response before any appreciable amount undergoes biotransformation or elimination, then the drug concentration in the fish is equal to:

$$C_B^* = Rt^* \qquad \text{(Eq. 2)}$$

where C_B^* is the drug concentration in the fish at the time (t^*) of the pharmacological response. Substituting for the absorption rate in Eq. 2 yields:

$$C_B^* = KCt^* \qquad \text{(Eq. 3)}$$

which, upon rearrangement, is equivalent to:

$$1/t^* = (K/C_B^*)C \qquad \text{(Eq. 4)}$$

Therefore, plots of reciprocal time of response as a function of drug concentration in the aqueous medium should be linear, pass through the origin, and have a slope equal to the absorption rate constant divided by the drug concentration in the fish necessary to produce the desired pharmacological response.

The Nightingale–Gibaldi (8) model extends the previous model to

Table III—Linear Least-Squares Regression of Reciprocal Time of Response as a Function of Concentration

Compound	Least-Squares Equation—Overturn	r	r^2	Least-Squares Equation—Death	r	r^2
I	$1/T = 0.5191C - 0.1704$	0.8761	0.7676	$1/T = 0.1190C - 0.0301$	0.9408	0.8851
II	$1/T = 0.0965C - 0.0670$[a]	0.9456	0.8942	$1/T = 0.0345C - 0.0233$[b]	0.9031	0.8156
III	$1/T = 0.0461C + 0.0217$	0.8360	0.6989	$1/T = 0.0211C - 0.0032$	0.9536	0.9094
IV	$1/T = 0.1112C - 0.0101$[b]	0.9791	0.9586	$1/T = 0.0532C - 0.0136$[b]	0.9591	0.9199
V	$1/T = 0.7221C - 0.1622$	0.8844	0.7822	$1/T = 0.1742C - 0.0334$[b]	0.9322	0.8690
VI	$1/T = 1.3680C - 0.0318$	0.9616	0.9247	$1/T = 0.4841C - 0.0521$	0.9799	0.9602

[a] Intercept significant, $p < 0.05$. [b] Intercept significant, $p < 0.01$.

include the minimum effective concentration (MEC) of drug in the bathing solution below which no pharmacological response occurs and in the vicinity of which there is a disproportionate increase in the time of the pharmacological response. With the assumption that the occurrence of the pharmacological response is absorption rate limited, Scheme I has been proposed:

$$A \underset{K_{21}}{\overset{K_{12}}{\rightleftharpoons}} B$$

Scheme I

where A is the drug solution bathing the fish, B represents the fish, K_{12} is the absorption rate constant into the fish, and K_{21} is the exsorption rate constant out of the fish. Therefore, the rate of change of drug in the bathing solution can be written as:

$$dX_A/dt = K_{21}X_B - K_{12}X_A \qquad \text{(Eq. 5)}$$

and the rate of change of drug in the fish is:

$$dX_B/dt = K_{12}X_A - K_{21}X_B \qquad \text{(Eq. 6)}$$

Integration of Eq. 6 yields:

$$X_B = \frac{K_{12}X_A^0}{(K_{12} + K_{21})}[1 - e^{-(K_{12}+K_{21})t}] \qquad \text{(Eq. 7)}$$

where X_B is the amount of drug in the fish at any time (t) and X_A^0 is the initial amount of drug in the bathing solution. Changing the amounts to concentrations and rearranging yield:

$$e^{-(K_{12}+K_{21})t} = \frac{KC_A^0 - C_B}{KC_A^0} \qquad \text{(Eq. 8)}$$

where C represents the drug concentration in the respective compartments, $K = [K_{12}V_A/(K_{12} + K_{21})V_B]$, V_A is the volume of the drug solution in the bathing solution, and V_B is the volume of the fish.

If the concentration of drug in the fish, C_B, reaches the level necessary to produce the C_B^* effect at time t^*, then Eq. 9 or 10 is applicable:

$$e^{-(K_{12}+K_{21})t} = \frac{KC_A^0 - C_B^*}{KC_A^0} \qquad \text{(Eq. 9)}$$

$$1/t^* = \frac{K_{12} + K_{21}}{\ln \dfrac{KC_A^0}{KC_A^0 - C_B^*}} \qquad \text{(Eq. 10)}$$

Equation 10 can be approximated by (8):

$$1/t^* = \frac{K(K_{12} + K_{21})C_A^0}{C_B^*} - \frac{K_{12} + K_{21}}{2} \qquad \text{(Eq. 11)}$$

which is equivalent to:

$$1/t^* = \frac{K_{12}V_A(K_{12} + K_{21})C_A^0}{(K_{12} + K_{21})V_BC_B^*} - \frac{K_{12} + K_{21}}{2} \qquad \text{(Eq. 12)}$$

It follows that:

$$1/t^* = \frac{K_{12}V_AC_A^0}{V_BC_B^*} - \frac{K_{12} + K_{21}}{2} \qquad \text{(Eq. 13)}$$

and:

$$1/t^* = \frac{K_{12}V_AC_A^0}{X_B^*} - \frac{K_{12} + K_{21}}{2} \qquad \text{(Eq. 14)}$$

where X_B^* is the amount of drug in the fish at the time of the pharmacological response. Therefore, a plot of $1/t^*$ as a function of the initial drug concentration in the bathing solution should be linear with a slope equal to $K_{12}V_A/X_B^*$ and an intercept equal to $-(K_{12} + K_{21})/2$.

RESULTS AND DISCUSSION

The concentrations of drugs employed in the study, number of fish used at each concentration, mean time of response, standard deviation, and reciprocal of the mean time of response are listed in Tables I and II for overturn and death, respectively. The data were analyzed by least-squares linear regression according to the relationship expressed in Eqs. 11–14; the values for the slope, intercept, correlation coefficient, and coefficient of determination are listed in Table III.

Figures 1 and 2 illustrate the relationship between the reciprocal of the mean time of response and the concentration of drug in the bathing solutions for overturn and death times, respectively. Table III indicates a strong relationship between the reciprocal time of response and the drug concentration in the bathing medium. Unexplained variability may have been due to variations in goldfish from different suppliers.

To investigate the effect of pH on overturn and death time, goldfish were placed in various concentrations of lidocaine solutions buffered to various pH values with 0.05 M phosphate buffers (Table IV). Goldfish were also placed in a pH 6.0 phosphate buffer (0.05 M) containing either a 1.0 or 1.2 mM lidocaine solution. No death responses were evident after 4 hr. Responses for overturn were elicited at the 1.2 mM concentration, but only three of five fish demonstrated overturn at the end of 4 hr at 1.0 mM.

Figure 3 is a plot of the reciprocal time of response as a function of pH for the 0.8 mM concentration of lidocaine. As the pH of the bulk solution decreased, the reciprocal time of response increased. At pH 6.0, 7.0, 7.4, and 8.0, lidocaine existed approximately as 2, 16, 32, and 65% unionized species, respectively. Plots of the reciprocal time of response as a function of the fraction unionized species present for lidocaine at the 0.8 mM

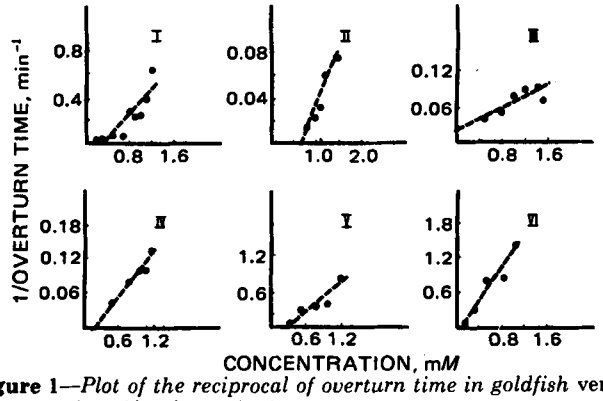

Figure 1—*Plot of the reciprocal of overturn time in goldfish* versus *concentration of local anesthetic.*

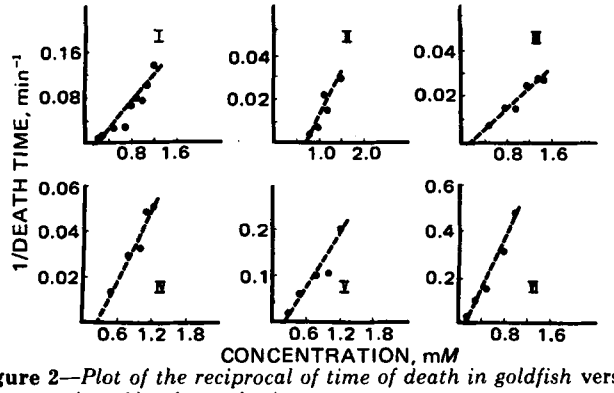

Figure 2—*Plot of the reciprocal of time of death in goldfish* versus *concentration of local anesthetic.*

Table IV—Effects of pH on Time of Response of Goldfish in Lidocaine Solutions

Concentration, m*M*	pH 7 Mean Overturn Time, min	*n*	*SD*	pH 7 Reciprocal Overturn Time, min^{-1}	pH 7.4 Mean Overturn Time, min	*n*	*SD*	pH 7.4 Reciprocal Overturn Time, min^{-1}	pH 8.0 Mean Overturn Time, min	*n*	*SD*	pH 8.0 Reciprocal Overturn Time, min^{-1}
0.5	—	—	—	—	16.26	13	8.19	0.0615	2.48	5	1.01	0.4033
0.8	6.96	5	1.78	0.1437	3.34	3	0.77	0.2994	1.82	5	0.84	0.5495
1.0	9.84	5	3.27	0.1016	4.16	26	3.62	0.2402	1.26	5	0.18	0.7937
1.2	8.52	5	3.01	0.1174	1.56	6	0.54	0.6397	3.55	5	2.62	0.2817

Concentration, m*M*	pH 7 Mean Death Time, min	*n*	*SD*	pH 7 Reciprocal Death Time, min^{-1}	pH 7.4 Mean Death Time, min	*n*	*SD*	pH 7.4 Reciprocal Death Time, min^{-1}	pH 8.0 Mean Death Time, min	*n*	*SD*	pH 8.0 Reciprocal Death Time, min^{-1}
0.5	—	—	—	—	38.64	13	15.90	0.0259	11.59	5	3.86	0.0863
0.8	30.23	5	1.85	0.0331	15.48	3	6.59	0.0646	7.35	5	1.36	0.1361
1.0	25.96	5	7.99	0.0385	13.20	26	8.91	0.0758	6.00	5	0.53	0.1667
1.2	16.15	5	2.34	0.0619	7.31	6	1.56	0.1369	5.24	5	0.77	0.1908

concentration (Fig. 4) suggest that the unionized drug may be responsible for producing the observed effects. Plots of the reciprocal time of response as a function of the concentration of unionized species present are shown in Fig. 5. The least-squares linear regression for the overturn data[10] is: 1/overturn time = 1.388 [concentration unionized] + 0.0949 ($r = 0.9334$, $p < 0.01$); that for death is: 1/death time = 0.2550 [concentration unionized] + 0.0025 ($r = 0.9689$, $p < 0.01$). To rule out the possibility that the observed effects were a result of pH-induced changes in membrane permeability rather than of unionized drug species, experiments were performed utilizing various concentrations of benzocaine over a pH 6–8 range (Table V and Fig. 6). Benzocaine exists virtually 100% as the unionized species in this pH range. No apparent trend exists in the data for the reciprocal time of response for equal concentrations of drug as a function of pH. This result indicates that pH changes are not affecting membrane permeability and that the unionized species is responsible for the effects observed.

The concentrations of drug found in the fish from three different bathing concentrations at the times of overturn and death are listed in Table VI. There was no significant difference ($p > 0.05$) in concentrations of drug in the fish at the time of the pharmacological response, regardless of the concentrations of lidocaine employed as the bathing solution.

The concentrations of derivatives found in the fish at the time of the pharmacological response to 1 m*M* concentrations of the derivatives are presented in Table VII. A significant difference ($p < 0.05$) existed between the amounts in the fish at overturn and death. Compound VI was the only drug whose concentration was significantly different from the others at the time of the pharmacological response. Compound VI differed significantly from all of the other compounds at the dosage causing overturn and from I, II, and III at the lethal dose.

The minimum effective concentration (MEC) of the bulk solution is the concentration below which no response will occur. Theoretically, this value should be the abscissa intercept of a reciprocal time of response as a function of concentration plot. The MEC of the bulk solution was calculated by setting the ordinate value to zero and calculating what the concentration of the bulk solution would be at that point. If the unionized drug molecule is responsible for the effects observed, then the MEC thus calculated are incorrect; the derivatives have different pKa values and, therefore, different fractions unionized at pH 7.4.

Table VIII contains the apparent MEC, the pKa of the compounds, the fraction unionized of the compounds at pH 7.4, and the MEC of unionized species. Some obvious discrepancies are apparent. The least-

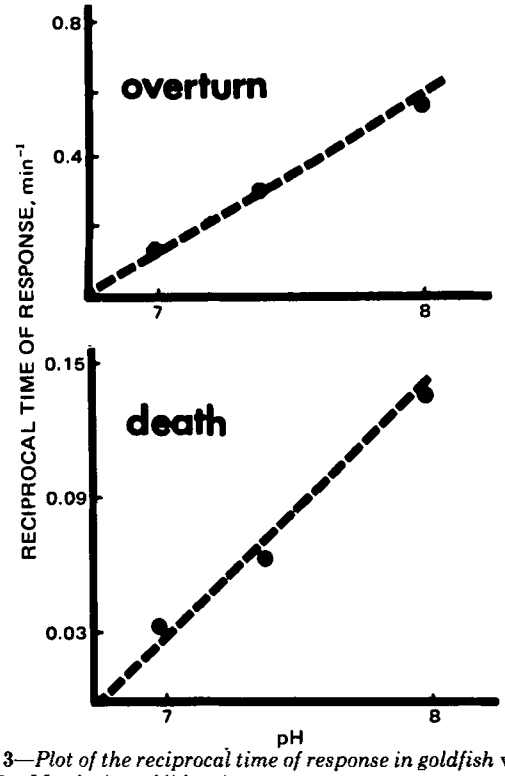

Figure 3—*Plot of the reciprocal time of response in goldfish* versus *pH for a 0.8 m*M *solution of lidocaine.*

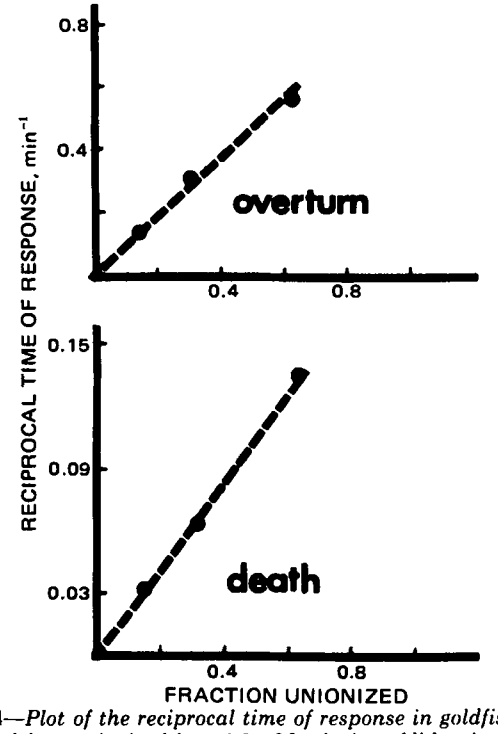

Figure 4—*Plot of the reciprocal time of response in goldfish* versus *fraction of drug unionized for a 0.8 m*M *solution of lidocaine.*

[10] The data point for the 0.78 m*M* concentration of unionized drug was omitted from the regression analysis.

Table V—Reciprocal of Overturn and Death Times for Benzocaine as a Function of Concentration [a] and pH

Concentration, m*M*	pH 6.0 Mean Overturn Time, min	*SD*	pH 6.0 Reciprocal Overturn Time, min^{-1}	pH 7.0 Mean Overturn Time, min	*SD*	pH 7.0 Reciprocal Overturn Time, min^{-1}	pH 8.0 Mean Overturn Time, min	*SD*	pH 8.0 Reciprocal Overturn Time, min^{-1}
0.1	31.63	4.16	0.0316	18.09	6.32	0.0553	23.83	8.57	0.0420
0.2	8.66	0.80	0.1155	10.12	2.54	0.0988	10.32	1.87	0.0920
0.3	5.79	0.99	0.1727	5.18	2.25	0.1931	5.86	2.63	0.1718
0.4	3.33	1.11	0.3003	1.98	0.53	0.5051	2.43	0.73	0.4115

Concentration, m*M*	pH 6.0 Mean Death Time, min	*SD*	pH 6.0 Reciprocal Death Time, min^{-1}	pH 7.0 Mean Death Time, min	*SD*	pH 7.0 Reciprocal Death Time, min^{-1}	pH 8.0 Mean Death Time, min	*SD*	pH 8.0 Reciprocal Death Time, min^{-1}
0.1	—[b]	—	—	—[b]	—	—	—[b]	—	—
0.2	28.58	9.26	0.0350	43.40	12.44	0.0230	39.43	9.25	0.0254
0.3	12.30	2.07	0.0813	15.20	4.92	0.0658	14.08	1.60	0.0710
0.4	9.68	1.80	0.1033	9.21	3.26	0.1086	8.50	2.31	0.1176

[a] Five fish used at each concentration. [b] No response at end of 4 hr.

Table VI—Concentration (C_B^*)[a] of Lidocaine in Goldfish at Time of Pharmacological Response

Concentration, m*M*	Overturn C_B^*, μm/g	*SD*	Number of Fish	Time of Response, min	*SD*	Death C_B^*, μm/g	*SD*	Number of Fish	Time of Response, min	*SD*
0.5	0.3072	0.0781	5	6.89	1.69	0.5664	0.1030	4	20.58	2.21
1.0	0.4984	0.1448	5	2.01	0.36	0.6545	0.3082	5	7.74	2.03
1.5	0.3395	0.1323	4	2.05	0.26	0.7185	0.3162	4	8.14	2.11

Analysis of Variance for Unequal Data Sets

Source of Variation	Overturn *df*	*SS*	*MS*	*F*	Significance	Death *df*	*SS*	*MS*	*F*	Significance
Between concentrations	2	0.1028	0.0514	3.52	NS ($p > 0.05$)	2	0.0468	0.0234	0.3297	
Within concentrations	11	0.1609	0.0146			10	0.7118	0.0718		NS ($p > 0.05$)
Total	13	0.2637				12	0.7586			

[a] $C_B^* = X_B^*/V_B$, where X_B^* is the amount of drug in the fish at the pharmacological end-point and V_B is the weight (grams) of the fish.

Table VII—Concentration of Derivatives (C_B^*) in Goldfish at Time of Pharmacological Response

Compound	Overturn C_B^*, μm/g	*SD*	Number of Fish	Time of Response, min	*SD*	Death C_B^*[a], μm/g	*SD*	Number of Fish	Time of Response, min	*SD*
I	0.4984	0.1448	5	2.01	1.69	0.6545	0.3082	5	7.75	2.03
II	0.2981	0.1460	5	61.82	8.92	0.5466	0.2583	5	94.08	38.02
III	0.3757	0.0974	5	16.68	6.71	0.6418	0.1047	4	42.80	10.46
IV	0.3235	0.0765	5	10.14	2.24	0.4975	0.1647	5	30.24	5.90
V	0.3805	0.1900	3	2.97	0.50	0.6689	0.1909	4	9.27	1.02
VI	0.0634	0.0513	4	0.75	0.14	0.2324	0.0812	5	2.19	0.53

Analysis of Variance for Unequal Data Sets[b]

Source of Variation	Overturn *df*	*SS*	*MS*	*F*	Significance[c]	Death *df*	*SS*	*MS*	*F*	Significance
Between drugs	5	0.4495	0.0899	6.07	$p < 0.05$	5	0.6537	0.13074	3.11	$p < 0.05$
Within drugs	21	0.3109	0.0148			22	0.9248	0.04201		
Total	26	0.7604				27	1.5785			

[a] $C_B^* = X_B^*/V_B$, where X_B^* is the amount of drug in the fish at the pharmacological end-point and V_B is the weight (grams) of the fish. [b] Homogeneity of variance tested using $F_{max} = S^2$ largest/Si^2 smallest at the 95% confidence level. For overturn, $F_{max} = 14.5$ ($p > 0.05$); for death, $F_{max} = 13.57$ ($p > 0.05$). [c] Newman-Keuls method with a harmonic mean to compensate for unequal data sets (9).

Table VIII—Minimum Effective Concentration (MEC) in Goldfish for Lidocaine and Derivatives

Compound	pKa	Fraction Unionized at pH 7.4	MEC, m*M*—Overturn	MEC, m*M*—Overturn, Unionized Species	MEC, m*M*—Death	MEC, m*M*—Death, Unionized Species
I	7.72	0.3246	0.328	0.106	0.253	0.082
II	7.22	0.6015	0.694	0.417	0.675	0.406
III	7.26	0.5798	—	—	0.152	0.088
IV	7.53	0.4273	0.091	0.039	0.256	0.109
V	7.98	0.2083	0.225	0.047	0.192	0.029
VI	7.88	0.2487	0.023	0.006	0.108	0.027

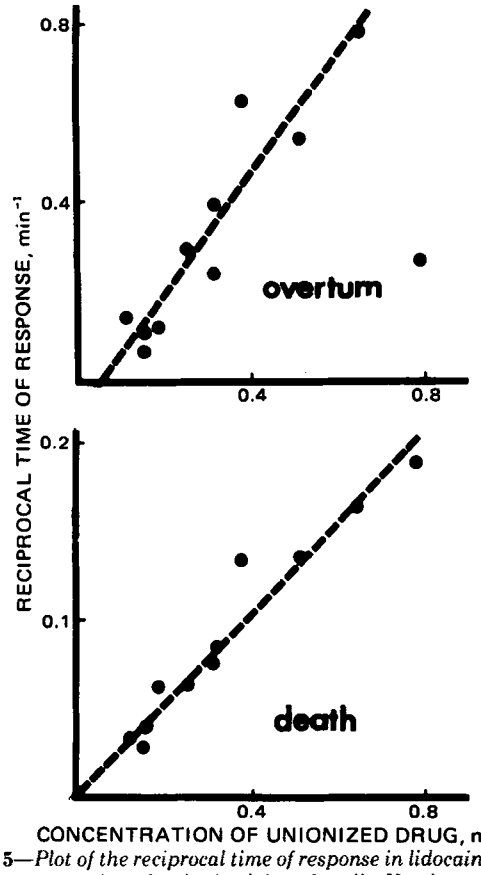

Figure 5—*Plot of the reciprocal time of response in lidocaine in goldfish versus concentration of unionized drug for all pH values studied.*

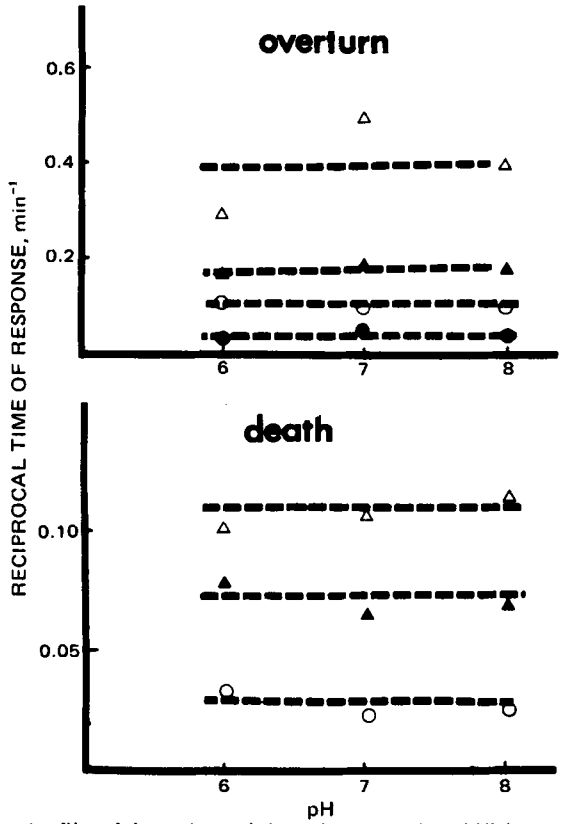

Figure 6—*Plot of the reciprocal time of response in goldfish versus pH for varying concentrations of benzocaine. Key: ●, 0.1 mM; ○, 0.2 mM; ▲, 0.3 mM; and △, 0.4 mM.*

Table IX—Least-Squares Analysis Reciprocal Time of Death as a Function of Reciprocal Time of Overturn

Compound	Least-Squares Equation	r	Significance
I	$1/T_D = 0.2077\ 1/T_O + 0.0136$	0.9732	$p < 0.01$
II	$1/T_D = 0.2944\ 1/T_O + 0.0032$	0.7852	N.S.
III	$1/T_D = 0.3205\ 1/T_O - 0.0034$	0.7975	N.S.
IV	$1/T_D = 0.4414\ 1/T_O - 0.0053$	0.9029	$p < 0.05$
V	$1/T_D = 0.2242\ 1/T_O + 0.0124$	0.9837	$p < 0.01$
VI	$1/T_D = 0.2309\ 1/T_O - 0.0246$	0.9528	$p < 0.05$

squares estimate of the MEC for III was omitted since it produced a physically impossible negative MEC. Furthermore, the MEC for overturn was apparently greater than that for death for I, II, and V, which again is physically impossible. And, finally, the MEC values calculated for overturn and death for I and for death for VI were larger than concentrations that produced a pharmacological response in the fish in this study. Thus, the MEC values of the drugs calculated in the present investigation are simply statistically generated numbers with high variability and questionable physical significance.

Overturn and death times were investigated to determine whether a relationship exists between the two end-points such that selection of one over the other would change the interpretation of the data. The possibility exists that these responses are mediated at physically and chemically distinct effective receptor sites. Overturn might be mediated by local muscle paralysis while death might be a result of central nervous system (CNS) depression.

Linear regression analysis was performed on individual data for the reciprocal of overturn time (Table IX). Reasonable predictions of the reciprocal of time of death can be obtained from the reciprocal of overturn

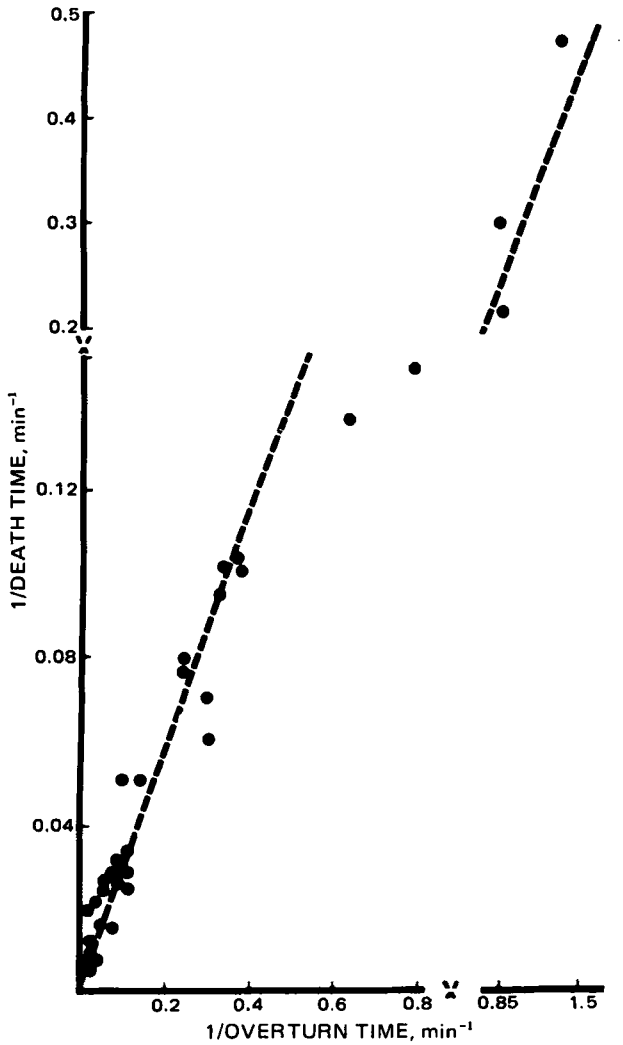

Figure 7—*Plot of the reciprocal of time of death in goldfish versus the reciprocal time of overturn in goldfish for all compounds studied.*

Table X—Ratio of Amounts in Fish at Overturn and Death

Compound	Ratio (X^*_{BO}/X^*_{BD})
I	0.590
II	0.545
III	0.585
IV	0.650
V	0.569
VI	0.273
Mean	0.535

time at that concentration. The apparent slopes do not differ much, and the intercepts are scattered about zero, indicating that all of the data may be grouped together. Linear regression of the combined data gave: $1/T_D = 0.2860(1/T_O) + 0.0001$ ($r = 0.9648$, $p < 0.01$), where T_D and T_O are the lethal and overturn times, respectively. A plot of $1/T_D$ *versus* $1/T_O$ is presented in Fig. 7.

For all data, there appears to be a linear relationship between the reciprocals of the time of response end-points at each concentration (Fig. 7). Pharmacologically, this finding suggests a simple relationship between the concentrations inducing narcosis and death. Where bulk solution concentrations are sufficiently high to make the MEC values negligible, the slope of $1/T_D$ *versus* $1/T_O$ is approximately equal to:

$$m = \frac{K_{12}V_AC^0_A}{X^*_{B_{death}}} \Big/ \frac{K_{12}V_AC^0_A}{X^*_{B_{overturn}}} \qquad \text{(Eq. 15)}$$

and:

$$m = \frac{X^*_{B_{overturn}}}{X^*_{B_{death}}} \qquad \text{(Eq. 16)}$$

Table X contains the ratio of the amount of each drug in the fish at the time of response, which may be regarded as a direct estimate of the narcosis to death ratio. The average value of the ratios is approximately 0.54. However, the value of the slope in Fig. 7, a second estimate, is only 0.29. The agreement between these two independent estimates is rough at best. The direct estimate assumes that the total fish concentration reflects the concentration in the critical CNS tissues at the moment the fish are removed from the drug solution, which may not be the case. Therefore, the pharmacological ratio would be the better estimate of the turnover to death concentration ratio. This fundamental point is worthy of more research.

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Synthesis and Antimicrobial Evaluation of Quaternary Salts of 4-Phenyl-1,2,3,6-tetrahydropyridine and 3,6-Dimethyl-6-phenyltetrahydro-2*H*-1,3-oxazine

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Abstract □ Fifteen predominantly alkyl bromide quaternary salts of 1-substituted 4-phenyl-1,2,3,6-tetrahydropyridine and 10 from 3,6-dimethyl-6-phenyltetrahydro-2*H*-1,3-oxazine were synthesized. None was effective against the parasitic protozoan *Eimeria tenella* and the helminth trichostrongyle nematode. Nearly all inhibited Gram-positive and Gram-negative bacteria; maximum efficiency was obtained with nonyl through dodecyl bromide salt derivatives. Antifungal effectiveness paralleled these results. The oxazinium salt analogs were inhibitory in an *in vitro* peridontal microorganism screen. The decyl bromide derivative at 0.05% in drinking water prevented dental plaque and reduced calculus deposition in rats but not in hamsters fed cariogenic diets. A 0.01% concentration of the tetrahydropyridinium analog caused increased plaque in rats compared to nonmedicated control animals.

Keyphrases □ 4-Phenyl-1,2,3,6-tetrahydropyridine quaternary salts—synthesis and antimicrobial evaluation □ 3,6-Dimethyl-6-phenyltetrahydro-2*H*-1,3-oxazine quaternary salts—synthesis and antimicrobial evaluation □ Antimicrobial activity—quaternary salts of 4-phenyl-1,2,3,6-tetrahydropyridine and 3,6-dimethyl-6-phenyltetrahydro-2*H*-1,3-oxazine

Two heterocyclic nitrogen bases, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine and 3,6-dimethyl-6-phenyltetrahydro-2*H*-1,3-oxazine, are readily accessible from the reaction of α-methylstyrene, formaldehyde, and methylamine salts (1) among other processes. Both compound types have served as moieties of synthetic drugs, *e.g.*, analgesics (2) and antineoplastic agents (3). Quaternary *N*-alkyl salt derivatives of the two bases appeared attractive as potential antimicrobials; in addition to the microbial cell wall degradative capability of the widely used cationic quaternary ammonium compounds (4), such novel products could have a possible mechanism for formaldehyde release initiated by Hofmann elimination. The inhibitory action of some preservatives, disinfectants, and antiseptics such as 1,3,5-trisubstituted hexahydro-*s*-triazines and methenamine compounds is considered attributable in part to a latent source of formaldehyde (5).

RESULTS AND DISCUSSION[1]

Thirteen quaternary salts of the tetrahydropyridine (Table I) were synthesized by alkylation with C-1–C-18 alkyl halides, decamethylene

[1] Tests for biological activities were carried out by divisions of Merck & Co., Rahway, N.J.

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