Keyphrases

Chlordan pretreatment—drug metabolism
Cyclophosphamide metabolism—chlordan effect
Hexobarbital metabolism—chlordan effect
LD_{50} values—cyclophosphamide and chlordan pretreated
Colorimetric analysis—spectrophotometer

Bile Salt Potentiation of Pharmacologic Effects and Drug Uptake in Goldfish

By MILO GIBALDI and CHARLES H. NIGHTINGALE

Concentrations of 1×10^{-4} *M* and 2×10^{-4} *M* sodium taurodeoxycholate (STDC), which demonstrate no intrinsic pharmacologic activity, significantly potentiate the pharmacologic effects (*i.e.*, time required to produce overturn) of pentobarbital and ethanol in goldfish. Quantitatively similar potentiation is noted when the fish is exposed to a bile salt-drug solution or when the fish is pretreated with STDC, rinsed, and immediately exposed to drug solution. The present results suggest that potentiation is independent of the duration of pretreatment of fish with STDC. The effects of STDC are mediated very quickly, reach a maximum level within minutes, and are slowly reversible. The results are consistent with a mechanism involving adsorption of bile salt molecules on the biologic membrane, consequent alteration of membrane permeability, and a resultant decrease in the time required to observe a pharmacologic response to ethanol and pentobarbital. A 1×10^{-4} *M* STDC solution also increases significantly the absorption of 4-aminoantipyrine in goldfish.

BILE SALTS are one of the most important groups of physiologic surface-active agents found in man. Although their role in fat absorption has been studied extensively (1, 2), little attention has been given to the possible effects of bile salts on drug absorption. Recent studies (3–6) have shown that components of bile significantly affect both the solubility and dissolution rate of a number of poorly water-soluble drugs. These studies suggest that bile may serve an important function in dissolution rate-limited drug absorption.

In addition to the solubilizing effects of bile salts, certain biologic effects may also be important in drug absorption. An example is the potential effects of bile salts on the gastrointestinal membrane and on drug transport. Parkinson (7) has observed inhibition of glucose, sodium, and amino acid active transport from the rat jejunum by certain bile salts. However, there is no literature available on the effects of bile salts on passive transport, the usual pathway in drug absorption.

Numerous studies suggest that surface-active agents can affect the integrity of biologic membranes and thereby enhance drug absorption (8). Most recently, Levy *et al.* (9) have observed a significant decrease in the time required for secobarbital-induced death in the goldfish in the presence of polysorbate 80. They attribute this effect to membrane alteration by the surfactant which permits more rapid absorption of secobarbital and decreases the time required to reach a lethal concentration in the fish.

The present study concerns the influence of bile salts on the pharmacologic effects of ethanol and pentobarbital and on the uptake of 4-aminoantipyrine in the goldfish.

EXPERIMENTAL

Goldfish, *Carassius auratus*, common variety, weighing about 3–6 g. were used as the test animal. All fish used in a given experiment were from the same lot. Five different lots of fish were used during the study.

The test drugs, ethanol and sodium pentobarbital, were dissolved in water or aqueous solutions contain-

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ing 1×10^{-4} or 2×10^{-4} *M* sodium taurodeoxycholate (STDC). The final solutions were adjusted to pH 6.1 with either 1 *N* HCl or NaOH. Solutions were prepared daily as required. All drugs and chemicals were USP or reagent grade. Chromatographically pure STDC, obtained from Maybridge Research Chemicals, Tintagel, N. Cornwall, England, was used.

The measured pharmacologic response was the time required for the fish to lose the ability to maintain itself upright after immersion in test solution, *i.e.*, the overturn time. The use of the overturn end point for the estimation of absorption and elimination kinetics in goldfish was the subject of a previous report (10). After each experiment the pH of the solution was redetermined and found to be 6.1 ± 0.1. Preliminary experiments indicated that the overturn times in ethanol in water and in water adjusted to pH 6.1 were the same.

Overturn time was determined with individual fish immersed in 200 ml. of solution at 26–28°, under the following experimental conditions: (*a*) immersion of fish in drug solutions; (*b*) immersion of fish in bile salt solutions; (*c*) immersion of fish in drug solutions containing bile salt; and (*d*) exposure of fish to bile salt solution, followed by rinsing with water and immersion in drug solutions.

Determination of Uptake of 4-Aminoantipyrine—Goldfish were placed in large beakers containing 250 ml. of solution per fish. The control solutions contained 250 mg.% 4-aminoantipyrine (Eastman) while the test solutions contained the drug and 1×10^{-4} *M* STDC. In each case the solutions were adjusted to pH 6.1. After a 15-min. immersion period, the fish were removed rapidly from the solutions, rinsed, and killed by freezing. The fish were stored in a freezer until assayed.

The analytical method for the determination of 4-aminoantipyrine in goldfish tissue was a modification of the colorimetric assay of Brun (11) as described by Levy and Miller (12). Tissue blanks averaged 22 mcg. 4-aminoantipyrine equivalent per g. of fish tissue. Prior exposure of the fish to 1×10^{-4} *M* STDC for 15 min. yielded identical blank values. Recovery of known amounts of 4-aminoantipyrine (300 mcg./g. of fish) added to homogenates averaged 97.1% (4 determinations, range 96.2 to 97.6%). Recovery of known amounts of 4-aminoantipyrine (400 mcg./g. of fish) added to homogenates of fish which were previously immersed for 15 min. in 1×10^{-4} *M* STDC averaged 94.5% (4 determinations, range 88.5–97.7%). Blank and recovery values were used to correct the analytical results.

RESULTS

Initial studies indicated that STDC showed significant toxicity in the goldfish at concentrations of 1×10^{-3} *M* and above. However, concentrations of 1×10^{-4} *M* and 2×10^{-4} *M* were without apparent effect. Fish from various lots were placed in 2×10^{-4} *M* bile salt solution for 24–48 hr. periods and in no instance was an effect upon the fish observed. Hence, it was concluded that these concentrations of STDC had no apparent intrinsic pharmacologic activity and these levels were used for further drug studies.

The overturn times of goldfish in pentobarbital and ethanol solutions, and the effect of STDC on overturn time, are shown in Table I. A significant potention of the apparent drug activity is observed in each case. The decrease in overturn time appears to be related to the STDC concentration.

The data in Table II represent the results of a number of experiments designed to test the interaction of individual aspects of the bile salt-drug-goldfish system. The following conclusions may be drawn from these results: (*a*) After exposure to pentobarbital to induce overturn, the fish recovers completely within 24 hr. when placed in fresh water and its subsequent response to drug + bile salt is the same as that of fish having no prior exposure. (*b*) After exposure to pentobarbital + STDC to induce overturn, the fish recovers completely within 24 hr. when placed in fresh water and its subsequent response to drug is the same as that of fish having no prior exposure. (*c*) Prolonged exposure to STDC (without observing overturn), followed by rinsing with distilled water and exposure to pentobarbital, results in an overturn time significantly shorter than that observed in Groups A_1 and B_2. In fact, the overturn time of goldfish in 5 mg.% pentobarbital solution after a 24-hr. exposure to STDC is almost identical to the overturn time found when the fish were exposed to drug + STDC.

This latter phenomenon was explored further by investigating the effect of duration of exposure to bile salt on drug-induced overturn time. In each case the fish were exposed to 1×10^{-4} *M* STDC for a period of time, rinsed, placed in either 5 mg.% pentobarbital or 1% ethanol solutions and observed

TABLE II—REVERSIBILITY OF PHARMACOLOGIC EFFECT IN GOLDFISH AND INFLUENCE OF STDC PRETREATMENT ON RESPONSE[a]

Group	Overturn Time,[b] min.	S.E.	Significantly Different[c] ($p < .05$) from Experiment
A_1	18.2	0.42	A_2, B_1, C
A_2	10.4	0.78	A_1, B_2
B_1	9.2	0.50	A_1, B_2
B_2	16.6	2.55	A_2, B_1, C
C	8.8	0.20	A_1, B_2

[a] Group A—Fish exposed to drug (5 mg. % sodium pentobarbital) (A_1); recovered 24 hr.; reexposed to drug plus bile salt (1×10^{-4}*M* STDC) (A_2). Group B—Fish exposed to drug plus bile salt (B_1); recovered 24 hr.; reexposed to drug (B_2). Group C—Fish exposed to bile salt for 24 hr., rinsed and exposed to drug, overturn time determined (C). [b] Values represent mean of 4 fish. [c] Determined by Student *t* test.

TABLE I—EFFECT OF STDC ON APPARENT DRUG-INDUCED OVERTURN TIME (T_0) IN GOLDFISH[a]

STDC, *M* Concn.	Ethanol (1.5% v/v) T_0 (min.)	S.E.	Significance of Difference[b]	Sod. Pentobarbital (5 mg.%) T_0 (min.)	S.E.	Significance of Difference[b]
Control	20.8	1.44	←	18.4	1.06	←
1×10^{-4}	16.0	0.64	←	14.9	1.39	←
2×10^{-4}	14.8	0.97		13.4	0.99	

[a] Values represent mean of 4 fish. [b] Determined by Student *t* test, arrows indicate significance at $p < 0.05$.

TABLE III—EFFECT OF DURATION OF EXPOSURE OF GOLDFISH TO 1×10^{-4} *M* STDC ON DRUG-INDUCED T_0 (MIN.)[a]

Exposure Time, min.	Ethanol (1% v/v) T_0	Ethanol (1% v/v) S.E.	Ethanol (1% v/v) Significance of Difference[b]	Pentobarbital (5 mg. %) T_0	Pentobarbital (5 mg. %) S.E.	Pentobarbital (5 mg. %) Significance of Difference[b]
0[c]	11.0	0.38	← ←	15.0	1.04	← ←
5	4.4	0.26	←	3.9	0.21	←
30	4.1	0.81	←	—	—	
60	—	—		4.9	0.28	←

[a] Values represent mean of 4 fish. [b] Determined by Student *t* test, arrows indicate significance at $p < 0.01$. [c] Control values determined by placing fish in distilled water (adjusted to pH 6.1) for 30 min. The fish were then rinsed and exposed to drug solutions.

for overturn. The results, shown in Table III, indicate that the potentiation of drug-induced overturn is independent of the duration of exposure to STDC. For example, 5-min. exposure to STDC reduces the apparent ethanol-induced overturn time to the same extent as exposure for a 30-min. period. Similar results are observed with pentobarbital.

Figure 1 shows the effect of 1×10^{-4} *M* STDC on the uptake of 4-aminoantipyrine by goldfish after a 15-min. immersion in 250 mg.% drug solution. The presence of bile salt results in a 30% increase in drug concentration in the fish tissue as compared to control values. This difference is statistically significant at the 95% level of confidence. These findings indicate clearly the ability of bile salts to modify membrane permeability.

DISCUSSION

The enhanced response of goldfish to ethanol and pentobarbital may be due to two effects. Firstly, STDC may produce an unspecified pharmacologic effect that is inadequate to promote overturn but makes the fish more susceptible to the pharmacologic action of ethanol and pentobarbital. The second possibility is that the bile salt increases the permeability of the fish membranes to the drugs and permits more rapid absorption. While both effects may be operative, the present findings are consistent with the hypothesis that the principal effect of STDC is alteration of membrane permeability.

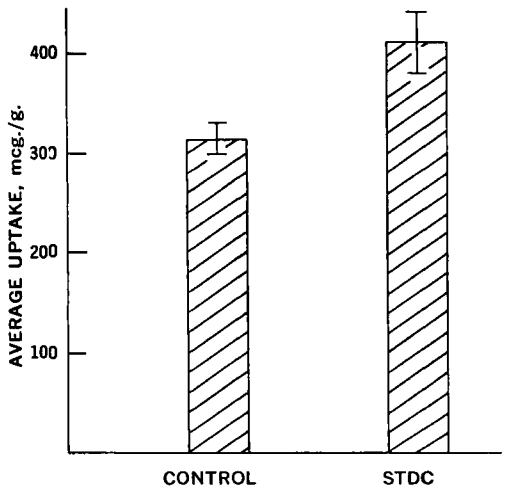

Fig. 1—Effect of 1 × 10⁻⁴ M STDC on the uptake of 4-aminoantipyrine by goldfish immersed in 250 mg.% drug solution. Each bar represents the mean of seven fish; vertical lines indicate standard error of the mean.

For example, if STDC potentiates drug effects in the goldfish by virtue of an intrinsic pharmacologic effect then when fish are first exposed to bile salt solution and immediately exposed to drug solution, one would anticipate a graded response to the drug, which is dependent on the time of exposure to the bilt salt. The data in Table III indicate that this is clearly not the case. The reduction in ethanol-induced and pentobarbital-induced overturn time is independent of the duration of preexposure to STDC solution.

Previous studies (9) of the effects of nonionic surfactants on drug absorption in the goldfish suggest an enhanced permeability to barbiturates but not to ethanol. In the present study this permeability discrimination was not observed. In fact, the observed potentiation was quantitatively similar for both ethanol and pentobarbital. This finding implies a possible difference in the mode of action of the nonionic polysorbate 80 and the anionic STDC.

The potentiation of drug effects in the absence of surfactant from the bulk drug solution, noted in the preexposure studies, has also been observed by Levy and Anello (13). These workers found that an absorption-enhancing effect occurs if the fish are first immersed in polysorbate 80 solution, washed, and then placed in a surfactant-free secobarbital solution. The rapid effects manifested by both polysorbate 80 and STDC are consistent with a mechanism involving adsorption of surfactant molecules on the biologic membrane and alteration of membrane permeability.

The use of the overturn end point rather than a death end point permitted the observation that the effect of STDC is reversible. As noted in Table II, fish exposed to STDC recover within 24 hr. in fresh water and then show the expected response to pentobarbital. Of particular interest is the fact that fish preexposed to 1×10^{-4} *M* STDC for 24 hr. gave the same enhanced response to pentobarbital as fish placed directly in an STDC-pentobarbital solution This suggests that the effect of STDC is mediated very quickly and is maximal within minutes. The persistence of potentiation even in the absence of STDC in the drug solution indicates that the effect of the bile salt is slowly reversible.

Studies with 4-aminoantipyrine suggest that extremely low concentrations of STDC can significantly alter membrane permeability. Levy (12) has previously shown that, under conditions similar to those used in the present study, 4-aminoantipyrine is absorbed by apparent zero-order kinetics. Accordingly the authors have determined an absorption rate of 21 mcg./g./min. in the absence of STDC and a value of 27 mcg./g./min. in the presence of the bile salt. The corresponding first-order absorption rate constants (k_a) were calculated from the relationship: k_a = absorption rate/drug concentration and

found to be 8.4 × 10^{-3} min.$^{-1}$ and 1.1 × 10^{-2} min.$^{-1}$, respectively.

The influence of bile salt on membrane permeability in the goldfish raises the interesting possibility of similar effects on the permeability of the intestinal membranes. The fluid bathing these membranes has significantly higher concentrations of bile salts (in the order of 10^{-2} M) than the concentrations employed in the present study. This possibility is now under investigation.

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Keyphrases

Bile salts—drug uptake, activity potentiation
Taurodeoxycholate, sodium—potentiating effect
Goldfish overturn time—activity analysis
4-Aminoantipyrine uptake—goldfish
Colorimetric analysis—uptake time

Synthesis of Tropine-Labeled Atropine IV

Labeling of the 2, 3, and 4 Positions of Atropine from Citric-^{14}C Acid

By THOMAS E. ELING, JOHN M. McOWEN, and GILBERT C. SCHMIDT*

Using a modification of the Robinson condensation, citric-3-^{14}C acid, citric-2,4-^{14}C acid, and citric-2,3,4-^{14}C acid were converted into correspondingly labeled tropine and atropine. The specific activities of the products varied from 0.62 to 1.0 depending on the labeled compound. This is the first reported syntheses of tropine or atropine labeled with ^{14}C in positions other than the *endo*-methyl group.

THE PROBLEM of selectively labeling the tropine moiety of atropine remains to be solved. For one reason or another, the biosynthetic compounds (1, 2), tritium-labeled atropine (3), and randomly labeled atropine (4) are inadequate for establishing the metabolic fate of the heterocyclic residue in atropine. Classical, microsynthetic methods for selectively labeling tropine and the tropine moiety of atropine are needed badly.

Excluding the authors' work, only Fodor (5) and Werner (6) have used classical methods to label tropine. In both instances, tropine-*N*-methyl-^{14}C and atropine-*N*-methyl-^{14}C were synthesized. Fodor's approach would not permit labeling of other positions, but Werner's use of the Robinson condensation is much more versatile. A lack of suitably labeled Robinson intermediates, other than methylamine hydrochloride, apparently prevented Werner from realizing the full potential of the condensation.

Prior to publication of Werner's work, the authors had been studying the Robinson condensation as a tool for labeling the carbon skeleton of tropine. The reaction was studied in detail, a reproducible microcondensation procedure developed, and the microesterification of tropine and tropic acid standardized (7). The overall procedure was checked by the synthesis of tropine-*N*-methyl-^{14}C and atropine-*N*-methyl-^{14}C, thereby confirming Werner's synthesis of these compounds (8). At the same time, a prototype procedure was developed for the microsynthesis of succindialdehyde and tropine from arabinose (9) which would be used to label the 1,5,6, and 7 positions of atropine.

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