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# ANTHELMINTICS I. THE EFFECT OF HYDROGEN PEROXIDE AND SOME OXYGENATED TERPENE HYDROCARBONS UPON *ASCARIS LUMBRICOIDES*.*

BY LEWIS W. BUTZ AND W. A. LA LANDE, JR.

The anthelmintic efficiency of oil of chenopodium in *Ascaris* infections of human beings is well established. The oil is also useful in other helminthiases (1). Its high toxicity for the host (2), (3), however, would seem to render unsafe its administration in doses sufficiently large and frequent to insure the desired anthelmintic effect. Henry and Paget (4) fractionated chenopodium oil into its constituents. These were later (5) examined separately for anthelmintic activity by Smillie and Pessoa who reported that this resided almost entirely in the ascaridole. It seems not unlikely that part of the toxicity of chenopodium oil for the human host may be due to the components other than ascaridole, *e. g.*, cymene and methyl salicylate, but apparently (2) ascaridole itself is quite toxic. It would be desirable therefore to study other substances chemically related to ascaridole with the hope of finding one with a higher therapeutic index. The work reported here represents the beginning of a proposed extended investigation having this aim in view.

The chemical structure of ascaridole (A) seems to be well established by analytic studies (6), (7), (8), although another formula has been suggested (9). It is interesting to consider whether the anthelmintic activity of this substance can be attributed to any one grouping in the molecule or whether this is due to the summation of its chemical and physical characteristics. As an approach to a solution of this question the action of hydrogen peroxide and of disuccinyl peroxide (alphozone) upon *Ascaris lumbricoides* has been studied and these substances have been found to be very toxic to the parasites. This indicates that the peroxide group, or the hydrogen peroxide or nascent oxygen arising therefrom under various conditions, has in itself pronounced anthelmintic properties. These findings logically lead to an examination of other peroxides some of which might have the desired property of low toxicity for the human host, and in addition be sufficiently stable to serve as therapeutic agents administrable *per os*.

Peroxides with these characteristics have first been sought among the terpene derivatives. It has long been known that terpenes upon exposure to air become altered and that peroxide formation is one of the processes that takes place. We have oxygenated a series of commercial and highly purified terpene hydrocarbons. In many cases products were obtained which were of the same order of toxicity to *Ascaris* as ascaridole although this toxicity probably cannot be attributed to

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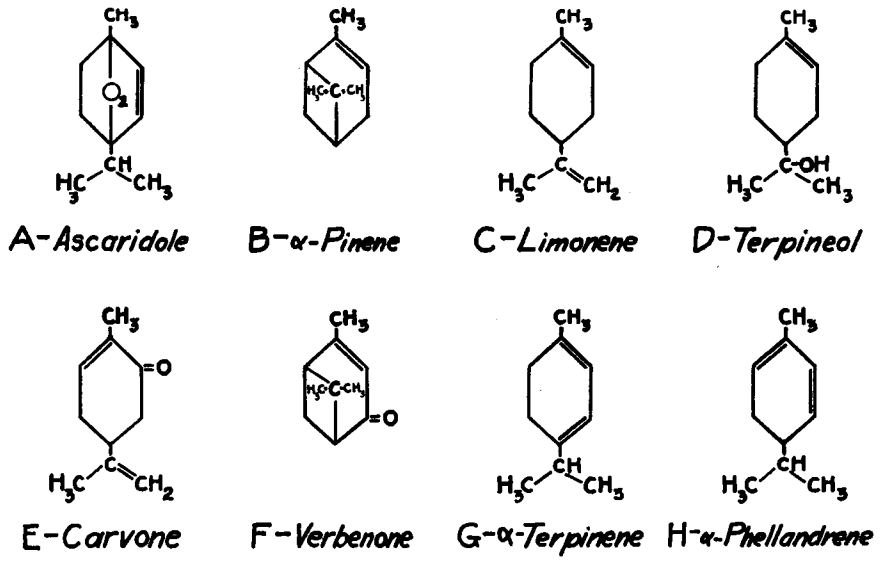
peroxides. The preliminary experiments indicated the wisdom of a more thorough study of α-pinene (B), turpentine (90–95% α-pinene), and *d*-limonene (C). The results of this study are reported in this paper. In order to determine whether any of the known oxidation products of these hydrocarbons were responsible for the activity we have tested so far the following compounds: Terpineol (D), carvone (E), verbenone (F) and formic acid. In addition we have included for comparison bioassay data obtained with chenopodium oil, ascaridole and formaldehyde.




EXPERIMENTAL.

*Materials.*—Seven specimens of commercial chenopodium oil were tested. These were all found to have the same anthelmintic potency and accordingly only one set of data is included in Table I. The two samples of ascaridole used in the tests were obtained from Eastman Kodak Co. at different times (A and B, Table I). Eighty per cent of B boiled between 92.5–4.5° at 8 mm. The hydrogen peroxide solutions were prepared from Merck's superoxol. The disuccinyl peroxide was Stearns' alphozone. All of the pinene products used in the experiments were prepared from Eastman Kodak "practical" pinene, b. p. 158–161°; the *d*-limonene was the best product obtainable from the same source, b. p. 67–68° at 20 mm., and was redistilled and used without further purification. One specimen of turpentine was obtained from a paint dealer (A, Table II); the other (B) was Merck's oil of turpentine U. S. P. X rectified. The two samples of terpineol (C and D, Table III) were obtained from Givaudan-Delawanna Co. and Eastman Kodak Co., respectively, and were used without further purification. The carvone, b. p. 113–114° at 16 mm., was obtained from Eastman Kodak Co. The verbenone, b. p. 98–101° at 16 mm., was prepared from *d*-α-pinene by oxidation with selenium dioxide according to the method of DuPont, *et al.* (10). The formic acid solutions were prepared by diluting Eastman Kodak formic acid (98–100%) m. p. 6–8°. The formaldehyde solutions were similarly prepared from Baker's C.P. 36%. The cobalt naphthenate catalyst[1] used in the oxidations was a commercial product. Tank oxygen was used for the oxygenations; purification was unnecessary since the gas contained only nitrogen and carbon dioxide as impurities.

*Oxidation of the Terpenes.*—Thirty-Gm. portions of the hydrocarbons were each treated with 0.4 cc. of the catalyst (equivalent to 0.05% cobalt) and placed in 250-cc. Erlenmeyer flasks which had previously been flushed with oxygen or air. The necessary atmosphere was maintained in

[1] Nuodex Cobalt, kindly furnished by Oliver Haun, D. H. Litter Co., Philadelphia.

each flask by passing into it a very slow stream of the oxygen or air. The loss by evaporation was inappreciable. All the oxidations were carried out at room temperature in diffused light. No attempt was made to exclude moisture. Although most of the cobalt was precipitated during the oxidation, it was nevertheless ascertained by blank experiments that the catalyst had no effect on the worms other than a slight stimulation. The oxidation products, which were usually yellow and more viscous than the original hydrocarbons, were kept in glass-stoppered bottles until needed. Active products can also be obtained without the catalyst (No. 17, Table II).

*Technique of Bioassay.*—In this work anthelmintic activity toward *Ascaris lumbricoides* was determined by immersion of vigorous specimens in a solution or emulsion of the substance to be tested. The apparatus consisted of a rectangular aquarium with glass windows which was filled with water and served as a constant temperature bath. An electrical heating unit, a thermostat and a mechanical stirrer maintained a uniform temperature of 37.5° ± 0.5°. The aquarium was provided with a sheet copper cover containing holes which supported 400-cc. beakers of the tall variety. Five worms were placed in each beaker which contained 300 cc. of the solution or emulsion. These were usually females and were always selected for uniformity of size and vigor. The worms used were 22–24 cm. long and weighed 2.0–2.35 Gm. They were all *Ascaris lumbricoides* obtained from the intestines of swine at the abattoir. The phenomena observed were the number of minutes after immersion required to produce cessation of normal vermiculatory movements, paralysis and death, respectively. For the last two observations it is necessary to remove the worms from the anthelmintic bath for 1–2 minutes. This was done about every 50–60 minutes with precaution to avoid changing the environmental temperature. Death was determined by means of an inductorium and electrode.[2] The worms were observed over a period of 5 hours.

When the worms are immersed in some substances rupture of the body wall occurs with extrusion of the viscera. Rupture is sometimes accompanied by a detonation. Death does not always follow soon after such ruptures. Rupture occurred principally with the unoxygenated terpenes and the more concentrated hydrogen peroxide solutions. Rigidity and increased pallor often accompany paralysis. In one experiment oxygen-free nitrogen gas was passed through the beakers containing the worms in water. They were apparently unaffected after 5 hours by this oxygen deprivation. When nitrogen was passed and the worms were immersed in emulsions of purified pinene the animals were somewhat affected, in fact to the same extent as they were when the nitrogen was omitted. Thereafter the hydrocarbons were tested without the precaution of using a nitrogen atmosphere.

As a method for determining *in vitro* the probable anthelmintic value of a given substance, we believe the foregoing procedure has considerable utility. It must be emphasized that the parasite for which an anthelmintic is sought and no other should be used in the test. This desideratum is almost attained in our method since the swine *Ascaris* is morphologically but not quite physiologically identical with the human *Ascaris*. It is not possible, however, to use a wholly unrelated species, *e. g.*, the earth worm, which we have found to be much more susceptible to some substances than *Ascaris*. This difference in susceptibility has also been observed by Munch (11) in the case of ethyl alcohol and some samples of chenopodium oil. Since, with proper care in handling, the results obtained with *Ascaris* are just as consistent as those obtained in other similar bioassays, there is no need to substitute a less satisfactory animal. It is perhaps desirable to add to the anthelmintic bath physiological amounts of substances constantly found in the intestine. In the present work these have been omitted for the sake of chemical simplicity. Also in most cases no emulsifying agent or other third constituent was ever added, the mixtures tested consisting only of the substance or product designated in the table and water. The terpineol (Sample D), carvone and verbenone were more difficult to emulsify, and some experiments are reported in which 0.1% or 0.2% of acacia gum was added. The emulsions were all made in a uniform way by mixing with distilled water in a mechanical shaking machine. It might be thought that the anthelmintic potency of a water-insoluble material at a given concentration would be related to the degree of dispersion of the material in the water. However, it was found that finer emulsions produced by a colloid mill were no more potent than those made with the shaking machine. The immersion tests here reported are being supplemented by a study of the

[2] Suggestion of Dr. James C. Munch.

effect of the same substances upon the movements of segments of *Ascaris* as recorded by a kymograph.

RESULTS.

The bioassay data in Table I show the relative anthelmintic potencies of chenopodium oil, ascaridole, hydrogen peroxide and disuccinyl peroxide as determined by the method just described. Although these results demonstrate the high toxicity of hydrogen peroxide to *Ascaris lumbricoides*, it cannot be stated with certainty just how much this finding contributes to an explanation of the anthelmintic action of ascaridole. Aqueous emulsions of ascaridole give with a vanadium pentoxide reagent,[1] after 15–20 minutes contact, the same intensity of color as hydrogen peroxide solutions of one-tenth the concentration. This color may or may not be due to hydrogen peroxide. Ascaridole, in the absence of complicating side reactions, might be expected to yield with water one-fifth its weight of hydrogen peroxide. But ascaridole and hydrogen peroxide seem to be of about equal toxicity to *Ascaris, i. e.*, ascaridole is more toxic than would be predicted if its activity were attributed solely to the hydrogen peroxide which it could generate. An explanation for this apparent anomaly must be sought by studying the decomposition of ascaridole under very mild conditions such as with water in contact with animal tissues. We have found that the hydrogen peroxide equivalent of a 1% ascaridole emulsion (as indicated by the $V_2O_5$ reaction) decreases very rapidly when successive groups of live *Ascaris* are immersed in the emulsion at 37° C. The hydrogen peroxide equivalent of a control emulsion kept at 37° C. decreases only slightly in comparison. Perhaps the anthelmintic activity of ascaridole is

TABLE I.—THE EFFECT OF HYDROGEN PEROXIDE, DISUCCINYL PEROXIDE, CHENOPODIUM OIL AND ASCARIDOLE ON *Ascaris lumbricoides*.[1]

| Substance. | Concn., %. | No. of Ascaris. | % Paralyzed. 2 Hrs. | 3 Hrs. | % Killed. 3 Hrs. | 4 Hrs. |
|---|---|---|---|---|---|---|
| Chenopodium oil | 0.4 | 85 | 74 | ... | 75 | 80 |
| Ascaridole A | 0.1 | 24 | 100 | ... | 91 | .. |
| Ascaridole B | 0.1 | 12 | 0 | 91 | 0 | 0 |
| Ascaridole B | 0.2 | 27 | 44 | 85 | 52 | 89 |
| Ascaridole B | 0.4 | 15 | 87 | ... | 87 | .. |
| Hydrogen peroxide | 0.033 | 20 | 0 | 0 | 0 | 0 |
| Hydrogen peroxide | 0.1 | 32 | 91 | ... | 100 | .. |
| Disuccinyl peroxide | 0.2 | 12 | 0 | 100 | 0 | 0 |
| Disuccinyl peroxide | 1.0 | 12 | 100 | ... | 100 | .. |

[1] All the worm tests reported in this paper were carried out by Miss Anna D. Ogden of the Jayne laboratory.

due in part to decomposition products other than hydrogen peroxide, or perhaps to the simple menthene structure itself. Also it may be supposed that the hydrogen peroxide arising from ascaridole is taken up more economically by the worm due to the fact that it is evolved slowly or is stabilized by the presence of the excess ascaridole. On the other hand when worms are immersed in pure hydrogen peroxide solutions, a part of the available hydrogen peroxide may be dissipated

[1] Prepared by dissolving 4 Gm. of vanadium pentoxide in 40 cc. of concentrated sulphuric acid with subsequent addition of enough water to make one liter. Two cc. of reagent were added to 1 cc. of solution or emulsion. Under these conditions ascaridole emulsions gave maximum color in about 20 minutes.

so far as opportunity to exert toxic action is concerned, *e. g.*, by combination with unimportant parts of the worm substance or by conversion to molecular oxygen.

The results of the worm tests with α-pinene, turpentine, limonene and their oxygenation products are given in Table II. It is seen from the table that oxygenated hydrocarbons can be prepared which are just as toxic as chenopodium oil or ascaridole. It is also apparent that the optimal conditions for producing these anthelmintic products are not yet worked out. In order to find these we are making a systematic study of factors which may affect the autoxidations concerned. It would appear likely from a consideration of the experimental procedure that the fraction of non-toxic terpene hydrocarbon converted is very small, and that therefore the toxicity of the substances formed during oxygenation must be very great indeed.

TABLE II.—THE EFFECT OF VARIOUS TERPENE SUBSTANCES ON *Ascaris lumbricoides*.

| Substance. | Preliminary Treatment. | Time of Oxidation in Hrs. By $O_2$. | By Air. | Concn., %. | No. of Ascaris. | % Paralyzed. 2 Hrs. | 3 Hrs. | % Killed. 3 Hrs. | 4 Hrs. |
|---|---|---|---|---|---|---|---|---|---|
| Pinene | None | ... | ... | 0.4 | 24 | 0 | 4 | 0 | 0 |
| Pinene | None | ... | ... | 0.8 | 24 | 0 | 0 | 0 | 0 |
| Pinene | Distd. over Na; stored in $N_2$ | ... | ... | 0.4 | 27 | 0 | 37 | 0 | 0 |
| Pinene | Distd. over Na; stored in $N_2$ | ... | ... | 0.8 | 12 | 0 | 50 | 0 | 0 |
| Pinene | None | 4 | ... | 0.4 | 25 | 0 | 36 | 0 | 0 |
| Pinene | None | 20 | ... | 0.4 | 25 | 80 | .. | 76 | 92 |
| Pinene | None | 70 | ... | 0.4 | 25 | ...[1] | .. | 80 | .. |
| Pinene | Distd. over Na; stored in $N_2$ | 20 | ... | 0.4 | 22 | 41 | 55 | 0 | 45 |
| Pinene | Distd. over Na; stored in $N_2$ | 40 | ... | 0.4 | 22 | 95 | .. | 0 | 27 |
| Pinene | Distd. over Na; stored in $N_2$ | 60 | ... | 0.4 | 22 | 27 | 82 | 0 | 0 |
| Pinene | Distd. over Na; stored in $N_2$ | 81 | ... | 0.4 | 22 | 27 | 73 | 14 | 23 |
| Pinene | Distd. over Na; stored in $N_2$ | 137 | ... | 0.4 | 22 | 14 | 73 | 0 | 0 |
| Pinene | None | ... | 46 | 0.4 | 25 | 84 | .. | 76 | .. |
| Pinene | None | ... | 113 | 0.4 | 15 | 0 | 0 | 0 | 0 |
| Pinene | None | ... | 137 | 0.4 | 15 | 73 | .. | 0 | 47 |
| Pinene | None | ... | 161 | 0.4 | 15 | 80 | .. | 60 | .. |
| Pinene | None | ... | 190[2] | 0.4 | 20 | 100 | .. | 60 | 70 |
| Turpentine A | None | ... | ... | 0.4 | 10 | 70 | .. | 0 | 60 |
| Turpentine B | None | ... | ... | 0.4 | 35 | 0 | 14 | 0 | 0 |
| Turpentine B | None | 120 | ... | 0.4 | 10 | 60 | .. | 0 | 60 |
| Turpentine B | None | ... | 47 | 0.4 | 20 | 0 | 55 | 0 | 0 |
| Turpentine B | None | ... | 120 | 0.4 | 25 | 100 | .. | 80 | .. |
| *d*-Limonene | None | ... | ... | 0.4 | 17 | 0 | 0 | 0 | 0 |
| *d*-Limonene | None | ... | ... | 0.8 | 12 | 0 | 0 | 0 | 0 |
| *d*-Limonene | None | 3 | ... | 0.4 | 15 | 0 | 0 | 0 | 0 |
| *d*-Limonene | None | 20 | ... | 0.4 | 40 | 95 | .. | 54 | 80 |
| *d*-Limonene | None | 24 | 21 | 0.4 | 25 | 80 | .. | 80 | .. |

[1] 64% were killed in two hours.

[2] No catalyst.

The chemical nature of these substances is not yet known. Examination reveals that the activity probably cannot be attributed to peroxides. These are demonstrable in the oxygenated hydrocarbons but are very quickly decomposed on shaking with water. The dilute emulsions which were so toxic to *Ascaris* gave no coloration with the $V_2O_5$ reagent. They therefore contained less than 0.0005% of hydrogen peroxide equivalent. Of course it is possible that peroxides are present which do not react with the $V_2O_5$ reagent under the conditions employed. It should be recalled here that Bodendorf (8) obtained peroxides toxic to earth worms by oxygenating $\alpha$-terpinene (G) and $\alpha$-phellandrene (H).

TABLE III.—THE EFFECT OF TERPINEOL, VERBENONE, CARVONE, FORMIC ACID AND FORMALDEHYDE ON *Ascaris lumbricoides*.

| Substance. | Concn. %. | No. of Ascaris. | % Paralyzed. 1 Hr. | 2 Hrs. | 3 Hrs. | % Killed. 2 Hrs. | 3 Hrs. | 4 Hrs. |
|---|---|---|---|---|---|---|---|---|
| Terpineol C | 0.1 | 12 | 0 | 0 | 0 | 0 | 0 | 0 |
| Terpineol C | 0.2 | 12 | 0 | 100 | .. | 0 | 100 | .. |
| Terpineol D[1] | 0.3 | 25 | 0 | 96 | .. | 0 | 72 | .. |
| Terpineol D[2] | 0.25 | 20 | 0 | 40 | 75 | 0 | 0 | 20 |
| Terpineol D[2] | 0.5 | 20 | 90 | 95 | .. | 45 | 85 | .. |
| Verbenone | 0.1 | 20 | 0 | 0 | 0 | 0 | 0 | 0 |
| Verbenone | 0.2 | 20 | 0 | 75 | 95 | 0 | 5 | 15 |
| Verbenone | 0.3 | 10 | 0 | 80 | .. | 0 | 40 | 60 |
| Verbenone[2] | 0.4 | 20 | 70 | 85 | .. | 25 | 40 | .. |
| Verbenone[3] | 0.5 | 20 | 0 | 45 | 75 | 0 | 15 | 25 |
| Carvone | 0.2 | 30 | 0 | 67 | .. | 0 | 0 | 0 |
| Carvone | 0.3 | 15 | 0 | 67 | .. | 0 | 0 | 45 |
| Carvone | 0.4 | 20 | 0 | 90 | .. | 0 | 75 | .. |
| Carvone[2] | 0.4 | 20 | 65 | ... | .. | 25 | 35 | 45 |
| Carvone[3] | 0.5 | 20 | 0 | 80 | .. | 0 | 70 | .. |
| Formic acid | 0.1 | 10 | 0 | 0 | 0[4] | 0 | 0 | 0 |
| Formic acid | 0.5 | 10 | 0 | 20 | .. | 80 | 100 | .. |
| Formaldehyde | 0.1 | 10 | 0 | 0 | 0 | 0 | 0 | 0 |
| Formaldehyde | 0.5 | 10 | 0 | 0 | 0 | 0 | 0 | 0 |
| Formaldehyde | 1.0 | 10 | 0 | 0 | 90 | 0 | 0 | 50 |

[1] Contained 2% of ethyl alcohol which in itself is not toxic to *Ascaris*.

[2] Contained 0.1% of acacia gum. [3] Contained 0.2% of acacia gum. [4] 30% paralyzed in 4 hrs.

We have observed that the oxygenated hydrocarbons give a strong Schiff reaction.[1] This is significant since it has been shown (12) that verbenone (F) is formed during the autoxidation of oil of turpentine, and that carvone (E) is similarly produced (13) from limonene. Both of these ketones give a positive Schiff reaction. We have investigated the effect of verbenone and carvone upon *Ascaris*. The results (Table III) show that, while both these substances are quite toxic, their toxicity is not sufficiently great to account for more than a part of the anti-ascaridic action of our oxygenated hydrocarbons. A number of other substances have been identified as products of oxidative transformations of $\alpha$-pinene and limonene. Two of these terpineol (D) and formic acid (14) have been bioassayed

[1] Reagent: Aqueous solution of basic fuchsin (0.25 Gm. per L.) treated with an excess of sulphur dioxide gas. One cc. of sample was added to 5 cc. of reagent and the mixture vigorously shaken.

with the results shown in Table III. Still others now being prepared will be studied later. Since the amount of free acid in the emulsions studied is small ($p_H$ 5.8) it is doubtful how much any free formic acid present contributes to the anthelmintic effect. Perhaps renewed analytical investigation of oxygenated $\alpha$-pinene and oxygenated limonene will give a clue to the identity of these very powerfully antiascaridic substances.

SUMMARY.

1. Hydrogen peroxide is very toxic to *Ascaris lumbricoides*. The relation of this observation to the anthelmintic activity of ascaridole has been discussed.

2. By passing oxygen or air over $\alpha$-pinene, turpentine or *d*-limonene products are formed of pronounced toxicity to *Ascaris lumbricoides*. The toxic substances in these products are probably not peroxides.

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## PHYSICAL PROPERTIES OF NEOARSPHENAMINE POWDER.*

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Very little has been written concerning the physical characteristics of neoarsphenamine powder. The United States Public Health Service specifications (1) for neoarsphenamine state that "Stability shall be determined by exposing the ampuled product to a temperature of 56° C. for a period of at least 24 hours, during which time it should show no marked change in color, consistency, or solubility." This statement does not, however, describe the various changes which can occur during the "heat-testing" of neoarsphenamines. The following discussion is based on a large number of observations made during the testing of a variety of neoarsphenamines all of which were sealed in evacuated ampuls.

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