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HALIDOHYDROLYTIC CONVERSION OF *N,N'*-BIS-(DICHLOROACETYL)-*N,N'*-DIETHYL-1,4-XYLYLENEDIAMINE IN RAT LIVER*

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Abstract—*N,N'*-bis-(dichloroacetyl)-*N,N'*-diethyl-1,4-xylylenediamine (WIN 13099) was synthesized with tritium on the xylylene carbon which did not exchange with body water. This compound was metabolized by 140,000 *g* supernatant liquid and the mitochondrial fraction of rat liver. Deproteinized 140,000 *g* supernatant liquid was required for the mitochondrial activity. Although WIN 13099 effectively inhibits drug metabolism by rat liver microsomes *in vitro* and *in vivo*, it is not metabolized by rat liver microsomes *in vitro*. The major metabolite *in vitro* of WIN 13099, *N*-dichloroacetyl-*N'*-hydroxyacetyl-*N,N'*-diethyl-1,4-xylylenediamine, was isolated and characterized and found not as effective as the parent compound in inhibiting electron transport by submitochondrial particles or drug metabolism by rat liver microsomes.

METABOLISM by halidohydrolase activity has been studied in mammalian and microbial systems.[1,2] Goldman *et al.*[2] showed that a Pseudomonad converted α-monohalogenated short-chain fatty acids to the corresponding hydroxy acids and α,α-dihalogenated short-chain fatty acids to the corresponding aldo or keto acids. Mass spectroscopy of the hydroxy acids formed from monohalogenated acids in the presence of $H_2{}^{18}O$, showed that the oxygen which was incorporated derived from water rather than molecular oxygen, in contrast to microsomal hydroxylations.[3] Heppel and Porterfield[1] demonstrated a similar conversion of dichloromethane and chlorobromomethane to formaldehyde *in vitro* using rat liver preparations. Chloramphenicol appears to be metabolized by a similar route, possibly involving a further reductive step, since its hydroxyacetyl analogue is found in the urine of cats and newborn infants medicated with this drug.[4] As part of our study on the biochemistry of antispermatogenic bis-dichloroacetamides, we undertook an investigation of the metabolism of an active member of this group of compounds and a structurally similar, but inactive, member of the group, hoping that these studies, together with others we have reported[5,6] and are now pursuing, would give us a better understanding of the biological activity of these potent but relatively nontoxic drugs. In this paper, we would like to present data which show that the major early metabolite of a particularly

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potent antispermatogenic agent, *N,N'*-bis-(dichloroacetyl)-*N,N'*-diethyl-1,4-xylylenediamine (WIN 13099), is an *N*-hydroxyacetyl derivative of the parent compound. Although this and many other bis-dichloroacetamides are quite potent inhibitors of microsomal mixed-function oxidase, the evidence presented here shows that this conversion is not microsomal.

MATERIALS AND METHODS

Chromatography

Thin-layer plates were coated with a 0·25-mm layer of Silica gel $HF_{254-366}$ (Brinkmann), air-dried and activated by heating at 115° for 1 hr. Reverse phase thin-layer chromatography (TLC) plates were prepared by developing the plates in a solvent system which was 3% DC 200 silicone oil (Applied Science) in chloroform (v/v). The mobile phases used were: 1, ethyl ether; 2, methanol; 3, chloroform; 4, benzene; and for the reverse phase chromatography, 5, water–acetone (7:3, v/v), silicone saturated. Plates were chromatographed 15 cm from the origin. Compounds of interest were visually detected by exposure to iodine vapor and radioactively detected with a Nuclear Chicago gas flow plate scanner.

Gas chromatography was carried out on a Varian Aerograph model 200 chromatograph with a flame ionization detector. The column was 3 feet by $\frac{1}{8}$ inch (o.d.) stainless steel packed with 3% OV-101 on Aeropak 30. The injector, column and detector temperatures were 235°, 220° and 275°, respectively, for isothermal gas liquid chromatography (GLC). For linear program GLC, the column temperature was adjusted from 115° (at time of injection) to 220° at a rate of 4° per min. The carrier gas was nitrogen at a flow rate of 60 ml per min.

Chemical synthesis

WIN 13099 was generously supplied by the Sterling–Winthrop Research Institute. Radioactive WIN 13099 with tritium on the xylylene carbons was synthesized according to a modification of the procedure of Surrey and Mayer[7] by reduction of the bisimine with sodium borotritiide.

Analytical methods

Infrared (i.r.) spectra were taken using a Perkin–Elmer model 337 spectrophotometer. For spectra of viscous liquids, a chloroform solution was dried on a KBr window. For crystalline compounds, 250 mg of a 1% mixture of the compound was used to make a KBr pellet.

Nuclear magnetic resonance (n.m.r.) spectra were taken with a Varian A-60 high resolution spectrometer at room temperature as a solution in deuterochloroform using tetramethylsilane as internal standard.

Mass spectra were taken using an AEI model MS-9 mass spectrometer with a 70 eV emission beam. Spectra were taken with and without heptacosafluorotributylamine as internal standard. Elemental analysis was performed by Micro-Analysis Inc. Melting points (uncorrected) were determined using a Fisher–Johns apparatus.

Tissue preparation

Liver from male Sprague–Dawley rats was homogenized in 2 vol. of cold 0·02 M

phosphate (K^+) buffered 0·25 M sucrose, pH 7·4, in a smooth glass-Teflon Potter–Elvehjem homogenizer. After ten passes, the whole-cell homogenate was centrifuged for 10 min at 750 *g*. The pellet (nuclear fraction) was resuspended in the above buffer and the supernatant liquid centrifuged for 10 min at 12,000 *g*. The pellet (mitochondrial fraction) was resuspended in the above buffer. The supernatant liquid of the second centrifugation was diluted 1:1 (v/v) with cold buffer and then centrifuged for 1 hr at about 140,000 *g*. The microsomal pellet was resuspended in the above buffer and the high-speed supernatant liquid utilized as such for metabolic studies.

An aliquot of the high-speed supernatant liquid was boiled and the denatured protein removed by centrifugation. The supernatant liquid of this treatment was used to determine whether a soluble, heat-stable cofactor would stimulate metabolism in any of the particulate fractions.

Metabolic reactions in vitro

Reaction mixtures incubated with shaking at 37° contained 200 μmoles phosphate (K^+), pH 7·4, 2·5 m-moles sucrose, and tissue equivalent to 1 g of rat liver in a total volume of 10 ml. Reactions were initiated by the addition of 5 μmoles of radioactive WIN 13099 in 0·1 ml of ethylene glycol dimethyl ether. A reagent blank, tissue blank, zero time control and boiled tissue control were run to supply control data for TLC and GLC analysis and to verify the metabolic nature of the conversion.

The anaerobic nature of the metabolism by 140,000 *g* supernatant liquid was investigated by carrying out experiments as above, except that the flasks were layered with 5 ml *n*-heptane and preincubated for 5 min at 37° before addition of substrate. Alternatively, the reactions were run in anaerobic Thunberg tubes or in Warburg flasks under nitrogen.

Reactions were terminated by extracting three times with 30-ml portions of ethyl acetate. The combined extracts were dried and dissolved in chloroform for GLC and TLC. Liquid scintillation counting of aliquots of the aqueous and organic phases after exctraction indicated that all of the radioactivity had been extracted from the various controls and 94 per cent from the flask in which the metabolism took place.

Extent of metabolism was estimated by comparing the areas of the peaks of the radiohistogram taken from TLC of the organic extract chromatographed in ethyl ether.

Metabolite isolation

Fifty mg of nonradioactive WIN 13099 was incubated with the 12,000 *g* supernatant liquid of three rat livers in a total volume of 200 ml at 37° for 2 hr to prepare sufficient metabolite for isolation and characterization. The ethyl acetate extract was filtered and taken to dryness by an air current at 60°. The residue was taken up in benzene and concentrated to 0·5 ml for column chromatography.

The benzene solution containing the metabolite was layered on a 300 × 19 mm column packed with 5 g of activated 100/120 mesh silicic acid in benzene. Material less polar than the subject compounds was washed off the column with 40 ml benzene. Chloroform–benzene (3:1, v/v) was then added and 5-ml fractions were taken until the amount of metabolite present in each fraction markedly diminished, as monitored by isothermal GLC. Impure fractions were combined and repeated through the process. In this manner, 30 mg of the major metabolite *in vitro* was recovered.

Biological activity

The ability of WIN 13099 and its major metabolite *in vitro* to inhibit electron transport in submitochondrial particles (ETP) prepared from heavy beef heart mitochondria was determined according to Merola and Brierley.[6] The rate of NADH oxidation was compared in the absence and presence of inhibitor and the results were expressed as concentration of inhibitor versus per cent inhibition (see Fig. 7a).

The ability of WIN 13099 and its major metabolite *in vitro* to inhibit *N*-demethylation of ethylmorphine and *O*-demethylation of *p*-acetanisidide was determined according to a modification of the procedure as described by Merola and Turnbull.[5] Inhibitor was added *in vitro* as a solution in ethanol before initiating the reaction by addition of substrate. Ethanol was found to have no effect on the reaction. Results are expressed as concentration of inhibitor versus per cent inhibition (see Fig. 7, b and c).

Inhibition of nitroreductase activity was measured in a similar manner, except that semicarbazide was omitted and the reaction was run under nitrogen. The reaction was terminated by the addition of 2 ml of 10% trichloroacetic acid (TCA), denatured protein was removed by centrifugation, and the concentration of *p*-aminobenzoic acid produced was determined according to Bratton and Marshall.[8] Results are expressed as concentration of inhibitor versus per cent inhibition (see Fig. 7d).

RESULTS

Synthesis

The infrared spectra of authentic and synthesized WIN 13099 were identical. Melting points of the two compounds and the mixed melting point were the same, i.e. 98–100°. TLC of synthesized WIN 13099 (with authentic WIN 13099 as the standard), using mobile phases 1, 2, 3 and 4, resulted in a single spot with the same R_f value as the standard in each system. No further spots were observed with ultraviolet light or by charring with sulfuric acid. R_f values observed were 0·79 for mobile phase 1, 0·62 for 2, 0·25 for 3, and 0·05 for 4. Radiohistograms taken of each plate produced a single peak whose R_f value corresponded to that of WIN 13099 in each case. Authentic and synthesized WIN 13099 were analyzed by isothermal and linear program GLC. A peak with a retention time of 15·5 min was observed isothermally and one of 34·0 min was seen utilizing a linear program. Only trace impurities were observed. The above data indicate that WIN 13099 synthesized with a tritium label is chemically identical to authentic WIN 13099 and is essentially chemically and radioactively pure, with a specific activity of 0·28 $\mu c/\mu$mole as determined by liquid scintillation counting.

Intracellular distribution of enzymatic activity

The intracellular distribution of enzymatic activity with regard to metabolism of WIN 13099 is shown in Table 1. Whole cell homogenate metabolized approximately one-half of the substrate within 30 min, and the activity was about equally divided between the supernatant and mitochondrial fractions. However, in the latter case, it was necessary to add heat-treated 140,000 *g* supernatant liquid to obtain appreciable conversion. In this and several other trials, the microsomal fraction was without

TABLE 1. INTRACELLULAR DISTRIBUTION OF WIN 13099 METABOLIZING ACTIVITY BY RAT LIVER*

Subcellular fraction	Per cent metabolism
Whole cell homogenate	47
Nuclear fraction	0
Nuclear fraction + boiled 140,000 *g* supernatant liquid	<3
Mitochondrial fraction	0
Mitochondrial fraction + boiled 140,000 *g* supernatant liquid	18
12,000 *g* supernatant liquid	30
Microsomal fraction + NADPH generating system	0
Microsomal fraction + NADPH generating system + boiled 140,000 *g* supernatant liquid	0
140,000 *g* Supernatant liquid	21
140,000 *g* Supernatant liquid incubated anaerobically	27

* Reactions were carried out for 30 min. The tissue for each is derived from 1 g rat liver. Metabolism of the tritiated drug was monitored by comparing the area of the peaks on radiohistograms taken of each incubation chromatographed in ethyl ether. One peak near the solvent front represents the parent compound and the other, near the origin, represents the metabolites.

activity in this conversion and perhaps consistent with this is the finding that in no case did there appear to be any requirement for molecular oxygen; the reaction proceeded as well under nitrogen in Thunberg tubes or in the Warburg apparatus under anaerobic conditions. Figure 1 shows TLC plates accompanied by radiohistograms of the organic extract of the complete reaction mixture. In addition to WIN 13099, other compounds (not present in the various controls) were observed with mobile phases 1, 2, 3 and 5. The R_f values of the metabolically produced compounds in various mobile phases were: overlapping spots at the origin and 0·05 in 1, 0·62 in 2, origin and 0·12 in 3, and 0·65 and 0·41 in 5. Additionally, an elongated spot was observed on the lower part of the TLC plate chromatographed in mobile phase 2, which appears as an elongated area of low level radioactivity on the accompanying radiohistogram. The peak at the origin of the radiohistogram taken of the plate chromatographed in mobile phase 5 is due to some radioactive WIN 13099, which crystallizes under these conditions.

The organic extracts of incubation mixtures were analyzed by isothermal GLC. Figure 2 shows a diagram of the GLC tracing of the zero time control and the incubation mixture in which the metabolic conversion took place. The peak with a retention time of 5·9 min is due to a compound in the sucrose–phosphate buffer extract and is probably derived from the extracting solvents. The small peaks at 0·9, 1·3 and 2·4 min are due to trace impurities in WIN 13099. The peak with a retention time of 15·9 min is due to WIN 13099 and the peak with a retention time of 7·4 min represents a metabolite of WIN 13099. The smaller peaks observed between those observed at 15·9 and 7·4 min appear to be due to metabolites less polar than the major metabolite.

Figure 3 shows the elution pattern of two metabolites which are eluted from a silicic acid column as analyzed by GLC. Relative peak height is plotted versus milliliters of

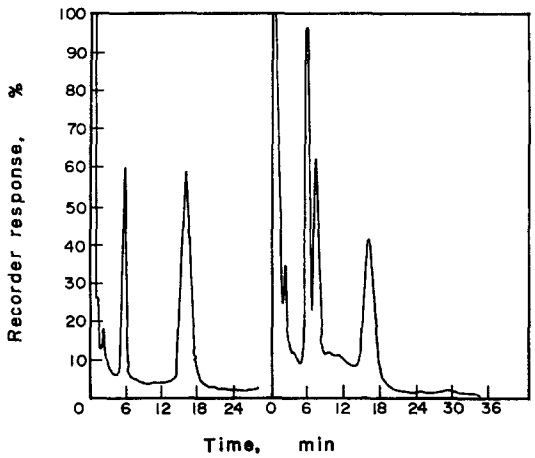

FIG. 2. Gas liquid chromatography of WIN 13099 and metabolites. The tracing on the left represents isothermal GLC of the ethyl acetate extract of the zero time control and the one on the right that of the metabolic conversion of WIN 13099 to more polar compounds on OV-101 at a column temperature of 220°. The peak appearing at 5·9 min is due to material in the zero time control and is probably derived from the extracting solvents.

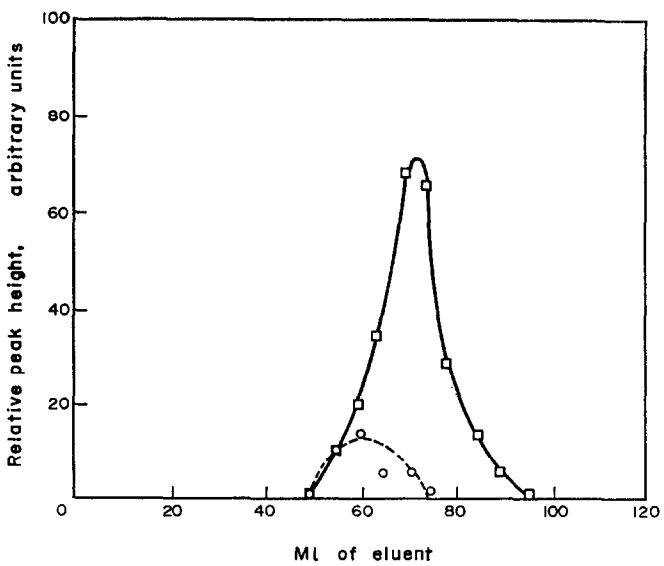

FIG. 3. Metabolite elution pattern from a 5-g silicic acid column. The first 40 ml of eluent was benzene and subsequent eluent was chloroform–benzene (3:1, v/v). Fractions (5 ml) were collected and elution was monitored by isothermal GLC on OV-101 at a column temperature of 220°. The more polar metabolite (□—□) had a retention time of 7·4 min and the less polar metabolite (○- - -○) had a retention time of 8·9 min. Other compounds were eluted before and after the indicated metabolites, but exhibited retention time on GLC unlike the major metabolite, and are omitted from the figure for clarity.

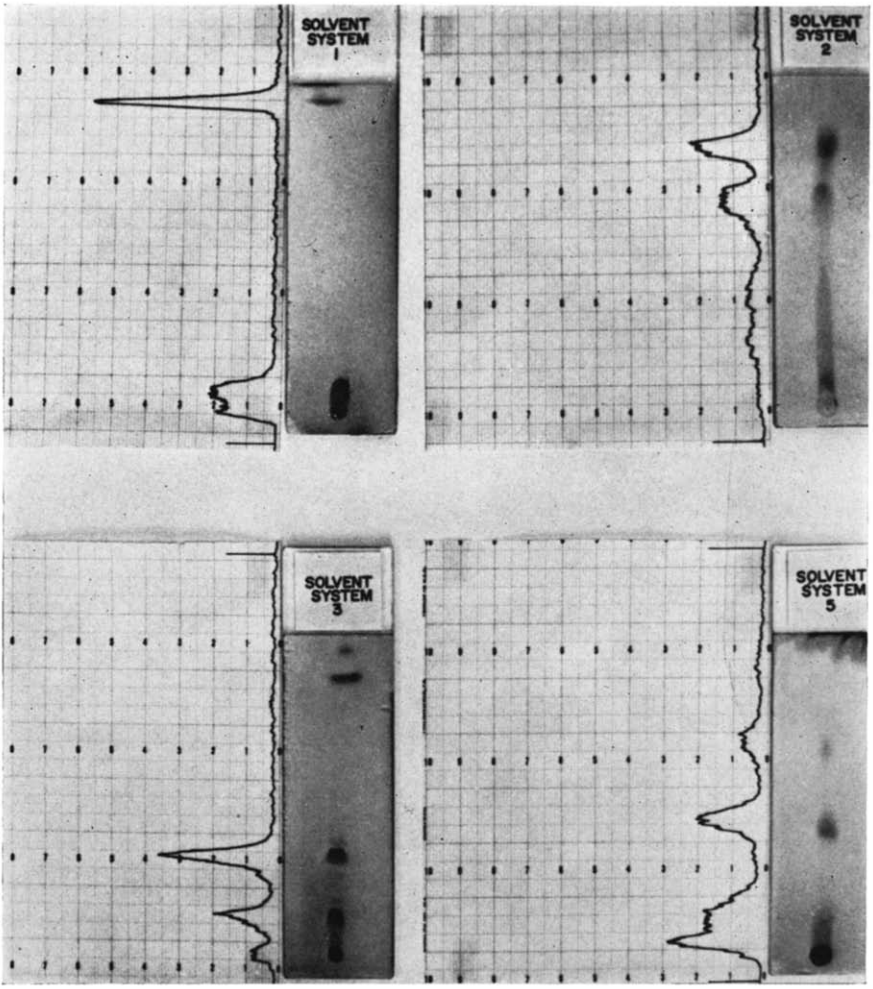

FIG. 1. Thin-layer chromatography of the ethyl acetate extract of the metabolic conversion of WIN 13099 to more polar compounds in mobile phases 1, 2, 3 and 5. Color was developed on the plates with I_2 vapor and the accompanying radiohistograms were taken with a Nuclear-Chicago Actigraph III TLC plate scanner.

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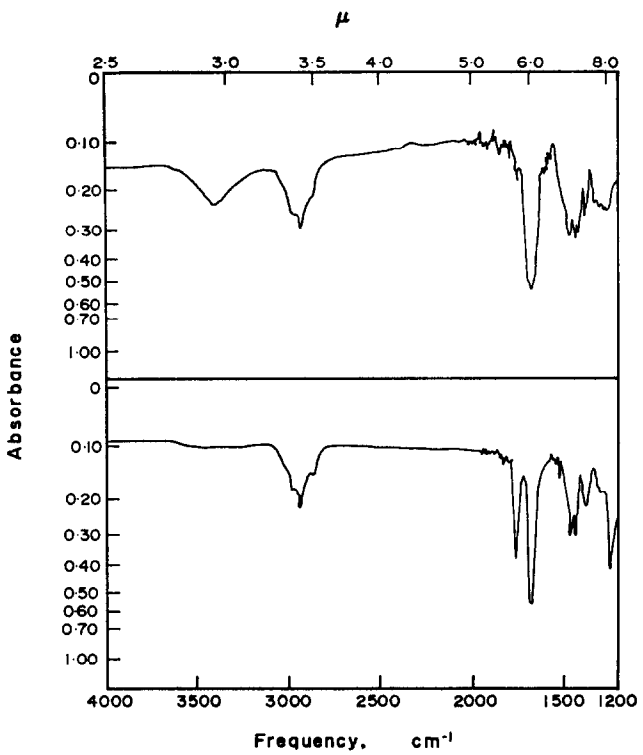


FIG. 4. Infrared spectra of the major metabolite *in vitro* of WIN 13099. The upper portion of the figure represents the partial i.r. spectrum of the major metabolite *in vitro* of WIN 13099. The lower portion represents the partial i.r. spectrum of the metabolite after acetylation with acetic anhydride. Spectra were taken as a film on a KBr window.

eluent. The fraction comprising 76–80 ml eluent contained the major metabolite, which appeared essentially pure as determined by isothermal and linear program GLC and by TLC. The amount of metabolite obtained was about 30 mg. It was isolated as a slightly yellow, viscous oil which decomposed at about 280°.

Qualitative tests for chlorine with silver nitrate and for sulfur with lead acetate after fusion of the metabolite with sodium indicated the presence of chlorine, but sulfur was absent. Qualitative tests for a phenolic hydroxyl group according to Millon's test and the α-nitroso-β-naphthol method of Ceriotti and Spandrio[9] were negative.

Figure 4 shows the partial i.r. spectrum (1200–4000 cm^{-1}) of the major metabolite *in vitro* of WIN 13099 and of the chemically acetylated metabolite. In addition to the peaks observed for WIN 13099, the metabolite has a rather wide band at 3400 cm^{-1}, suggesting an amine or hydroxyl group. This band at 3400 cm^{-1} disappears upon acetylation of the metabolite with acetic anhydride, but a sharp band appears at 1750 cm^{-1} which is not present in the metabolite or WIN 13099. A band at this wave number is indicative of an ester carbonyl group.

Figure 5 shows the n.m.r. spectrum of the major metabolite *in vitro* of WIN 13099 at 40°. The quadruplet at 1·0–1·4 ppm is due to the protons of the methyl groups on the *N*-ethyl moieties. The asymmetry of this quadruplet is also evident in the n.m.r.

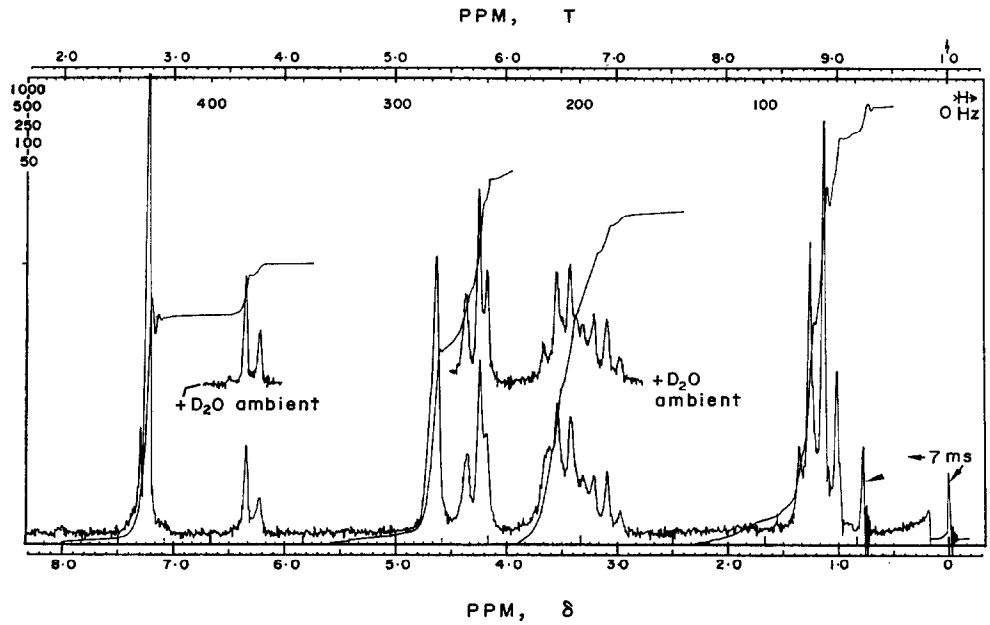


FIG. 5. Nuclear magnetic resonance spectrum of the major metabolite *in vitro* of WIN 13099 taken as a solution in deuterochloroform using tetramethylsilane as internal standard. The upper trace represents the spectrum obtained in the presence of D_2O.

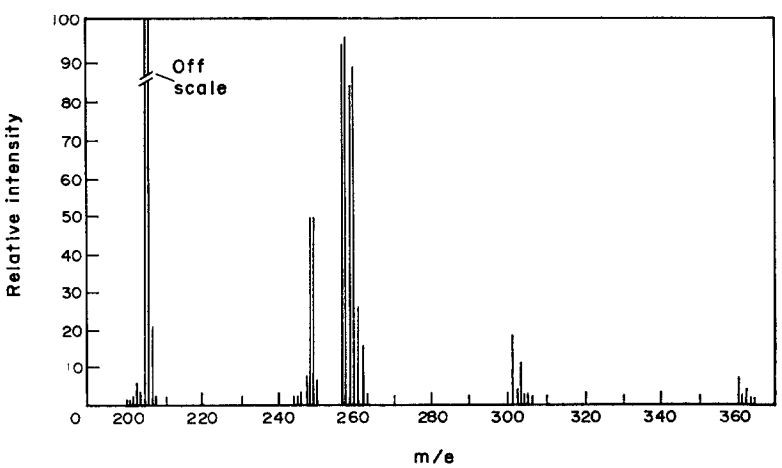


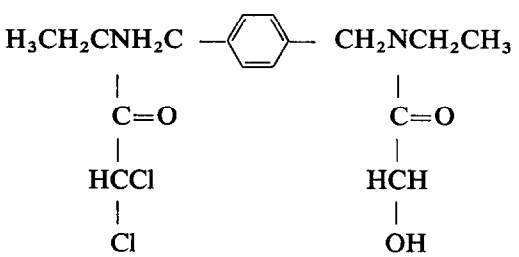
FIG. 6. Partial mass spectrum (m/e = 200 to m/e = 370) of the major metabolite *in vitro* of WIN 13099.

spectrum of the parent compound and may be due to hindered rotation. This quartet becomes more symmetrical at 60°. The absorption at 7·22 ppm is due to the benzene protons. The doublet at 6·21 and 6·32 ppm due to the methine proton is also present in the parent compound, but the relative intensity of the peaks suggests that, whereas two such protons are present in the parent compound, one is present in the metabolite. The doublet absorption characteristic of this proton becomes less apparent at 60°, suggesting that it also is due to hindered rotation. The absorption at 4·63 ppm is due to the xylylene protons. Additional peaks not present or less pronounced in the n.m.r. spectrum obtained with the parent compound appear at 4·23 and 3·62 ppm. These are interpreted as being due to the methylene and hydroxyl protons of a hydroxyacetyl moiety. Diminution of the peak at 3·62 ppm in the presence of D_2O supports this view. Integration of the spectrum was not entirely satisfactory, due probably to some impurity concentrated from solvent in the noncrystalline sample submitted to n.m.r. With this reservation in mind, the absorption at 4·37 ppm is believed due to an impurity and the absorption at 4·23 ppm due to two methylene protons. The entire complex at 2·97–3·62 ppm, which includes the four methylene protons of *N*-ethyl moieties and the hydroxyl proton, integrates to yield six protons instead of the expected five and we take this to mean that the peaks at 2·97–3·22 ppm represent an impurity. Taken alone, the n.m.r. data indicate loss of one of the dichloromethyl methine protons and the presence of an absorption peak due to a hydroxyl group proton, and also an absorption peak due to protons on a methylene group between a hydroxyl group and the carbonyl of a tertiary amide. This suggests that one of the dichloromethyl groups has been converted to a hydroxymethyl group.

Figure 6 shows the partial mass spectrum (m/e = 200–370) of the major metabolite *in vitro* of WIN 13099. The molecular ion and certain fragments contain two chlorine atoms. The isotopes of chlorine give rise to P, P + 2, and P + 4 peaks whose relative intensities are dependent on the amount of ^{35}Cl and ^{37}Cl present in each molecule. In these instances, the peak at the lowest mass unit (2 ^{35}Cl's) will be referred to. The molecular ion is at m/e = 360, loss of the hydroxyacetyl radical 301, loss of the *N*-ethyl-*N*-hydroxyacetamide radical 258, loss of the dichloroacetyl radical 249, and loss of the *N*-ethyl-*N*-dichloroacetamide radical 206. A peak is observed at m/e = 257, indicative of an ion similar to that observed at m/e = 258 but which has lost a proton. A similar phenomenon is observed at m/e = 205.

Elemental anal. Calc. for $C_{16}H_{22}Cl_2O_3$: C, 53·20; H, 6·4; N, 7·75; Cl, 19·63. Found: C, 53·64; H, 6·18; N, 7·18; Cl, 18·56.

Consideration of the i.r., n.m.r. and mass spectroscopy and the elemental analysis leads to the identification of the major metabolite as being *N*-dichloroacetyl-*N'*-hydroxyacetyl-*N*,*N'*-diethyl-1,4-xylylenediamine:



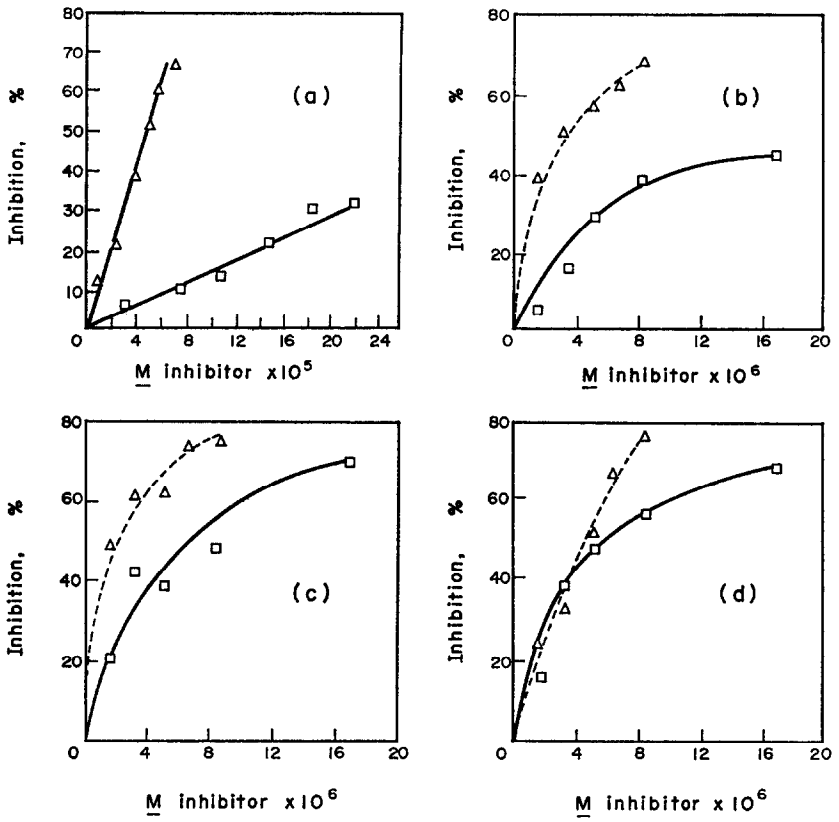


FIG. 7. (A) Inhibition of ETP catalyzed oxidation of NADH by WIN 13099 and by metabolite. Inhibition by WIN 13099 (Δ) and by metabolite (□) was measured in 5-ml incubations maintained at 37°, utilizing a Clark oxygen electrode (YSI). Each incubation contained 1·25 m-moles sucrose, 100 μmoles phosphate (K^+), pH 7·4, 30 μmoles $MgCl_2$ and 0·1 ml ETP (1·5 mg protein). The rate of oxidation of 1 μmole NADH was measured in the absence and then in the presence of inhibitor (added in ethanol) and the slopes of the linear portion of the curves were compared.

(B) Inhibition of *N*-demethylation of ethylmorphine by WIN 13099 and by metabolite. The ability of WIN 13099 (Δ) and metabolite (□) to inhibit *N*-demethylation of ethylmorphine was determined by carrying out reactions in 6-ml systems incubated in 25-ml Erlenmeyer flasks maintained at 37° with shaking. Each incubation contained 1·5 m-moles sucrose, 120 μmoles phosphate (K^+), pH 7·4, 30 μmoles $MgCl_2$, 2 μmoles NADP, 10 μmoles glucose 6-phosphate (G-6-P), 1 unit G-6-P dehydrogenase (Sigma), 4·5 μmoles semicarbazide, and 0·2 ml microsomes (6 mg protein). Inhibitor was added in ethanol and the reaction was initiated by the addition of 12 μmoles ethylmorphine in buffer. Reactions were terminated at 30 min by the addition of 2 ml of 10% TCA. Denatured protein was removed by centrifugation and formaldehyde was determined by the procedure of Cochin and Axelrod.[10]

(C) Inhibition of *O*-demethylation of *p*-acetanisidide by WIN 13099 and by metabolite. The ability of WIN 13099 (Δ) and metabolite (□) to inhibit *O*-demethylation was determined as in B, except that 12 μmoles *p*-acetanisidide was added in buffer to initiate the reaction.

(D) Inhibition of reduction of *p*-nitrobenzoate by WIN 13099 and by metabolite. The ability of WIN 13099 (Δ- - -Δ) and metabolite (□—□) to inhibit microsomal reduction of *p*-nitrobenzoic acid was similarly determined, except that the semicarbazide was omitted and the reaction was run under nitrogen. Inhibitor was added in ethanol and then 12 μmoles sodium *p*-nitrobenzoate was added to initiate the reaction. The reactions were terminated at 30 min by the addition of 2 ml of 10% TCA and denatured protein was removed by centrifugation. The concentration of *p*-aminobenzoic acid produced was determined according to the procedure of Bratton and Marshall.[8]

Figure 7a shows the inhibition of ETP catalyzed oxidation of NADH by WIN 13099 and by the metabolite. At all concentrations studied, the parent compound was about seven times as effective as metabolite in inhibiting the reaction.

Figure 7b shows the inhibition of ethylmorphine *N*-demethylase activity of rat liver microsomes by WIN 13099 and by the metabolite. Figure 7c shows the inhibition of *p*-acetanisidide *O*-demethylase activity of rat liver microsomes by WIN 13099 and by the metabolite. These data indicate that WIN 13099 is about five times as effective as the metabolite up to concentrations which cause about 30 per cent inhibition of *N*-demethylation of ethylmorphine or 40 per cent inhibition of *O*-demethylation of *p*-acetanisidide by rat liver microsomes.

Figure 7d shows the inhibition of *p*-nitroreductase activity of rat liver microsomes by WIN 13099 and by the metabolite. This figure indicates that the two compounds are similar in their ability to inhibit this reaction up to concentrations which cause about 50 per cent inhibition. Beyond this concentration, there was a diminution in the ability of the metabolite to inhibit nitroreductase activity of rat liver microsomes as compared to WIN 13099.

DISCUSSION

Heppel and Porterfield[1] carried out the 5-fold purification of an enzyme from rat liver which mediated formation of formaldehyde from dihalogenated methane derivatives. This enzyme was inhibited by sulfhydryl reagents and stimulated by cyanide and either cysteine or reduced glutathione. Optimal activity was observed at pH 7 and incubation under anaerobic conditions caused a 3-fold stimulation of the conversion.

Halidohydrolytic conversion of WIN 13099 occurs under similar conditions in the 140,000 *g* supernatant liquid of rat liver homogenate, except that the dichloromethyl group is converted to a hydroxymethyl group rather than an aldehyde, presumably by an additional reductive step. Corroboration for this type of conversion is supplied by Dill *et al.*,[4] who found the hydroxyacetyl analogue to chloramphenicol in the urine of cats and newborn infants given this drug. Our data indicate that this type of conversion is non-microsomal in nature. That the conversion occurs readily under anaerobic conditions implies that, like the halidohydrolytic microbial enzymes, the oxygen of the hydroxyl group derives from water. The requirement for deproteinized high-speed supernatant liquid for activity by the mitochondrial fraction might involve supplying a soluble, heat-stable cofactor.

Metabolic conversion has diminished the ability of WIN 13099 to inhibit *N*-demethylase, *O*-demethylase and *p*-nitroreductase activity of rat liver microsomes. Binding of WIN 13099 to rat liver microsomes is indicated by a spectral shift similar to that caused by type II substrates.* A possible reason why the parent compound, WIN 13099, is so effective as an inhibitor of microsomal drug metabolism is that this very lipid-soluble compound is capable of binding to the microsomal drug-metabolizing enzymes, but is not itself metabolized by them. Metabolism of WIN 13099 has resulted in a compound which is about one-seventh as effective as WIN 13099 in inhibiting electron transport. A chemically synthesized analogue of WIN 13099 in which both acyl groups are hydroxyacetate [*N*,*N*′-bis-(hydroxyacetyl)-*N*,*N*′-diethyl-

* J. D. Turnbull, unpublished observations.

1,4-xylylenediamine] was not active as an inhibitor of any of the above reactions.* This suggests the requirement of the dichloroacetyl group for optimal lipid solubility, as a binding site, as an active site or some combination of these to be effective as an inhibitor of drug metabolism[5] or electron transport.[6] Studies are underway to determine whether these hydroxyacetyl derivatives cause the alcohol intolerance effect exhibited by WIN 13099[11] and preliminary experiments suggest that they are not as effective as WIN 13099 in inhibiting aldehyde dehydrogenase *in vivo*. Perhaps a study of equal importance would be to determine whether there would be a further separation of the alcohol intolerance effect from the effects on drug metabolism and spermatogenesis. The latter study would require larger quantities of the metabolite than are available at this time. In any case, the study reported here is an example of a drug metabolism inhibitor which is bound to but not metabolized by rat liver microsomes. This offers a variation of the hypothesis that inhibitors of drug metabolism such as SKF 525-A act mainly by competing as substrates for the microsomal mixed-function oxidase system.[3]

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* D. A. Knowlton, unpublished observations.

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