In conclusion, the data indicate that the relative potency of certain phenolic drugs as uncouplers of oxidative phosphorylation may be determined primarily by their ability to cross a boundary membrane and to bind to a cationic site on a protein normally associated with energy-conservation, rather than merely by their relative acidity or lipid solubility. This is further exemplified by the hydroxybenzoic acids, of which salicylic acid most readily partitions into yeast[15] and is the most potent in uncoupling phosphorylation.[16]

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The inhibition of drug metabolism by antispermatogenic N,N′-bis(dichloroacetyl) diamines

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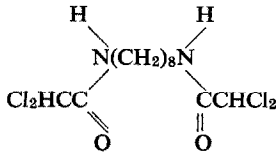
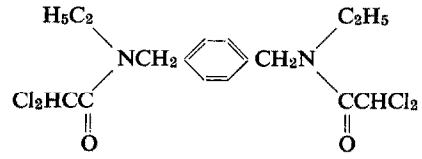
THAT one drug may alter the pharmacological potency of another is by now a well-established fact,[1–4] and the problem of the interaction of drugs has become a subject of widespread interest in recent years.[5, 6] Perhaps the best known of the inhibitors of drug metabolism, SKF 525-A (β-diethylaminoethyl-3-3-diphenylpropylacetate) has been shown to be firmly bound to subcellular membrane systems, and it has been proposed that this binding is related to the effects on microsomal metabolism by this compound.[7] In this communication we would like to present evidence for the inhibition of drug metabolism by two dichloroacetylamines, N,N′-bis(dichloroacetyl-1-8) octamethylenediamine (WIN 18,446), and N,N′-1,4-xylylene-bis(N-ethyldichloroacetamide) (WIN 13,099) which, like SKF 525-A, are firmly bound to subcellular membrane systems* and which have been shown to

* A. J. Merola, unpublished observation.

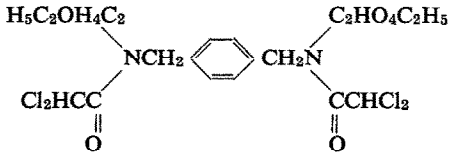
possess potent antispermatogenic activity.[7] Data are also presented from studies using N,N′-1,4-xylylene-bis(N-ethoxyethyldichloroacetamide) (WIN 13,146), a close structural analog of WIN 13,099 which lacks the potent antispermatogenic activity of the latter but is highly effective as an amebicidal compound both *in vivo* and *in vitro*.[8] The structures of these compounds are as follows:



WIN 18,446



WIN 13,099



WIN 13,146

Table 1 shows that both antispermatogenic compounds significantly prolonged sleeping time in rats, whereas the ethoxyethyl-substituted amebicidal compound produced only a slight increase which

TABLE 1. THE EFFECT OF TWO ANTISPERMATOGENIC BIS-DICHLOROACETYLAMINES (WIN 13,099; WIN 18,446), AND A NON-ANTISPERMATOGENIC BIS-DICHLOROACETYLAMINE (WIN 13,146) ON HEXOBARBITAL SLEEPING TIME IN RATS

Drug treatment	No. of animals	Sleeping time (min ± S.E.)
None (gum tragacanth)	9	20·2 ± 2·4
WIN 13,146	9	26·9 ± 4·7 (n.s.)
WIN 13,099	10	82·6 ± 24·0
WIN 18,446	7	65·5 ± 11·1*

Male Sprague–Dawley-derived rats weighing approximately 180 g were medicated by stomach intubation for 3 days with the compounds indicated at doses of 50 mg/kg. Control animals received only the 1% gum tragacanth vehicle. Approximately 20 hr after the last medication, the animals were given sodium hexobarbital i.p. (80 mg/kg), and sleeping time was measured as the duration of the loss of the righting reflex.

* One rat in this group had a sleeping time of over 4 hr and was discarded as an outlier.

was not significant ($P > 0{\cdot}25$). In this experiment, sleeping time, measured as the duration of loss of the righting reflex, was tested with sodium hexobarbital, 80 mg/kg i.p., 18–20 hr after the last of three daily doses of the drugs (50 mg/kg) administered in the form of suspensions in 1% gum tragacanth by stomach intubation. The animals were fed and watered *ad libitum*. This level of drug is far below the massive levels used in toxicity studies where it was reported that these dichloroacetylamines were well tolerated by mice and rats.[7, 8]

Table 2 shows the results of a test of sleeping time in mice 1 hr after a single i.p. injection of drug suspension. Just as in the above test the non-antispermatogenic ethoxyethyl derivative (WIN 13,146) resulted in little or no increase in hexobarbital sleeping time; however, the antispermatogenic compounds produced a two- to three-fold increase in sleeping time. It may be of interest that N,N′-bis-(dichloroacetyl)-N,N′-diethylalkylene diamines with alkyl chains of 5, 7, and 9 carbons separating nitrogen atoms, are also powerful potentiators of hexobarbital sleeping time in mice. These data

appear in Table 3. This test was performed in a manner identical with that of Table 2 except that hexobarbital was given at a level of 100 mg/kg. Assumed that hexobarbital potentiation is a reflection of the inhibition of hexobarbital metabolism, this experiment suggests that inhibitory activity does not depend on any one specific chain length in this series of compounds.

TABLE 2. THE EFFECT OF TWO ANTISPERMATOGENIC BIS-DICHLOROACETYLAMINES (WIN 13,099; WIN 18,446) AND A NON-ANTISPERMATOGENIC BIS-DICHLOROACETYLAMINE (WIN 13,146) ON HEXOBARBITAL SLEEPING TIME IN MICE

Drug treatment	No. of animals	Sleeping time (min ± S.E.)
None (gum tragacanth)	10	29·5 ± 4·5
WIN 13,146	10	38·4 ± 3·0 (n.s.)
WIN 13,099	10	55·6 ± 5·9
WIN 18,446	10	87·7 ± 6·2

Swiss mice weighing 30–35 g were injected i.p. with the drugs indicated at a level of 50 mg/kg body weight. One hour after this injection, each mouse was given hexobarbital (80 mg/kg), and the duration of the loss of righting reflex was measured.

TABLE 3. THE EFFECT OF SOME N,N′-BIS(DICHLOROACETYL)-N,N′-DIETHYLALKYLENEDIAMINES OF VARYING METHYLENE CHAIN LENGTHS ON HEXOBARBITAL SLEEPING TIME IN MICE

Drug treatment*	Methylene carbon length	No. of animals	Sleeping time (min. ± S.E.)
None (1% gum tragacanth)		10	65 ± 5
I	5	10	346 ± 21
II	7	10	244 ± 28
III	9	10	273 ± 39

Swiss mice weighing 20–25 g were injected i.p. with the drugs indicated at a level of 50 mg/kg body weight. One hour after this injection, each mouse was given hexobarbital (100 mg/kg), and the duration of the loss of the righting reflex was measured.

* Drugs used were, I: N,N′-bis(dichloroacetyl)-N,N′-diethylpentamethylenediamine; II: N,N′-bis(dichloroacetyl)-N,N′-diethylheptamethylenediamine; and III: N,N-bis(diethylnonamethylenediamine).

In order to verify that the effects in the above tests *in vivo* were a reflection of interference of drug metabolism, the 10,000-*g* supernatant solutions of liver homogenates prepared from medicated rats were tested *in vitro* for the ability to metabolize several drug substrates. In these experiments adult male rats were medicated for 3 days with drug suspensions in gum tragacanth. Drug levels were the same as those used in the sleeping-time experiments. On the day after the third medication, the rats were killed by a blow on the head and their livers were removed to cold homogenizing medium composed of 0·08 M phosphate buffer at pH 7·4, 0·004 M $MgCl_2$, and 0·03 M nicotinamide. After chilling, the livers were pooled by group, and duplicate or triplicate homogenates were prepared in two volumes of the homogenizing mix in a glass–Teflon Potter Elvehjem homogenizer. Care was taken to ensure that each preparation received the same treatment. The entire supernatant fraction obtained by decantation after centrifugation for 10 min at 10,000 *g* was used in reaction mixtures containing 1 μmole NADP, 10 μmoles glucose 6-phosphate, 6·7 μmoles semicarbazide, 20 μmoles substrate, 460 μmoles phosphate (K^+), pH 7·4, and 1·0 ml of the 10,000-*g* supernatant fraction in a total volume of 6·0 ml. The mixtures were incubated at 37° for 1 hr. The results of these experiments appear in Table 4 and are compatible with the barbiturate sleeping-time tests. In order to compare the results of different experiments, the data are expressed relative to a control activity of 100. It is clear that the ethoxyethyl compound (WIN 13,146) has little effect on the N-demethylation of 4-dimethylaminoantipyrine and the O-demethylation of *p*-acetanisidide, whereas the antispermatogenic compounds consistently

lowered drug metabolism in these systems (Table 4). In contrast to these results, we have failed to note any marked inhibitory effects by any of these compounds on the S-demethylation of 2-(methylthio)-benzothiazole even when homogenates were used which showed inhibition with respect to the other drug substrates.

TABLE 4. THE EFFECT OF ADMINISTRATION OF SOME DICHLOROACETYLAMINES ON DRUG METABOLISM *in vitro*

Drug substrate	Compound administered	Average activity (±S.D.)
4-Dimethylaminoantipyrine	Control (4, 18)	100 (±13)
	WIN 13,146 (4, 18)	87 (±22)
	WIN 13,099 (2, 10)	59 (±8)
	WIN 18,446 (4, 18)	49 (±15)
p-Acetanisidide	Control (3, 10)	100 (±19)
	WIN 13,146 (3, 10)	86 (±10)
	WIN 13,099 (2, 7)	67 (±3)
	WIN 18,446 (3, 10)	68 (±13)
2-(Methylthio)benzothiazole	Control (3, 10)	100 (±7)
	WIN 13,146 (3, 10)	91 (±6)
	WIN 13,099 (2, 7)	99 (±4)
	WIN 18,446 (3,10)	104 (±2)

Each of the drugs was administered as a suspension in 1% gum tragacanth at a dose level of 50 mg/kg to male Sprague–Dawley-derived rats. Control rats received the drug vehicle. On the day following the last of three daily medications, the animals were killed by a blow on the head, and the metabolism of the drugs indicated in the table was measured in the 10,000-*g* supernatant fraction. Formaldehyde formation was used as an estimate of drug metabolism.[9] All activity values are expressed relative to a control value of 100. Numbers in parentheses refer to the number of experiments averaged and the number of animals used, in that order.

Although WIN 13,099 and WIN 18,446 are poorly soluble in aqueous systems, both show fairly potent inhibitory activity on the demethylation of aminopyrine when added directly to liver homogenates and incubated for 30 min as described above. Under these conditions, 50 per cent inhibition obtains at drug concentration of approximately 10^{-4} M.

In addition to inhibiting spermatogenesis, WIN 18,446 and WIN 13,099 produce a reaction to alcohol resembling that elicited by tetraethylthiuram disulfide* and, like the latter compound, both drugs inhibit liver NAD-linked aldehyde dehydrogenase.[10] The ethoxyethyl derivative (WIN 13,146) is an exception in this respect also.

Further studies of the interaction of these compounds and other members of this series with subcellular enzyme systems are in progress; however, the data presented here indicate that members of this group of drugs inhibit drug metabolism, and this property may explain in part the report of potentiation of the effects of exogenous estrogen administration in rats.[11]

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Hepatic microsomal drug-metabolizing enzyme activity in the opossum*

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THE LEVEL of hepatic microsomal drug-metabolizing enzyme activity is species as well as strain dependent.[1–3] The activity of microsomal azo and nitro reductase in livers from fish, amphibia, reptiles, birds, and mammals was studied by Adamson *et al.*,[4] and they suggested that microsomal reductive pathways were more "primitive" (were detected earlier in the phylogenic scheme) than were the oxidative ones. The importance of an aquatic or amphibian versus terrestial habitat of animals in regard to oxidative liver microsomal drug-metabolizing enzyme activity was suggested in a review by Brodie and Maickel.[5] Hepatic drug metabolism, according to these investigators, increased as the phylogenic scale was ascended from fish and mammals. Hepatic drug-metabolizing enzymes in birds and mammals were located in the microsomal fraction of the cell. The metabolism of aminopyrine in the toad, however, was qualitatively different from that of the mammal, and several oxidative drug-metabolizing enzyme systems in the toad were found in the soluble fraction of the liver cell.

The opossum, a marsupial, is considered to be an offshoot or terminal branch of the main phylogenic line of mammals as the toad is an offshoot of the line of amphibia. Since some differences in hepatic drug metabolism between toads and other animals were found by Brodie and Maickel,[5] the amount of enzyme activity and the location of hepatic enzymes that metabolize drugs were studied in the opossum.The effect of phenobarbital or benzpyrene pretreatment on these enzyme activities in opossum liver was also investigated.

METHODS

Animals. Male and female opossums (2–2·5 kg) were used. These animals were obtained from local trappers. "Treated" animals received an intraperitoneal (i.p.) injection of phenobarbital sodium 15, 25, or 35 mg/kg twice daily for 4 days, or one injection of benzpyrene, 25 mg/kg. All determinations were made 12 hr after the last dose of phenobarbital or 48 hr after benzpyrene pretreatment.

Enzyme assays. Livers were excised and homogenized (1 g liver and 2 ml of 1·15% KCl) in the cold with a glass homogenizer having a plastic pestle, or with a Waring Blendor. The liver homogenate was centrifuged at 9000 *g* for 20 min at 4° to obtain the 9000-*g* supernatant fraction. The liver 9000-*g* fraction was centrifuged at 104,000 *g* for 1 hr to produce the pellet of microsomes and a supernatant designated as the "soluble" fraction of the cell. The liver microsomes were washed once in 1·15% KCl, centrifuged at 104,000 *g*, and resuspended in 1·15% KCl such that each ml of suspension contained microsomes from $\frac{1}{3}$ g of liver. Each ml of liver 9000-*g* fraction or soluble fraction was considered as equivalent to 0·33 g liver.

One ml of the liver fraction (e.g. 9000-*g* fraction) was added to an incubation mixture containing cofactors [triphosphopyridine nucleotide (NADP) $1{\cdot}1 \times 10^{-4}$ M, glucose 6-phosphate 5×10^{-3} M, nicotinamide 2×10^{-2} M, and $MgSO_4$ 5×10^{-3} M] and substrate. The substrates used and their

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