Effect of Edetate Disodium on Goldfish Membrane

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Abstract □ The effect of edetate disodium on the absorption of secobarbital sodium was examined in goldfish. An inverse relationship was observed between the concentration of edetate disodium to which fish were exposed and the time of death induced by secobarbital sodium. This inverse relationship is postulated to be due to an enhanced absorption of the ionized form of secobarbital in goldfish. There appears to be a period of time after exposure of the fish to the chelating agent in which the enhanced permeability of the membrane is absent in most studies. However, once the enhanced permeability effect is obtained, it is present 24 hr after exposure.

Keyphrases □ Edetate disodium—effect on absorption of secobarbital sodium in goldfish □ Secobarbital sodium—absorption in goldfish, effect of edetate disodium □ Permeability—effect of edetate disodium on goldfish membrane

Edetic acid is a sequestering agent capable of forming chelates with alkaline earth and heavy metal ions. Tidball (1) reported a 10-fold increase in the absorption of phenolsulfonphthalein (phenol red) in rats in the presence of edetic acid. Enhanced absorption of heparin and synthetic heparinoids from the GI tract of rats and dogs in the presence of edetic acid was observed (2). Feldman and Gibaldi (3) reported an ~20% increase in the transfer rate of salicylate across the everted rat intestine after exposure to edetic acid but found no apparent change in the transfer rate of salicylamide. They suggested that edetic acid may alter the rate-limiting barrier for water-soluble compounds but not for lipid-soluble compounds.

The purpose of this investigation was to examine the effect of edetate disodium on the permeability of the goldfish membrane. Levy and coworkers (4, 5) utilized goldfish to study the effects of surfactants on the absorption of alcohols and barbiturates. Nightingale *et al.* (6) used goldfish to assess structure–toxicity relationships of some substituted phenothiazines. Feldman *et al.* (7) used goldfish to study various local anesthetic agents and found an apparent relationship between the minimum effective concentration of drug to produce a response in goldfish and the minimum blocking concentration of these agents in isolated nerve or muscle fibers (8).

Therefore, it was of interest to examine the effects of edetate disodium on the goldfish membrane to determine if the permeability effects were similar to those reported previously (1–3) in other biological models.

EXPERIMENTAL

Concentration and Recovery Time Studies—Goldfish (*Carassius auratus*) (1–3 g) were placed in groups of five in tanks containing 2 liters of a solution of edetate disodium[1] at 0.1, 0.2, and 0.4 mg/ml. The solutions were made with glass-distilled[2] water and were adjusted to pH[3] 7.4 using 0.1 N NaOH or HCl. The fish were left in the solutions for 24 hr and then were placed for 30 min or 24 hr in recovery tanks containing 2 liters of glass-distilled water. Individual fish then were placed into beakers con-

taining 200 ml of 0.1 mM secobarbital sodium[4] in 0.05 M phosphate buffer (pH 7.4). Cessation of gill and mouth movements was noted, and the time to death was computed.

Controls were run for each group following the procedure for the edetate disodium-treated fish, except that the chelating agent was omitted from the treatment tank. All investigations were performed at 20 ± 2°.

pH Study—Five fish were placed in tanks containing 2 liters of 0.2 mg/ml edetate disodium in glass-distilled water. The solutions were adjusted to pH 7.4 using 0.1 N NaOH or 0.1 N HCl. The fish were left in the tanks for 24 hr and then were placed for 30 min or 24 hr in a recovery tank containing 2 liters of glass-distilled water. Individual fish were placed in 200 ml of a 0.1 mM secobarbital sodium solution buffered to pH 6.4, 7.4, or 8.4 with 0.05 M phosphate buffer. Cessation of gill and mouth movements was noted, and the time to death was computed.

Controls were run in the same manner as described previously. All experiments were performed at 20 ± 2°.

RESULTS AND DISCUSSION

The results of the concentration and recovery time study are summarized in Table I. Comparisons were made within each block using a Student t test. There was no significant difference between the treated animals and the controls for the 24-hr treatment, 30-min recovery study, except at the highest concentration of the chelating agent. In the 24-hr treatment, 24-hr recovery study, there was a significant difference between the treated and control fish at all concentrations of edetate disodium. If it is assumed that the amount of secobarbital necessary to produce death is the same for treated and control animals, edetate disodium apparently increases membrane permeability to the barbiturate, as indicated by the shorter times until death for the treated animals.

Although the reason is unknown, there appears to be a time period after exposure in which the enhanced permeability of the membrane is absent, except at the highest concentration (0.4 mg/ml) of the chelating agent. This conclusion is suggested by the fact that there was no significant difference between treated and control animals in the 24-hr treatment, 30-min recovery study for edetate disodium at 0.1 and 0.2 mg/ml, while a significant difference was found for the 24-hr treatment, 24-hr recovery study. The fact that the apparent increased permeability of the membrane, once established, exists 24 hr after exposure suggests that the effect on membrane permeability is relatively long, at least in the system investigated.

It was of interest to determine whether a relationship exists between the concentration of the chelating agent and the increased membrane permeability. Since the condition of the fish employed may have varied, an analysis of variance was performed on the control fish for each concentration of the chelating agent used in the 24-hr treatment, 24-hr recovery study. The results indicated that a significant difference existed between the three control groups ($p < 0.05$). An analysis of the means using the least significant difference method indicated that the control run with the fish treated with 0.2-mg/ml edetate disodium was significantly different ($p < 0.05$) from the controls run with the fish treated with 0.1- and 0.4-mg/ml edetate disodium. A comparison of the means for the fish treated with 0.1- and 0.4-mg/ml edetate disodium using a Student t test showed a significant difference ($p < 0.05$) between the two means. Therefore, the higher the concentration of chelating agent to which the fish are exposed, the greater is the apparent increase in permeability.

Studies also were performed in which the fish were treated with 0.2-mg/ml edetate disodium solutions and then exposed to secobarbital sodium solutions buffered to pH 6.4, 7.4, and 8.4 (Table II). There again appeared to be a period of time after exposure of the fish to the chelating agent in which the enhanced permeability of the membrane was absent. This effect was evident in the 24-hr treatment, 30-min recovery study, in which no significant difference was observed between the treated and the control groups. With the 24-hr treatment, 24-hr control, there was

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Table I—Effect of Concentration of Edetate Disodium and Recovery Time on Time of Death of Goldfish in 0.1 mM Secobarbital Sodium [a]

| | Concentration of Edetate Disodium | | | | | |
| | 0.1 mg/ml | | 0.2 mg/ml | | 0.4 mg/ml | |
	Mean Time ± SD, min	Significance	Mean Time ± SD, min	Significance	Mean Time ± SD, min	Significance
Treatment [b]	16.56 ± 6.63	NS [c]	20.21 ± 3.74	NS	17.66 ± 2.23	$p < 0.05$
Control [d]	18.93 ± 8.51		23.89 ± 5.80		21.09 ± 4.68	
Treatment [e]	18.88 ± 3.85	$p < 0.05$	23.03 ± 1.79	$p < 0.05$	14.26 ± 3.16	$p < 0.05$
Control [f]	25.07 ± 5.88		32.27 ± 8.07		20.99 ± 2.77	

[a] Results given are for 10 determinations. [b] 24-hr treatment, 30-min recovery. [c] Not significant ($p > 0.05$). [d] 24-hr blank, 30-min recovery. [e] 24-hr treatment, 24-hr recovery. [f] 24-hr blank, 24-hr recovery.

Table II—Effect of pH and Recovery Time on Time of Death of Goldfish Exposed to 0.2-mg/ml Edetate Disodium [a]

| | pH 6.4 | | pH 7.4 | | pH 8.4 | |
	Mean Time ± SD, min	Significance	Mean Time ± SD, min	Significance	Mean Time ± SD, min	Significance
Treatment [b]	14.88 ± 0.99	NS [c]	20.21 ± 3.74	NS	52.29 ± 9.70	NS
Control [d]	13.49 ± 2.51		23.89 ± 5.80		62.30 ± 16.93	
Treatment [e]	18.73 ± 5.92	NS	23.03 ± 1.79	$p < 0.05$	58.83 ± 17.31	$p < 0.05$
Control [f]	15.80 ± 3.51		32.27 ± 8.07		97.84 ± 22.19	

[a] Results given are for 10 determinations except for the 24-hr blank, 24-hr recovery study at pH 6.4, for which the results of five determinations are given. [b] 24-hr treatment, 30-min recovery. [c] Not significant ($p > 0.05$). [d] 24-hr blank, 30-min recovery. [e] 24-hr treatment, 24-hr recovery. [f] 24-hr blank, 24-hr recovery.

a significant difference between the treated fish and the controls for secobarbital sodium at pH 7.4 and 8.4. However, there was no difference between the treated fish and the controls for the secobarbital sodium solution buffered at pH 6.4.

Since secobarbital is a weak acid with a pKa of 7.9, it exists ~3, ~24, and ~76% ionized at pH 6.4, 7.4, and 8.4, respectively. Thus, the chelating agent apparently affected the transport of the ionized barbiturate form. However, the increase in the transport rate of the ionized form apparently was not enough to compensate for the decrease in the unionized concentration as the pH of the secobarbital sodium solutions increased. This result was evident from the apparent increase in time necessary to produce death as the pH of the secobarbital sodium solutions increased.

With a model proposed by Levy and Gucinski (9) (see *Appendix*) for the absorption of both the unionized and ionized drug forms in goldfish, a plot of the reciprocal of the response time ($1/t$) as a function of the fraction of drug unionized should be linear with a slope equal to $K_b(K_u - K_i)$ and an intercept equal to $K_b K_i$:

$$1/t = K_b K_i + K_b(K_u - K_i)f \qquad \text{(Eq. 1)}$$

where K_b is a constant equal to the quotient of the secobarbital concentration to which the fish were exposed divided by the amount of barbiturate in the fish at death and K_i and K_u are the first-order rate constants for the ionized and unionized forms of the drug. Figure 1 is the least-squares regression of the reciprocal of the response time *versus* the fraction of drug unionized for the 24-hr treatment, 24-hr recovery and the 24-hr treatment, 30-min recovery studies. The regression lines are $1/t = 0.0050 + 0.0500f$ ($r = 0.9999$) and $1/t = 0.0029 + 0.0644f$ ($r = 0.9960$), respectively.

Dividing the slope by the intercept yields:

$$\frac{\text{slope}}{\text{intercept}} = \frac{K_b(K_u - K_i)}{K_b K_i} = \frac{K_u}{K_i} - 1 \qquad \text{(Eq. 2)}$$

For the 24-hr treatment, 24-hr recovery study, Eq. 2 yields:

$$11 = K_u/K_i \qquad \text{(Eq. 3)}$$

For the 24-hr treatment, 30-min recovery, it yields:

$$23.3 = K_u/K_i \qquad \text{(Eq. 4)}$$

A rather dramatic change in the magnitude of the unionized to ionized ratio is observed between the two studies. With the assumption that the amount of drug in the fish is the same at the end-point:

$$\frac{\text{intercept 1}}{\text{intercept 2}} = \frac{\dfrac{C}{\text{amount}} K_i}{\dfrac{C}{\text{amount}} K_i} = \frac{K_{i1}}{K_{i2}} = 1.7 \qquad \text{(Eq. 5)}$$

Thus, there appears to be an ~70% increase in the rate constant for the

ionized drug in the 24-hr treatment, 24-hr recovery study as compared to the 24-hr treatment, 30-min recovery study. Substituting the apparent increase in the rate constant for the absorption of the ionized form into Eq. 2 for the 24-hr treatment, 24-hr recovery study yields $11 = K_u/1.7$ K_i or $18.7 = K_u/K_i$. Although the apparent increase of the ionized rate constant obtained from the intercept values does not explain completely the observed ratio for the 24-hr treatment, 30-min recovery study ($K_u/K_i = 23.3$), it does account for ~80% of the value.

Unfortunately, the controls for both recovery time studies do not appear to fit the model. Although the exact reason for this result is unknown,
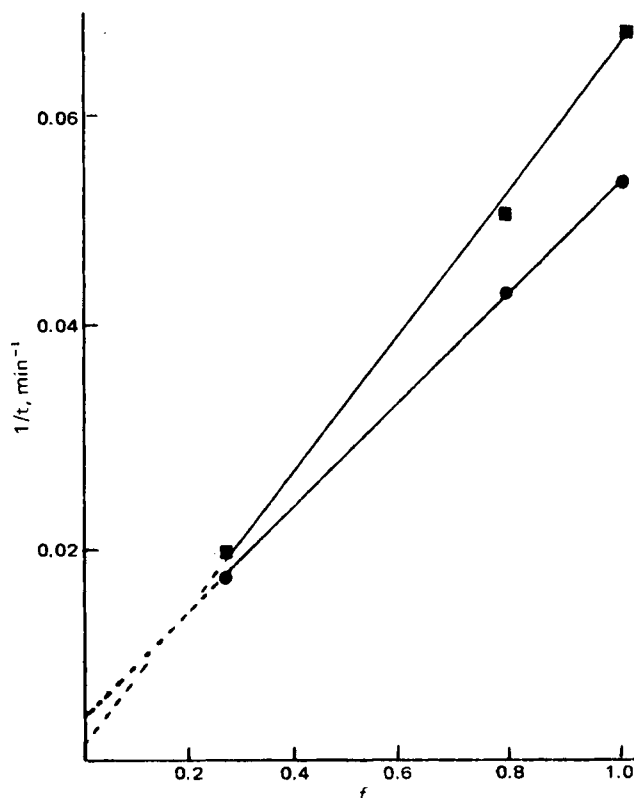

Figure 1—*Plot of the reciprocal of the time of death of goldfish versus the fraction of secobarbital unionized for the 24-hr treatment, 24-hr recovery study (●) and the 24-hr treatment, 30-min recovery study (■).*

in the experiment by Levy and Gucinski (9) the fish exposed to seco-barbital apparently did fit the model, which led these investigators to conclude that secobarbital was absorbed in both the unionized and ionized forms. For the most part, that experiment was similar to the controls in this experiment.

In summary, there appears to be a period of time after exposure of fish to the chelating agent in which the enhanced permeability of the membrane to the barbiturate is absent. However, once the effect is obtained, it is present 24 hr after exposure. The chelating agent appears to affect the transport of the ionized drug species, but the increase in the transport of the ionized form is not enough to compensate for the reduction of the unionized concentration in the pH study. There also appears to be a direct relationship between the change in permeability and the concentration of chelating agent to which the fish are exposed. Further studies are underway concerning the effects of edetate disodium on the goldfish membrane.

APPENDIX

The model proposed by Levy and Gucinski (9) concerning the absorption of both the unionized and ionized drug forms in the goldfish is:

$$R = K_i C_i + K_u C_u \qquad \text{(Eq. A1)}$$

where R is the absorption rate, K_i and K_u are the first-order rate constants, and C_i and C_u are the concentrations of the ionized and unionized species, respectively. The amount of drug in the fish is equal to the product of the absorption rate and the time the fish is exposed to the bathing solution:

$$\text{amount} = Rt \qquad \text{(Eq. A2)}$$

Substituting Eq. A1 for R in Eq. A2 yields:

$$\text{amount} = (K_i C_i + K_u C_u)t \qquad \text{(Eq. A3)}$$

which is equivalent to:

$$1/t = \frac{K_i C_i + K_u C_u}{\text{amount}} \qquad \text{(Eq. A4)}$$

Since the sum of C_i and C_u represents the total drug concentration, which was constant for these experiments, then:

$$1/t = \frac{C}{\text{amount}} [K_i(1 - f) + K_u f] \qquad \text{(Eq. A5)}$$

where C represents the total concentration of drug and f and $1 - f$ represent the fraction of the drug present in the unionized and ionized forms, respectively. Therefore:

$$1/t = \frac{C}{\text{amount}} (K_i - K_i f + K_u f) \qquad \text{(Eq. A6)}$$

and:

$$1/t = \frac{C K_i}{\text{amount}} + \frac{C(K_u - K_i)f}{\text{amount}} \qquad \text{(Eq. A7)}$$

If it is assumed that the amount of drug necessary to produce death is constant regardless of the pH employed:

$$1/t = K_b K_i + K_b(K_u - K_i)f \qquad \text{(Eq. A8)}$$

where K_b is a constant and equals C/amount. Therefore, a plot of the reciprocal of the response time $(1/t)$ as a function of the fraction of drug unionized should be linear with a slope equal to $K_b(K_u - K_i)$ and an intercept equal to $K_b K_i$.

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Stability of Apomorphine in Solutions Containing Ascorbic Acid and Bisulfite and Effects of Antioxidants on Apomorphine-Induced Cage Climbing and Hypothermia in Mice

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Abstract □ Ascorbic acid (100 mg/ml) and sodium bisulfite (0.5 and 20 mg/ml) prevented more than 10% oxidation of apomorphine hydrochloride in water maintained at room temperature over 1–3 days. Refrigeration at 5° prevented oxidation of apomorphine hydrochloride in aqueous solutions for 1 week. Neither ascorbic acid nor sodium bisulfite affected murine stereotyped cage climbing or hypothermia induced by apomorphine.

Keyphrases □ Apomorphine—stability in solutions containing ascorbic acid and sodium bisulfite, effects of antioxidants on apomorphine-induced cage climbing and hypothermia in mice □ Antioxidants—ascorbic acid and sodium bisulfite, stability of apomorphine in solutions containing antioxidants, effects of antioxidants on apomorphine-induced cage climbing and hypothermia in mice □ Stability—apomorphine in solutions containing ascorbic acid and sodium bisulfite

Current interest in apomorphine (I) stems from its activity as a dopaminergic agonist and its consequent antiparkinsonian activity (1–3). Its clinical unity has been demonstrated when used alone and in combination with other agents such as levodopa (4). In addition to parkinsonism, recent studies indicated potential new uses for I