REFERENCES

(1) Brodie, B. B., Mark, L. C., Papper, E. M., Lief, P. A., Bernstein, E., and Rovenstine, E. A., *J. Pharmacol. Exptl. Therap.*, **98**, 85(1950).
(2) Brodie, B. B., Bernstein, E., and Mark, L. C., *ibid.*, **105**, 421(1952).
(3) Price, H. L., Kovnat, P. J., Safer, J. N., Conner, E. H., and Price, M. L., *Clin. Pharmacol. Therap.*, **1**, 16(1960).
(4) Mark, L. C., and Brand, L., *Bull. N. Y. Acad. Med.*, **40**, 476(1964).
(5) Saidman, L. J., and Eger, E. I., *Anesthesiology*, **27**, 118(1966).
(6) Bischoff, K. B., and Brown, R. G., *Chem. Eng. Prog. Symp. Ser. No. 66*, **62**, 32(1966).
(7) Bischoff, K. B., in "Chemical Engineering in Medicine and Biology," Hershey, D., ed., Plenum Press, New York, N. Y., 1967, p. 417.
(8) Mapleson, W. W., *J. Appl. Physiol.*, **18**, 197(1963).
(9) Rackow, H., Salanitre, E., Epstein, R. M., Wolf, G. L., and Perl, W., *ibid.*, **20**, 611(1965).
(10) Perl, W., Rackow, H., Salanitre, E., Wolf, G. L., and Epstein, R. M., *ibid.*, **20**, 621(1965).
(11) Gillette, J. R., in "Drugs and Enzymes," *Proc. 2nd Intl. Pharmacol. Mtg.*, 1965, p. 9.
(12) Krüger-Thiemer, E., Diller, W., Dettli, L., Bünger, P., and Seydel, J., *Antibiot. Chemotherap.*, **12**, 171(1964).
(13) Krüger-Thiemer, E., Bünger, P., Dettli, L., Spring, P., and Wempe, E., *Chemotherapia*, **10**, 61, 129, 325(1965–1966).
(14) Krüger-Thiemer, E., Diller, W., and Bünger, P., in "Antimicrobial Agents and Chemotherapy—1965," Hobby, G. L., ed., Am. Soc. Microbiol., Ann Arbor, Mich., 1966.
(15) Goldbaum, L. R., and Smith, P. K., *J. Pharmacol Exptl. Therap.*, **111**, 197(1954).
(16) Bellman, R., Jacquez, J. A., and Kalaba, R., *Bull. Math. Biophys.*, **22**, 181, 309(1960).
(17) Mark, L. C., *Clin. Pharmacol. Therap.*, **4**, 504(1963).
(18) Mark, L. C., Brand, L., Kamvyssi, S., Britton, R. C., Perel, J. M., Landrau, M. A., and Dayton, P. G., *Nature*, **206**, 1117(1965).
(19) Mark, L. C., Burns, J. J., Brand, L., Campomanes, C. I., Trousof, N., Papper, E. M., and Brodie, B. B., *J. Pharmacol. Exptl. Therap.*, **123**, 70(1958).
(20) Bischoff, K. B., and Dedrick, R. L., *Proc. Ann. Conf. Eng. Med. Biol.*, **1967**, p. 8.6.
(21) Dedrick, R. L., and Bischoff, K. B., *Chem. Eng. Prog. Symp. Ser.*, No. 84, **64**, 32(1968).

Keyphrases

Pharmacokinetics—thiopental
Thiopental—distribution
Protein binding—thiopental
Model, mathematical—thiopental distribution

Effect of Chlordan Pretreatment on the Metabolism and Lethality of Cyclophosphamide

By ROBERT L. DIXON

Cyclophosphamide is converted to a cytostatic agent by enzymes present in the hepatic microsomes. Prior treatment of young rats with the insecticide, chlordan, resulted in an increased cyclophosphamide toxicity. Chlordan pretreatment also increased the *in vitro* metabolism of cyclophosphamide and hexobarbital, increased the excretion of the alkylating metabolite of cyclophosphamide *in vivo*, and decreased the sleeping times of animals treated with hexobarbital *in vivo*. It is most probable that increased amounts of cytostatic metabolite occurred as a result of chlordan induced microsomal drug-metabolizing enzyme stimulation.

CYCLOPHOSPHAMIDE[1] [*N,N*-bis(β-chloroethyl)-*N'*, *O*-propylene phosphoric acid ester diamine monohydrate, NSC 26271] is a potent antineoplastic agent of the nitrogen mustard class and has been used in the treatment of patients with many types of neoplastic diseases. Cyclophosphamide is especially valuable in the treatment of patients with malignancies arising from the hematopoietic tissues. The compound can be administered by all routes (1).

In contrast to the nitrogen mustards ordinarily used in cancer chemotherapy, cyclophosphamide is inert when placed in direct contact with bacteria, leukocytes, and most tumor cells in culture. *In vivo* activation occurs in the liver and perhaps in other sites (2). The subcellular site of enzymic activation in the liver is the microsomes. These hepatic microsomal drug-metabolizing systems require oxygen and NADPH (TPNH) and are capable of altering a number of drugs (3). The microsomal enzyme that activates cyclophosphamide is influenced by a number of various drugs (3). For instance, SKF-525a (β-diethylamino-ethyldiphenylpropylacetate), an inhibitor of microsomal drug metabolism, will decrease serum levels of metabolite capable of alkylation after cyclophosphamide (4), while pretreatment of animals with phenobarbital increases the concentration of this metabolic agent three to fourfold by enzyme induction (5).

The fact that the concentration of metabolite capable of alkylation after cyclophosphamide treatment can be increased by enzyme induction

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[1] Marketed as Cytoxan by Mead Johnson & Co., Evansville, Ind.

might have therapeutic possibilities. Surely it is of interest to those testing new compounds for toxic effects or action against transplantable tumors in animals. There are a large number of drugs and foreign compounds that have been shown to be effective stimulators of microsomal drug-metabolizing enzymes (2). This paper reports enhancement of cyclophosphamide metabolism and toxicity in rats after exposure to the insecticide, chlordan, which is commonly used in laboratory quarters.

METHODS

Cyclophosphamide was obtained commercially. Technical chlordan, a mixture containing 60–75% of the pure compound and 25–40% of related compounds, was used in these experiments.

Young male rats, 50–60 g., were treated with either 10 mg./kg. of technical chlordan dissolved in corn oil or received corn oil alone. The technical chlordan was dissolved in corn oil to make a 0.1% solution and each chlordan treated animal received subcutaneously 0.01 ml. of this solution per gram of body weight. Animals were pretreated with chlordan twice, 144 and 72 hr. prior to oral administration of cyclophosphamide.

Cyclophosphamide was administered orally to groups of 10–12 rats at a dose of 90, 125, 180, or 250 mg./kg. Cyclophosphamide was administered at a concentration such that each animal received 0.01 ml. of aqueous solution per gram of body weight. Deaths were recorded daily for 30 days following a single dose of cyclophosphamide.

In vitro metabolism of hexobarbital[2] (side-chain oxidation) was determined using methods, conditions of incubation, cofactors, and concentrations described previously (6).

The *in vitro* metabolism of cyclophosphamide was studied using the same conditions described above. The substrate concentration was 10 mg./5 ml. incubation. The active metabolite of cyclophosphamide was estimated by the spontaneous alkylation of α-(4-nitrobenzylpyridine) (NBP) (7, 8). Five milliliters of 6.67% trichloroacetic acid was added to the beaker following incubation. The solutions were mixed and poured into a heavy walled centrifuge tube and centrifuged prior to taking a 3.0-ml. aliquot for assay.

In vivo metabolism of hexobarbital was estimated using sleeping times after hexobarbital treatment. Hexobarbital, 150 mg./kg., was administered intraperitoneally and the time in minutes from loss of righting reflex to recovery of reflex was recorded.

In vivo metabolism of cyclophosphamide was estimated by collecting and analyzing urine for metabolites capable of alkylation after cyclophosphamide treatment. Urine was collected from each of six animals (three chlordan treated, three corn oil treated) for the first 4 hr. following cyclophosphamide treatment. Animals were placed in individual plastic metabolism cages which allowed collection of urine. Each animal received 500 mg./kg. of cyclophosphamide orally. After the 4-hr. collection period, the urine was obtained and the cage rinsed with distilled water. Urine plus cage rinsings were brought to a final volume of 25 ml. with distilled water. Three-milliliter aliquots from each total volume were then assayed for material capable of alkylating the NBP reagent.

Statistical methods used are described by Snedecor (9) and Litchfield and Wilcoxon (10).

RESULTS

Table I demonstrates the effect of chlordan pretreatment on the metabolism of hexobarbital *in vitro* and *in vivo*. It can be seen that chlordan pretreatment increased the rate of metabolism *in vitro*, and decreased the duration of sleep after hexobarbital. These data are in agreement with those of Hart *et al.* (11) and serve only to confirm the effect of chlordan on the injected animals.

In vitro studies of the metabolism of cyclophosphamide by hepatic supernatant fraction from chlordan and corn oil pretreated animals revealed that the chlordan pretreated animals produced 76% more of an alkylating substance during a 30-min. incubation than did corn oil treated control animals. The mean absorbance reading for the metabolite–NBP reagent complex formed by the chlordan treated animals was 0.294 ± 0.096 *A* units/hr./g. of tissue compared to 0.167 ± 0.052 *A* units/hr./g. of tissue for the corn oil pretreated animals. These values are significantly different ($p \leq 0.05$). Five animals were used in the chlordan treated group and four animals were used in the control group. The colored complex was developed from a 3-ml. aliquot taken from the supernatant of the incubation system (5 ml.) plus 5 ml. of TCA, and the color subsequently extracted with 7 ml. of the nonpolar phase. The absorbance readings (*A*) were converted to absolute values by using a standard curve prepared after hydrolyzing cyclophosphamide as described by Philips *et al.* (12). The reactivity of the hydrolyzed solutions of cyclophosphamide averages 99% of that of molar equivalent amounts of nor-NH_2HCl. In these studies a concentration of activated product from 10 mcg. of cyclophosphamide per ml. of the nonpolar extraction phase (acetone-ethyl acetate) had an absorbance of 0.613 when measured at 600 mμ in a 1-cm. square cell. Using this standard curve the above absorbance values represent 112 and 64 mcg. cyclophosphamide metabolized per gram liver per hour, respectively. The absorbance values were multiplied by $^{10}/_{3}$ to correct the value for the aliquot, and by 7 in order to express micrograms of metabolite in the 7 ml. of nonpolar solvent.

In vivo studies of the excretion of a metabolite capable of alkylating the NBP reagent (activated

TABLE I—EFFECT OF CHLORDAN PRETREATMENT ON THE METABOLISM OF HEXOBARBITAL

	Corn oil Treated	Chlordan Treated
In vitro metabolism[a]	3.87 ± 0.85 (4)	5.37 ± 1.17 (5)
In vivo sleeping times[b]	106 ± 14	84 ± 21

[a] Micromoles of hexobarbital metabolized per gram of liver during 45-min. incubation. Values are means ± standard deviation. Number of individual animals is indicated in the parentheses. [b] Minutes of loss of righting reflex after 150 mg./kg. of hexobarbital i.p. Values are means ± standard deviation for six animals per group.

[2] Evipal, Winthrop Laboratories, New York, N. Y.

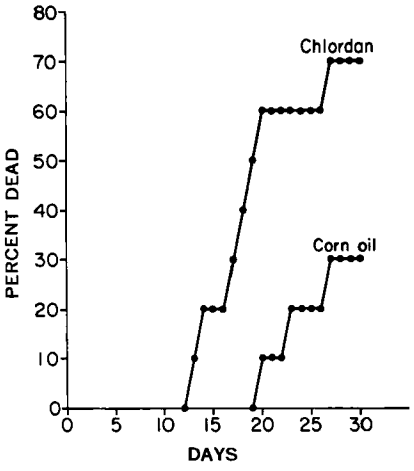

Fig. 1—Percent of animals (10 per group) which died following a single oral dose of cyclophosphamide (125 mg./kg.). The chlordan group was treated prior to cyclophosphamide administration with chlordan. Control animals received only corn oil.

metabolite) after cyclophosphamide treatment revealed that the chlordan pretreated animals excreted more of this substance in 4 hr. than did the corn oil treated controls.

Chlordan pretreated rats excreted 61% more of this metabolite than did corn oil treated controls. The average final readings determined from a 3-ml. aliquot of urine from each total volume of 25 ml. was 0.255 (0.215, 0.283, 0.267) for chlordan pretreated animals compared to a mean reading of 0.158 (0.155, 0.145, 0.173) for the corn oil treated control animals. The metabolite from the 3.0 ml. was extracted into 7.0 ml. of a nonpolar phase prior to reading the *A* with a colorimeter. Using the standard curve referred to in the *in vitro* results, the above values correspond to 242 and 148 mcg., respectively.

Figure 1 expresses the lethality data seen at one dose, 125 mg./kg., of cyclophosphamide. The same trend seen here, that of earlier and greater lethality, was seen with each of the higher doses studied.

Table II presents lethality data for cyclophosphamide for groups of corn oil and chlordan pretreated animals. It can be seen that the LD_{50} for the chlordan pretreated group is lower, 145 (125–166) mg./kg., than that for the corn oil pretreated animals which was 170 (142–204) mg./kg. Neither of the two slopes was significantly heterogeneous and they were parallel. However, the potency ratio of 1.17 was not significant at the 95% level of probability. Nevertheless, it should be pointed out that after chlordan pretreatment a dose of cyclophosphamide normally lethal to 50% of the animals tested resulted in approximately 80% dead due to the very steep dose-response slope associated with this drug.

DISCUSSION

Results presented here demonstrate that exposure to chlordan, an insecticide commonly used in animal quarters, can result in changes in drug metabolism which alter the toxicity (weight loss and diarrhea) and lethality of cyclophosphamide. Animals receiving two subcutaneous injections of chlordan,

TABLE II—EFFECT OF CHLORDAN ON LETHALITY OF CYCLOPHOSPHAMIDE

Oral Dose, mg./kg.	% Alive, 30 days	
	Corn Oil Treated	Chlordan Treated
250	10	0
180	42	20
125	70	30
90	100	100
LD_{50}	170 (142–204)	145 (125–166)

144 and 72 hr. prior to cyclophosphamide, showed signs of toxicity and died earlier and in greater number than the corn oil pretreated animals.

This increase in lethality can be explained by an induction of microsomal enzymes which convert cyclophosphamide to an active form and accounts for the increase in the levels of the cytotoxic form. Data were presented which demonstrated that animals pretreated with chlordan metabolized both hexobarbital and cyclophosphamide to a greater extent *in vitro* than corn oil treated controls. Also, animals pretreated with chlordan excreted more metabolite capable of alkylating NBP reagent in 4 hr. following cyclophosphamide than did corn oil treated control rats. The effect of chlordan was further demonstrated by a decreased sleeping time after hexobarbital administration in the chlordan treated group. This effect was similar to the increased cytotoxic action described for cyclophosphamide after phenobarbital pretreatment (5).

These data indicate that spraying animal quarters with the chlorinated hydrocarbon insecticides can result in spurious testing results concerning toxicity and perhaps effectiveness against transplantable tumors. In most cases increased metabolism of a drug would result in its decreased effectiveness and lethality. However, increased metabolism of cyclophosphamide resulted in an increase of active metabolite and enhanced drug effect.

The clinical implications for therapeutic treatment of such findings is probably less important than possible effects on laboratory animals. However, it might be possible to increase the effectiveness of this drug in patients who for some reason have a less than normal ability to metabolize cyclophosphamide. In such patients cyclophosphamide would be less effective. A decreased metabolism of drugs could possibly occur in patients who are very young, or as a result of various pathological conditions (2). These suggestions are based on findings in animals, and must be extrapolated to the clinical situation with care.

REFERENCES

(1) Calabresi, P., and Welch, A. D., "The Pharmacological Basis of Therapeutics," Goodman, L. S., and Gilman, A., Eds., Macmillan Co., New York, N. Y., 1965, p. 1354.
(2) Remmer, H., *Ann. Rev. Pharmacol.*, **5**, 405(1965).
(3) Gillette, J. R., *Progr. Drug Res.*, **6**, 11(1963).
(4) Brock, N., and Hohorst, H. J., *Arzneimittel.-Forsch.*, **13**, 1021(1963).
(5) Rauen, H. M., and Kramer, K. P., *ibid.*, **14**, 1066 (1964).
(6) Dixon, R. L., Hart, L. G., Rogers, L. A., and Fouts, J. R., *J. Pharmacol.*, **142**, 312(1963).
(7) Epstein, J., Rosenthal, R. W., and Ess, R. J., *Anal. Chem.*, **27**, 1435(1955).
(8) Friedman, O. M., and Boger, E., *ibid.*, **33**, 906(1961).
(9) Snedecor, G. W., "Statistical Methods," Iowa State College Press, Ames, Iowa, 1956.
(10) Litchfield, J. T., and Wilcoxon, F. A., *J. Pharmacol.*, **96**, 99(1949).
(11) Hart, L. G., Shultice, R. W., and Fouts, J. R., *Toxicol. Appl. Pharmacol.*, **5**, 371(1963).
(12) Philips, F. S., Sternberg, S. S., Cronin, A. P., and Vidal, P. M., *Cancer Res.*, **21**, 1577(1961).

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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