Metabolism of Trichloroethylene in Liver Microsomes

II. Identification of the Reaction Product as Chloral Hydrate[1]

KEITH H. BYINGTON[2] AND KENNETH C. LEIBMAN

Department of Pharmacology and Therapeutics, University of Florida College of Medicine, Gainesville, Florida

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SUMMARY

The product of metabolism of trichloroethylene in rat liver microsomes has been characterized as chloral hydrate. In common with chloral hydrate, the metabolite is converted to dichloroacetic acid upon reaction with basic cyanide; to chloroglyoxal bis-2,4-dinitrophenylhydrazone upon treatment with 2,4-dinitrophenylhydrazine in acid; to trichloroethanol by the action of liver alcohol dehydrogenase; and to trichloroacetic acid upon oxidation mediated by chloral hydrate dehydrogenase. Possible mechanisms of the conversion of trichloroethylene to chloral hydrate are discussed.

INTRODUCTION

The pathway of conversion of trichloroethylene in mammals to its excreted metabolic products, trichloroacetic acid, trichloroethanol, and the glucuronide of the latter, has not been elucidated. Daniel (2) has shown that these products are formed without mixing of the chlorine atoms of trichloroethylene with the body chloride pool. Butler's postulation that chloral hydrate is an intermediate in this process (3) was based on the similarity between the final products of metabolism of chloral hydrate and trichloroethylene. Although Butler was unable to detect chloral hydrate after administration of trichloroethylene, his assumption has erroneously been presented as proved fact in several major textbooks of pharmacology and by other authors. The demonstration that trichloroethylene is converted to a nonvolatile polyhalogenated substance by liver microsomes (4) has provided a means of testing Butler's hypothesis.

GENERAL METHODS

Amounts of the trichloroethylene metabolite sufficient for identification were isolated from large volumes of the *in vitro* trichloroethylene metabolizing system previously reported (4). The following solutions were mixed in a 2800 ml Fernbach culture flask and gently shaken for 2 hr at 37° in a Dubnoff metabolic shaking incubator: 160 ml of a solution containing (at the indicated concentrations) Tris-HCl buffer, pH 7.5 (0.5 M), $MgSO_4$ (2.5×10^{-2} M), disodium EDTA[3] (3.0×10^{-3} M), NADP (3.0×10^{-5} M), ATP (1.5×10^{-3} M), nicotinamide (5.0×10^{-2} M) and sodium fumarate

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[2] Postdoctoral trainee, U. S. Public Health Service training Grant No. GM-760. Present address: Institute for Enzyme Research, University of Wisconsin, Madison, Wisconsin.

[3] Abbreviations used are EDTA, ethylenediamine tetraacetate; NAD and NADH, oxidized and reduced forms, respectively, of nicotinamide-adenine dinucleotide; NADP and NADPH, oxidized and reduced forms, respectively, of nicotinamide-adenine dinucleotide phosphate; ATP, adenosine triphosphate; Tris, tris(hydroxymethyl)aminomethane.

(1.0×10^{-2} M); 240 ml of an emulsion of trichloroethylene, cottonseed oil, Tween 80® polysorbate, and water (4); and 80 ml of a suspension containing 75 mg/ml of lyophilized 9000 *g* supernatant fraction of rat liver from rats which had been pretreated with phenobarbital (5). The incubated mixture was titrated to pH 1.5 with 6 N HCl and steam distilled; the volume of distillate collected was 750 ml. After bubbling air through the distillate for 1 hr to remove trichloroethylene, colorimetric analysis usually indicated from 25 to 30 μg of chloral hydrate per milliliter. A more concentrated solution of the metabolite could be prepared by evaporating part of the water at temperatures less than 50° under a stream of air or by extracting the metabolite into diethyl ether and then evaporating the ether over a small volume of water.

Infrared spectra were recorded using a Beckman IR-5 infrared spectrophotometer and the potassium bromide pellet technique.

Chloral hydrate was routinely determined in the steam distillate by the modified Fujiwara reaction of Leibman and Hindman (6) or by the quinaldine ethiodide method of Archer and Haugas (7). Two additional methods for chloral hydrate were briefly studied: the reaction with alkaline phloroglucinol and the formation of the diphenylformazan upon oxidation of the phenylhydrazone. Optimum conditions for these procedures were found to be as follows:

Phloroglucinol reaction. To 2 ml of sample were added 2 ml of 1% aqueous phloroglucinol and 0.5 ml of 1 M ammonium hydroxide. The components were mixed, and the mixture was allowed to stand at room temperature for 1 hr. The absorbance at 460 mμ was then determined, using a reagent blank. Absorbance per micromole of chloral hydrate was 1.16.

Diphenylformazan formation. This was modified from the method described by Kramer *et al.* (8) for formaldehyde, glyoxal, and glyoxylic acid. A mixture of 2 ml of sample and 2.5 ml of 1% phenylhydrazine hydrochloride was heated in a boiling water bath for 10 min. The solution was cooled and 2.5 ml of concentrated HCl was added, followed by 2.5 ml of 1% potassium ferricyanide. The absorbance was determined at 367 mμ against a reagent blank. Absorbance per micromole of chloral hydrate was 0.85.

PROCEDURES AND RESULTS

Colorimetric Reactions

In Table 1 are shown the results of five different colorimetric analytical procedures applied to the same solution of metabolite from the microsomal oxidation of trichloroethylene. Although none of these methods are absolutely specific for chloral hydrate, comparable quantitative results were obtained. The Fujiwara alkaline-pyridine reaction, and its modification, are specific for compounds containing polyhalogenated carbon atoms. The quinaldine ethiodide method has been suggested to be specific for the $CH(OH)_2$ group (7). The reaction with phloroglucinol and the formation of diphenylformazans are characteristic of aldehydes.

TABLE 1

Assay of the trichloroethylene metabolite by five colorimetric methods

References for the first three methods are indicated. The latter two procedures are described under General Methods.

Method	Chloral hydrate (μg/ml)
Fujiwara reaction (9)	52
Fujiwara-benzidine modification (6)	53
Quinaldine ethiodide method (7)	59
Phloroglucinol reaction	63
Diphenylformazan formation	67

Reaction of the Metabolite with Basic Cyanide Solution

The trichloroethylene metabolite reacted with a basic cyanide solution to yield dichloroacetic acid; this is a demonstrated reaction of chloral hydrate (10). Dichloroacetic acid was identified by infrared spectroscopy and by thin layer chromatography.

A 645 ml sample of a solution of the trichloroethylene metabolite, which con-

tained 22 μg of chloral hydrate per milliliter, was treated with 10 g of $CaCO_3$ and 12 mg of NaCN. The slurry was then heated at 50–60° for 2 hr. Most of the water was then evaporated by increasing the temperature and directing a stream of air over the surface of the slurry. Drying was completed on a steam bath and in an oven at 110°. The dry residue was thoroughly washed with ether, dissolved in excess 1 N HCl and extracted four times, each time with one volume of ether. The combined ether extracts were evaporated to about 50 ml and extracted with dilute, carbonate-free potassium hydroxide. The aqueous phase was titrated to pH 4.0 and evaporated to dryness on the steam bath, and the residue was dried at 110°.

A reference solution of chloral hydrate also was treated as described above. Figure 1 is a tracing of the infrared spectra of

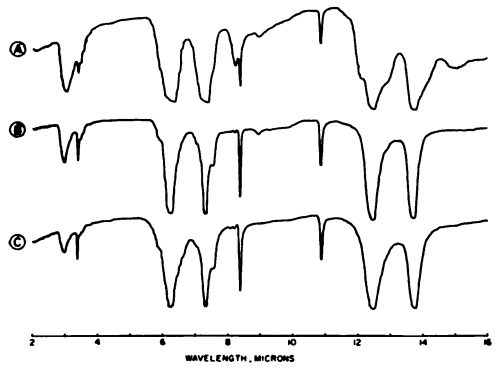


FIG. 1. *Infrared spectra of reaction product of alkaline cyanide*

(A) With the trichloroethylene metabolite. (B) With chloral hydrate. (C) Spectrum of the potassium salt of dichloroacetic acid (Calbiochem).

potassium dichloroacetate and of the potassium salts of the compounds obtained from the reaction of cyanide with the metabolite and with reference chloral hydrate.

Thin layer chromatography on silica gel G was performed with *n*-propanol:concentrated NH_4OH (70:30 v/v). Spots were located with bromocresol green indicator. The R_F values (0.46) of the products obtained from the reactions of the metabolite and known chloral hydrate with basic cyanide were identical to that of authentic dichloroacetic acid. Dichloroacetic acid was easily separated from trichloroacetic acid (R_F 0.53) with this system; the latter was not found in the reaction mixtures.

Reaction of 2,4-Dinitrophenylhydrazine with the Metabolite

Chloral hydrate and 2,4-dinitrophenylhydrazine react to yield numerous products (11). One of these compounds, chloroglyoxal bis-2,4-dinitrophenylhydrazone, crystallizes from the reaction mixture if an acidic solution of chloral hydrate (40–80 μg/ml) is treated with two equivalents of 2,4-dinitrophenylhydrazine in phosphoric acid–ethanol (Johnson's reagent) (12).

The steam distillates from several preparations were pooled and concentrated, by evaporating part of the water, to obtain 1650 ml of a solution containing about 45 μg/ml (as chloral hydrate) of metabolite. This solution was made 0.3 M in phosphoric acid, heated to 85° and treated with 4.7 ml of Johnson's reagent. The product, a few milligrams of orange powder, was filtered after the solution had been cooled for 6 hr. This material melted at 207–210°. After prolonged extraction with two 20-ml portions of ethanol at room temperature, the ethanol-insoluble residue melted with decomposition at 258–260°. Treating a reference solution containing 40 μg/ml of chloral hydrate in the same manner yielded a product which melted, with decomposition, at 256–258°. A decomposition point of 259° has been reported for chloroglyoxal bis-2,4-dinitrophenylhydrazone (11).

Figure 2 shows the infrared spectra of these products. In Fig. 3 are illustrated the absorption maxima at 385 and 435 mμ of chloroform solutions of these compounds. These absorption maxima are characteristic of 1,2-dicarbonyl-bis-2,4-dinitrophenylhydrazones (13).

Reduction of the Metabolite with Alcohol Dehydrogenase and NADH

The reduction of chloral hydrate to trichloroethanol, mediated by liver alcohol dehydrogenase, has been demonstrated (14). In this system, the metabolite from

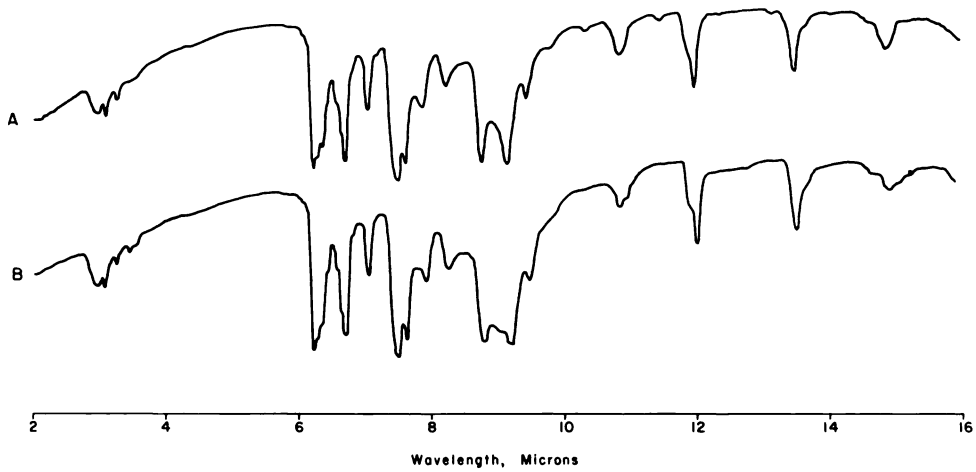


FIG. 2. *Infrared spectra of chloroglyoxal bis-2,4-dinitrophenylhydrazone prepared from chloral hydrate (A) and from the trichloroethylene metabolite (B)*

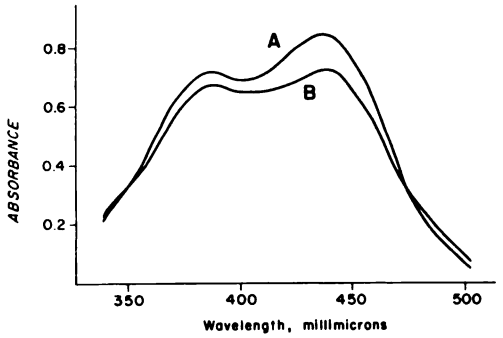


FIG. 3. *Absorption spectra of chloroglyoxal bis-2,4-dinitrophenylhydrazone prepared from chloral hydrate (A) and from the trichloroethylene metabolite (B)*

trichloroethylene was converted to a substance identified by gas chromatography as trichloroethanol.

The solution of the trichloroethylene metabolite prepared as described above contained amounts of trichloroethanol detectable by gas chromatography, presumably due to the presence of alcohol dehydrogenase in the 9000 *g* supernatant fraction; therefore, a solution of metabolite was prepared for reduction with alcohol dehydrogenase by incubating the trichloroethylene emulsion with isolated liver microsomes and added NADPH (4). The incubation mixture was similar in composition to that described above except that NADP was omitted, and isolated microsomes equivalent to 4 ml of 9000 *g* supernatant fraction were used as the enzyme source per 12 ml of total volume. At zero time 3.0 μmoles of NADPH was added and a further 1.5 μmoles was added every 15 min during the 2-hr incubation. After incubation, the contents of five such flasks were combined, acidified, steam-distilled, and bubbled free of trichloroethylene. The volume of the distillate from the five flasks was 100 ml. Of this solution, 10 ml was reserved for gas chromatography. The remaining solution was concentrated by the ether extraction method to yield 6 ml of a solution assayed to contain 125 μg/ml of chloral hydrate. Five milliliters of the concentrated metabolite solution, 0.25 ml of 1 M sodium phosphate buffer, pH 7.4, and 14 mg of equine liver alcohol dehydrogenase (Worthington Chemical Co.) were incubated at 37°. At the beginning of the incubation, 0.6 ml of 7.5 mM NADH was added, and 0.1 ml of the same solution was added each 15 min throughout the incubation. After 2 hr, the reaction mixture was analyzed as described by Garrett and Lambert (15); after addition of 5 g of sodium chloride, the mixture was extracted with 6 ml of benzene, and aliquots of the extract were subjected to gas-liquid chromatog-

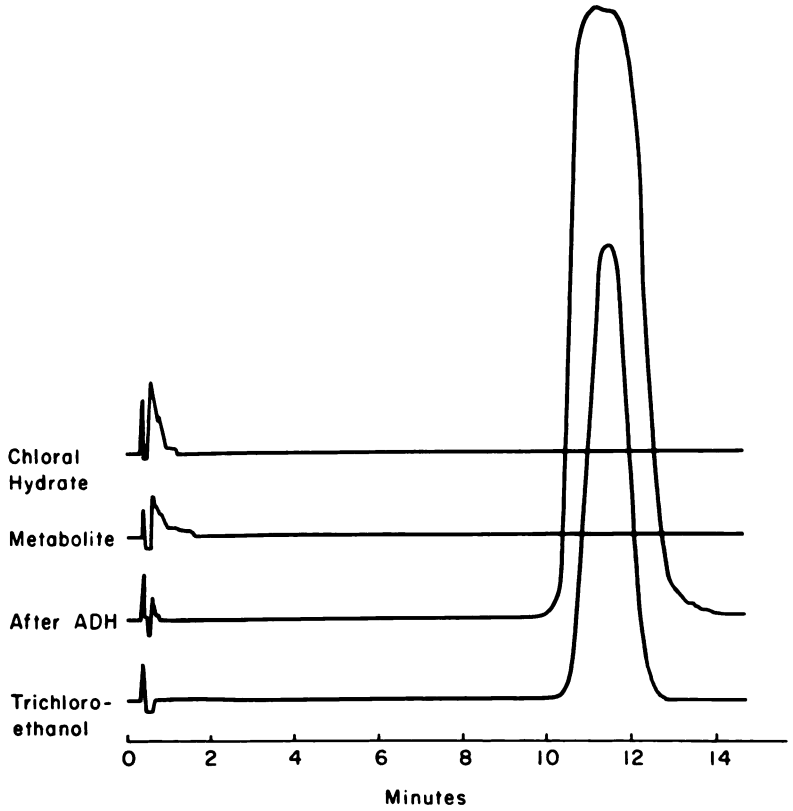


FIG. 4. *Gas chromatograms illustrating conversion of the trichloroethylene metabolite to trichloroethanol by alcohol dehydrogenase*

Tracings (from the top) are: chloral hydrate; trichloroethylene metabolite; product of incubation of trichloroethylene metabolite with alcohol dehydrogenase and NADH; trichloroethanol. F&M Model 700 gas chromatograph was used with electron capture detector. Column: 4 ft stainless steel containing 20% Carbowax 20M on 60–80 mesh Chromosorb W, 130°. Injection port, 120°. Carrier and purge gas flows, 60 and 140 ml/min, respectively.

raphy. Figure 4 shows the chromatograms of chloral hydrate, the microsomal trichloroethylene metabolite, the product of enzymic reduction of this metabolite, and reference trichloroethanol. The metabolite showed the same retention time as did chloral hydrate, while after enzymic reduction the chromatographic peak corresponding to chloral hydrate was much reduced in area, and a new peak of retention time identical to that of trichloroethanol appeared.

Oxidation of the Metabolite with Chloral Hydrate Dehydrogenase and NAD

Cooper and Friedman (16) have described an enzyme which mediates the oxidation of haloacetaldehydes to the corresponding haloacetic acids. The metabolite from trichloroethylene was found to act as a substrate for this enzyme.

The enzyme was prepared from rabbit liver as described by Cooper and Friedman (16). To a solution of metabolite estimated to contain 13 μmoles of chloral hydrate were added 50 mg of the lyophilized redialyzed enzyme preparation and 160 μmoles of NAD in 0.05 M glycine–pyrophosphate buffer, pH 9.5. Trichloroacetic acid was estimated by the modified Fujiwara method (procedure B) (6), and NADH from the absorbance at 340 mμ. After 100 min, 3.3 μmoles of trichloroacetic acid and 3.0 μmoles of NADH had been formed. After

the mixture had stood at room temperature overnight, 8.2 μmoles of trichloroacetic acid were found in it.

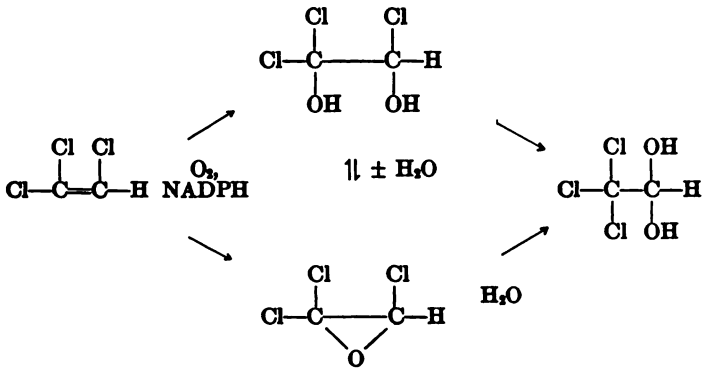
DISCUSSION

The present identification of the product of metabolism of trichloroethylene in liver microsomes as chloral hydrate justifies Butler's hypothesis (3). Butler was unable to demonstrate the presence of chloral hydrate in the plasma of dogs after the administration of trichloroethylene by inhalation. Chloral hydrate, however, has a very short biological half-life in dogs (17, 18), mice (19), and men (18). Thus, Marshall and Owens could find no chloral hydrate in human plasma at intervals from 5 to 30 min after ingestion of large hypnotic doses (30 mg/kg) of chloral hydrate, although both trichloroethanol and trichloroacetic acid were measurable in the plasma during this time. After much larger doses (250 mg/kg) intravenously in dogs, chloral hydrate disappeared from the plasma with a half-life of approximately 10 min (16). Scansetti *et al.* (20) reported surprisingly high (>200 mg/liter) plasma concentrations of chloral hydrate in workers exposed to trichloroethylene, with a plasma half-life of 13 hr. Thus, according to these data, the blood plasma alone of these individuals contained approximately a hypnotic dose of chloral hydrate. It is difficult to reconcile these reports. It should be noted that in all these studies, chloral hydrate was estimated by a difference in absorption between two relatively insensitive and nonspecific colorimetric procedures, and therefore greatly subject to error. Using a more sensitive analytic technique, McKay and Cooper (19) found the half-life of chloral hydrate in mouse brain to be 20–40 min.

The demonstration that the conversion of trichloroethylene to chloral hydrate is similar to a large number of drug-metabolic reactions requiring NADPH and oxygen and mediated by liver microsomes (4) leads to interesting speculation on the pathway of this conversion. This group of microsomal enzymes have been characterized as hydroxylating systems (21). It would appear likely, then, that trichloroethylene glycol or trichloroethylene oxide, or both, might be intermediates in the conversion of trichloroethylene to chloral hydrate. Both glycols and epoxides have been shown to be products of microsomal oxidation of olefinic compounds. Thus, dihydronaphthalene is converted in such a system to the corresponding glycol (22), and epoxides are produced from the insecticides aldrin, heptachlor, and isodrin (23). In the case of trichloroethylene, both the glycol and the epoxide would be expected to be quite unstable. The epoxide-carbonyl rearrangement of trichloroethylene oxide could give rise to either chloral or to dichloroacetyl chloride, depending on whether the chlorine or hydrogen atom migrated. McDonald and Schwab (24) have obtained evidence of the preferential migration of the chlorine atom in the rearrangement of α-chloroepoxides. Thus, unsymmetrical ring-substituted α-chlorostilbenes, on attempted epoxidation with peracids, yielded only that α-chloroketone which would have resulted from rearrangement of the assumed intermediate epoxide with migration of the chlorine atom. A photocatalytic oxygenation product of trichloroethylene, assumed to be the epoxide, was converted under anhydrous conditions to either chloral or dichloroacetyl chloride, depending on the catalyst employed (25). Tetrachloroethylene oxide rearranges to trichloroacetyl chloride (26), a process requiring chlorine atom migration.

Trichloroethylene glycol would undoubtedly be quite short-lived; the great instability of α-haloalcohols has been reported (27–29). In an intramolecular rearrangement similar to the pinacol rearrangement (30), with migration of the chlorine atom, chloral hydrate would be formed. The epoxide and the glycol might possibly be interconverted by hydration and dehydration.

Citing the work of Daniel (2), who showed that there is no dilution of the chlorine atoms of trichloroethylene with chlorine atoms from other body pools during conversion to trichloroethanol and trichloroacetic acid, Van Dyke and Chenoweth (31) have very recently rejected Butler's hypothesis, claiming that such dilution would be an expected consequence of the rearrangement of an epoxide inter-



mediate. They propose instead an acid-catalyzed rearrangement of trichloroethylene to a cyclic chloronium ion, which upon attack by water or by glucuronic acid forms trichloroethanol or its glucuronide, respectively, as the first stable product. However, the present work shows that although in the presence of both microsomes and soluble supernatant fraction of liver, both chloral hydrate and trichloroethanol are found after incubation with trichloroethylene, no trichloroethanol is formed when the soluble fraction is omitted, and NADPH is supplied directly to the microsomes. Any proposed mechanism for the microsomal oxidation of trichloroethylene must therefore account for chloral hydrate, not trichloroethanol, as the first stable intermediate. Furthermore, migration of the halogen atom during pinacol rearrangement of a glycol intermediate or during epoxide-carbonyl rearrangement of a haloepoxide, need not involve removal of the halogen atom as a halide ion, any more than in the rearrangement proposed by Van Dyke and Chenoweth. Indeed, such a chlorine bridge may be approached as a step in the intramolecular rearrangement of the glycol or epoxide in a process which may be represented crudely as follows:

Apparently the only attempt prior to the present work to demonstrate metabolism of trichloroethylene *in vitro* was made by Fabre and Truhaut (32), who incubated trichloroethylene with homogenates of rat brain, liver, lung, spleen, and kidney for up to 8 hr, and measured, by the nonspecific Fujiwara reaction, the "trichloroacetic acid" formed. One of us has shown (4) that only liver microsomes form chloral hydrate from trichloroethylene, and that those of brain, spleen, lung, and kidney are inactive. In addition, pretreatment of rats with phenobarbital, which greatly enhances the ability of liver microsomes to oxidize trichloroethylene, does not cause the induction of such activity in microsomes of other tissues (5).

The present work, together with that of Cooper and Friedman (14, 16), provides a complete *in vitro* pathway for the conversion of trichloroethylene to its major metabolites, trichloroethanol and trichloroacetic acid.

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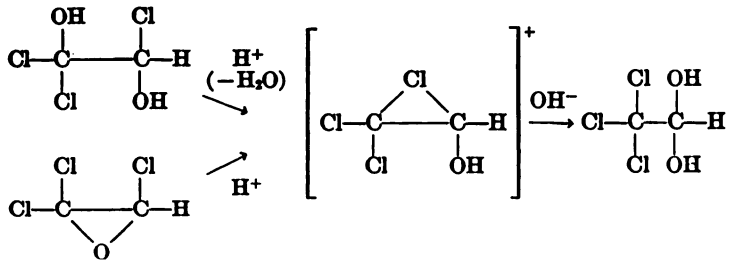

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REFERENCES

1. K. H. Byington and K. C. Leibman, *Federation Proc.* **24**, 300 (1965).
2. J. W. Daniel, *Biochem. Pharmacol.* **12**, 795 (1963).
3. T. C. Butler, *J. Pharmacol. Exptl. Therap.* **97**, 84 (1949).
4. K. C. Leibman, *Molec. Pharmacol.* **1**, 239 (1965).
5. K. C. Leibman and W. J. McAllister, Jr., *Pharmacologist* **7**, 159 (1965).
6. K. C. Leibman and J. D. Hindman, *Anal. Chem.* **36**, 348 (1964).
7. A. W. Archer and E. A. Haugas, *J. Pharm. Pharmacol.* **12**, 754 (1960).
8. D. N. Kramer, N. Klein and R. A. Baselice, *Anal. Chem.* **31**, 250 (1959).
9. P. J. Friedman and J. R. Cooper, *Anal. Chem.* **30**, 1674 (1958).
10. C. Rosenblum, C. Taverna and N. L. Wendler, *Chem. Ind. (London)* 718 (1960).
11. L. A. Jones, C. K. Hancock and R. B. Seligman, *J. Org. Chem.* **26**, 228 (1961).
12. G. D. Johnson, *J. Am. Chem. Soc.* **73**, 5888 (1951).
13. L. A. Jones and C. K. Hancock, *J. Am. Chem. Soc.* **82**, 105 (1960).
14. P. J. Friedman and J. R. Cooper, *J. Pharmacol. Exptl. Therap.* **129**, 373 (1960).
15. E. R. Garrett and H. J. Lambert, *FLACS* (Florida Sect. Publ., Am. Chem. Soc.) **18**(8), 16 (1965).
16. J. R. Cooper and P. J. Friedman, *Biochem. Pharmacol.* **1**, 76 (1958).
17. T. C. Butler, *J. Pharmacol. Exptl. Therap.* **92**, 49 (1948).
18. E. K. Marshall, Jr. and A. H. Owens, Jr., *Bull. Johns Hopkins Hosp.* **95**, 1 (1954).
19. F. J. MacKay and J. R. Cooper, *J. Pharmacol. Exptl. Therap.* **135**, 271 (1962).
20. G. Scansetti, G. F. Rubino and G. Trompeo, *Med. Lavoro* **50**, 743 (1959).
21. B. B. Brodie, J. R. Gillette and B. N. LaDu, *Ann. Rev. Biochem.* **27**, 427 (1958).
22. E. Boyland and P. Sims, *Biochem. J.* **77**, 182 (1960).
23. D. T. Wong and L. C. Terriere, *Biochem. Pharmacol.* **14**, 375 (1965).
24. R. N. McDonald and P. A. Schwab, *J. Am. Chem. Soc.* **85**, 4004 (1963).
25. F. W. Kirkbride, U. S. patent 2,292,129 (1942).
26. D. M. Frankel, C. E. Johnson and H. M. Pitt, *J. Org. Chem.* **22**, 1119 (1957).
27. M. Hanriot, *Ann. Chim. Phys.* [5] **25**, 219 (1882).
28. F. M. Litterscheid, *Liebig's Ann.* **316**, 157 (1901).
29. A. Stepanow, N. Preobraschensky, and M. Schtschukina, *Ber. Deut. Chem. Ges.* **59**, 2533 (1926).
30. C. J. Collins, *Quart. Rev.* **14**, 357 (1960).
31. R. A. Van Dyke and M. B. Chenoweth, *Anesthesiology* **26**, 348 (1965).
32. R. Fabre and R. Truhaut, *Brit. J. Ind. Med.* **9**, 39 (1952).