sufficiently rapid permeation rates, needless to say, will exhibit no pressure or surfactant effects, and yield a maximum solubility dependence with low tortuosities. These characteristics apply very well to salicylic acid release in 0.05% AOT as shown by Table II and Fig. 9.

It appears that the techniques developed and presented here lend themselves very well to a definitive description and characterization of the particular matrix system one is dealing with, and in particular, permit the selection of the proper variables to alter in order to remedy nonideal matrix systems to provide the desired release profiles.

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Keyphrases

Drug release rates
Matrices-inert
Wetting effect–drug release
Surfactant effect–wetting
Contact angle measurements
Pressure effect–drug release
Diffusion coefficients
Permeation rates-solvent

Use of the Overturn End Point for the Estimation of Absorption and Elimination Kinetics in Goldfish

By MILO GIBALDI and CHARLES H. NIGHTINGALE

The advantages of overturn time as a pharmacologic end point for studies of biologic membrane permeation in goldfish are presented. Studies with both pentobarbital and ethanol showed good reproducibility. Redeterminations of overturn time after 18 hr. provided excellent agreement with initial determinations with both drugs. Evidence is presented to suggest a complex mechanism of action of ethanol in goldfish. A kinetic model is derived which relates overturn time and recovery time and permits the estimation of an elimination rate constant from pharmacologic response data alone.

FISH, particularly goldfish, have been used since 1867 as test animals for determining the toxicity of various substances, and a number of techniques have been developed for this purpose (1). A useful approach has been measurement of the time required to produce a well-marked stage of toxicity such as death.

Powers (2), after an extensive series of experiments with goldfish using a variety of drugs and chemicals, concluded that for every toxic substance, over a certain concentration range, the survival time is inversely related to the concentration. For many years the idea prevailed that the magnitude of the slope of a reciprocal time of death-concentration plot was indicative of intrinsic toxicity (1). Recently, Levy and Gucinski (3) have demonstrated that the slope of such a plot is actually a complex function of both the intrinsic toxicity and the absorption rate of the drug. Furthermore, these workers proposed that the reciprocal time of occurrence of any suitable pharmacologic response for a given drug will be directly proportional to the absorption rate of the drug under certain specified conditions (*viz.*, absorption occurs by passive diffusion, drug concentration gradient across the absorbing membranes remains essentially constant, drug metabolism in the fish is negligible, and the pharmacologic end point occurs without significant delay after a given amount of drug is absorbed). Knowledge of the amount of drug in the goldfish body required to induce a pharmacologic response permits the calculation of the absorption rate constant from reciprocal time of response-concentration plots. Even in the absence of such knowledge, relative absorption rates of a single drug under different experimental conditions may be assessed from the slopes of reciprocal time-concentration plots (3).

Another apparently well-marked pharmaco-

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logic response that has been used in the goldfish is the so-called overturn time (1). The overturn end point marks the time, after exposure to a given substance, at which the fish appear to lose their sense of balance and float on their side or upside-down. This end point seems somewhat analogous to the loss of righting reflex in the rat.

Use of the overturn time rather than time of death as a pharmacologic end point offers certain advantages in that the fish survive the study and may, after a suitable period of time, be used again in other experiments. In well-designed experiments a fish or a group of fish may serve as its own control, thereby affording a decided statistical advantage. Furthermore, the ability to reuse fish in a series of experiments allows conservation of test animals which minimizes the problems of lot-to-lot variation (3). In certain toxicity studies the overturn time end point has been claimed to be better defined than the time of death end point since with some drugs the fish enter a deep anesthetic stage which may be mistaken as death (1).

A further advantage of the use of overturn time as a pharmacologic criterion of drug absorption is that the duration of pharmacologic response may be determined. The duration of response should be related to the body drug levels in the fish as well as the rate of drug elimination from the fish.

It is the purpose of the present report to demonstrate the utility of the overturn time end point for the determination of drug absorption and exsorption rates in the goldfish.

EXPERIMENTAL

Goldfish, *Carassius auratus*, common variety, weighing from 5 to 10 Gm. were used. All fish utilized in a given experiment were from the same lot. Three different lots were used during the study. Solutions were prepared daily, as required, from reagent grade or USP materials. The test drugs, ethanol and sodium pentobarbital, were dissolved in 0.05 *M* phosphate buffer, adjusted to pH 6.1.

Evaluation of Pharmacologic Response—All measurements of overturn time (T_0) and duration of effect (T_R) were made at 24–26°. The overturn time was taken as the time required for the fish to lose the ability to maintain itself upright after immersion in drug solution. When the first signs of overturn were observed the end point was tested by attempting to turn the fish on its side with a stirring rod. Unaffected fish will either not allow themselves to be placed on their side or will immediately right themselves. The end point was judged as the time when a fish placed on its side did not right itself immediately. The parameter T_R was defined as the time required (from $t = T_0$) for a fish to regain the ability to right itself immediately after being placed on its side.

The determination of T_0 was carried out with individual fish in 200 ml. of drug solution in 250-ml. beakers. At $t = T_0$ the fish was immediately (or after a timed interval of 2 or 4 min.) placed in approximately 5 gal. of distilled water. The time required from immersion of the fish in the reservoir to recovery of righting reflex is T_R.

At one concentration with each drug the fish were removed from the drug solution after determination of T_0 and placed in a holding tank containing 10 gal. of distilled water. These fish were restudied under identical conditions after 18 hr.

RESULTS AND DISCUSSION

It was found in preliminary experiments that immersion of goldfish in the buffer solution for 24 hr. had no apparent effect on the fish. In addition the overturn time found in 3% ethanol solutions with and without buffer was virtually identical. Hence, it was concluded that the buffer system had no direct effects on pharmacologic response.

According to the model proposed by Levy and Gucinski (3), the time required for a pharmacologic response (T) is related to drug concentration (C) in the following manner:

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

where $K = ka/A_B$. The term ka is the apparent absorption rate constant (which includes the absorptive surface area) and the term A_B is the amount of drug required in the fish to elicit the pharmacologic response. The requisite assumptions for Eq. 1 have been detailed (3).

Levy and Miller (4) have found that the relationship between time of death and concentration of various barbiturates including pentobarbital and various alcohols is in agreement with Eq. 1. The results of the present study with pentobarbital (shown in Fig. 1) using overturn time as the pharmacologic end point rather than time of death are also in good agreement with the biologic model. A plot of $1/T_0$ as a function of concentration demonstrated linearity over a concentration range from 10 to 50 mg.%. The significance of a least-squares treatment on a reciprocal plot, without appropriate weighting factors, is questionable. Accordingly, the

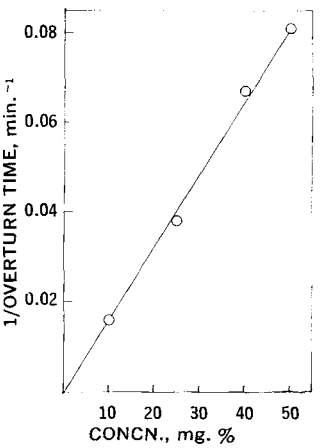

Fig. 1—Reciprocal time of overturn of goldfish as a function of pentobarbital concentration in pH 6.1 buffer.

TABLE I—OVERTURN TIMES OF GOLDFISH IN VARIOUS CONCENTRATIONS OF PENTOBARBITAL

Concn., mg. %	No. of Animals	Av. Overturn Time, min.	S.E.
10	5	62.8	4.18
25	4	26.4	1.28
40	5	15.0	1.25
50	10	12.3	0.69

TABLE II—OVERTURN TIMES (MIN.) OF INDIVIDUAL GOLDFISH IN SODIUM PENTOBARBITAL AND ETHANOL, INITIALLY (*A*) AND AFTER 18 HR. (*B*)

	Na Pentobarbital 50 mg. %		Ethanol 3% (v/v)	
	A	*B*	*A*	*B*
	7.5	8.0	4.2	4.0
	10.5	11.5	4.6	4.2
	11.0	12.0	5.2	5.7
	13.0	13.5	6.7	6.0
	14.0	14.5	6.8	7.0
Mean	11.2	11.9	5.5	5.4
S.E.	1.12	1.11	0.53	0.56

TABLE III—OVERTURN TIMES OF GOLDFISH IN VARIOUS CONCENTRATIONS OF ETHANOL

Concn., % v/v	No. of Animals	Av. Overturn Time, min.	S.E.
1.5	4[a]	58.1	4.78
2.0	5	28.0	4.02
3.0	5	13.6	1.87
4.0	5	8.4	0.32

[a] A total of 5 fish was studied but one fish showed no response over a 2-hr. period and was not included in the calculation.

data in Fig. 1 were fitted by eye and the resulting line appears to approximate the origin. The mean data obtained at each concentration of pentobarbital along with standard error calculations are summarized in Table I.

The results of retesting a group of fish after exposure to 3% ethanol or 50 mg.% pentobarbital are shown in Table II. The excellent agreement of the mean values, range, and standard errors between the initial study and the second study, conducted 18 hr. later, indicates clearly the statistical advantages to be gained by the use of a pharmacologic end point other than time of death.

The results of the overturn time–ethanol concentration studies are shown in Table III. The reciprocal time-concentration plot shown in Fig. 2 clearly indicates that the data do not adhere to the theoretical model. Although a linear relationship exists between $1/T_0$ and ethanol concentrations between 1.5 and 4.0% (v/v), a clearly defined intercept is observed at a concentration of about 1%. These findings are similar to those reported recently by Hall and Hayton (5) using overturn time of goldfish and guppies in ethanol solutions.

The model proposed by Levy and Gucinski (3) requires conditions where the fish body functions as an infinite sink, *i.e.*, drug concentration in the fish never exceeds 10 to 20% of drug concentration in the bathing solution. Accordingly, if the concentration required to elicit a pharmacologic response in the fish is $1/5$ to $1/10$ the concentration of the drug

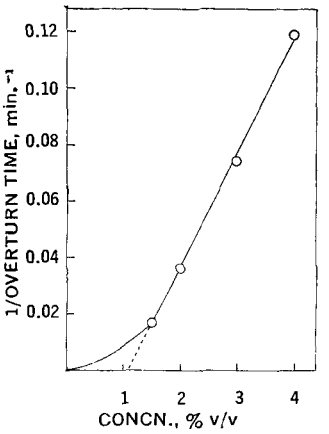

Fig. 2—Reciprocal time of overturn of goldfish as a function of ethanol concentration. Data suggest a concentration intercept of 1% (v/v).

solution, one would anticipate a change in the order of absorption kinetics and a lack of agreement with Eq. 1 at these low concentrations.

On the basis of the present findings it would appear that the minimum body level of ethanol required to elicit overturn in the goldfish is in the order of 150 to 300 mg. %. Interestingly, this estimate approximates the toxicity level of ethanol in man. Sollmann (6) reports that an ethanol blood level of 300 mg. % is required to induce a stuporous condition or sedation in man.

An alternative possibility to explain the intercept is that the fish is capable of metabolizing ethanol. If this were the case, the minimum body level of ethanol required to induce overturn may be significantly lower than 150 mg. % and one would still observe a concentration intercept of approximately 1%.

The ethanol studies also indicated lack of agreement with the biologic model at concentrations above 4% (v/v). Experiments conducted at 5% and 7.5% ethanol gave reciprocal times of overturn that were significantly higher than those predicted by extrapolating the results in Fig. 2. These tentative findings suggested a change in the mechanism of penetration or pharmacologic effect of ethanol in the goldfish as a function of concentration.

On the basis of these findings, it was decided to restudy a second lot of fish and to determine overturn times over a 2 to 8% concentration range at 1% intervals.[1] The results of this second study are shown in Table IV and Fig. 3. Once again a concentration intercept was noted at about 1%. In this particular lot of fish a linear relationship between $1/T_0$ and concentration was found from 2 to 5% ethanol.

The data in Fig. 3 clearly indicate a discontinuous increase in $1/T_0$ above 5% ethanol, *i.e.*, time of overturn is significantly shorter than can be predicted from the low concentration data. The present results suggest the absence of a concentration intercept if high ethanol concentration data are extrapolated to zero concentration. This finding is in agreement with that of Levy and Miller (4).

[1] Concentrations above 8% ethanol gave rise to T_0 values of less than 1 min. and corresponding $1/T_0$ values are subject to large errors.

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extracted with portions of methanol. The methanolic extracts (2 L.) were concentrated to about 50 ml. and passed through 20 Gm. Amberlite IRA 400 (OH) resin column. The resin was washed with 150 ml. 50% aqueous methanol. The effluents were evaporated to yield 123 mg. of choline. The base was identified by converting it to picrate and crystallizing the product from absolute ethanol. The melting point of the picrate, 242–244°, was not depressed by mixing the compound with reference choline picrate and IR spectra of the two samples were identical.

The unidentified polar base, which had zero R_f value on the preparative paper chromatograms, was isolated by extracting the corresponding band with 50% aqueous methanol acidified with formic acid. The product obtained after evaporation was saved.

DISCUSSION

An investigation of *Lophophora williamsii* constituents resulted in isolation and identification of four crystalline compounds. Three bases, which were new peyote alkaloids, were designated—anhalotine, lophotine, and peyotine—and were identified as metho derivatives corresponding to anhalidine, lophophorine, and pellotine, respectively. A fourth base which was obtained as a picrate was identified as choline.

Of the three quaternary tetrahydroisoquinolines isolated and identified, lophotine and peyotine are expected to be optically active. Since only small amounts of the two compounds were available, the optical rotations of the compounds could not be determined. However, the melting points of lophotine iodide isolated and that of the methiodide prepared from the natural lophophorine were the same and the mixed melting point of the two showed no depression. This suggested that the isolated compound was identical with the prepared.

The melting point of crystalline peyotine iodide was lower than the melting point of the methiodide prepared from *dl*-pellotine. The isolated compound softened at 114° and thereafter solidified and then melted at 185–186°. It is possible that the isolated compound is an optically active substance or that the compound in part may have racemized during the isolation. According to Spaeth and Kesztler (18) pellotine base itself undergoes racemization and this had led the authors to suggest that the optically active base is present in the plant; the compound is racemized during extraction or aging of the drug. As indicated earlier, small amounts of peyotine iodide isolated did not permit any further work. The R_f values, IR, and UV spectra of the isolated and prepared compounds were, however, identical.

With the isolation and identification of anhalotine, lophotine, and peyotine the expected occurrence of the quaternary bases corresponding to the known peyote secondary and tertiary bases—namely, anhalamine, anhalidine, anhalonine, lophophorine, anhalonidine, and pellotine—is realized. However, the isolated quaternary bases—namely, the three tetrahydroisoquinolines, choline, and the other two unidentified basic compounds—occur only as minor constituents of peyote.

The quaternary compound corresponding to mescaline, the major and important peyote base, appears not to be present in a detectable amount in the plant. In connection with this, it may be indicated that *N*-methylmescaline has been isolated as a minor alkaloid of peyote (19) and *N*,*N*-dimethylmescaline (≡ tricocereine) which though known to be present in the cactus plant, *Trichocereus terscheckii* (Perm.) (20), has not yet been isolated from the mescal buttons.

As indicated earlier in a recent study thin-layer chromatographic identification of candicine has been reported (5) from peyote quaternary base fraction. In connection with the identification of this compound, the following may be noted.

In Table I are listed the R_f values of anhalotine, lophotine, and peyotine iodides and choline chloride. For comparison R_f values of candicine iodide have also been given. The results recorded in the table indicate that the R_f values of candicine iodide are either too close to or the same as that of the quaternary alkaloids which have been isolated in our studies. Even on alumina G plate and the solvent system carbon tetrachloride-methanol-glacial acetic acid (12:28:1) used (5), candicine iodide has the same R_f value as peyotine iodide. Wagner's reagent a nonspecific reagent, was used for location of the quaternary bases on the chromatogram by these authors. Therefore, distinction of the compound(s) present on the spot having an R_f value same as candicine iodide, into phenolic and nonphenolic quaternary bases, could not be made. In this study tetrazotized di-*o*-anisidine was used for the location of the phenolic bases. The nonphenolic and the phenolic quaternary bases were further detected and distinguished by the use of the modified Dragendorff's reagent. The former agent yielded distinct violet colored spots with the phenolic tetrahydroisoquinoline bases anhalotine, peyotine, and also other nonquaternary phenolic bases anhalamine, anhalidine, anhalonidine, and pellotine. On the other hand candicine, a phenolic quaternary β-phenylethylamine and other related compounds namely, tyramine, *N*-methyltyramine, and hordenine, gave yellow colored spots. In the chromatographic examination of the quaternary base fraction of peyote, no yellow colored spot at the candicine R_f location was observed. This observation and the fact that the R_f values of candicine iodide are the same as, or too close to, other quaternary bases, suggest that the candicine cannot be identified by the method reported and its occurrence in peyote has not been proven.

In the present studies, in addition to the quaternary bases, the known peyote alkaloids namely, lophophorine, pellotine, and anhalonine were obtained by a method differing from that reported previously (8–11). The modified method is based on the fact that from an aqueous extract acidified with hydrochloric acid, the three known bases can be extracted as hydrochlorides with chloroform. Inasmuch as the isolation of the three compounds is accomplished in earlier stages of extraction, the modified method reported herein can be considered as a convenient procedure for the isolation of at least lophophorine and pellotine.

One noteworthy point regarding isolation of pellotine that needs to be pointed out is that, even though this is a phenolic alkaloid, it can be extracted from an aqueous potassium hydroxide

solution with chloroform and ether. Spaeth and Becke (8) also have reported such a behavior for the peyote phenolic alkaloids. According to them, these compounds were at least in part extracted with ether from an aqueous potassium hydroxide solution even though they used concentrated solution (50%) of aqueous potassium hydroxide.

In conclusion, it may be indicated that although the β-phenylethylamine quaternary alkaloids, candicine and coryneine (≡ 3-hydroxycandicine), have been isolated from some cacti (1,20), *Lophophora williamsii* appears to be the first cactus plant from which simple quaternary tetrahydroisoquinolines have been isolated and identified. Occurrence of such compounds in other cacti could be expected, and as in peyote, these compounds may be present at least as minor basic constituents.

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Keyphrases

Peyote alkaloids
Alkaloids, quaternary–*Lophophora williamsii*
Anhalotine, lophotine, and peyotine–isolation
IR spectrophotometry–structure
UV spectrophotometry–structure
NMR spectrometry

Thalictrum Alkaloids V

Isolation

By MAURICE SHAMMA and BERNARD S. DUDOCK

The experimental procedure which led to the isolation of the new alkaloids thalifendlerine (I), thalifendine (II), thalidastine (III), thaliporphine (IV), preocoteine (V), and thalidezine (VI), from *Thalictrum fendleri* is described in detail. Known alkaloids also found in *T. fendleri* are berberine (VII), jatrorrhizine (VIII), glaucine (IX), magnoflorine (X), ocoteine (XI), hernandezine (XII), and thalicarpine (XIII). A completely new dimeric aporphinebenzylisoquinoline alkaloid of only partially elucidated structure is thaldimerine (XIV). A novel procedure for the separation of tertiary alkaloids consists of partition chromatography of the mixture of hydrochloride salts using a cellulose column in the system methyl ethyl ketone–water.

THE STRUCTURAL ELUCIDATION of a variety of new isoquinoline-type alkaloids from *Thalictrum fendleri* has previously been reported. The alkaloids involved were thalifendlerine (I), thalifendine (II), thalidastine (III), thaliporphine (IV), preocoteine (V), and thalidezine (VI) (1–4). In addition, the presence of the following known alkaloids in *T. fendleri* was indicated: berberine (VII), jatrorrhizine (VIII), glaucine (IX), magnoflorine (X), ocoteine (XI), hernandezine (XII) (3), and thalicarpine (XIII). The purpose of the present paper is to describe the isolation procedure employed to obtain these 13 compounds. Additionally, the isolation of the completely new alkaloid thaldimerine (XIV) will also be discussed, although lack of sufficient material has so far precluded the complete structural elucidation.

Coarsely ground *T. fendleri* was extracted with methanol and the solvent was evaporated. The oily residue obtained was taken up in dilute acetic acid and filtered. The filtrate was extracted with chloroform and ether to remove acidic and neutral components. The aqueous acidic layer was then made just basic with concentrated ammonium

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