RELATION OF THE CARBONYL GROUP TO VESICIDAL ACTIVITY

BY

L. Levan Mahoney

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ProQuest LLC.
789 East Eisenhower Parkway
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INTRODUCTION AND HISTORY

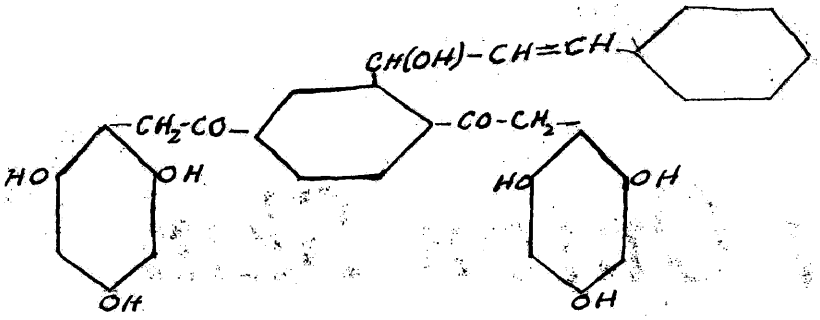
The attempt to identify atomic groups as specific carriers of vermicidal activity is an instance of the continuing effort of pharmacologist and chemist to interpret pharmacological activity from chemical constitution. The work reported in this paper was undertaken to determine if or to what extent the carbonyl group is responsible for vermicidal effectiveness. The preparation of compounds exhibiting this functional characteristic and a method for their pharmacological evaluation will be described.

The application of chemotherapeutic concepts to anthelmintic drugs has been made possible by the isolation and identification of a variety of plant principles. Over 360 crude vegetable drugs said to possess value as anthelmintics have been listed by Lillig[1]. The vermicidal compounds of known constitution, both synthetic and of vegetable origin, differ so widely in structure that complete chemical classification has not been possible. Greene[2], however, recognizes five chemical types to which the constitutions of these substances may be referred, viz., (a) thymol, (b) phloroglucinol, (c) lactone, (d) alkaloids, (e) heterogeneous.

Within recent years the structural characteristics of a limited number of active vermicidal principles from plant sources have been elaborated sufficiently to give support to more comprehensive studies of this phase of chemotherapy. While the structural details of many principles have not been established definitely, it is possible to say with certainty that the carbonyl group

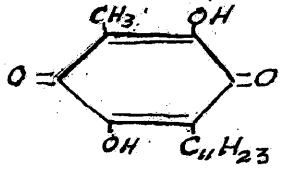
exists in the molecules of many compounds of different origins. In this category may be mentioned rottlerin, embelin (embelic acid), aspidium compounds, santonin, artemisin and tanacetin.

The formula for rottlerin given by Dutt and Goswami[3] is



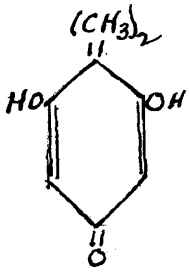
although the analyses of Hoffmann and Pari[4] of rottlerin and some of its derivatives require two carbon atoms, one oxygen atom and one double bond less than the formula of Dutt and Goswami.

Embelin, a taenicide from the fruit of Embelia glandulifera, E. robusta, or E. ribes, according to Abderhalden's Biochemisches Handlexikon[5] is assigned the structure

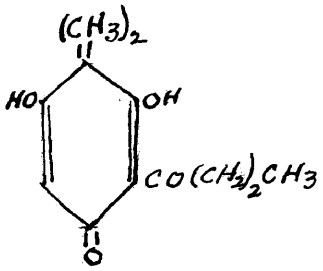


but elsewhere[6] it is said to be 2,5-dioxy-3-lauryl-p-benzoquinone.

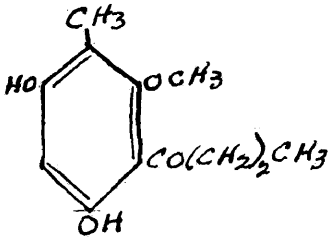
Filicinic acid, one of the structural units found in male fern constituents and which, in itself, is ineffective becomes active against tapeworms with the appearance of a butyryl side chain in butyryl filicinic acid. Its isomer aspidinol, which also contains the butyryl radical is held to be completely ineffective.



Filicinic acid

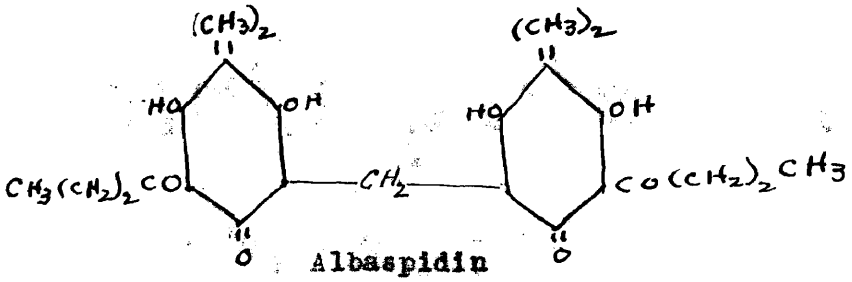


Butyryl filicinic acid

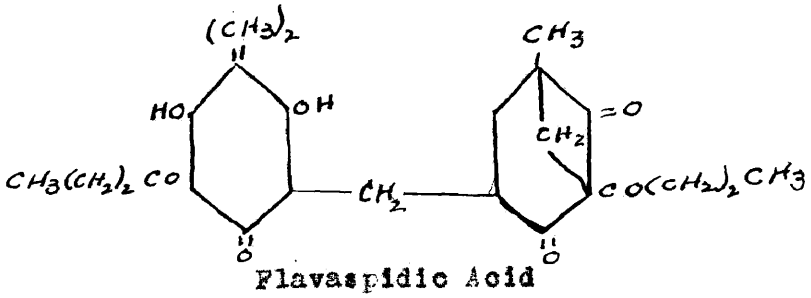


Aspidinol

Albaspidin and flavaspidic acid, two active constituents of male fern, have been assigned the formulas

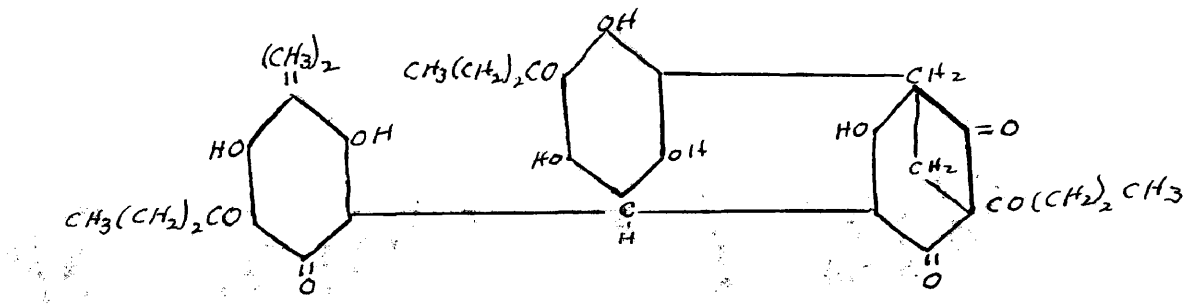


Albaspidin



Flavaspidic Acid

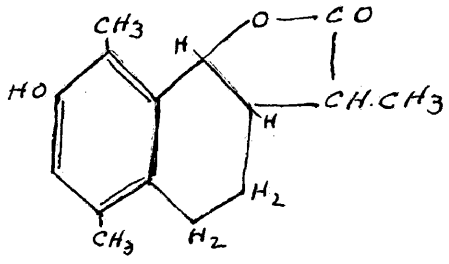
Filicic acid, another effective constituent of male fern is probably



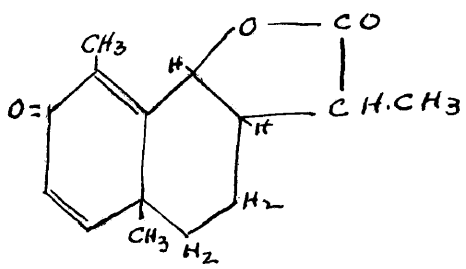
Filicic acid

Schlossberger[7] metions as compounds that are probably similarly constituted: kosotoxin, pannic acid, albopannin, flavopannin, pannol, flemingin, homoflemingin, and tanacetin.

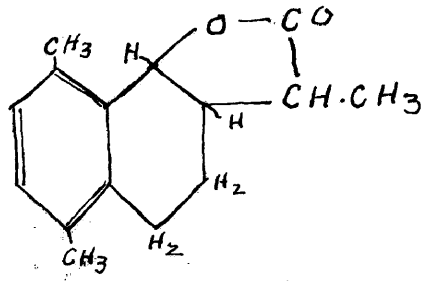
In the santonin series of compounds, the question of the structure of santonin has been settled by the determination of the position of the lactone ring in the synthesis of desmotroposantonin[8] by Clemo, Haworth and Walton.



Desmotroposantonin

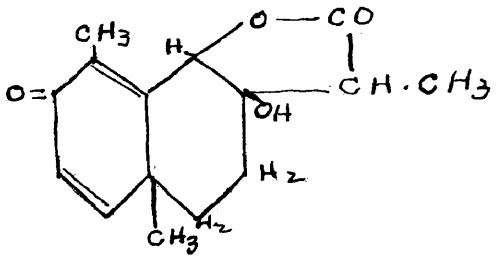


Santonin

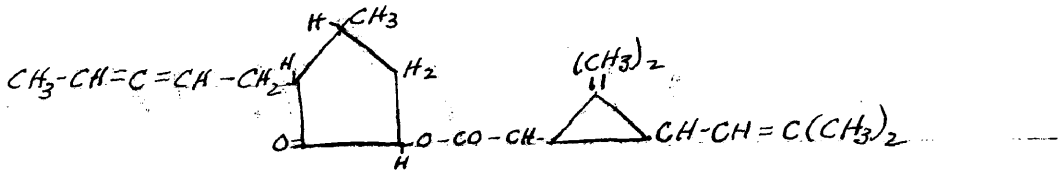


Hyposantonin
according to Gluschke[9]

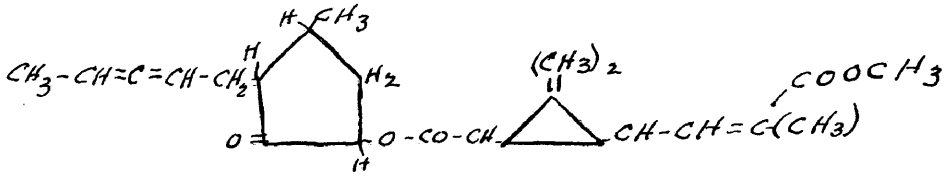
Artemisin, a principle which accompanies santonin in species of Artemesia but which is less active than santonin, has been given, by Wedekind[10], the formula



Staudinger and Ruzicka[11] isolated from Dalmatian insect powder (Chrysanthemum cinerariaefolium) two principles, Pyrethrin I and Pyrethrin II which Chevalier and Pouchet[12] tested and found to be effective against hog ascarids, dog tapeworms, oxyuris, trichocephalus and ankylostoma.



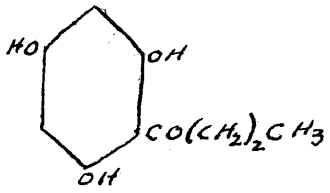
Pyrethrin I



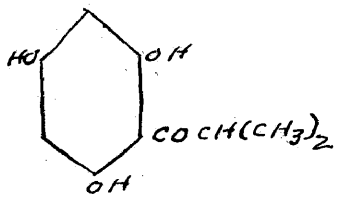
Pyrethrin II

It is stated in Frankel's Arzneimittel Synthese that the activity of phloroglucinol derivatives does not appear until after the introduction of a butyric acid residue into the filicinic acid molecule. The belief that vermicidal activity is connected with the phloroglucinol nucleus and a butyric acid residue comes from the observation that filix substances, upon decomposition, yield phloroglucinol, butyric acid and isobutyric acids. Rottlerin decomposes into trimethylphloroglucinol and butyric acid; tanacetin into catechol and butyric acid. These observations led Karrer and co-workers[13,14] to synthesize and study a series of acyl substituted compounds analogous in structure to filix derivatives. This group comprised: methylphlorbutyrophenone (isoaspidinol)

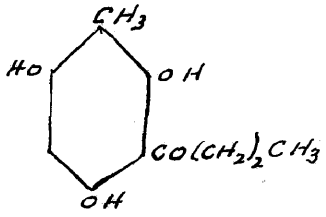
methylene-di-(methylphlorbutyrophenone), phlorbutyrophenone, phlorisobutyrophenone, methylene-di-(phlorbutyrophenone), methylene-di-(phlorisobutyrophenone) and a number of similar resorcinol phenones.



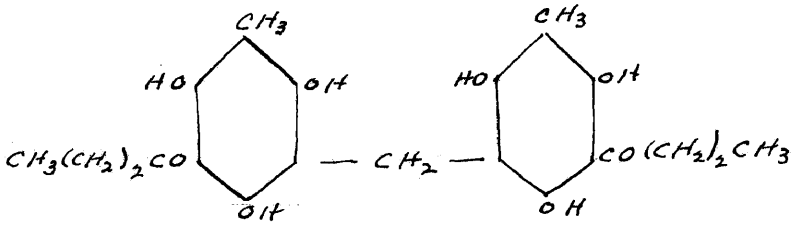
Phlorbutyrophenone



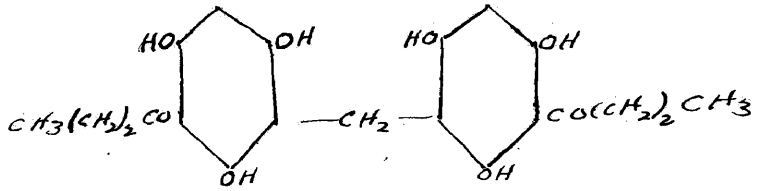
Phlorisobutyrophenone



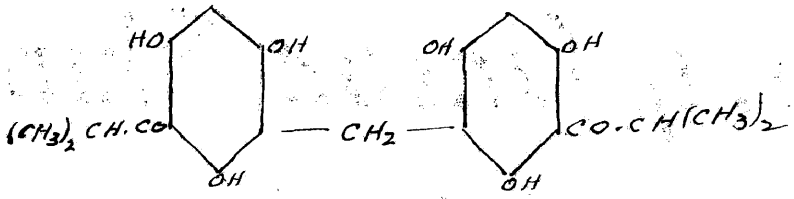
Methylphlorbutyrophenone



Methylene-di-(methylphlorbutyrophenone)



Methylene-di-(phlorbutyrophenone)



Methylene-di-(phlorisobutyrophenone)

Their pharmacological studies indicate that taenicidal activity is associated with the phloroglucinol nucleus and butyryl side-chain, but that such activity is a specific attribute of neither the nucleus nor side-chain, since on the one hand resorcinol is an effective nucleus and on the other the butyryl and isobutyrl radicals may be substituted by other higher acid remnants without destruction of activity. Karrer found maximum effectiveness to appear with introduction of the isocaproic acid residue and to disappear again with further increase in the length of the side-chain. Additional studies on the synthesis of hydroxyphenones have been conducted by Hoesch[15], Rosenmund and coworkers[16,17] and by Wojahn[18]. The greater number of members of this group of compounds exhibit <u>in vitro</u> germicidal or vermicidal

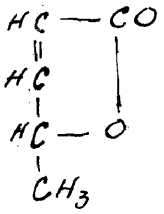
properties. The derivatives of the phloroglucinol and resorcinol series are the most active, but the phenones of catechol and hydroquinone likewise possess activity[19]. The weakest phenones are those of phenol. It is thus seen that activity in this series is augmented by the presence and accumulation of hydroxyl groups in the nucleus. p-Hydroxyacetophenone is said to be about twenty times more active than acetophenone.

With regard to the activity of ketones as a class Fränkel makes the following generalizations. Even the simplest ketones have a vermicidal activity, but their oximes are almost completely inactive; nuclear substitution in the phenones decreases their toxicity; conversion of phenolic hydroxyl to ether or ester linkages decreases vermicidal properties; the introduction of hydroxyl groups into the acyl radical decreases activity.

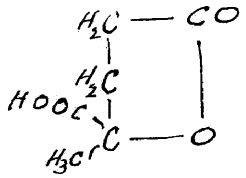
Interest was first aroused in the santonin series by the efforts of Cannizzaro, Wedekind, Straub, Kobert and Sieburg. Desmotroposantonin, according to Straub[20], is non-toxic to ascarids and marine worms, but Trendelenburg[20] finds it is equally as irritant to worm muscle as santonin. Coppola[20] states that the oxime is less toxic than the ketone. Tetrahydrosantonin and artemisin, in which the ketone and lactone characters are retained, are irritant to worm muscle.

In the santonin series of compounds greater emphasis has been attributed to the lactone ring as a factor in anthelmintic activity than upon any other constitutional characteristic. Trendelenburg, Lautenschläger, Gluschke and others adhere to this

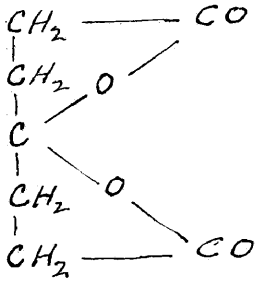
view. Lautenschläger[21], after a study of synthetic lactones, believed the activity to depend upon the lactone ring in conjunction with the presence of a cyclic radical, the purely aliphatic lactones investigated by him being indifferent. Von Oettingen and Garcia[22], on the other hand detected activity in beta-angelica lactone, valerolactone carboxylic acid, and in the dilactone of acetone diacetic acid against Lumbricus terrestis. In vivo experiments against ascaris in cats were 100 per cent successful in seven of ten experiments in the case of the dilactone.



Beta-angelica lactone



Valerolactone carboxylic acid



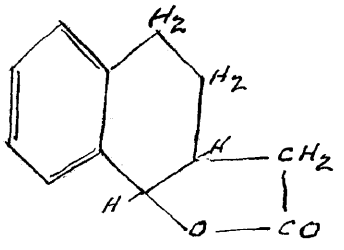
Dilactone of acetone diacetic acid

Trendelenburg and his supporters base their view of the lactone group as the carrier of activity upon the fact that activity

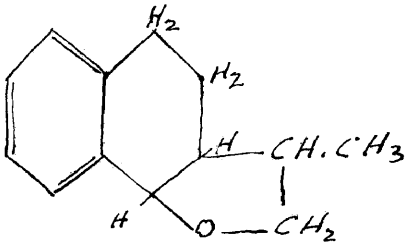
disappears upon cleavage of the lactone ring. Oshika[23] studied the methyl and ethyl esters of santoninic acid and observed, contrary to Trendelenburg, that the disappearance of activity from santonin and its derivatives by opening the lactone ring is dependent not upon the disappearance of lactone character, but to the appearance of acid character, in as much as esterification restores the activity.

Caius and Bhaskar[24], from evidence presented in twenty-two reports covering data accumulated in their extensive clinical researches in the control of hook worm disease, express a disbelief in the significance of the lactone ring and attach greater importance to the ketone group. According to them, the dominant group is $-CO-CH_2$, while $-CO-CH=$ has a weaker effect and the groups $-COH=CH-$ and $=C=CH$ are ineffective.

Gluschke[20] has studied the influence of nuclear substitution in two structural analogs of santonin, viz., syntonin-a (5-tetralol-6-acetic acid lactone) and syntonin-b (5-tetralol-6-propionic acid lactone).



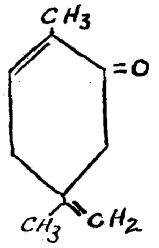
Syntonin-a



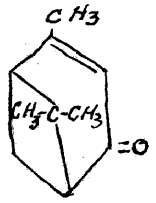
Syntonin-b

with the result that substitution in the benzenoid nucleus is superfluous and that the introduction of reactive grou s appears to diminish rather than augment activity.

Butz and LaLande Jr.[25] have demonstrated that oxygenation of limonene and certain other terpene hydrocarbons increases their ascaricidal properties. They point significantly to the autoxidation[26] of limonene to form the ketone, carvone, and likewise to the fact that verbenone is produced[27] similarly from oil of turpentine. The ketones and the oxygenated terpenes studied by them give a strong Schiff reaction.



Carvone



Verbenone

PHARMACOLOGY SECTION

Discussion

This investigation has been conducted with the object of correlating vermicidal activity with molecular constitution, without special regard for the ultimate values of the compounds in therapeutics. It was required that a biological method might be found or evolved which would afford an expression for molecular activity referable to a suitable standard. A survey of the literature showed that a number of methods have attracted recognition, but that none are wholly adaptable to the requirements of all investigators. Where practicability and simplicity are offered to vindicate a procedure, these advantages prevail usually at the sacrifice of accuracy and utility.

To submit synthetic products to clinical trial without thorough preliminary study of their properties is neither expedient nor wise. The different classes of laboratory workers who assume the task of pre-clinical evaluation embrace collectively a variety of objectives in research. Each group is likely to sanction such methods as are suited to the pecularities of its special occupation. The chemist and the collaborating pharmacologist are often restricted in choice of methods by having at their disposal limited quantities of material for study. In particular, the chemist who must work without the co-operation of a pharmacologist

is subject to the temptation of a cursory biological treatment. In this connection, an abundance of reports are found in which the chemist has contented himself with very crude approximations to relative activities.

The position held by the clinician, in contrast to this attitude, has been rightly, as always, to reserve judgment until actual clinical trial. Unfortunately, in the past, the clinical evaluation of anthelmintics has had an empirical basis. The clinical observations sometimes gave false indications of the true value of the substance being studied. For example, the fact that a number of parasites had been eliminated from the body was recognized as evidence of merit although it furnished no proof that numerous other parasites did not remain behind.

Following the researches of Hall, Shillinger, Foster and others[28,29,30] upon dogs, it became more widely recognised that the greatest precision for determining the activity of an anthelmintic demands control of the experiment through sacrifice of the test animal. By this method, following administration of the drug, a count is made of the number of parasites eliminated and after a stipulated time, the number of parasites not eliminated is determined. The efficiency is represented as per cent of total number of parasites eliminated. It is not contested that this method does furnish a reliable index to the usefulness of an anthelmintic in the treatment of various types of worm diseases. Unfortunately, in view of the circumstances of many laboratories, it is

not suitable to all conditions of experiment. It is costly and, moreover, infested animals are not always conveniently or immediately available. Most important, the expression "per cent efficiency" does not reflect the activities of individual molecules -- a knowledge of which chemotherapeutic research demands as the most valid datum for comparing activities.

It is not surprising, then, that the search for a suitable test object for use in an accurate yet feasible in vitro method continues. Leeches, earthworms, fish, frogs, and the endoparasites (especially ascarids) have been employed under various experimental conditions. Recently, Rath[31] has introduced the vinegar eel (Anguillula aceti), claiming for it advantages over the earthworm. Its responses are alleged to be more comparable with hematodes than with cestodes.

In vitro methods employing worms as experimental animals have been applied according to two general concepts. Either strips of worm muscle are mounted in a tissue chamber and the muscular responses are recorded with the aid of a kymograph, or the effect of the agent is determined by its direct action on the intact object. The worm muscle-strip method was devised originally by Trendelenburg[32] for the evaluation of santonin and some of its derivatives after Straub[33] had observed that strips of worm muscle were effected by aspidium. Trendelenburg used earthworm muscle-strips from which the nerve cord and ventral ganglia had been dissected. Schneider[34] claims that more accurate results are obtained by the use of whole

sound worms in the tissue bath in place of the muscle strips. Toscano Rico[35] recommends the use of segments of ascarids with organs attached as the most suitable tissue. Sollmann[36,37] demonstrated that all clinical vermicides are markedly toxic to earthworms. Since then, this animal has been the classical test object among the methods using the intact worm. The effectiveness of the vermicide is usually based upon the survival times of the worms when they are exposed to the action of the substance in a suitable solvent. Schneider[34], however, uses as criteria of activity the rates of contraction and relaxation and the degree of shortening of the worm and clitellum.

The choice of a biological method for the present investigation hangs upon a conjecture. That is whether the activities, as determined by any of the current methods, are strictly comparative in terms of individual molecules. As regards the method approved by Hall and co-workers, previously referred to, it has already been intimated that in so far as it embraces both the parasitotropic and organotropic factors it is the most conclusive test for applied therapy, but that its data are inadequate for correlating pharmacological activity with chemical structure. Turning to the muscle strip method in either its original or modified forms, it seems obvious that the responses of the tissue measure the irritant properties of a substance to a greater extent than they do any specificity of action attributable to atomic groups or linkages of the molecule. Here, the refinements of measurement depend

upon an accurate interpretation of the relation between the tonic and clonic phases of the tissue contractions and upon the reversibility of the processes. The likelihood of these phases being functions of concentration and time of exposure rather than specific action is apparent. As Gluschke[38], who used this method in a study of santonin-like compounds has said: "It would, indeed, be remarkable if substances such as phenols, volatile oils, camphor, tartar emetic, etc. did not affect the extremely irritable worm muscle". The authority for the method depends upon the opinion, long held, that anthelmintics are effective by virtue of a specific action on worm muscle. Gluschke, by highly critical analysis involving careful considerations of concentrations of solutions, character of contractions, and tonus increase, concluded that substances such as phenol, cresols, xylenols, thymol, picric acid, and napthols do not possess a specific santonin-like action. This argument does not dispose of the fact that a number of substances, such as thymol, which Gluschke says are not santonin-like in action, have proven their merit in the hands of field-workers combating helminthic infestations. It points only to the limitation of the method in failing to provide a means of comparing the activities of those substances which exert their toxic properties through some tissue other than muscle or are effective because they act through some other route or by some other mechanism.

Recognizing this weakness of the muscle strip method, it seems probable that the use of the intact worm should provide for a wider latitude of comparison. The desirability of a more extensive

study of the use of the earthworm is indicated. The most common objection to the use of this animal is that it is more sensitive to active substances than the pathological parasites.

While the earthworm is more sensitive to chemical agents than the endoparasitic worms, the latter are more susceptible to environmental influences. In Ringer's solution, or in sugar solutions they seldom live more than two days. In nutrient solutions, the medium undergoes putrefaction at the optimum temperature for the parasite. It is, therefore, difficult to determine whether the response of the parasite is caused by its unnatural environment or by the medicinal agent. Still another factor may be recognized in the differences in solubility and absorption in the intestinal juices as compared with these properties under the conditions of in vitro experimentation. Kudice and Borchardt[39] have demonstrated such differences in noting that the effective doses of alkyl halides as anthelmintics is reduced somewhat by pancreatic juice and to a greater extent by bile.

In view of the objections cited, the skepticism directed against the use of the earthworm as a test object for the evaluation of anthelmintics is to a great measure justified. Recently, Butz and LaLande[40] have again emphasized the importance of using the parasite for which an anthelmintic is sought in determining the probable anthelmintic value of a given substance. In a study of the effects of some oxygenated terpene hydrocarbons upon Ascaris lumbricoides, they have minimized the objectionable influence of

environmental conditions upon ascarids by maintaining a constant temperature of 37.5°C ± 0.5°C during the experiment and by limiting the period of observation to five hours. While their method introduces a refinement in technic, the limitation of the observation period imposes the necessity of emulsifying many substances in order to afford concentrations sufficiently high to produce paralysis or death within the stipulated time. It does not seem likely that data obtained by the use of emulsions is accurately comparative with that of true solutions unless the stability of an emulsion, the degree of dispersion, and the emulsifying agent do not influence the measured activity.

From consideration of the many aspects presented in the foregoing critical discussion of biological methods a number of inferences may be drawn in favor of further study of the quantitative nature of the responses of the earthworm to anthelmintics:

1. No scientifically valid expression for vermicidal activity, based upon the use of the intact earthworm, either in terms of absolute or comparative values, has been utilized.
2. The greater susceptibility of earthworms over ascarids and other parasites provides for a more sensitive method.
3. A more sensitive test object will detect weaker activity. The use of solutions holding lower concentrations of solute is made possible, thus tending to

obviate the necessity of emulsifying many substances.

Earthworms, with regard to their wide availability and the ability to withstand environmental changes are well adapted for routine laboratory experimentation. They may be kept for months in moist earth and will live during the winter months in a warm room if the ground in which they are placed is moistened frequently with water.

Experimental

During the month of August 1934, a preliminary study was undertaken to determine in a general way the susceptibility and sensitivity of the earthworm to chemical agents and to environmental conditions. Particular attention was directed to the nature of the responses observed. It was found that earthworms will live in distilled water for from several days to a week or longer, but that they survive for much longer periods in 0.2 to 0.4 per cent solutions of sodium chloride. In normal saline solution they show irritation and severe distress symptoms. Six out of six worms were still alive three weeks following immersion in 0.3 per cent saline solution; three out of three worms had not died at the end of the second week in a control experiment during

the month of December.

The greater number of compounds used consisted of cyclic and non-cyclic ketones, but some chemical variety was provided in the use of thymol, vinegar, and philocarpine hydrochloride. All of the substances which proved to be highly toxic to the earthworm produced similar effects along the course of action, irrespective of their structural characteristics. The phenomena of toxicity appeared always in the same order, here mentioned; i.e., excitement and irritation, passing directly to a stage of clonic or tonic-clonic convulsive movements, followed by loss of irritability, paralysis, impaired dorsal circulation and death. If the convulsive movements are very severe, rupture of the body wall is likely to occur. In the paralysis stage, the segments posterior to the clitellum are the first to be affected. The segments anterior to the clitellum are affected in ascending order. Feeble movements of the prostomium persist for some time after the worms have become otherwise immobile. The convulsant stage is relatively short in duration and may not be observed if the different phases follow one another in rapid succession. Complete cessation of movement is not an evidence of death, since by transferring completely paralyzed worms to very dilute saline solutions the power of movement is almost invariably restored to some extent and revival is often complete in several hours, although death may ensue.

It was concluded that the death point is a more suitable and exact criterion of vermicidal activity than any of the other phenomena of toxicity noted. A technic was then developed which

made possible the determination of the death point with fair accuracy.

Attempts to relate the data obtained by different experimenters by use of the earthworm have been unsatisfactory for a number of reasons: There is lack of uniformity as regards the vehicle or solvent chosen for the substance tested; the reported survival times correspond to a wide range of concentrations; the modes of expressing activities vary and are frequently vague.

For these reasons an effort was made to apply the more soluble substances to the test objects at equal concentrations.

Technic of Method.- The test objects were sound vigorous specimens of Lumbricus terrestris or L. rubellus of fairly uniform size, measuring 8 to 12 cm. in a relaxed state.

The object of the method is to determine the time in which a vermicide is just fatal at a given concentration to a definite number of earthworms. In our studies, the death-point was chosen as the time which would just permit the survival of one of the three objects at the concentration used. The procedure follows: The worms are immersed in a solution of the substance to be tested, ten cc. of solution being allotted to each worm used. At intervals, depending upon the concentration employed, three worms are removed at one time by means of a blunt hook, adhering mucoid secretion is removed carefully with cloth or blotting paper and they are then placed in 150 cc. beakers containing 100 cc. of 0.3 per cent sodium chloride solution prepared with distilled water. After 18 hours,

the worms in each beaker are examined. By noting the number that have died or recovered in each case, it is possible to determine at a given concentration approximately within what time range the death-point or survival time lies.

Two or three series or groups of twelve worms each are required for each concentration. The first group is used for ordinal tests. Three worms are transferred from the solution of the vermicide to the resuscitation bath* at 1, 2, 4, and 8 hours respectively.

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* In the resuscitation baths, the substances that have caused paralysis are washed from the tissues and muscular contractility is rapidly regained. The renewed activity appears to be purposeless and, therefore, independent of cerebral function. It may be dependent upon the intrinsic contractile function of worm muscle and should not be interpreted as an indication of permanent recovery. In the final examination to decide upon the number of worms that have recovered, a beam of intense light or direct mechanical stimulation are useful in initiating movements in the recovered test-objects. It is helpful also to examine the dorsal blood vessel for the condition of the circulation. A turbid appearance of the solution or the detection of putrefactive odor are certain indications of death.

The second and third groups of worms are used to define more accurately the time limits within which the true survival time lies. Closer time intervals for removing the worms are observed for solutions

which cause death speedily than for solutions which require longer periods to be effective.

The results of the first group of experiments is given in Table I. In obtaining these data it became apparent that it would be impossible to utilize the same concentrations for all vermicides owing to the fact that many of the less soluble ones do not afford solutions of sufficiently high concentration to exert the activity of the agent, and also because of the difficulty of distinguishing, in such cases, between the effect of the solution and the influence of environmental conditions. A saturated solution of santonin, for example, did not kill any of three worms in thirty hours. Evidence of the error in assuming effectiveness at high dilutions was subsequently found and illustrated in the Time-Concentration curves (Fig. I) for thymol and carvone, from which it may be seen that the ordinates, representing survival times at different concentrations, approach a Y-asymptote. Below certain concentrations these substances will not cause death in any time.

The inability to find a suitable common concentration for the preparation of all solutions is responsible, no doubt, for the inconsistent and unsatisfactory systems in vogue for reporting experimental data. Symbols such as "+", "-+", "++", etc., exist chiefly for need of a more accurate and descriptive designation.

It seemed desirable, therefore, to study the survival time-concentration relation for several substances with the object of finding a mathematical expression which would permit the calcu-

lation of activities under either identical conditions of time or concentration. Thymol was adopted tentatively as a standard or reference substance because it possesses recognized value as anthelmintic and has wide range of effective concentration. The results of this study are given in table II and are expressed graphically in Fig. I.

In Fig. II, the function, $\log_{10} C/t$, plotted as ordinates and C, the abscissae, graphed from the data in table II, are seen to be in linear relation. C is concentration (moles/liter); t, the survival time in minutes.

$$\frac{C}{t} = \frac{\text{concentration}}{\text{survival time}} = \frac{\text{moles/liter}}{\text{minutes}} = \text{"apparent" speed of fatality}$$

C/t is not the absolute speed of fatality. It is designated "apparent" speed of fatality, because it is not known how much of the dissolved agent is directly responsible for the death of the best objects.

The empirical linear equation is

$$\log_{10} C/t = A + BC \tag{1}$$

where B is the slope $\left(\frac{\Delta \log_{10} C/t}{\Delta C}\right)$ and A the intercept on the Y-axis. In exponential form, (1) becomes

$$C/t = ae^{bC} \tag{2}$$

The relation between the constants A and B of equation (1)

to a and b of equation (2) is

$$A = \log_{10} a$$

$$B = b \log_{10} e = .4343\ b$$

The values for constants A and B are obtained from the graphs (Fig. II). A is the apparent speed of fatality at zero concentration, obtained by extrapolation; B is computed according to the expression

$$B = \frac{\Delta \log_{10} C/t}{\Delta C}$$

Substitution of the computed values for the thymol curve in equation (1) gives

(3) $$\log C/t = -5.6 + 10400$$

For carvone, it is found

(4) $$\log C/t = -5.3 + 250\ C$$

The usual criterion of potency in biological assay methods is the dose that will cause a definite physiological effect in a stipulated time. Time is usually fixed as one of the conditions of experiment. Potency varies in an inverse sense with the minimum effective dose. The potency of an unknown substance in relation to a reference substance is expressed by the ratio:

$$\frac{\text{weight of standard } (s)}{\text{weight of unknown } (r)}$$

In the present study, the concentrations of the standard (C_s) and of the unknown (C_r) may be substituted for weight of standard and weight of unknown because the same volume of solution per worm is used in each test.

$$\frac{\text{potency of r}}{\text{potency of s}} = \frac{(\text{weight})_s}{(\text{weight})_r} = \frac{\frac{(\text{weight})_s}{(\text{volume})_s}}{\frac{(\text{weight})_r}{(\text{volume})_r}} = \frac{C_s}{C_r}$$

If the relative activities of two vermicides are in proportion to the reciprocals of the concentrations at which they act at equal apparent speeds in producing identical physiological effects, then

(5) $$\frac{\text{activity of r}}{\text{activity of s}} = \frac{\frac{1}{C_r}}{\frac{1}{C_s}} = \frac{C_s}{C_r}$$

and when, apparent speed of standard (s) = apparent speed of vermicide (r)

$$(C/t)_s = (C/t)_r$$

$$\log(C/t)_s = \log (C/t)_r$$

From equation (1),

$$A_s + B_sC_s = A_r + B_rC_r$$

$$B_sC_s = (A_r - A_s) + B_rC_r$$

For active substances, (A_r-A_s) is insignificant.

$$B_sC_s = B_rC_r$$

$$C_s/C_r = B_r/B_s$$

Substituting in (5),

$$\frac{\text{activity of r}}{\text{activity of s}} = \frac{B_r}{B_s} \quad \text{(at equal apparent speeds of fatality)}$$

It is thus shown that the activities of two vermicides are to each other as the ratio of their rates of change of apparent speed of fatality with change in molar concentration, and that B, the slope, is a suitable criterion of activity.

$$(6) \quad \frac{\text{activity of carvone}}{\text{activity of thymol}} = \frac{B\ (\text{carvone})}{B\ (\text{thymol})} = \frac{250}{1040} = \frac{0.24}{1.00}$$

If the relative activities are determined from the graphs by comparing the concentrations corresponding to equal values for log (C/t), the co-ordinates should be extrapolated to higher concentrations to obtain the most nearly consistent values. The higher concentrations conform more nearly with the doses given in actual treatment and give more consistent values over a wider range of concentration.

It becomes more evident that the constants B_r and B_s in equation (5) are expressive of molecular activity, if the ratio 0.24/1.00 is derived in another way.

If the "molar vermicidal activity", (V), be defined as log apparent speed of fatality (log C/t) when the vermicide is acting at unit molar concentration, then, when C = 1, let the

molar vermicidal activities of two substances, r and s, be to each other as $V_r : V_s$.

$$\frac{V_r}{V_s} = \frac{\log(C/t)_r}{\log(C/t)_s} = \frac{(\log C_r - \log t_r)}{(\log C_s - \log t_s)}$$

When $C_r = 1$
and $C_s = 1$

(7) $$\frac{V_r}{V_s} = \frac{0 - \log t_r}{0 - \log t_s} = \frac{\log t_r}{\log t_s}$$

For thymol, at unit molar concentration,

$$\log (C/t) = -5.6 + 1040 \times 1$$

When $C = 1$,

$$\log (C/t) = -\log t$$

(8) $$-\log t = 5.6 + 1040$$

Likewise for carvone at unit molar concentration

(9) $$-\log t = -5.3 + 250 \times 1$$

Letting r represent carvone and s thymol, and then substituting in (7),

$$\frac{V_r}{V_s} = \frac{-5.3 + 250}{-5.6 + 1040} = \frac{0.236}{1.000}$$

If the constant A, is dropped,

$$\frac{V_x}{V_s} = \frac{250}{1040} = \frac{0.240}{1.000}$$

The computed values for the slope $\left(\frac{\Delta \log C/t}{\Delta C}\right)$ and for the molar vermicidal activities as referred to thymol are given collectively in table III.

Biological Evaluation of Acyclic and Alicyclic Ketones.-

A series of ketones consisting of acetone, diethyl ketone, methyl propyl ketone, methyl isopropyl ketone, cyclopentanone, and cyclohexanone were evaluated by the method previously outlined. In this series, all of the ketones possessed activities of an extremely low order, ranging from 0.0014 that of thymol in the case of acetone to 0.045 that of thymol for methyl isopropyl ketone; the values obtained for cyclopentanone and cyclohexanone fell within the upper and lower limits. The pattern followed during the course of activity did not show the convulsant stage alluded to elsewhere, the test objects passing through a stage of irritation directly to a dimished motility suggestive of hypnosis.

It may be seen from table III that for the straight-chain ketones, activity increases with molecular weight. With ring closure, the activity drops off and there is then again exaltation with increase in molecular weight after cyclisation. The order of activity of the ketones included in this study is related to their solubilities in an inverse sense. In consequence

no conclusion is drawn which would imply a direct relation between activity and functionally active groups or linkages.

Many of the compounds, the preparation of which may be found recorded in a following chemical section, have not been evaluated pharmacologically, owing to the difficulty of obtaining a sufficient number of test specimens during the winter months and because too little time remained to complete the study after they became available in sufficient number. Such preparations will be studied systematically at a later date and the findings reported elsewhere.

The experimental evidence reported in this paper is not extensive enough in scope to permit any generalization whatsoever which endeavors to relate vermicidal activity with chemical constitution.

In our studies, the method of biological evaluation developed was adopted tentatively as the most accurate and feasible method yet found to inter-relate vermicidal toxicities from a molecular concept and which, at the same time, may afford an index to the probable anthelmintic value of the compounds under consideration. From casual observation, it appears likely that to the extent that ascaricidal properties, in contradistinction to any other vermicidal activity, are involved, the activities as determined by our earthworm method bear a relation to each other of approximately the same order as has been observed by their evaluation against *Ascaris lumbricoides*. Unfortunately the number of cases where such comparison

has been possible have been limited, so that an assumption that such a relation exists between all compounds by both methods of evaluation remains unproved until additional data can be gathered.

TABLE I

Compound	Concentration, moles/liter	Survival time, minutes
Acetophenone[1]	0.005	105-120
Thymoquinone	0.005	75-90
Carvone[1]	0.005	50-60
Pulegone[1]	0.004	40-50
Menthone[2]	0.005	15-25
Cyclohexanedione-2,5-dicarboxylic acid-1,4-diethyl ester[3]	0.005	Footnote 4
1-methyl-cyclohexane-dione-2,5-dicarboxylic acid-1,4-diethyl ester	0.005	130-160
1,4-dimethyl-cyclohexane-dione-2,5-dicarboxylic-acid-1,4-diethyl ester	0.005	120-135
Alpha-napthaquinone[3]	0.005	2-4
Beta-napthaquinone[3]	0.005	15-20
Santonin	Saturated	Footnote 4
Benzophenone	"	"
Benzoin	"	"
3-Aminophthalhydrazide	"	"
Philocarpine hydrochloride	(1%)	Footnote 5

1. Eastman Kodak Co.
2. Turbid dispersion
3. Suspension in acacia solution
4. Specimens apparently unaffected after 30 hours.
5. Specimens active and hardy after 3 hours.

TABLE II

Compound	Minutes (t)	Moles/liter (C)	C/t	log C/t
Thymol	3	0.0025	0.000833	-3.08
"	7	0.0020	0.000286	-3.54
"	15	0.0015	0.000100	-4.00
"	35	0.0010	0.0000286	-4.54
Carvone	25	0.007	0.00028	-3.55
"	35	0.006	0.000171	-3.77
"	55	0.005	0.0000909	-4.04
"	110	0.003	0.0000273	-4.56
"	180	0.002	0.00001110	-4.95
Pulegone	40	0.004	0.0001	-4.00
"	80	0.003	0.0000375	-4.43
"	135	0.002	0.0000148	-4.83
Acetone	56	1.00	0.0178	-1.75
"	150	0.50	0.00333	-2.48
Diethylketone	15	0.30	0.020	-1.70
"	30	0.20	0.00666	-2.18
"	60	0.10	0.00166	-2.78
Me.pr.ketone	28	0.20	0.00714	-2.15
"	58	0.10	0.00172	-2.76
Me.isopr.ketone	40	0.04	0.001	-3.00
"	140	0.02	0.000143	-3.84
"	240	0.01	0.0000416	-4.38
Cyclopentanone	22	0.40	0.0182	-1.74
"	60	0.20	0.00333	-2.48
"	80	0.10	0.00125	-2.90
Cyclohexanone	15	0.20	0.0133	-1.88
"	95	0.10	0.00105	-2.98

TABLE III
Showing Relative Vermicidal Effectiveness

Compound	log C/t / C	Molar Vermicidal Activity Referred to Thymol
Thymol	1040	1.00
Pulegone	415	0.40
Carvone	250	0.24
Acetone	1.5	0.0014
Diethyl ketone	5.4	0.0052
Me. pr. ketone	6.2	0.0061
Me. isopr. ketone	45.0	0.043
Cyclopentanone	3.8	0.0037
Cyclohexanone	11.0	0.0100

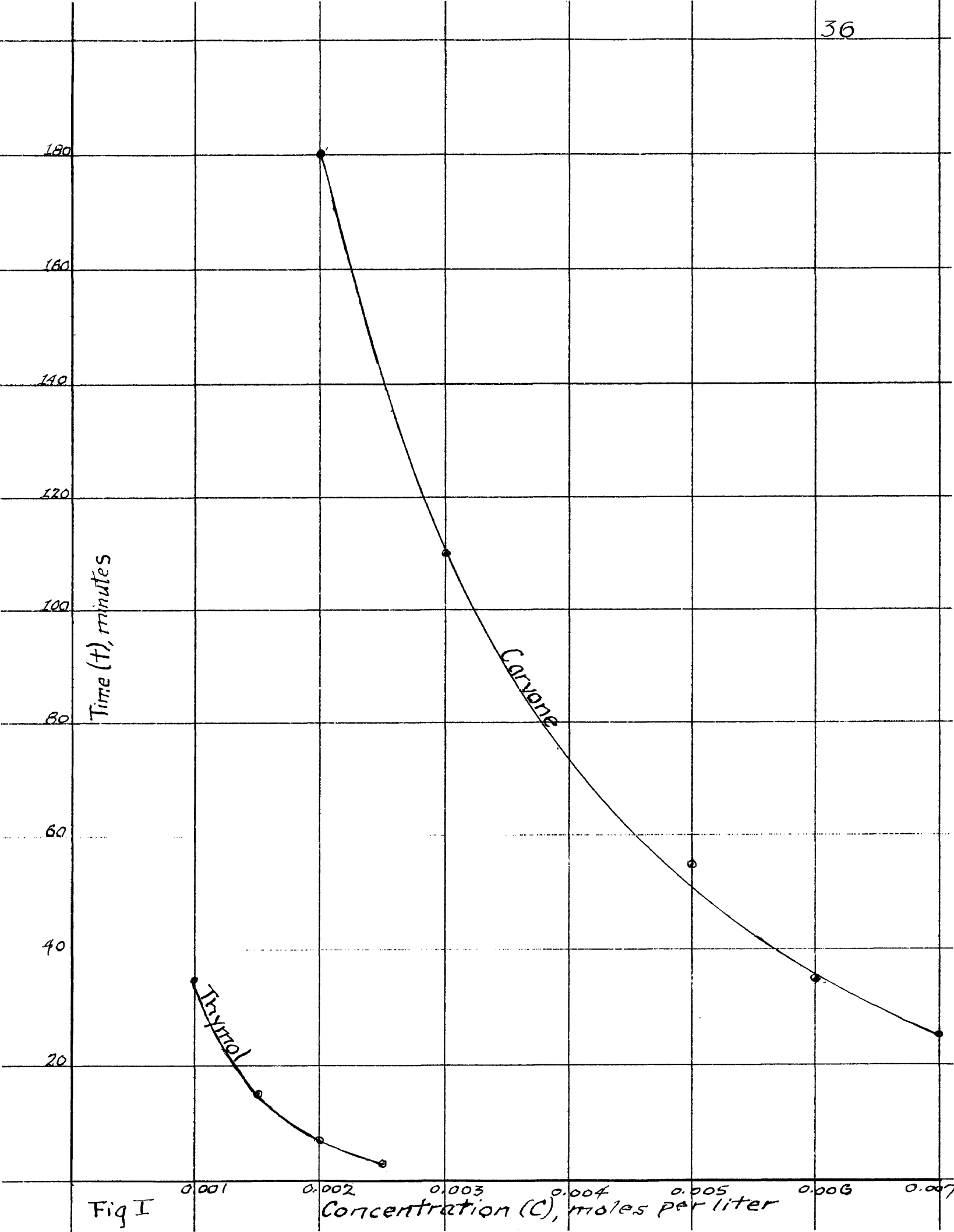

180
160
140
120
100
80
60
40
20
Time (t), minutes
Carvone
Thymol
0.001
0.002
0.003
0.004
0.005
0.006
0.007
Concentration (C), moles per liter

Fig I

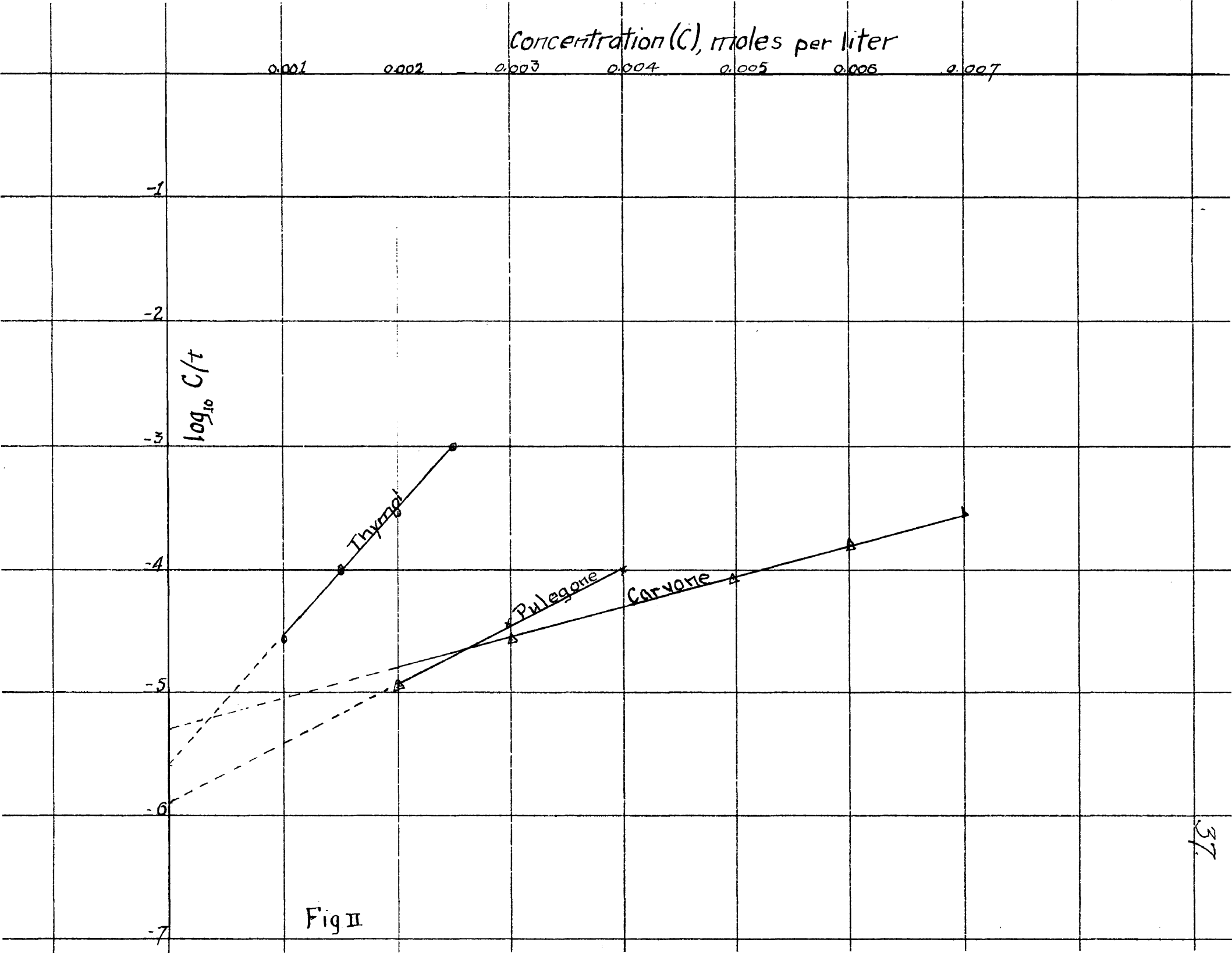

Concentration (C), moles per liter
0.001
0.002
0.003
0.004
0.005
0.006
0.007
-1
-2
-3
-4
-5
-6
-7
log10 C/t
Thymol
Pulegone
Carvone

Fig II

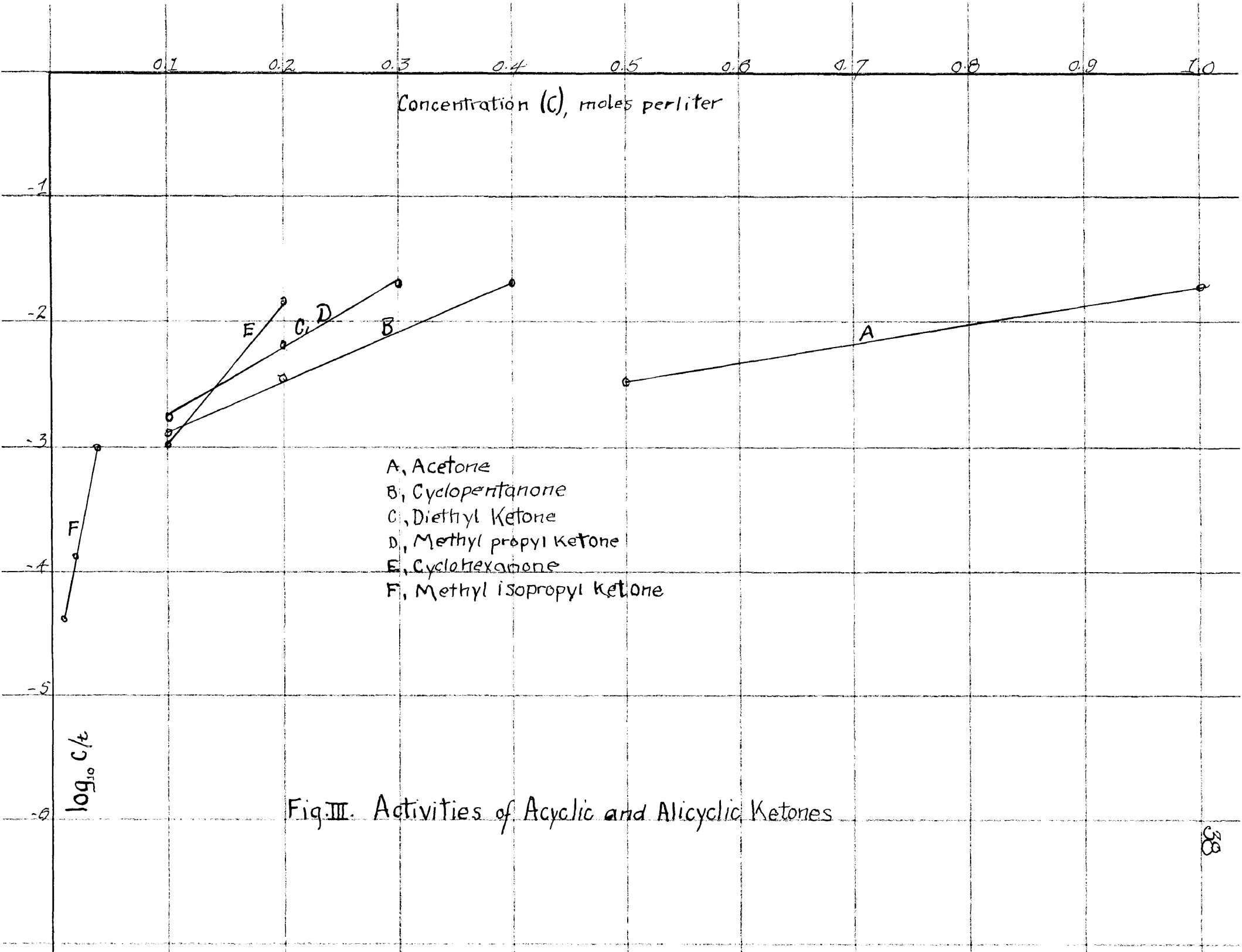
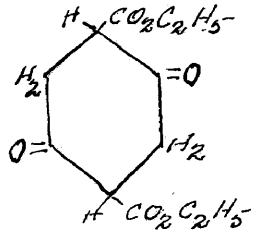


Fig. III. Activities of Acyclic and Alicyclic Ketones

CHEMISTRY SECTION

Experimental

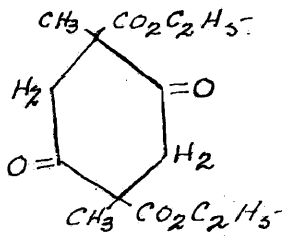
Cyclohexanedione-2,5-dicarboxylic acid-1,4-diethylester.-



This compound, according to Hantzsch[41], does not exist as the diketone[42,43,44,45,46] nor in keto-enol equilibrium, but entirely as the enol form, 2,5-dihydroxy- $\Delta^{1,4}$ - dihydroterephthalic acid diethyl ester. It was prepared after the technic of Upsenski and Turin[47] with a slight modification in the method of purification: 100 g. of diethyl succinate, 27 g. of metallic sodium in fine granules (prepared in hot anhydrous xylene) and 4 cc. of absolute ethyl alcohol was placed in a 1 liter Erlenmeyer flask equipped with a mercury seal to exclude air and moisture. The mixture was allowed to stand at room temperature for a week and then heated in an oven at 70° C. for 8 hours, at 90° C. for 16 hours and at 110 C. for 16 hours. At the end of this time the mixture had become a dry powder and possessed a deep carmine color. It was transferred to a 4 l. beaker and was treated slowly, while cooling in an ice bath, with sufficient 75 per cent alcohol to remove the unaffected sodium

pellets, and to the paste, thus formed, was added enough water to completely dissolve the sodium derivative. The sodium derivative was decomposed by the addition of hydrochloric acid, the precipitate was collected on a Büchner funnel, washed well with water, dried, and crystallized from hot ethyl alcohol. The yield of purified product was 48 g., corresponding to 65.3 per cent of the theoretical. M.P., 127-128° C.

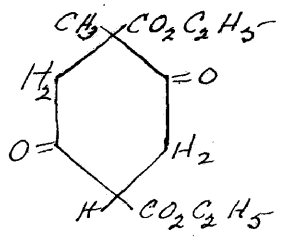
1,4-Dimethyl-cyclohexanedione-2,5-dicarboxylic acid-1,4- diethylester.-



Fifteen grams of cyclohexanedione-2,5-dicarboxylic acid-1,4-diethylester was dissolved in 400 cc. of hot absolute ethyl alcohol (preserved over calcium oxide and distilled from sodium) placed in a liter round-bottomed flask attached to a reflux condenser in which was fitted a calcium chloride tube. 3 g. of finely chipped sodium was added at one time to the solution and after the first vigorous reaction, a gentle heat was applied until the sodium had completely disappeared. The disodium derivative separated as a bulky carmine colored solid producing a thick somewhat pasty mixture. Frequent agitation was required to keep the sodium distributed through the mixture. After all of the sodium had disappeared, the mixture was refluxed for one hour on a water bath. Methyl iodide was then added in 5 cc. portions directly to the cooled liquid at intervals of one hour and heating was resumed after each addition

until the mixture became homogeneous. An additional quantity of methyl iodide equal to the total amount previously added was introduced into the mixture and heating continued for seven hours more. The excess halide and most of the alcohol were removed by distillation from a water bath under reduced pressure, using a water pump. The residue was treated with 50 cc. of 5 per cent sodium hydroxide and the aqueous solution extracted with ether until the ether layer showed only a weak fluorescence. The combined ether extracts were washed with water, then dried over anhydrous sodium sulfate. After distilling off the ether, the residue was distilled at 15 mm. pressure and the fraction passing over up to 190° C. collected. The oily distillate congealed upon cooling, yielding colorless crystals which gave a hydrazone, melting at 206° C. The melting point of the hydrazone given by Baeyer[48] is 207° C. The yield of the diketone was 6-7 g.

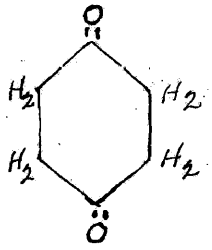
<u>1-Methyl-cyclohexanedione-2,5-dicarboxylic acid-1,4-diethyl ester.-</u>



The same procedure was followed as in the preparation of the dimethyl derivative, using 1.5 g. of sodium and 15 g. of cyclohexanedione-2,5-dicarboxylic acid-1,4-diethyl ester. The compound was purified after the removal of the excess halide and the solvent by direct vacuum distillation, eliminating the treatment

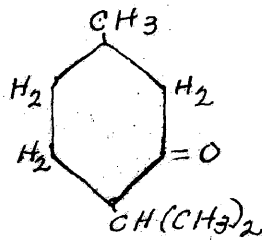
with sodium hydroxide. The finished product had the properties reported in Beilstein[49].

Cyclohexanedione-1,4.-



The method of Baeyer[50] was followed: Twenty grams of cyclohexanedione-2,5-dicarboxylic acid-1,4-diethyl ester was dissolved, with cooling in an ice bath , in 85 cc. of concentrated sulfuric acid. The mixture was poured upon 390 g. of cracked ice, 8 cc. of alcohol was added and the mixture was refluxed over a direct flame until all of the solid had disappeared and there was no more evolution of carbon dioxide. The solution was then brought to near neutrality with powdered sodium carbonate in an ice bath with care to avoid an excess of carbonate. Too much carbonate is indicated by the appearance of a brown color. The sodium sulfate was frozen out and removed by filtration and the filtrate extracted seven times with chloroform. Upon evaporating the chloroform from the combined extracts, 7 g. of diketone was obtained, which upon recrystallization from absolute alcohol melted at 78° C.

l-Menthone.-



The method of Beckmann[51] was used: To a mixture consisting of 110 g. of sodium dichromate, 80 cc. of concentrated sulfuric acid and 500 cc. of water was added 90 g. of menthol, all in one portion. The mixture was agitated vigorously with a mechanical stirrer while permitting the temperature to rise spontaneously to 55° C. and maintaining this temperature during 40 minutes without interrupting the stirring. The mixture was cooled, extracted three times with ether, the ether extracts were washed with water and after removal of the ether by distillation, the residue was steam distilled. The menthone was extracted from the distillate with ether, the ether solution dried over anhydrous sodium sulfate, then distilled and the fraction boiling at 208° C. collected. The yield was 50 g. The product was redistilled adn the fraction passing over sharply at 208° C. reserved for the biological tests.

Napthaquinone-1,4.- The method of Groves[52] was employed. Two hundred grams of chromium trioxide was dissolved in a mixture of 280 cc. of glacial acetic acid and 75 cc. of water. This solution was added slowly over a period of several hours to 50 g. of napthalene dissolved in 475 cc. of glacial acetic acid contained in a 2 l.,

three-necked flask equipped with a dropping funnel, thermometer and efficient stirrer. The chromium trioxide solution was added at such a rate as to maintain a temperature of 20-30° C., the reaction flask being immersed in a bath of ice water. The mixture was allowed to stand for four days at room temperature, then poured into 4 l. of water and the precipitate collected on a suction filter. The yield was 22 g. For the pharmacological tests, the compound was recrystallized from petroleum benzine until it melted sharply at 125° C.

Naphthaquinone-1,2.

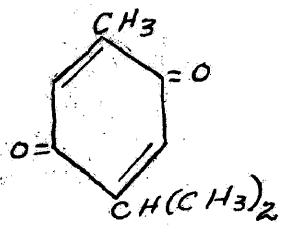
(A) Orange II.[53]- Thirty four grams of sulfanilic acid was diazotized at 5° C., using 14.4 g. of sodium nitrite in 10 per cent solution and 60 cc. of hydrochloric acid. The diazonium solution was added to 28.8 g. of beta-napthol dissolved in 30 cc. of water containing 9 g. of sodium hydroxide and was then diluted to 360 cc. After stirring for about an hour, the dye began to separate and the separation was then completed by the addition of a little salt.

(B) Alpha-amino-betanapthol[54].- The Orange II from (A), amounting to about 25 g., was dissolved in 200 cc. of boiling water and to this was added a solution of 38 g. of tin dissolved in 190 cc. of concentrated hydrochloric acid. When the solution became decolorized it was filtered rapidly, whereupon the hydrochloride separated in the filtrate in the form of needles.

(C) Naphthaquinone-1,2[55].- The amino-napthol from (B) was suspended, without drying, in 100 cc. of 30 percent sulfuric acid and a slight

excess of potassium dichromate in 10 per cent solution added at 0° C., with mechanical stirring. The precipitate was collected on a filter, washed well with water and crystallized several times from petroleum benzine. Approximately 5 g. of purified product was thus obtained.

THYMOQUINONE.



This compound was prepared by the method outlined by Kremers, Wakeman and Hixon[56].

(A) Nitrosothymol.- To a solution of 100 g. of thymol in 500 cc. of 95 per cent ethyl alcohol was added 500 cc. of concentrated hydrochloric acid. This mixture was cooled to 0° C. and 72 g. of sodium nitrite was then added in 5 g. portions, with stirring and cooling in an ice-salt bath. The resulting pasty mixture was poured into about 8 l. of cold water, the precipitate was collected on a suction filter and washed well with water.

(B) Aminothymol.- The entire quantity of nitrosothymol from (A) was treated with 2500 cc. of 15 per cent ammonia water and the solution filtered free of undissolved resinous matter. Hydrogen sulfide was passed into the filtrate until precipitation of amino-thymol was complete. The precipitate was collected at once on a

suction filter and washed well with water.

(C) Thymoquinone.- Fifteen grams of the moist amino-thymol was dissolved in 250 cc. of concentrated sulfuric acid diluted to 1 l. with water and oxidized at room temperature by the gradual addition of 19 g. of sodium nitrite. The mixture was heated for 1/2 hour on a water bath and then steam distilled to remove the thymoquinone. The product, amounting to 7 g., when recrystallized from petroleum benzine, was obtained in yellow crystals melting at 43-45 C.

Benzalacetophenone[57].-

$$C_6H_5CHO \quad C_6H_5COCH_3 \xrightarrow{NaOH} C_6H_5CH{=}CHCOC_6H_5 \quad H_2O$$

A solution of 36 g. of sodium hydroxide in a mixture of 327 cc. of water and 167 g. of 95 per cent alcohol was stirred vigorously for three hours with 87 g. of acetophenone (fraction boiling at 201-202) and 77 g. of U. S. P. benzaldehyde. The temperature was kept around 25 C. It was not permitted to drop below 15 C. or rise above 30 C. The mixture was set aside for 36 hours in an ice box, after which it was cooled in a freezing mixture, then filtered through a Büchner funnel, washed with water until the washings were neutral to litmus, and finally washed with 50 cc. of cooled alcohol. The yield of crude, thoroughly air-dried product was 186 g., affording 130 g. of purified light yellow crystals which melted at 505-507 C. The crude material was purified by saturating alcohol with it at 50 C., allowing the solution to cool gradually, and finally cooling it in a freezing mixture.

Benzalacetophenone-dibromide[58] ($C_6H_5CHBr.CHBrCOC_6H_5$). -

Twenty-six cc. of bromine was added, with cooling and stirring, to 104 g. of benzalacetophenone dissolved in 500 cc. of carbon disulfide. The dibromide was collected on a Büchner funnel and washed with two 125 cc. portions of hot alcohol. The product, dried in air, weighed 145 g. and melted at 155.5-156.6° C. An additional quantity of impure product, amounting to 15 g. and melting at 105-115° C. was obtained from the carbon disulfide filtrate and alcohol washings. Recrystallization of the impure tailings yielded 11 g. of product melting at 105-135° C.

DIBENZOYLMETHANE: (Phenyl-alpha-hydroxystyryl ketone)[59] .-

$$C_6H_5CHBr.CHBrCOC_6H_5 + 2NaOCH_3$$

$$\longrightarrow C_6H_5C(OCH_3)=CHCOC_6H_5 + CH_3OH + 2NaBr$$

$$C_6H_5C(OCH_3)=CHCOC_6H_5 + H_2O$$

$$\xrightarrow{HCl} C_6H_5C(OH)=CHCOC_6H_5 + CH_3OH$$

$$C_6H_5C(OH)=CHCOC_6H_5 \rightleftharpoons C_6H_5COCH_2COC_6H_5$$

In a 1 l., three-necked, round-bottomed flask, fitted with condenser, stirrer, and dropping funnel was placed 140 g. of benzalacetophenone-dibromide (m.p., 155.5-156.5° C.) and 126 cc. of absolute methyl alcohol (refluxed and distilled over calcium turnings. A solution of sodium methylate, prepared from 17.5 g. of sodium and 154 cc. of absolute methyl alcohol was added rapidly,

with stirring, and the mixture was then refluxed for one hour. Sufficient concentrated hydrochloric acid was added to neutralize the mixture, then 2 cc. of acid additional. After refluxing for not longer than five minutes, 122 cc. of cold water was added with stirring and cooling by ice water. The crystals were collected on a Büchner funnel, washed once with 50 cc. of 50 per cent methyl alcohol, and then with water until free of acid. The air-dried product weighed 68 g. By recrystallization from 110 cc. of hot methyl alcohol, 43 g. of light yellow crystals, melting at 77-78° C., were obtained.

Dibenzoyldibromomethane[60] ($C_6H_5COCBr_2COC_6H_5$) .-

In a 500 cc. three-necked flask, fitted with stirrer, dropping funnel, and thermometer, were placed 26 g. of dibenzoylmethane and 7 cc. of chloroform. The flask was surrounded with an ice bath, and 14 cc. of bromine which had been dried previously with concentrated sulfuric acid and then dissolved in 115 cc. of chloroform, was run into the dibenzoylmethane slowly, with stirring, during 40-50 minutes (a 30 minute period is recommended by Bigelow and Hanslick). The temperature was kept below 15° C. during the bromination; the hydrogen bromide evolved was continously removed by aspirating a gentle stream of air over the surface of the solution. Stirring was continued for 15 minutes after all of the bromine had been added and the solvent was then under diminished pressure at room temperature. The colored residue was treated with an equal volume of 95 per cent ethyl alcohol, the solid collected on a filter

and washed with a small volume of alcohol. The yield of crude, white cyrstals (m.p., 90-95° C.) was 44 g., corresponding to 92 per cent of the theoretical. Recrystallization from 60 cc. of hot ethyl alcohol afforded 38 g. of pure product, melting at 94 -95° C.

Diphenyl Triketone Hydrate[61]. $(C_6H_5COC(OH)_2COC_6H_5)$.-

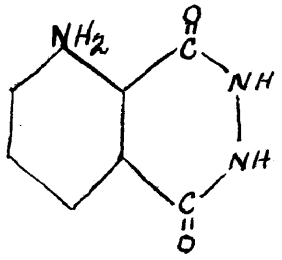
Thirty-six grams of dibenzoyldibromomethane was refluxed with a solution of 17.5 g. of fused sodium acetate in 70 cc. of glacial acetic acid until precipitation of sodium bromide ceased. About one hour was required. The mixture was cooled to room temperature and 100 cc. of water was added with constant shaking to dissolve the inorganic salt and to precipitate the triketone hydrate. This was separated by filtration, washed with water until the washings were no longer acid to litmus, and then dried in an oven at 60° C. The yield amounted to 20 g.

Diphenyl Triketone[61]. $(C_6H_5COCOCOC_6H_5)$.-

The entire yield (20 g.) of triketone hydrate was distilled at 5 mm. from a Claisen flask heated by means of a sand bath. The first fraction collected, distilling over up to 150° C. and amounting to about 2 cc., was rejected. Most of the product distilled between 163° C. and 165° C. and solidified to a yellow crystalline mass which weighed 15 g. This yellow material was crystallized from 20 cc. of hot gasoline and was thus obtained in the form of small yellow needles which, after standing in a vacuum desiccator until

the odor of gasoline was no longer noticeable, melted at 70-72° C. The melting point reported by Bigelow and Hanslick is 68-70° C. The yield was 7 g. The product was preserved in sealed tubes until required for the biological tests.

3-AMINOPHTHALHYDRAZIDE



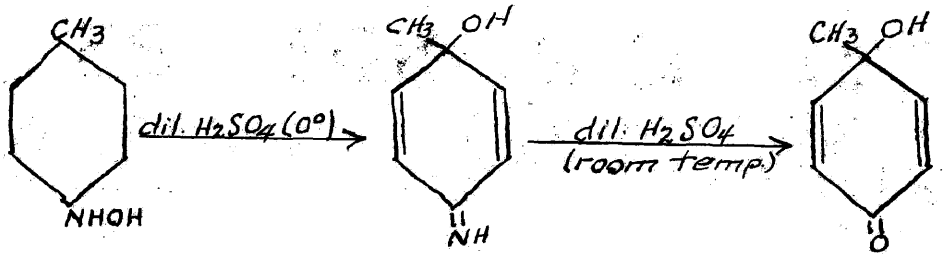
This compound was prepared by the procedure given by Huntress, Stanley, and Parker[62].

(A) 3-Nitrophthalhydrazide.- A solution of 130 g. of hydrazine sulfate and 272 g. of crystallized sodium acetate in 400 cc. of hot water was added to 211 g. of 3-nitrophthalic acid contained in an 8 inch porcelain evaporating dish. The mixture was evaporated rapidly over a direct flame to a dry solid residue with continuous stirring. The dry, powdered residue was then placed in a beaker and heated for three hours in an oil bath at a temperature of 160 ± 10° C. When heating was completed, the solid was extracted twice with 350 cc. portions of hot water to remove sodium sulfate, and finally dried at 105° C. to constant weight.

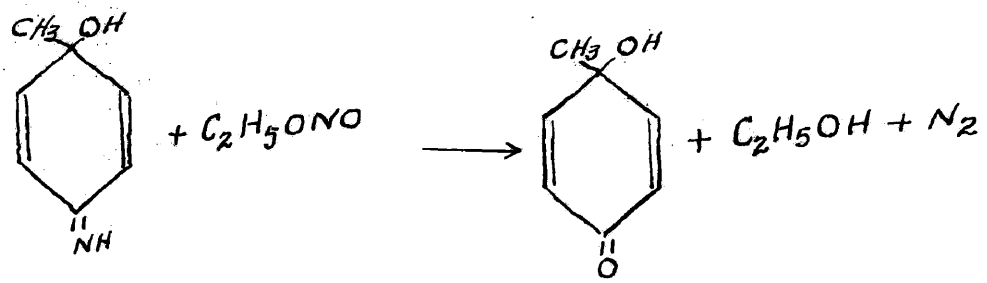
(B) 3-Aminophthalhydrazide. - A solution of ammonium sulfide was prepared by passing hydrogen sulfide gas into 200 cc. of ammonium hydroxide (sp. gr., 0.90) with cooling in an ice bath until the gas was no longer absorbed; 200 cc. more of concentrated ammonium hydroxide solution was then added and the solution diluted to one liter. To the ammonium sulfide solution, thus prepared, was added in small increments the entire yield of crude 3-nitrophthalhydrazide. The resulting suspension was boiled for an hour and additional hydrogen sulfide passed in while heating. After this treatment, the mixture was boiled for an hour more and then set aside to cool. The precipitated 3-aminophthalhydrazide mixed with the sulfur was collected on a suction filter, washed with water and dried. The desired product remaining dissolved in the original filtrate was precipitated with a slight excess of glacial acetic acid and was collected mixed with sulfur on a suction filter, washed and dried. The amino compound was removed from the crude sulfur containing mixtures by stirring with a 5 per cent solution of sodium hydroxide slightly in excess of the theoretical requirement. After stirring and slight warming, the mixture was filtered and the filtrate set aside to permit the sodium salt to separate. This was collected, washed well with water, and sparingly with alcohol and ether. The free aminophthalhydrazide, when required for use in the pharmacological tests, was dissolved in water and reprecipitated with a slight excess of glacial acetic acid.

OXIDATION OF p-TOLYLHYDROXYLAMINE BY ETHYL NITRITE.

The oxidation of aminothymol by nitrous acid to thymoquinone supports the assumption that p-tolylhydroxylamine, by similar treatment, should be converted to toluquinole and that a convenient method for the synthesis of higher homologs of toluquinole might be perfected possessing advantages over the method of Bamberger[63]. The mechanism of the transformation of p-tolylhydroxylamine to toluquinole is depicted by Bamberger in the following manner:



The method described by him is objectionable when applied to the synthesis of a series of compounds because of the tedium and time factor involved in the isolation and purification of products. The chief difficulty to be overcome is the complete elimination of sulfuric acid without decomposition of the unstable toluquinole. An attempt was, therefore, made to prepare toluquinole by direct treatment of p-tolylhydroxylamine with ethyl nitrite, as illustrated:



p-Tolylhydroxylamine.- was first prepared by the method of Bamberger and Rising[64]. Fifty grams of p-nitrotoluene, dissolved in 50 cc. of hot alcohol, was added with vigorous stirring to a solution of 26 g. of commercial ammonium chloride in 800 cc. of water and placed in a 2-l, three-necked flask equipped with a thermometer and an efficient stirrer. 65 g. of zinc dust was then added in small increments until the temperature rose spontaneously to 50-60° C. This temperature was maintained by carefully regulating the rate of addition of the metal. About 30 minutes were required for the addition of all of the zinc. The mixture was stirred for 15 minutes longer after all the zinc had been added and the mixture was then filtered warm with suction. The zinc oxide and the hydroxylamine collected on the filter were washed rapidly with 500 cc. of water warmed to 60° C.; the primary filtrate was saturated with salt, then chilled in an ice-box and the white or slightly yellow plates of the hydroxylamine collected with suction and washed with a little petroleum benzin. The last aqueous filtrate was extracted with ether, the ether solutions were combined with the petroleum benzin washings, dried over calcium chloride, the excess solvent removed by evaporation from a steam bath, and the residue poured into several volumes of petroleum benzin and chilled. The total yield was 30 g., corresponding to 75 per cent of the theoretical. The melting point of the product after recrystallizing once from petroleum benzine was 92-93° C.

The addition of ethyl nitrite, either in alcoholic or hydro-alcoholic solution, to the hydroxylamine prepared in the manner

described gave, instead of toluquinole, advanced oxidation products of p-toluidine, viz., p,p'-azoxytoluene, which crystallized from petroleum benzin in yellow plates, melting at 69-70° C.; and p-nitrosotoluene, a dark green oil which solidified upon cooling in an ice-box and which, when crystallized from petroleum benzin, was obtained as a white crystalline solid melting at 48° C. The extent of oxidation or the particular product obtained is determined by the amount of nitrite used, by the reaction time and the reaction temperature.

The use of finely divided copper to catalyze the reaction resulted, after the first violent reaction, in the separation of a light to dark green, water-insoluble material which proved to be either p,p'-azo-cupric-benzoate or p,p'-azoxy-cupric-benzoate, or a mixture of the two. The copper derivative was prepared for study in the following manner: To a solution of 2 g. of p-tolylhydroxylamine in 20 cc. of ethyl alcohol cooled to 20° C. was added slowly, with stirring, 2.5 cc. of ethyl nitrite in 30 cc. of alcohol. During the addition, the temperature was maintained between 15 and 20° C. The mixture was transferred to an Erlenmeyer flask and heated slowly and cautiously on a water bath under a reflux condenser until the reaction began. At the first indication of the exothermic reaction, the heat was withdrawn until the vigor of the reaction diminished somewhat. When separation of the solid appeared to be complete, the mixture was refluxed for 15 minutes longer, after which it was cooled, the green crystalline solid collected on a filter and purified by extraction with ethyl alcohol in a Soxhlet apparatus, the extractive

being replaced by pure solvent several times to avoid overheating the copper derivative. The yield of copper salts collected from the combined extracts amounted to 1.3 g. The melting point was 195-196° C. (decomposition) and remained unchanged after another crystallization from alcohol. The product was dried at 110° C. for analysis.

Analysis. Required for $C_{14}H_8O_4N_2Cu$: Cu, 19.16 %.
N, 8.48 %

Required for $C_{14}H_8O_5N_2Cu$: Cu, 18.28 %
8.06 %

Found: Cu, 18.82%; N, 8.24%

SUMMARY AND CONCLUSIONS

1. The literature pertaining to synthetic and naturally occuring anthelmintic compounds exhibiting one or more carbonyl groups as a structural characteristic has been reviewed.

2. The literature pertaining to the biological evaluation of anthelmintics has been reviewed.

3. A number of keto-compounds have been prepared, purified and identified, some of which have been tested by a new earth-worm method; others of which have been preserved after purification for future evaluation.

4. A method, employing the intact earth-worm (Lumbricus terrestris or L. rubellus) as test animal, by which the criterion of molecular toxicity is based upon the rate of change of apparent speed of fatality with change in molar concentration has been developed.

5. The toxicities of a series of acyclic and alicyclic ketones have been determined by this biological method.

6. The evidence sought and gathered for deciding upon the importance of the carbonyl group as a functional attribute in certain types of anthelmintic drugs is neither sufficiently adequate nor extensive in scope to permit generalization.

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