Comparative Depressant Activity and Interaction of Ethanol and Pentobarbital in Goldfish

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Abstract □ Overturn as produced in goldfish by various concentrations of ethanol and/or sodium pentobarbital was studied in tromethamine-buffered solutions at pH 7.1 and a solution temperature of 23°. The results indicate that ethanol and pentobarbital interact in an additive manner to produce overturn. Tolerance or cross-tolerance was not observed, with one exception, when fish maintained in low concentrations of ethanol or pentobarbital for 48 hr. were tested against two concentrations of the same drug or the opposite drug. Determination of the ratio of death time/overturn time for the two agents indicates a greater margin of safety for ethanol than for pentobarbital.

Keyphrases □ Goldfish—comparative depressant (overturn) activity of ethanol and pentobarbital in tromethamine □ Ethanol depressant activity—goldfish overturn, compared to pentobarbital □ Pentobarbital, sodium, depressant activity—goldfish overturn, compared to ethanol □ CNS depression—ethanol and pentobarbital in goldfish

Numerous reports concerned with the CNS depressant effect of ethanol or barbiturates on goldfish have appeared in the recent literature (1–4). However, the nature of the pharmacological relationship existing with combinations of the two types of agents in this species has not been reported. It is widely believed among the lay and professional public alike that ethanol and barbiturates possess supraadditive CNS depressant effects when both are present in a biological system. Support for a supraadditive interaction has appeared in the literature (5–8); however, the results of other studies indicate that only an additive relationship exists between the two types of CNS depressants (9–12).

The primary purpose of this investigation was to determine the nature of the pharmacological interaction of ethanol with a short-acting barbiturate, pentobarbital, utilizing overturn as an end-point indicative of CNS depression in the goldfish. Secondary objectives were to study tolerance and margin of safety as additional comparative characteristics of the two drugs in this species.

METHODS

A population of 120 goldfish, *Carassius auratus*, purchased in a single lot from a local pet supplier and weighing 3.5–7 g., was employed in this study. The fish were housed in tanks of aerated tap water thermostatically controlled at 23°. Overturn time and death time determinations similar to those employed by other workers (2–4, 13) were utilized as end-points for quantitating pharmacological activity. Overturn was judged to have occurred when one could turn the fish completely over with a glass rod without resistance; cessation of mouth, gill, and fin movements was considered indicative of death.

All experiments were conducted at 23° in 0.05 *M* tromethamine buffer[1] adjusted with hydrochloric acid to pH 7.1. Preliminary experiments and the results from other studies (1, 2) prove that tromethamine is pharmacologically inert in such a system. A pH of 7.1 was selected because pentobarbital (pKa 8.1) exists 90% in the readily absorbable unionized form at this pH. The activity of ethanol, a nonionizable substance, has been shown to be unaffected by pH changes (1).

Experiments were performed by placing individual fish in 200 ml. of the test solution in 250-ml. capacity beakers until overturn or death ensued. Following overturn time determinations, the fish were placed in a recovery tank. All test solutions were used only once, and all end-point determinations were made by the same individual. Although overturn-tested fish were subsequently reused, all fish were allowed a minimum of 72 hr. for recovery; the results of Gibaldi and Nightingale (4) indicate that a recovery period of 18 hr. is adequate.

The investigation was divided into three phases: (*a*) interaction studies, (*b*) tolerance studies, and (*c*) toxicity studies.

Pharmacological Interaction between Ethanol and Pentobarbital—The method employed to determine the nature of the interaction between ethanol and pentobarbital was suggested by Gaddum (14) and is based upon the extensive work of Loewe (15).

By applying this method, overturn times produced by selected doses (concentrations) of ethanol and pentobarbital alone were compared with the overturn times produced by combining one-half of the selected dose of each drug. The concentrations selected were taken from two activity levels on the experimentally determined dose-activity plots (Figs. 1 and 2). With both drugs the concentrations predicted to provide depressant activity represented by overturn times of 10 min. (1/overturn time = 0.1) and 20 min. (1/overturn time = 0.05) were utilized. Actual overturn times for these concentrations were then experimentally determined, and the results were averaged for ethanol and pentobarbital at each of the

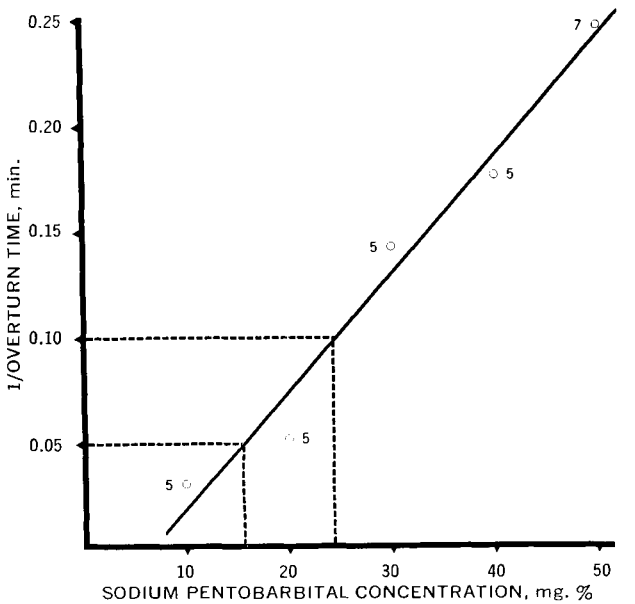

Figure 1—*Reciprocal plot of overturn time (see Table I) as a function of pentobarbital concentration (pH 7.1, temperature 23°). This plot was utilized to obtain the concentrations of pentobarbital predicted to provide the levels of activity (overturn time) selected for the pentobarbital–ethanol interaction study. Key: predicted overturn time concentration—10 min., 24.5 mg.%; and 20 min., 16.0 mg.%. ○ = mean.*

[1] Fisher Scientific Co.

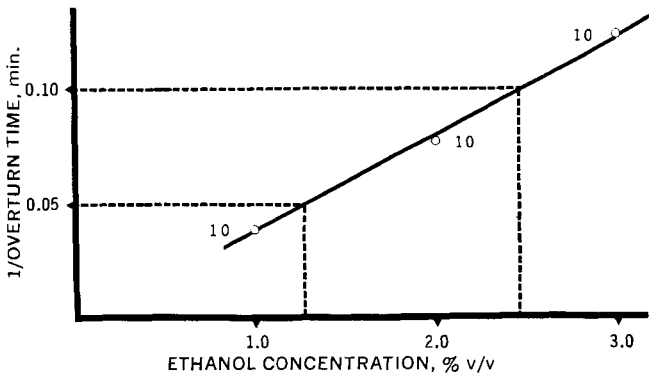

Figure 2—*Reciprocal plot of overturn time (see Table I) as a function of ethanol concentration (pH 7.1, temperature 23°). This plot was utilized to obtain the concentrations of ethanol predicted to provide the levels of activity (overturn time) selected for the pentobarbital–ethanol interaction study. Key: predicted overturn time concentration—10 min., 2.5%; and 20 min., 1.3%. O = mean.*

two points. These values, which were essentially identical to the predicted values, served as controls for the overturn times produced by the drug mixtures. Comparisons were made employing Student's *t* test, with a *p* value of 0.05 or less accepted as indicative of real difference.

If the overturn time produced by the ethanol–pentobarbital mixture was shown to be shorter than the average of the two drugs alone, one could state that a supraadditive drug interaction was involved; if the effect did not differ from control, then an additive relationship was present; and if the overturn time of the drug combination was longer than the average of the two drugs alone, then some form of negative synergism or antagonism was demonstrated.

Tolerance Studies—These studies utilized 48–52 hr. of exposure of fish to either 0.1% ethanol or 1 mg./100 ml. pentobarbital concentration in 0.02 *M* tromethamine buffer adjusted to pH 7.1. Preliminary experiments indicated that no discernible depression was produced by these concentrations whereas slightly higher concentrations, while not producing overturn, did produce a state of sluggishness. Following the 2-day exposure period, the fish were then tested against one of two doses of either ethanol or pentobarbital to ascertain whether such exposure would produce tolerance and/or cross-tolerance.

Toxicity Studies—These studies were performed to determine the ratio of death time/overturn time for each of the two drugs. Overturn and death were produced at three dose levels with each of the drugs, and an average ratio for each drug was calculated.

RESULTS AND DISCUSSION

Initial experiments were conducted to determine dose–activity plots with both test drugs. With pentobarbital (Fig. 1) and with ethanol (Fig. 2), relatively linear relationships were established when the concentration of each drug was plotted against the reciprocal of the overturn time produced. These results and those in Table I closely resemble those obtained by other workers (4, 13); the slight differences are probably due to difference in the pH or temperature of the drug solutions or to the exact nature of the end-point employed. Several other groups of fish were exposed to higher concentrations of ethanol, and it was observed that concentrations greater than 4% produced overturn more rapidly than would be predicted by extrapolation of the results obtained with 1, 2, and 3% ethanol. Similar modification of the ethanol dose–activity plot was reported by Gibaldi and Nightingale (4).

Table I—Overturn Times Produced by Progressive Concentrations of Either Sodium Pentobarbital or Ethanol

Drug and Concentration	Number of Fish	Overturn Time ± *SE*, min.	1/Overturn Time
Pentobarbital:			
10 mg.%	5	33.04 ± 2.05	0.0302
20 mg.%	5	19.21 ± 0.80	0.0521
30 mg.%	5	6.99 ± 0.24	0.1430
40 mg.%	5	5.68 ± 0.24	0.1764
50 mg.%	5	4.06 ± 0.48	0.2463
Ethanol:			
1%	10	25.44 ± 2.10	0.0393
2%	10	12.93 ± 0.72	0.0774
3%	10	8.10 ± 0.39	0.1231

Table II—Comparison of Overturn Times Produced by Pentobarbital or Ethanol Alone with Those Produced by Predicted Equipotent Combinations of the Two Drugs

Drug and Concentration	Number of Fish	Predicted Overturn Time, min.	Actual Overturn Time ± *SE*, min.
High dose levels:			
A. Pentobarbital, 24.5 mg.%	10	10.0	9.77 ± 0.57
B. Ethanol, 2.5%	10	10.0	9.88 ± 0.72
C. Average of A and B	20	10.0	9.82 ± 0.45
D. Pentobarbital, 12.25 mg.%, plus ethanol, 1.25%	10	10.0	8.84 ± 0.54
t test for difference between C and D: $p > 0.10$ (n.s.)			
Low dose levels:			
E. Pentobarbital, 16.0 mg.%	10	20.0	18.25 ± 1.45
F. Ethanol, 1.3%	10	20.0	19.26 ± 1.68
G. Average of E and F	20	20.0	18.75 ± 1.08
H. Pentobarbital, 8.0 mg.%, plus ethanol, 0.65%	10	20.0	16.97 ± 1.21
t test for difference between G and H: $p > 0.10$ (n.s.)			

The primary purpose of the dose–activity plots was to allow selection of equipotent concentrations of the two drugs for utilization in the ethanol–pentobarbital interaction phase of the investigation. From these plots, an overturn time of 10 min. (1/overturn time = 0.1) was predicted for 2.5% ethanol or 24.5 mg.% pentobarbital, and an overturn time of 20 min. (1/overturn time = 0.05) was predicted for 1.3% ethanol or 16.0 mg.% pentobarbital (Figs. 1 and 2). Each of the four concentrations was then tested, and the resulting overturn times were found to be close to the predicted value. When the predicted 10-min. overturn time concentrations of ethanol and pentobarbital were averaged, a value of 9.82 min. resulted; a value of 18.75 min. was obtained for the average of the two concentrations predicted to provide the 20-min. overturn time (Table II). As can be seen, the results obtained using mixtures of one-half the concentration of each drug provided overturn times that were not significantly different from the average of the two drugs used separately. Consequently, it appears that, in this study, ethanol and pentobarbital demonstrate an additive relationship in terms of their pharmacological characteristics responsible for overturn production (presumably CNS depression).

Our conclusion is in agreement with that of Graham (11), who found that the combinations of ethanol and pentobarbital required to produce 50% lethality in mice followed an isobole constructed between the LD_{50} dose of either drug alone, thus indicating an additive interaction. In a recent study, Gebhart *et al.* (12) employed combinations of one-half paralyzing doses ($^1/_2$ PD_{50}) of ethanol and phenobarbital in mice and were able to demonstrate only an additive relationship between the two agents. As mentioned in the review by Veldstra (10), earlier workers who reported supraadditive interactions probably did so because of experimental designs that did not include appropriate selection of doses nor a consideration of the drugs' varying onsets of activity and peak times of effect.

Table III presents the results obtained when fish that had been maintained for 2 days in low concentrations of either ethanol or pentobarbital were then subjected to overturn time determinations. Tolerance was observed only in the experimental group exposed to ethanol and tested against the low concentration of ethanol. In no case could cross-tolerance (from ethanol to pentobarbital or vice versa) be demonstrated. Indeed, the pentobarbital-exposed fish tested against 16 mg.% pentobarbital were shown to overturn sooner (higher reciprocal value) than the control fish. However, one should recognize the fact that this apparent sensitivity may be explained by a partial, undetectable response of the fish to the maintenance concentration of pentobarbital and may not represent true sensitivity.

Table III—Overturn Time Determinations in Fish Maintained in Solutions of Sodium Pentobarbital or Ethanol for 48 hr.

Treatment	Agent and Concentration Employed to Produce Overturn[a]			
	Sodium Pentobarbital		Ethanol	
	24.5 mg.%	16 mg.%	2.5%	1.3%
Control	10.40 ± 0.53 (20)	18.40 ± 0.89 (20)	9.80 ± 0.50 (20)	19.26 ± 1.68 (10)
Pentobarbital exposed[b]	11.92 ± 1.00 (10)	13.51 ± 1.02[c] (10)	8.57 ± 0.57 (10)	18.32 ± 1.13 (8)
Ethanol exposed[d]	10.67 ± 0.54 (9)	17.20 ± 1.15 (10)	10.93 ± 0.96 (10)	25.28 ± 1.70[c] (10)

[a] All results are expressed as overturn time, in minutes, ± *SE* (number of fish). [b] 48 hr. in sodium pentobarbital, 1 mg./100 ml. of 0.02 *M* tromethamine. [c] Significantly different from control value at 0.05 level. [d] 48 hr. in ethanol, 0.1% v/v in 0.02 *M* tromethamine.

The fact that tolerance was not demonstrated, with one exception, under the conditions employed does not preclude the possibility that tolerance or cross-tolerance could be shown if more prolonged exposure periods or higher exposure concentrations of the two drugs were employed.

The lack of apparent pharmacological effect at low concentrations of ethanol (0.1%) and pentobarbital (1 mg.%) and the fact that the plots shown in Figs. 1 and 2 cannot be extrapolated through the origin indicate that a critical concentration must be exceeded if a pharmacological effect is to be elicited. This finding is in agreement with the work of Nightingale and Gibaldi (16) and Nightingale (17).

Although a study of recovery time following overturn with the two drugs was not an objective of this investigation, it was consistently observed that the ethanol-treated fish recovered more rapidly from overturn than did the pentobarbital-treated fish and that many more inadvertent deaths occurred in the pentobarbital-treated groups during the recovery periods. For these reasons, a study was undertaken to determine the margin of safety between the exposure time required to produce overturn and that required for death with several concentrations of each drug. This margin was quantitated as the ratio between death time and overturn and as such is similar to the standard pharmacological calculation of a "therapeutic index" (18). Overturn could be considered analogous to the loss of righting end-point employed to study CNS depressants in laboratory mammals.

As seen in Table IV, the margin of safety (death time/overturn time ratio) is consistently greater with ethanol than with pentobarbital or, stated in another manner, the pentobarbital fish are nearer death at a given level of CNS depression (overturn) than are the ethanol fish. Notice that at equivalent overturn time concentrations (24.5 mg.% for pentobarbital, 2.5% for ethanol) it was impossible to kill all of the fish with ethanol and those that did die survived approximately 40% longer than did pentobarbital fish which turned over at approximately the same time. Efforts were also made to obtain a death time/overturn time ratio with a 1.3% concentration of ethanol, but no fish died in the 6-hr. observation period, although overturn was produced at approximately the same point in time as with pentobarbital, 16 mg.%, which did produce 100% fatalities.

Table IV—Determination of Ratio of Death Time/Overturn Time for Pentobarbital and Ethanol in the Goldfish

Drug and Concentration	Overturn Time[a]	Death Time[a]	Death Time/ Overturn Time
Pentobarbital:			
16.0 mg.%	18.55 (10)	84.12 (10)	4.54
24.5 mg.%	11.03 (10)	50.77 (10)	4.54
33.5 mg.%	8.35 (10)	40.33 (10)	5.20
	Average of three dose levels = 4.76		
Ethanol:			
2.5%	9.73 (10)	71.38(7[b])	7.35
3.7%	4.43 (10)	41.30 (10)	9.30
4.9%	3.16 (10)	20.57 (10)	6.50
	Average of three dose levels = 7.72		

[a] Results expressed in minutes (number of fish). [b] Three fish did not die (6-hr. observation).

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