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THE ANALYTICAL DETERMINATION OF CANTHARIDIN IN CANTHARIDES

BY

NYDIA MARGARITA KING

A Thesis Submitted in Partial Fulfillment

of the Requirements for the Degree of

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INTRODUCTION

Purpose of this Investigation

The methods that have been developed thus far for the determination of cantharidin in Cantharides have not proved satisfactory. The present assay process in the Ninth Revision of the National Formulary has some disadvantages, namely, that in the final step the cantharidin is associated with inert material which is not easily removed, therefore the results are not accurate especially since the determination is a gravimetric one; also its value is questionable on the basis of incompleteness of extraction of cantharidin.

The present investigation was undertaken to develop a convenient and efficient method for the estimation of cantharidin which could be applied to the assay of Cantharides. The problem was approached from two different angles: first, separation of cantharidin from fat, pigment, and other inert material by means of partition chromatography; secondly, the determination of the amount of cantharidin by titration.

History of Cantharides

The use of Cantharides in medicine dates back as far as the early Greek civilization and even earlier. Hippocrates (1) mentioned it in his writings and it is known that Galen (1) and Dioscorides (2) as physicians, prescribed it.

In the National Formulary, Ninth Revision, Cantharides is defined as the dried insects, Cantharis vesicatoria (Linne) de Geer (Fam. Meloidese). It is popularly referred to as Spanish Flies or Russian Flies, and occurs extensively in southern areas of Europe, especially Spain, Russia, and France.

The first step in the production of the drug is the collection of the insects. These are found on the ground in larva form, and during the month of May they are transformed into the adult stage; they infest trees and plants, especially the pivet, ash, lilac, and honey-suckle. At this time the insects are gathered by shaking them from the infected bushes onto spread blankets. They are killed by exposing them to acid fumes, or chloroform, then dried quickly in the sunlight, or by artificial means (3).

In medicine the principal action of Cantharides is that of a vesicatory, and this effect determines the uses to which it has been applied. Used externally, it produces redness and vesication, depending upon the mode of application and length of time of contact. As a blistering agent it is preferred to all other substances. It has been

used for sciatica, local chronic inflammations, and diseases of the abdomen and chest; also in hair tonics for stimulating the hair follicles and thereby preventing alopecia. In liniments it serves as an efficient rubefacient. In small medicinal doses it acts as a stimulant and diuretic, and has been employed in such conditions as chronic gonorrhea, amenorrhea, and other urinary afflictions. Internally administered Cantharides is a powerful irritant which has caused its use to be almost completely abandoned in favor of less harmful remedies. As an internal cure, Cantharides is of little practical value.

In this country, the drug has been used internally in the form of a tincture and externally as a plaster or cerate. In other countries, particularly in England, it has been used in liniments, ointments, lotions and plasters.

Constituents of Cantharides

After several investigations had been conducted to determine the composition of Cantharides, a white crystalline substance was finally obtained by Robiquet in 1810 (4). This principle proved to be the active vesicatory constituent and was termed "cantharidin" by Thompson in his system of Chemical Nomenclature (5) It was obtained from the powdered drug by extraction with ether. Evaporation produced a green residue which was then extracted with hot alcohol. The crystals from the latter solution

were purified by recrystallization.

Cantharidin occurs as colorless, rhombic prisms, or laminae, stable in air, and melting at 218°C (corr.). At higher temperatures it readily sublimes; even at lower temperatures it is somewhat volatile. It is insoluble in water, carbon disulfide, and petroleum ether; slightly soluble in cold alcohol, and readily soluble in hot alcohol, acetone, chloroform, ethyl acetate, acids, and oils. It is present in Cantharides as both the anhydride and the salts of the acid (6).

The other constituents of Cantharides such as fat, oil, organic acids, salts, etc., are therapeutically inert.

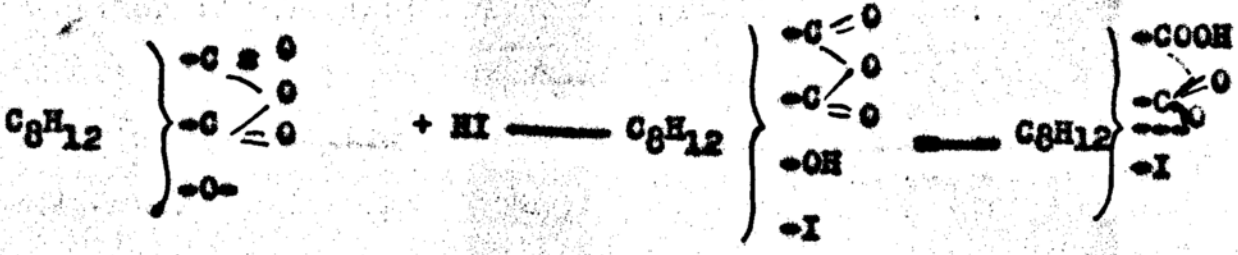
Chemistry of Cantharidin

It was not until 1877 that any important work on the chemistry of cantharidin was accomplished. Previously Dragendorff and Masing (7) showed that the simplest formula was $C_5H_6O_2$ and that it reacted with alkali, when heated, to form salts. In 1877 Picard (8) determined the molecular weight and proved the formula to be $C_{10}H_{12}O_4$ rather than $C_5H_6O_2$. He then made an intensive study of the pyrogenic decomposition of cantharidin obtaining a hydrocarbon by dry distillation of the barium salt. This product was termed "cantharene", and had the formula C_8H_{12}. Further analysis showed it to be a di-hydro-o-dimethyl benzene.

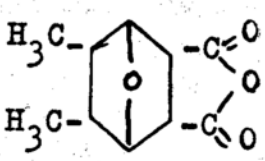
The dibasicity of cantharidin was first observed by Homolka (9) who prepared the dimethyl ester, $C_{10}H_{12}O_5(CH_3)_2$.

the empirical formula of which indicated the existence of an acid anhydride.

It was observed by Picard (10) that when hydrogen iodide and cantharidin were heated, a product, $C_{10}H_{12}O_3I_2$, was formed, which was designated as the "di-iodide". Gadamer viewed this reaction as one typical of the others. He also reasoned that if cantharidin contained an ether structure, the action of hydrogen iodide should, in addition, result in the formation of an alcohol. Accordingly this last compound was both an alcohol and a halogen derivative, and should have an empirical formula $C_{10}H_{13}O_4I$. He attempted to isolate the compound from the reaction mixture (11). The compound finally obtained was the lactone corresponding to it.



With the functional groups thus determined, i.e. ethereal oxygen, two carboxy groups as an acid anhydride, and hexahydro O-xylene as the underlying structure, three possible formulas were advanced by Gadamer:



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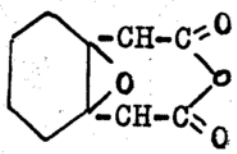

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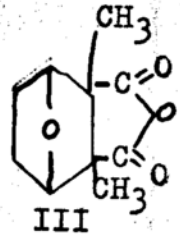
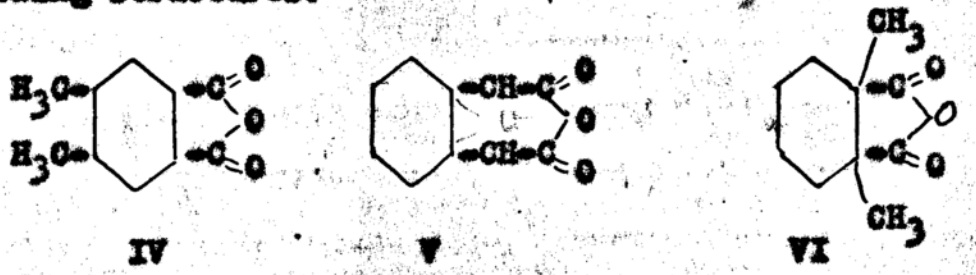


III

A study concerning the reduction product obtained by treating cantharidin with sodium and alcohol showed that there was loss of one atom of oxygen (12) As the reduced compound was still dibasic, it was the ethereal oxygen which must have been lost by this treatment. According to Gadamer this desoxy compound can be represented by one of the following structures:

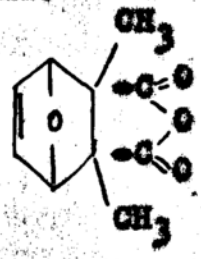


IV V VI

Rudolph (13) considered that the compound represented by formula V should be exceedingly unstable as it contains a seven membered ring. He prepared desoxy cantharidin in order to compare its stability with that of structure V. It was found that the desoxy cantharidin was very stable, even resisting the action of alkaline permanganate. This evidence was sufficient to eliminate Gadamer's second formula. The first formula advanced by him was likewise ruled out when a compound having the structure of IV was synthesized by Coffey (14). A comparison made of the properties immediately revealed that this was not identical with desoxycantharidin.

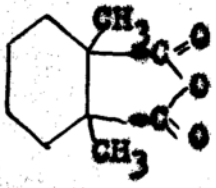
Von Bruchhausen and Berach (15) decided in favor of III rather than I upon obtaining small amounts of dimethyl maleic anhydride as a result of passing cantharidin over

palladium-asbestos at 280°C. These investigators assumed that the anhydride was formed by an inverse Diels-Alder reaction of the hypothetical dehydrocantharidin, represented by the following formula:



They considered this a reasonable assumption in view of the ready reversibility of the furan-maleic anhydride reaction and the failure of numerous attempts to add dimethyl maleic anhydride to furan to obtain dehydrocantharidin. But the experiments of Bruchhausen and Berndt did not afford unequivocal proof of the structure of cantharidin. According to Woodward (16), applying to structure I the breakdown mechanism adduced by these authors, the initial products would be 3,4-dimethyl furan and maleic anhydride. Under the conditions of the experiment it is not inconceivable that partial oxidation of 3,4-dimethyl furan could account for the small amounts of dimethyl maleic anhydride observed.

Woodward and his co-workers (16) proved the structure of cantharidin indirectly by way of desoxycantharidin. They synthesized cis-1,2,dimethyl-cyclohexane-1,2-dicarboxylic anhydride



and established its full identity with desoxycantharidin obtained from the natural product, thus favoring structure III. However, there still remained a stereochemical point to be considered.

In 1942 Ziegler, Schenck, et al, succeeded in effecting the total synthesis of cantharidin - but by a route which included an unsatisfactory last step resulting in a complex mixture from which cantharidin was eventually isolated in a 2% yield (17). Later efforts to render the synthesis stereospecific were unsuccessful (18). Stork and his collaborators achieved a stereospecific synthesis of cantharidin whose identity they established by its melting point, infra red spectrum and X-Ray powder diffraction pattern (19). The path taken in this synthesis confirmed the structure of cantharidin as being represented by formula III.

History of the Assay of Cantharides

Since the efficacy of Cantharides as a drug depends upon its chief constituent cantharidin, it has been necessary to determine the content of the latter in evaluating the drug.

The methods generally used for the determination of cantharidin are similar in principle although frequently differing in detail. They consist of extracting the acidified powdered beetles with an organic solvent, usually chloroform or benzene, which in addition to the cantharidin

also removes fat and coloring matter. The solvent is then removed by evaporation and the fat and coloring matter are washed away with another solvent in which cantharidin is insoluble. Petroleum ether is generally used for this purpose. The purified residue is then weighed. Since there is usually difficulty in purifying the extracted cantharidin, the results are consequently inaccurate.

Throughout the literature there are numerous references to modifications introduced for the purpose of improving the extraction process or modifications to avoid losses through solubility in the purifying solvents or through volatilization. Outstanding among these, are the work of Boudin (20), Nagelvoort (21), Gunn (22), Greenish and Wilson (23), Leger (24), Sing and Nagai (25), and Kneip, Ney, and Reimer (26). Hecht and Parks (27) and Guthrie and Brindle (28) (29), have proposed volumetric methods for the determination of pure cantharidin. Listroy (30) devised a color reaction but this test proved unreliable by reason of interfering substances. An adsorption chromatographic assay for the Tincture of Cantharides has been described by Franck and Valentin (31).

The official assay for Cantharides was introduced in the United States Pharmacopoe of 1910. This method has remained unchanged throughout the subsequent revisions, but in 1942 it was transferred from the USP XI to the National Formulary, Seventh Revision. The method is reproduced here as follows:

> Place 15 grams of Cantharides in moderately coarse powder in a pressure bottle of not less than 250 ml capacity, add 150 cc of a mixture of benzene = two volumes and petroleum ether = one volume, and then add 2 cc of hydrochloric acid. Stopper the bottle tightly, shake it well, and allow to stand for about ten hours. Gradually warm the bottle and its contents to about 40°C and maintain it at approximately that temperature with frequent shaking for three hours, avoiding evaporation. Cool the mixture, decant or filter off 100 cc of the clear solution, and evaporate this rapidly in a tared beaker to a volume of about 5 cc. Add 5 cc of chloroform to the residue and set it aside in a moderately warm place. When the solvent has all evaporated, add to the crystals 10 cc of a mixture of equal volumes of dehydrated alcohol and petroleum benzin, which has been saturated with pure cantharidin, allow the mixture to stand during 15 minutes, and then decant the liquid through a pledget of purified cotton. Then wash the cotton with a small quantity of warm chloroform to dissolve any adhering crystals, evaporate the solvent with the aid of a current of air, dry the crystals at 60°C for thirty minutes and weigh. The resulting weight represents the amount of cantharidin from ten grams of Cantharides. It should contain not less than 0.6%.

Guthrie and Brindle (28) omitted the heating at 40°C and obtained results not significantly below the average in ten assays. This would indicate that this heating is not necessary.

The possible errors in this process, apart from the contamination of the final residue with fat are:

1. Incomplete extraction of the powdered beetles.
2. Loss of cantharidin during evaporation of the solvents.
3. Loss of cantharidin in drying at 60°C.
4. Loss of cantharidin in the removal of the fat.

These different stages have been examined by Guthrie and Brindle (28). In the extraction of the drug, they concluded that benzene is as efficient as chloroform, a fact confirmed by Hecht and Parks (27). They also found that Soxhlet extraction offered no advantage over maceration, and that there was no appreciable loss of cantharidin on drying at 60°C. Hecht and Parks have presented evidence to indicate that rapid evaporation of the solvent results in less loss of cantharidin.

Since cantharidin is soluble in fats and oils there is loss of it during the removal of the fat. Hecht and Parks proved this by adding castor oil to a solution of cantharidin in chloroform, evaporating the chloroform and washing away the castor oil with petroleum ether. A loss of cantharidin occurred. Although solvents in which cantharidin is practically insoluble are used, there is evidence that the oil increased the solubility of the cantharidin in the solvent. Attempts have been made by several workers to guard against the possibility of this loss, (23) (32), however, none of the methods suggested has been successful enough to justify their use.

Cantharides is official in the German and French Pharmacopoeas and in the British Pharmaceutical Codex. A summary of the assays given in these books follows:

German Pharmacopoea

The powder is extracted by shaking frequently during twenty four hours with chloroform in the presence of hydrochloric acid. Then ether is added and after half-an-hour an aliquot portion is filtered off. The solvent is evaporated and the residue washed with a mixture of light petroleum and absolute

alcohol, filtered through cotton wool, and the crystals washed back into the tared flask with chloroform. The chloroform is then evaporated and the residue dried for twelve hours in a desiccator. If the residue is unsatisfactory the cantharidin is purified by dissolving in sodium hydroxide and re-extracting with chloroform after acidifying with hydrochloric acid. The residue left after evaporation of the chloroform is washed with the light petroleum and alcohol mixture as before and then dried.

French Pharmacopea (1949)

The extraction process differs from the German and British in that the powder is extracted with benzene in the presence of hydrochloric acid in a Soxhlet apparatus. The solution is evaporated and the residue washed with light petroleum and absolute alcohol mixture, the crystals being collected in a porous glass filter and then dried.

British Pharmaceutical Codex (1949)

The method of extraction is similar to that of the German Pharmacopea, but the chloroform solution, without the addition of ether, is evaporated and the residue washed with light petroleum alone. After treatment with chloroform and evaporation of the solvent the residue is purified by treatment with sodium hydroxide solution and potassium permanganate. The alkaline solution is then acidified with sulfuric acid and extracted with chloroform in the presence of ferrous sulfate. The chloroformic solution is evaporated and the residue dried below 60°C.

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DISCUSSION OF EXPERIMENTAL WORK

PART ONE

ISOLATION OF CANTHARIDIN FROM EXTRACT OF CANTHARIDES BY PARTITION CHROMATOGRAPHY

Introduction

The method of partition chromatography was introduced by Martin and Synge in 1941 (1). In the resolution of mixtures by this method, the substances are distributed between two liquid phases, one of which is adsorbed on a support while the other is mobile. The individual partition coefficients of the components of the mixture between the two liquids are the determining factors in their separation.

Two fundamental conditions must be fulfilled in partition chromatography:

(1) the support should be able to bind a substantial quantity of the non-mobile liquid phase, and

(2) it should not disturb the partition between the two liquids by acting as an ordinary adsorbent for the solute.

The theory of partition chromatography was proposed by Martin and Synge.(2) The relative rate of movement of a certain zone is defined as R = movement of position of maximum concentration of solute / simultaneous movement of surface developer above the column. The connection between R and the partition coefficientα (g of solute per ml of non-mobile phase/ g solute per ml of mobile phase) is expressed by the terms:

$$R = \frac{A}{A_L + \alpha A_g} = \frac{A_L + A_g + A_I}{A_L + \alpha A_g}$$

from which the partition coefficient

$$\alpha = \frac{A}{R A_g} + \frac{A_L}{A_g}$$

where,

A = area of cross-section of the column
A_g, A_L and A_I = respective areas of cross section of the non-mobile phase, mobile phase, and inert solid
$A = A_g + A_L + A_I$

Separation based on different partition coefficients of solutes is, therefore, to be expected despite the fact that α is not a constant but depends on the absolute concentration (2) (3). Furthermore, the interaction of the respective solutes alters the theoretically forecast picture. Nevertheless, in many instances, calculated and determined partition coefficients are in good agreement.

The method of partition chromatography has been applied to the separation of amino acids, fatty acids, and other substances.

In view of the wide application of partition chromatography in the analysis of mixtures, it seemed worthwhile to attempt the quantitative isolation of cantharidin from Cantharides by this technique. As mentioned previously, there is loss of cantharidin on removal of the fat from the extract of Cantharides. An adequate partition column would avoid this loss by permitting the internal phase to retain the cantharidin while the fat is being eluted with the proper solvent. Therefore, it appeared that this technique would be more advantageous than the methods advanced by

other workers.

A method employing adsorption chromatography was described in 1936 by Pyanek and Valentin (4) to determine the contents of cantharidin in the Tincture of Cantharides. In this work alumina was used as the adsorbent and a mixture of acetone and chloroform as eluent. The cantharidin was recovered impure from the column, purified by washing with petroleum benzin-absolute alcohol, dried and finally weighed.

A. Partition Chromatography Using Methanol-Water as Internal Phase

1. Experiments on Pure Cantharidin

Before attempting any separation using Cantharides extract, the first step was necessarily to determine the behavior of pure cantharidin in partition columns. This had to be preceded by the determination of the partition coefficients of this substance between different pairs of solvents. Ordinarily it would be necessary to determine the partition coefficients of the individual substances to be separated, and by the relationship between them, establish which is the best pair of solvents for the separation. It is known, for example, that as the partition coefficients of two substances approach each other, their separation becomes more difficult. In the present case, in which it was possible to determine the partition coefficient of the desired component only, this value was used as an indication of the facility with which this particular component would

be removed from the column. A high value would suggest that elution might take an impracticably long time, while a low value would predict that the substance would be removed too soon, that is, before the undesired material had had a chance to be removed. A suitable value is obtained when the amount of material in the immobile phase is about twice the amount in the mobile phase, i.e., $\frac{\text{immobile phase}}{\text{mobile phase}} = 2$.

The cantharidin available at the time this work was started consisted of white platelets melting at 214.5°C - 215°C (uncorr.). The first pair of solvents chosen was 70% aqueous methanol and Skelly C. Twenty-five ml of each solvent (mutually saturated) was placed in a small separatory funnel and a weighed amount of cantharidin was added. The funnel was shaken for five minutes and allowed to come to equilibrium. After this, the layers were separated and poured into tared aluminum dishes, and the solvent allowed to evaporate; the dishes were then dried and weighed; the increase in weight of the dish with the aqueous phase divided by the increase in weight of the dish with the organic phase gave the partition coefficient. This pair of solvents gave a partition coefficient of 1.84, a value suitable for partition chromatographic purpose. In the next pair of solvents used, the concentration of methanol was brought up to 85%, and a value of 19.5 was obtained, which was much too high.

In view of the favorable partition coefficient obtained with the first pair of solvents, i.e., 70% methanol and

Skelly C, the first column was run using these two solvents as the non-mobile phase and mobile phase respectively. Twenty-five grams of silicic acid was moistened thoroughly with 25 ml of 70% aqueous methanol. The moistened powder was mixed with Skelly C to form a slurry which was packed into the chromatographic tube. A sample of cantharidin weighing 14.1 mgms was dissolved in a mixture of 20 ml of Skelly C and 8 ml of chloroform and poured in the column. As shown in Fig. 1a, 10.1 mgm of material was recovered in fractions four to eight. The column developed air bubbles after a time and was not homogenous. Owing to these physical changes and the fact that the sample was in part recovered too soon, it was necessary to try other conditions. The somewhat large volume of solvent used to dissolve the sample was considered as a possible source of error. Also, the column turned yellow on addition of the Skelly C. Hence, this solvent was purified in the following manner: the Skelly C was shaken with several portions of concentrated sulfuric acid until the acid remained colorless, then it was washed with distilled water until the washings were no longer acidic to litmus paper, and finally it was dried over anhydrous sodium sulfate and distilled.

The second column was packed in the same way as the first, but only 3 ml of chloroform was used to dissolve a sample of cantharidin weighing 26.7 mgm. Fifteen ten-ml fractions of Skelly C were collected. Figure 1b shows that 19.1 mgm of material was recovered in the third and

fourth fractions. Obviously the sample was not retained in the column. It was evident that the system was not working as predicted by the partition coefficient. The next consideration was to increase the concentration of methanol in the non-mobile phase; this seemed logical because a higher concentration of methanol favored the partition coefficient with respect to this phase. Hence, the next column was prepared using 85% methanol as the internal phase; a sample of cantharidin weighing 15.1 mgms was dissolved in 4 ml of methanol, instead of chloroform; Silica Gel G was used as eluent. But this attempt was unsuccessful because the solvent would not flow. Assuming that this behavior was due to the high percentage of methanol present, its concentration was diminished but to no avail. The concentration was decreased in one case to 70%, in another to 50%, and finally only distilled water was used. In all cases, 3 ml of methanol was employed to dissolve the sample, and the same breaking of the column and stopping of the flow occurred.

2. Experiments on Extract of Cantharides

Although the experiments on pure Cantharidin were not successful, it was desired to determine the behavior of the system when using the crude extract.

The Cantharides used for extraction showed 0.581% of cantharidin when assayed by the N.F. procedure (p.10). The extract used in this experiment was obtained by the N.F. procedure and represented 10 Gm of drug. Only one column

was run on the extract. The internal phase consisted of 20 ml of methanol. Fifteen ten-ml fractions of petroleum ether (b.p. 30°-75°)* were collected, and then chloroform was used as the second eluent, of which thirty-five ten-ml fractions were collected. As seen in Figure 2 the petroleum ether removed the fat, and on changing the eluent to chloroform, it was followed immediately by the cantharidin plus other material. The total amount of material in the second peak was 84.4 mgm. or 0.844%, which is very high. Apparently there was no clear-cut fractionation.

B. Partition Chromatography Using 66% Sulfuric Acid as Internal Phase

1. Experiments on Pure Cantharidin

In selecting another pair of solvents to work with, it was necessary to consider, first, the partitioning of cantharidin between them; and secondly, the mutual immiscibility of the two solvents. Sulfuric acid was one of the substances which seemed suitable for the internal phase because cantharidin is readily soluble in it, and because it is immiscible with Skelly C, chloroform or benzene. It was determined experimentally that 66% sulfuric acid was the lowest concentration of acid that would dissolve a weight of cantharidin convenient to work with, i.e., 10 mgms in 20 ml of acid; hence, this concentration of acid

* It was found that petroleum ether, unlike Skelly C, did not color the column, and hence it was unnecessary to purify it. Although the use of Skelly C was resumed in some of the preliminary experiments which followed, this phase of the investigation was finished using petroleum ether (b.p. 30°-75°).

was used in the experiments which followed.

The partition coefficient between 66% sulfuric acid and chloroform was first determined. A 20 ml-aliquot containing 8.8 mgms of cantharidin in 66% sulfuric acid was shaken for five minutes with 20 ml of chloroform in a separatory funnel. The layers were separated, and the chloroform layer was washed with distilled water until the washings were no longer acidic to litmus paper; it was dried over anhydrous sodium sulfate and poured into a tared beaker; the chloroform was evaporated off and the residue was dried and weighed. The amount present in the acid layer was determined by difference. This weight divided by the weight in the chloroform gave a partition coefficient of 0.12, which was too low. Since benzene decreases the solubility of cantharidin in chloroform, it was assumed that a mixture of benzene and chloroform would give a higher partition coefficient with respect to the acid than would chloroform alone. This was found to be true when the process was repeated using 50% benzene-chloroform. A value of 1.76 was obtained, which is suitable for partition chromatographic purposes.

The first column in this series was prepared using 30 Gm of silicic acid, 20 ml of 66% sulfuric acid, and benzene to form a slurry. Five ml of a mixture of equal volumes of chloroform and thiophene-free benzene* was used to dissolve

* Ordinary benzene produced darkening of the column, a situation which was avoided by using the thiophene-free grade.

a sample weight of 19.6 mgms. The eluent was 50% benzene-chloroform* of which eighteen ten-ml fractions were collected. A total weight of 10.7 mgms of sample was recovered in fractions 3 to 6, as represented in Figure 3a. It seemed that the recovery of the sample could be delayed by increasing the volume of sulfuric acid used as internal phase, and decreasing the concentration of chloroform (in which cantharidin is readily soluble) in the eluent. Accordingly, the next column was packed using 30 ml of internal phase and 80% benzene in chloroform as external phase. A sample weighing 14.7 mgms was dissolved in chloroform and placed in the column. The rate of flow of the eluent was extremely fast, yielding 12.9 mgs of the sample scattered throughout nineteen ten-ml fractions. This is shown in Figure 3b. The solvent coming out of the column was acidic and reacted with the aluminum dishes in which the fractions were collected, as evident from the formation of a white solid insoluble in chloroform, and from a marked increase in weight of the dishes. This was avoided in later work by shaking the chloroform with concentrated sulfuric acid; washing it with water until the acidity was completely removed, drying it over calcium chloride, and finally distilling it. Several other columns were tried using 30 ml of 66% sulfuric acid as the internal phase, but in none of them did it prove

* The chloroform was previously shaken with portions of concentrated sulfuric acid until the latter was no longer colored by the chloroform.

possible to control the rate of flow of the eluent, except by using a stopcock. There seemed to be no advantage in using that much volume of internal phase, so the use of a 20 ml-volume was resumed.

A column was packed using 20 ml of internal phase, and 80% benzene in chloroform was used as eluent. A sample weighing 13.9 mgms of cantharidin was dissolved in 5 ml of the eluent and poured into the column. Thirty ten-ml fractions were collected, with the recovery of 14.8 mgms of material in fractions 10 to 21 as represented in Figure 4a. The excess of 0.9 mgm is within experimental error. The appearance of the cantharidin in fraction ten seemed appropriate, but it tailed into a rather large number of fractions. Nevertheless, this system was investigated further.

2. Effect of Fat on Elution of Cantharidin

As cantharidin exists in Cantharides together with a considerable amount of fat, the effect of fatty material in the elution of cantharidin had to be determined. A sample weighing 19.3 mgms of cantharidin was dissolved in 2.5 Gm of olive oil and placed in a column like the one just described. Twenty-nine ten-ml fractions of 80% benzene-chloroform were collected. Figure 4b is a plot of the weight of oil and cantharidin which was recovered starting in the fourth fraction. This result led to the possibility of eluting the column first with Skelly C to remove the fat and other material, and then with the benzene-chloroform

solvent. This procedure was carried out using a sample weight of 10.1 mgms dissolved in a few ml of benzene-chloroform. The results are graphically represented in Figure 5a. Twenty-five ten-ml fractions of Skelly C were collected, and then the eluent was changed to 80% benzene-chloroform, for the next twenty-five fractions. A total of 12.8 mgms of material was recovered in fractions six and seven of the benzene-chloroform eluate. The fact that cantharidin was not eluted by the Skelly C was favorable, hence, an attempt was made to reproduce the results using 10.6 mgms of sample dissolved in 2.05 Gm of olive oil. Most of the oil was removed from the column by the Skelly C, starting in fraction five. It was evident, however, that the oil contained crystalline cantharidin. When the eluent was changed to 80% benzene-chloroform, the rest of the cantharidin, together with more fatty material, was recovered in fraction four of the latter. This is shown in Figure 5b. The failure of this experiment may lie in the use of an excessive amount of oil, exceeding the capacity of the column.

It was desired to investigate whether an amount of cantharidin comparable to the content of this substance in Cantharides would behave in the columns in the same manner as the smaller amounts used thus far. The possibility that the cantharidin might be removed because the amount used exceeded the capacity of the column had to be considered.

One column was run into which 50.7 mgms of cantharidin dissolved in benzene was added. The first elution was with petroleum ether (b.p. 30°-75°) of which thirty-three ten-ml fractions were collected. The second eluent used was thiophene-free benzene of which twenty-seven ten-ml fractions were collected. Figure 6a represents the elution of 50.8 mgms of cantharidin in fractions seven to ten of the second elution. This result shows that the conditions of the column are appropriate for the amount of sample used.

3. Experiments on Extract of Cantharides

The powdered Cantharides used here had been obtained some time before this investigation was started from S. B. Penick and Company. Two samples were assayed by the N.F. method described on page 10. The results obtained were 0.185% and 0.205%, which are quite below the official standard of 0.6%. However, this batch of drug was considered suitable for the preliminary work.

At this point the primary objective of the experiments was to achieve the complete separation of the pure cantharidin from the fat, pigment, and other material, and to determine the cantharidin gravimetrically. The system used consisted of 30 Gm of silicic acid as supporting phase, 20 ml of 66% sulfuric acid as internal phase, Skelly C as first eluent, and 80% benzene-chloroform as the second eluent. This system produced favorable results with the

pure cantharidin, as shown in Figure 5a, but with the extract certain modifications were found necessary.

For the first column the extract was obtained by macerating 15 Gm of the powdered Cantharides with 50 ml of chloroform for one hour. The mixture was then filtered, the filtrate was concentrated to a volume of 5 ml with the aid of mild heat and a current of air, and finally was poured into the column. Twenty-three ten-ml fractions of Skelly C were collected at a rate of 6 drops per minute. The eluent was changed to 80% benzene-chloroform for the next twenty-two ten-ml fractions. Figure 6b shows the elution of the fatty material and of the cantharidin following the change of eluent. Six and nine tenths of a milligram of cantharidin, corresponding to 0.066%, was recovered in fractions 5 to 9 of the second eluent. On examination of the fraction, the presence of some fatty material preceding the cantharidin became obvious, which is not a desirable situation.

The extracts for all the other columns which were run with this batch of Cantharides were obtained by extracting with chloroform in a Soxhlet apparatus for six hours. The extracts were concentrated to about 5 ml before placing in the columns. It has already been pointed out that other workers have found this type of extraction to be as effective as the official procedure.

The experiments which followed were designed to test

the total elution of cantharidin from the column and to determine whether this material would be eluted together with the fat. By adding a definite weight of cantharidin to the extract and eluting through column (A), then comparing the amount recovered from this column with the amount of material obtained from a column (B) through which the extract only had been eluted, then total elution can be assumed if the weight of residue from column (A) equals the weight of residue from column (B) plus the added cantharidin. The system used was exactly the same as described earlier in this section.

The extract corresponding to 15 Gm of drug was placed in a column (A). Thirty-eight ten-ml fractions of Skelly C were collected, which contained most of the fatty material. The eluent was changed to 80% benzene-chloroform and twenty ten-ml fractions were collected. Figure 7a shows the elution of 27.9 mgm of cantharidin in fractions four to six of the second eluate. The amount corresponded to 0.18%. The experiment was repeated but adding 15.7 mgms of pure cantharidin to the extract before placing it in the column (B). Thirty ten-ml fractions of Skelly C and twenty-five ten-ml fractions of 80% benzene-chloroform were collected. A total weight of 21.6 mgms of cantharidin was recovered in fractions three to six of the second eluate. The elution of this column is shown in figure 7b. Subtraction of the amount of cantharidin added, i.e., 15.7 mgms, from the total

weight recovered, i.e., 21.6 mgms, revealed 5.9 mgms of cantharidin, or 0.039%, as present in the extract. This value was too low when compared to the 0.18% obtained for the column represented in figure 7a. The experiment was, therefore, repeated to determine if better results could be obtained. The extract from fifteen grams of drug was divided in two portions, each corresponding to 7.5 Gm of Cantharides. One of the portions was placed in column (C) and was first eluted with 250 ml of Skelly B* in ten-ml fractions, and then with an equal volume of 80% benzene-chloroform, also in ten-ml fractions. A weight of 13.5 mgms of cantharidin was recovered in fraction 7 to 9 of the benzene-chloroform eluate, corresponding to 0.18%. Twelve and seven tenths of a milligram of cantharidin was added to the second portion of extract and poured into column (D). The elution was carried out as in column (C). A total of 23.3 mgms of material was recovered in fractions 6 to 8 of the second eluate. Subtraction of the weight of cantharidin added (12.7 mgm) from the total weight recovered (23.3 mgms) left 10.6 mgms of cantharidin, which corresponded to 0.14%. The elutions of columns (C) and (D) are shown in Figures 8a and 8b.

Another pair of columns (E and F) were run in a similar manner, but with the extract from 9 Gm of drug. From the

*It had been found that Skelly B was easier to purify than Skelly C, that is, it required less shaking with sulfuric acid, and since their behavior in the columns was identical, it was used here and in some of the other experiments.

column containing only the extract, 14.7 mgms or 0.16% of material was recovered in fractions five to eight of the benzene-chloroform eluate. From the other column, in which the extract containing 17.8 mgs of added cantharidin had been placed, 29.0 mgms was recovered in fractions five to eight of the second eluate. A subtraction similar to the first two pairs resulted in a value of 10.2 mgms of cantharidin or 0.11%. Figures 9a and 9b give a graphical representation of the results.

A study of the results of these columns showed that they were extremely low in one case, namely column (B), 0.039% not being comparable to the result of 0.18% in column (A) which contained the extract only. Column (D), 0.14%, approached column (C), 0.18%, more closely, and columns (F), 0.11%, and (E), 0.16%, were closely related though to a lesser degree. Columns (A), (C), and (E), that is, the columns containing the extract only, gave results which checked fairly closely, i.e., 0.18%, 0.18%, and 0.16% respectively, and approached the lower values obtained by the official assay of the same batch of drug, i.e., 0.185% and 0.205%. On the basis of these results alone it can be said that elution was complete.

The results of columns (B), (D), and (F), that is, of columns which contained the extract plus added cantharidin, were not consistent (0.039%, 0.14%, 0.16%) and were farther off from the assay value. This might be due to the fact that during the manipulation of the samples, considerable

difficulty was encountered in dissolving the added cantharidin in a small volume of extract which was already saturated with this substance. This resulted in loss of cantharidin during its transfer to the columns. Then, the evidence obtained cannot be considered valid as far as total elution is concerned. These experiments were repeated in a different batch of drug with some modifications and the results were more satisfactory.

In the preparation of the extracts used in these columns, A to F no hydrochloric acid was employed as stipulated in the National Formulary. Perhaps this explains the low values obtained. To confirm this, the extract from 15 Gm of drug was prepared following the official procedure and an aliquot corresponding to 7.5 Gms was placed in a column. The elution, as shown in Figure 10a, yielded 0.284% of cantharidin. The result is high in comparison to all the others. Another observation made in the elution of these columns was that the cantharidin was preceded by certain impurities which rendered difficult an accurate quantitative determination of the recovered material. This is apparent from an examination of the figures, which show a peak just before the cantharidin.

In an attempt to retain these impurities in the column, or to fractionate them from the cantharidin, the elution was effected, after removal of the fat, by increasing the concentration of benzene in Skelly B gradually from 20%, to 40% and 80%. As shown in Figure 10b, this resulted in

tailing of the peak which is not desireble, especially since visual examination of the peak fraction showed that the material was apparently impure. Figure 11a is the plot of another column run in a similar manner but in which the second eluent was 50% benzene-Skelly B. Although the peak is not clear-cut, the impurities were apparently retained. In both these columns, Figures 10b and 11a, the extracts were obtained by the National Formulary procedure.

It was considered possible that some of the cantharidin was being eluted by the Skelly B together with the fat. This assumption was based on the work of Haeht and Parks in which it was shown that oils and fats increase the solubility of cantharidin in petroleum ether. On the other hand, theoretically, the concentration of sulfuric acid in the internal phase should be enough to retain the cantharidin and permit removal of the fat. To ascertain which assumption was correct, the Skelly B eluate from one of the columns was concentrated to a volume of about 5 ml and placed in another column. The elution was carried out first with 250 ml of Skelly B in ten-ml fractions and then with 200 ml of 50% benzene-Skelly B. Figure 11b shows that no cantharidin was obtained on the second elution, which indicated the absence of cantharidin in the fat originally present.

C. Partition Chromatography Using 78% Sulfuric Acid as Internal Phase

1. Experiments on Pure Cantharidin

The partition coefficients between 78% sulfuric acid and

benzene (acid/benzene) were found to be 30.19, 28.41, and 25.26. Although these values were high, suggesting that the cantharidin would not be eluted from the column after the addition of a reasonable volume of eluent, an exploratory column was run, with surprising results. The column was prepared using 20 ml of 78% sulfuric acid as the internal phase. A sample weighing 52.1 mgms was dissolved in 5 ml of thiophene-free benzene and poured onto the column, and thirty-seven ten-ml fractions of petroleum ether (b.p. 30°-75°) were collected. The eluent was changed to benzene for the collection of twenty-three ten-ml fractions. As seen in Figure 12a the cantharidin was recovered in fractions ten to fifteen of the benzene eluate. This seemed an ideal situation, as the cantharidin peak did not appear immediately after the addition of the benzene. The experiment was repeated with duplicate results. Figure 12b shows that the peak appeared in the tenth fraction of the second elution. Fifty-three mgms of cantharidin was placed in the column, and this same amount was recovered.

2. Experiments on Extract of Cantharides

The extract for these columns was prepared in the following manner: Fifty grams of powdered Cantharides (N.F. assay 0.581%) was mixed well with 4 ml of concentrated hydrochloric acid, then moistened with chloroform and packed into a Soxhlet apparatus, where it was extracted for ten hours with 200 ml of chloroform. This volume of extract

was made up to 250 ml with chloroform in a volumetric flask and 50 ml fractions were used, representing 10 Gm of drug. Before use, the desired volume of chloroform extract was pipetted into a 100 ml beaker and evaporated to dryness by mild heat and a current of air, the residue was dissolved in five ml of benzene and this solution was poured onto the column.

The first column of this group was run with extract corresponding to 20 Gm of Cantharides. Twenty-five ten-ml fractions of petroleum ether (b.p. 30°-75°) were collected, followed by twenty-two ten-ml fractions of benzene. Figure 13 shows the removal of the fat immediately after addition of the petroleum ether, starting in fraction two. After the change of eluent, there was a peak of fatty material followed by another peak of crystalline cantharidin. The latter, which appeared approximately at the same place as shown in Figures 12a and 12b for pure cantharidin, weighed 88.6 mgms or 0.443%. This value is somewhat low - the more so since the cantharidin recovered in this peak was not absolutely pure.

An attempt was made to effect a more clear-cut separation between the second and third peaks, by running a column in which the second eluent was 20% benzene in petroleum ether, followed by 40% benzene in petroleum ether, and finally benzene. In this case the cantharidin peak, as shown in Figure 14, appeared when the benzene only was used as eluent; however, this fraction still contained traces of

fatty material, so there was no point in prolonging its elution. It weighed 169.6 mgms or .848%.

A third column was run containing the extract from 10 Gm of drug. The first elution was with 400 ml of petroleum ether (30°-75°) to remove the fat, and the total volume of eluate was collected in a beaker. The column was then transferred to the automatic fraction cutter, where twenty-five ten-ml fractions of benzene eluate were collected. The first peak of fatty material appeared on fraction two, i.e., as soon as benzene started coming through. The cantharidin peak was contained in fractions 12 to 24. It was obviously impure, as shown by inspection and by the weight, which was 90.1 mgms or 0.901%. There was also considerable tailing which was not desireble. Figure 15a represents the elution of this column.

D. Partition Chromatography Using 85% Sulfuric Acid as Internal Phase

1. Experiments on Pure Cantharidin

Only one column was run, in which 50.0 mgs of cantharidin was used. As shown in figure 15b, the cantharidin was not recovered after thirty-two ten-ml fractions of benzene had been collected. There was a peak of liquid material as soon as the benzene started coming through.

E. Partition Chromatography Using Concentrated Sulfuric Acid as Internal Phase

It was not possible to recover the cantharidin which was

added (49.7 mgm) to a column containing concentrated sulfuric acid as the internal phase. The shape of the curve in figure 16a was due to the presence of benzene-insoluble material in the dishes. This material was formed from the acid being carried through the column on elution and reacting with the aluminum dishes.

F. Chromatography Using Silicic Acid as Adsorbent

Due to the fact that the behavior of the columns was not as predicted by the partition coefficients, a column was run using silicic acid as adsorbent, petroleum ether (30°-75°) as first eluent and benzene as the second eluent. A sample weighing 52.4 mgms was dissolved in 5 ml of benzene and placed in the column. Figure 16b shows that no cantharidin was eluted by the petroleum ether or by the benzene. This indicated that there was adsorption and the mode of operation of these systems was not true partition. The elution of the cantharidin should be expected immediately after addition of benzene since there was no internal phase to retain it.

Summary and Conclusions

An unsuccessful attempt was made to find an appropriate partitioning system using methanol (or aqueous methanol) as internal phase, which could be applied to the separation of cantharidin from extracts of Cantharides.

The use of different concentrations of sulfuric acid as internal phase was investigated. 66% sulfuric acid and

78% sulfuric acid worked rather well with the pure cantharidin. When using the extract, 78% sulfuric acid gave better results, although the cantharidin was always preceded by fatty material. 85% and concentrated sulfuric acid (98%) did not permit elution of the pure cantharidin.

A column using silicic acid only, did not yield the pure cantharidin upon elution with petroleum ether followed by benzene. This indicated that there was adsorption, which may explain why in the columns containing an internal phase, the behavior is not as predicted by the partition coefficients.

It was shown that the cantharidin was not eluted together with the fat, i.e., the fat was removed leaving the cantharidin in the column.

According to the experiments thus far, it is possible to separate cantharidin from Cantharides extract by partition chromatography. But it is not possible to determine whether the separation is quantitative, as there are traces of impurities present which greatly increase the weight of the fractions.

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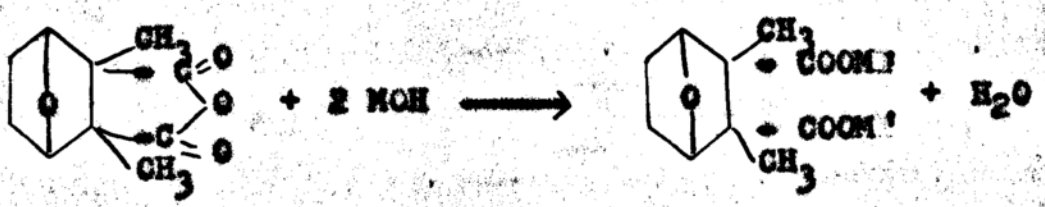
Discussion of Experimental Work

Part Two

The Titration of Cantharidin

A. Introduction

Homolka (1) demonstrated the dibasicity of cantharidin by preparing the dimethyl ester whose empirical formula, $C_{10}H_{12}O_5(CH_3)_2$, indicated the existence of an acid anhydride. Using the formula now accepted for cantharidin, its neutralization may be represented by the following equation:



It seems possible and practicable to determine cantharidin by acidimetric titration. However, Seeville (2) showed that cantharidin could not be titrated quantitatively and concluded that it does not combine with alkali in definite proportions. He used solutions in alcohol, acetone, and benzene, and employed a residual technique. The acetone solution and the alcohol solution gave varying results. The benzene solution seemed more promising, but still constancy could not be secured. Gadamer (3) has explained the discrepancy involved in the titration as being due to hydrolysis of the salt formed according to the following equation:

$$C_{10}H_{12}O_5K_2 + 2\ H_2O \longrightarrow C_{10}H_{12}O_5H_2 + 2\ KOH$$
$$2\ KOH \longrightarrow K^+ + 2\ OH^-$$

Then, he said, the presence of excess hydroxyl ions accounts for an end-point before all the anhydride could be titrated. Danckwortt (4) also presented this explanation. Hecht and Parks (5) however, showed that the failure to titrate cantharidin in the presence of organic solvents is due to some effect exerted by the latter. This effect is apparently a depression of the ionisation or neutralisation of cantharidin, due possibly to the low dielectric constant of the solvent and its inability to ionize. These workers developed a volumetric method in which the cantharidin was dissolved in acetone and an excess of alcoholic potassium hydroxide was added. After removing the organic solvents by boiling, they titrated the excess alkali with N/10 hydrochloric acid using phenolphthalein indicator. Guthrie and Brindle (6) had difficulty in obtaining concordant results with this procedure. Their main difficulty was one of determining end-points. Jonsson and Parks (unpublished work) also obtained low and erratic results.

Guthrie and Brindle (6) have presented a volumetric method whereby the cantharidin is precipitated with excess standard dichromate, the excess of this reagent then being determined with potassium iodide and thiosulfate. This method is, however, inapplicable to the determination of the content of cantharidin in the crude drug because various

impurities are precipitated along with the cantharidin.

B. Determination of Cantharidin by a Pyridine-Sodium Hydroxide Titration

The main difficulty in the titrimetric method for the determination of cantharidin has been the difficulty in breaking the anhydride structure to form cantharidic acid which then can be titrated as an ordinary weak acid. Anhydrides like phthalic anhydride have been hydrolyzed by the catalytic action of 10% sodium iodide in pyridine or with pyridine alone (7). Jonsson and Parks (unpublished work) applied this technique to the titration of cantharidin in a method which gave encouraging results. They used samples weighing about 0.1 gm and the per cent error in the determination using the sodium iodide in pyridine reagent varied from 0.0% to 0.9%. With pyridine alone the per cent error was from 0.0% to 0.4%. The procedure used by them is given here, since the method employed in the present work is essentially a modification of it.

> A sample weighing 0.1 gm of cantharidin was dissolved in 10 ml of pyridine and allowed to stand for one-half hour. Then ten ml of tenth normal alkali and ten ml of water was added and allowed to stand for one-half hour. Three drops of phenolphthalein test solution was added and the excess alkali was titrated with tenth normal hydrochloric acid until the pink color disappeared. A blank was also run.

It was specified that if some salt of cantharidin was precipitated initially or the mixture became cloudy to add water until a clear solution would result. Added fat did not interfere with the titration, a fact which might be

useful in the titration of the crude extract.

Analytical Determinations on Pure Cantharidin

When titrating cantharidin in the presence of pyridine, Jonsson and Parks obtained satisfactory results with samples weighing about 100 mgm. Cantharides contains about 60 mgm. of cantharidin for each 10 Gm sample of crude drug. Keeping in mind that the final purpose of this investigation was to develop an assay procedure for the drug (10 Gm samples can be conveniently handled and simplify the calculations), it was thought convenient to test the accuracy of the titration using these smaller amounts.

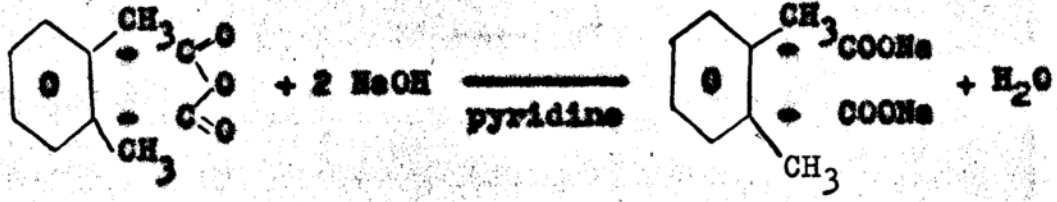
Jonsson and Parks had indicated that the titration by a direct method was as effective as by the residual technique. Therefore, the work was started using the direct titration.

Direct Titration

In Determination No. 1 five samples were titrated all weighing between 19.2 and 24.3 mgms. Since these samples were smaller, the volume of pyridine was reduced from ten ml to five ml. The error in mgms. varied from +0.6 to -0.9. An intense red color, which disappeared very slowly, was produced every time a drop of alkali was added. This prolonged the process and made the detection of the end-point somewhat uncertain. Although Jonsson and Parks did not mention this uncertainty in the determination of the end-point , they did point out that the titration was slow.

and hence used the residual technique, which shortened the process even though a half hour of standing was necessary. Also the end-points were sharper in the latter case, as they were in the present analytical work. The slow titration suggests the resistance of the anhydride structure to the action of alkali, even in the presence of pyridine. Although the results obtained as shown in Table I, are within acceptable values, the procedure in itself is subject to the introduction of higher deviations.

The reaction which takes place may be represented by the following equation:



Residual Titration

In Determination No. 1 of this series of experiments, ten samples of cantharidin were titrated by the residual technique. Their weights varied from 19.5 mgm to 37.2 mgms and the error in mgms varied from $^{+}0.1$ to -2.37. Five ml each of pyridine and standard sodium hydroxide were used. The results in Table II show that the deviations cover a rather wide range. Of the ten samples, four had errors of only ± 0.2 and ± 0.1 mgms which is very good, three samples had errors of ± 0.6 to $^{+}0.9$, which also may be considered within experimental error. The remainder of the samples had errors of -1.4, -1.7, and -2.37 mgm.

Considering that the weight of the samples might be responsible for the variation, a sample weighing 126.1 mgms was titrated. Here, the error was +1.9 mgms, falling within the range for the smaller samples. Therefore, evidently sample weight had no effect on the determination. In general these results were not considered satisfactory due to the fact that there was a marked deviation within each set of samples.

Determination No. 2 was carried out on cantharidin recovered from a column containing 20 ml of 66% sulfuric acid as the internal phase. The results in Table III show excellent agreement between the volumetric and gravimetric determinations, as well as between these two values and the amount of cantharidin added to the column. It was noticed in this and some of the determinations which followed, that sharper end-points and more accurate results were obtained when the use of water was avoided in the titration, and the phenolphthalein in acetone indicator was used.

In Determination No. 3 the cantharidin was recovered from columns containing 78% sulfuric acid as the internal phase. Here the agreement between the amount of material added to the column and the amount recovered volumetrically was not as good as in the preceding case, but still the results were within experimental error.

It has been shown that cantharidin may be titrated fairly accurately using pyridine to catalyze the hydrolysis of the anhydride structure. The residual technique proved

more satisfactory, especially in the absence of water and using phenolphthalein in acetone as indicator.

Analytical Determinations On Cantharidin Obtained From Extract of Cantharides

In the titration of cantharidin obtained from extract of Cantharides, regardless of the method, i.e., direct or residual, the volumetric values should be lower than the gravimetric ones. This should be so especially in cantharidin obtained from the N.F. assay. It has been stated that the residues thus obtained contain traces of impurities which naturally increase the value of the gravimetric determination. In cases where the cantharidin is isolated by partition chromatography, the material recovered should be sufficiently pure to render it suitable for titration, and the gravimetric and volumetric results should then be in fairly close agreement. It will be seen that in the present investigation this ideal situation was not attained and the residues recovered from the columns had to be purified further.

In establishing comparisons between the two determinations it is only possible to notice that the volumetric values should be lower, within reasonable limits. A better judgment of the accuracy of the analysis is obtained by comparing the values obtained from several samples by use of the same method, e.g., the gravimetric or volumetric method. In deciding what are the "reasonable limits" and

what is "too high" or "too low" the fact that there is no standard of comparison makes the decision arbitrary. The N.F. standard (not less than 0.6%) should not be used because as already mentioned, this value will generally be higher than the real value. How much higher will depend on the effectiveness of the purification process.

In Part One of this work it was concluded that in the separation of cantharidin by partition chromatography, the best results were obtained when 66% or 78% sulfuric acid was used as internal phase. Hence, the columns that were run in the work described in Part Two used one or the other of these.

Direct Titration

The results shown in Table V were very poor, except possibly for the column represented by Fig. 11a where a difference of only 1.9 mgms between the two determinations was obtained. The errors in this experiment were much higher than the determination represented in Table I where the same type of titration was applied. In the former, however, there exist experimental errors which were not present in the latter, such as those inherent in the extraction of the drug and the elution of the columns. The detection of the end-point was more difficult here due to the fact that the solution which was titrated was yellowish in color.

The cantharidin residue from an assay determination by the N.F. procedure was also titrated by this method and

there was a difference of 0.026% between the two results, the volumetric being lower.

The direct titration of cantharidin was not found recommendable, due mainly to the prolonged procedure and the uncertainty in detecting the end-point, which naturally lead to unreliable results. The remainder of the titrations using sodium hydroxide in the presence of pyridine were done by the residual technique.

Residual Titration

Although it had been observed that the titration of pure cantharidin in the absence of water and using phenolphthalein in acetone as indicator, gave more accurate results, it was desired to try the method as presented by Jonsson and Parks without modification, in the extract of Cantharides.

The purpose of Determination No. 1 was to isolate cantharidin from Cantharides and to determine it by titration. The batch of Cantharides used was below the N.F. standard but it was considered suitable for a preliminary determination. The volumetric result, 0.267%, was high in comparison to the N.F. assay value, i.e., 0.195% (average of two results). This is contrary to what has been pointed out before, that the gravimetric result should be higher than the volumetric value, due to the presence of impurities in the cantharidin as obtained in the N.F. assay procedure. The present result, as shown in Table VII, suggested that acidic impurities were present in the material obtained from the column which definitely increase the volumetric value.

Determination No. 2 was carried out, first, to compare the N. F. extraction procedure with the chloroform extraction by gravimetric determination, and secondly, to compare the gravimetric and volumetric values of the material obtained by the two methods of extraction. The results presented in Tables VIIIa and VIIIb show good agreement between the two methods of extraction, which justified the use of chloroform in most of this work. There was also good agreement between the gravimetric and volumetric values, the former being higher as expected.

In order to test the accuracy of the titration procedure a weighed amount of pure cantharidin was added to chloroform extracts which were then purified according to the N.F. procedure, the residue first weighed and then titrated. The results in Table IX show that the gravimetric results agree quite closely among themselves, while there is a more marked variation between them and the volumetric results, especially in those cases in which pure cantharidin was added to the extracts. These variations are as follows, the gravimetric value always being higher:

Sample	No. 1	4.9 mgms
	No. 2	6.5 "
	No. 3	7.2 "
	No. 4	7.5 "

These differences between the two methods may be accepted, since it has been stated that the gravimetric determinations are higher because of impurities difficult to remove.

Determination No. 4 was similar to the one preceding

it, except that the extraction and purification of the extracts was done according to the N.F. procedure. Here again, the gravimetric results were higher than the volumetric, as shown in Table X. Although the variations between the two methods was within acceptable values, i.e., 3.6 mgm, 4.1 mgm, 7.4 mgm, and 4.7 mgm, the results for the two samples to which pure cantharidin was added were extremely low in both cases. One of these samples yielded 35.2 mgm (0.352%) by titration and 42.6 mgms (0.426%) by gravimetry. The other resulted in 43.0 mgm (0.430%) and 47.7 (0.477%) respectively. This batch of drug assayed 0.581% by the N.F. method.

In Determination No. 5 the cantharidin obtained from columns containing 66% sulfuric acid as internal phase was titrated as recovered, and the results compared with cantharidin obtained from a similar column but treated with an alcohol-petroleum ether mixture (as in N.F.) before titration. Table XI indicates that the untreated fractions gave much higher results than the treated ones, therefore suggesting the presence of acidic impurities in the former. Samples no. 2 (0.658%) and 3 (0.611%) were the only ones to fall within the range of the values obtained in the other determinations. It must also be noticed that in this table the volumetric result obtained for sample No. 8 was too low (0.485%). In this determination the material isolated should be pure enough to give fairly close values between the volumetric and gravimetric determinations. There was a

difference of 5.5 mgm which is rather high. This determination proved that some kind of purification of the residues is necessary before titration.

In the following determination (No. 6) then, all the residues were washed with the absolute alcohol-petroleum ether mixture. Weighed samples of pure cantharidin were added to three of the extracts. As in other determinations in which pure cantharidin was added to the extracts, the purpose here was to determine the completeness of elution from the columns. The results as shown in Table XII were inconsistent. In the first sample there was a difference of 12.6 mgm between the two values. In the second sample the volumetric value was higher than the gravimetric, which should not be expected. Samples 4, 5, and 6 gave differences of 6.4 mgm, 7.5 mgm, and 7.8 mgm, respectively. While these values would be acceptable in residues purified by the M.F. method, they are not satisfactory in residues purified by partition chromatography. Sample No. 3 was the only one that gave close enough values, with a difference of 1.2 mgm. It is to be noticed that the values obtained for columns with added cantharidin were always lower. In regard to this point, attention is called to the explanation given on page 30.

The difference between the volumetric and gravimetric results which appear on Table XIII for Determination No. 7 are extremely high, 27.8 mgm, 24.4 mgm, 14.0 mgm, and 25.2 mgm for the four samples. The gravimetric values were

higher than had been obtained so far.

During the process of the isolation of cantharidin in Part One of this work, the use of 78% sulfuric acid as internal phase was investigated for the purpose of finding a system whereby a minimum amount of impurities would be obtained together with the cantharidin. The results of the columns in Figs. 14 and 15a (Table XIV) were rather high by the gravimetric determination, but not as high by titration. This showed once more that the residues obtained from the columns were highly impure, although in this case the impurities did not seem to be acidic to any appreciable extent. It was thought then, that even if the impurities interfered with the gravimetric determination their presence would be of no consequence if they would not affect the volumetric determination.

Inspection of the graphs in Fig. 14 and 15a revealed that a peak fraction of fatty material was obtained immediately before the elution of the cantharidin, and after approximately 90 ml of the benzene eluant had been passed through the column. Determination No. 9 was planned as a result of this observation. After the first 90 ml of benzene eluate had been collected the next 200 ml of the eluate was collected in a separate beaker. The residue obtained from the latter was titrated. The results shown in Table XV (0.759% and 0.812%) differ from each other by 5.3 mgm which is somewhat high, and also these values are higher than the N.F. assay (0.721%) which is not as

expected. These results suggested the presence of acidic impurities, a fact confirmed by testing with moist litmus paper. This situation was not apparent in Determination No. 8. In an attempt to remove the acidity from the benzene eluates containing the cantharidin, they were washed with distilled water, dried, evaporated, and the residues obtained were weighed, as indicated in Determination No. 10. The results in Table XVI show excellent agreement. The presence of acidic impurities was confirmed by comparing the values with the one obtained for a residue titrated as obtained from the column, that is from an unwashed eluate. The latter yielded 0.865% in contrast to 0.613%, 0.616%, and 0.614% for the other three samples. In view of these favorable results in the remainder of the determinations elutions were carried out in a similar manner and the benzene eluates received identical treatment.

It had been observed that in determinations where cantharidin was added to the extracts before placing in the columns, the results were generally low (Determination No. 6) and an explanation was offered on p. 30. In Determination No. 11 it was desired to add pure cantharidin to the extracts in order to check the effectiveness of the elution of the columns. Extract from 5 Grams of drug was used to which approximately 30 mgms of cantharidin was added. Thus the extract to be poured in the columns would contain an amount of cantharidin comparable to the normal content in

Cantharides, i.e., about 60 mgm per 10 Gm. The cantharidin then, would not separate from the extract during the concentration of the latter before placing it in the columns. The results shown in Table XVII were fairly good, although, even in this case the values obtained from the columns containing added cantharidin were slightly lower by about 0.020%. The values for the extract containing no added cantharidin agree closely with those appearing in Table XVI.

In Determination No. 12 the pure cantharidin was added to the 200 ml portions of benzene eluates. This was done to check the accuracy of the titration. In this case as shown in Table XVIII, the results were much higher than in the two previous determinations, but lower than for the unwashed residues. One of the eluates containing the added cantharidin gave lower results, but the difference was only between 1.8 mgm and 2.8 mgm.

Determination No. 13 was carried out using the extract obtained with the M.F. solvent in a Soxhlet apparatus. Comparing the results in Table XIX with those in Table XVI, in which chloroform was the solvent used for extraction, it was observed that those in Table XIX were slightly lower, i.e., by about 3 mgm. Hecht and Parks (5) and Guthrie and Brindle (6) had concluded that both solvents are equally efficient.

Up to this point the determinations were carried out on aliquots of the extracts. Determination No. 14 was done on extract obtained by the method of the National Formulary.

in which 15 Gm samples were extracted, purified by partition chromatography and titrated. The results in Table XX checked well with each other and with the residue purified by the N.F. method. They were lower than the values obtained by extraction with the same solvent in a Soxhlet extractor (Table XIX) and by extraction with chloroform in a Soxhlet apparatus (Table XVI).

In Determination No. 15 the procedure was changed only in that the 15 Gm samples were extracted with 150 ml of chloroform (instead of benzene-petroleum ether). Here again, the results were higher than when using the benzene-petroleum ether solvent, there being a difference as high as 9.2 mgm (Tables XX and XXI). The method of extraction seemed to make a difference also, since the results in Table XVI, in which aliquots were used, varied by as much as 5.3 mgm with those of Table XXI in which the extract was obtained from individual samples. The results in Table XVI show less variation with each other than those in Table XXI. This might suggest that the use of aliquots is more satisfactory in running a set of samples.

In Determination No. 16 ten gram samples were extracted with chloroform in a Soxhlet extractor and analyzed individually. The results in Table XXII check well with each other and with the value for the residue purified by the N.F. method. The values were lower than when 15 Gm samples were used (Table XXI), varying by as much as 3.2 mgm.

Summary and Conclusions

In the volumetric determination of pure cantharidin it was found that fairly accurate values were obtained by dissolving the sample in pyridine, adding excess aqueous sodium hydroxide and water, and titrating the excess alkali with 0.1N hydrochloric acid to a phenolphthalein in alcohol indicator. Still better results were obtained by omitting the water and using a 1% solution of phenolphthalein in acetone as indicator. In the latter case, a difference of +0.1 mgm, +0.3 mgm, and +0.7 mgm was obtained in three different samples weighing about 50 mgm.

In the titration of cantharidin obtained from the extract of Cantharides the direct technique gave inconsistent results, hence only the residual type of titration was investigated further.

The isolation of cantharidin from extract of Cantharides by partition chromatography using 66% sulfuric acid as internal phase and benzene as eluant yielded the cantharidin contaminated with acidic impurities which interfered with the titration. The same situation was encountered in columns using 78% sulfuric acid as the internal phase. In the latter case the situation was not improved by discarding the first 90 ml of benzene eluate which contained fatty material. The difficulty was overcome by washing the benzene eluate containing the cantharidin with distilled water.

The elution of cantharidin from the column was checked by adding a weighed amount of pure cantharidin to the extract of the crude drug and chromatographing it. The recovered cantharidin was determined volumetrically with satisfactory results.

In order to check the titration of the cantharidin eluted from the column, a weighed sample of the pure compound was added to the benzene eluates before washing them. The total material recovered from these eluates was titrated. The results were high in comparison to the two preceding determinations, i.e. Determinations No. 10 and 11.

The extraction of Cantharides with chloroform in a Soxhlet extractor gave higher results, volumetrically as well as gravimetrically, than either extraction by the official N.F. method or extraction with the benzene-petroleum ether solvent in a Soxhlet apparatus.

When aliquots of the extracts were used for chromatographic purposes the results were higher and more consistent than when individual small samples were extracted.

A table comparing the results obtained by the different methods of extraction used follows:

COMPARISON OF METHODS OF EXTRACTION

Table No.	Source of Extract	Method of Extraction	Solvent	Results (Mgms) Volum Part. Chrom (ave)	H.F.	Grav
XVI	10 Gm aliquots	Soxhlet	Chloroform	61.4	*	*
XIX	10 Gm aliquots	Soxhlet	Benzene-pet. ether (2:1)	58.3	58.3	70
XX	15 Gm samples	HF	Benzene-pet. ether (2:1)	57.4	57.8	79.9
XXI	15 Gm samples	Soxhlet	Chloroform	65.4	66.3	73.2
XXII	10 Gm samples	Soxhlet	Chloroform	64.2	64.0	68.7

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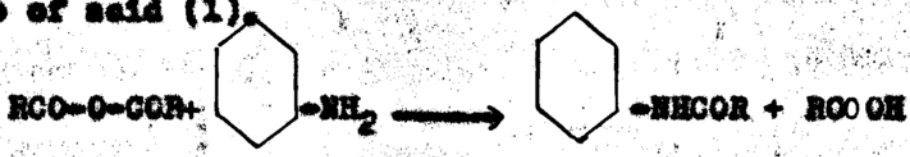
Determination of Cantharidin by a Sodium Methylate Titration Together with a pyridine-sodium Hydroxide Titration

Organic anhydrides are estimated analytically either by hydrolysis followed by titration of both acidic groups, or by the use of a reaction which converts one acidic residue into a neutral substance leaving only one carboxyl group as a measure of the anhydride present. By using the two principles in combination, it is possible to determine the anhydride content of a mixture in the presence of free acid. Most of these procedures make use of the fact that the anhydride reacts with aniline to form a mole of amide and a mole of acid (1).



The action of alcoholates of the alkali metals upon anhydrides is analogous to that of aniline, a mole of ester and a mole of alkali salt resulting from the reaction carried out as a titration. (2)

Smith and Bryant (3) used a standard solution of sodium methylate in place of aniline since it offered the advantage of combining in a single step the cleavage and neutralization processes of the older method while sharing its high reactivity. They determined the total acidity by titration of a separate sample with aqueous sodium hydroxide. In this connection they added a large amount of pyridine just before the titration in order to accelerate the

hydrolysis of the anhydride. This is a principle which has been applied in the first part of the present work, i.e., in the titration with sodium hydroxide in the presence of pyridine. The two basic reactions involved are represented by the following equations:

$$RCO\text{-}O\text{-}COR + CH_3ONa \longrightarrow RCOOCH_3 + RCOONa$$

$$RCO\text{-}O\text{-}COR + 2NaOH \longrightarrow 2RCOONa + H_2O$$

In a mixture with other acidic materials the distinctive measure of an anhydride present is the amount of acyl radical rendered unavailable as ester, or the equivalent differences between the titers of the above reactions. Smith and Bryant (3) indicated that the initial presence of acid was without effect upon the determination. Their procedure for solid samples is briefly summarized:

> A sample weighing 1 - 3 Gms was dissolved in dry methanol or dry acetone, warming if necessary. The solution was titrated directly with 0.5 N sodium methylate in methanol to a phenolphthalein or thymol blue end-point. The indicator was made up in dry dioxane or acetone.* This titer is a measure of the anhydride plus the free acid. A second sample was mixed with 25 ml of pyridine (C.P. reagent) and titrated with 0.5 N aqueous sodium hydroxide to the indicator previously used. This titer is a measure of the total acidity of the sample. The true anhydride content was measured by difference between the two titers expressed in moles per gram of sample.

The following table reproduces the results obtained by these authors:

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* An alcoholic solution would be unsuitable for use in the next step because of ester formation.

Anhydride	Millimoles per Gm of Sample NaOH (a)	$NaOCH_3$ (b)	-CO-O-CO- (a-b)	% of Theoretical -CO-O-CO-
acetic	19.61	9.88	9.73	99.3 ±0.2
propionic	15.40	7.79	7.61	99.0 ± .2
n-heptylic	8.21	4.21	4.00	96.9
succinic	19.83	10.35	9.48	94.8 ± .2
maleic	20.20	10.20	9.98	97.8 ± .0
glutaric	17.42	8.67	8.75	99.8 ± .1
benzoic	8.84	4.45	4.39	99.3 ± .1
phthalic	13.50	6.75	6.75	99.9 ± .3
furoic	9.61	4.97	4.64	95.6 ± .2

Camphoric anhydride was found to be unreactive because of steric hindrance arising from the tertiary carbon atom. However, if heated with an excess of either alkali and back titrated in the cold with standard acid the following results were obtained:

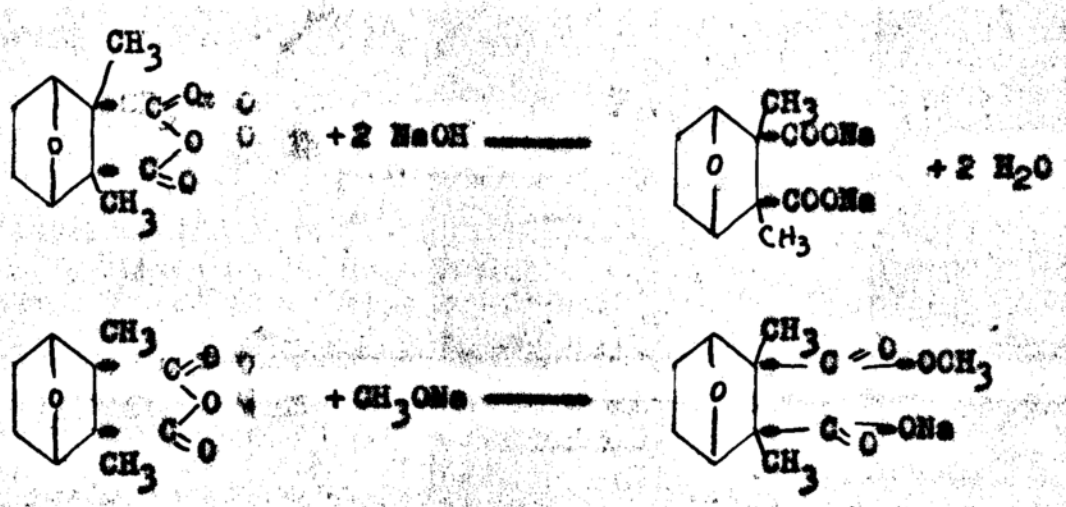
Millimoles per Gm of Sample NaOH (a)	$NaOCH_3$ (b)	-CO-O-CO- (a-b)	% of Theoretical -CO-O-CO-
10.99	5.49	5.50	100.2 ± 0.4

In view of the results obtained by Smith and Bryant, the titration of cantharidin with sodium methylate seemed applicable. It has been shown in the present work that the cantharidin residues obtained from the partition columns contained varying amounts of acidic impurities which gave high and unreproducible results by the pyridine-sodium

hydroxide titration alone. A method of this nature would then avoid the additional manipulation required in washing the benzene eluates obtained from the columns, as described earlier in this report.

Analytical Determinations on Pure Cantharidin

The total acidity of the sample was determined by titrating with sodium hydroxide in the presence of pyridine. This affects both carboxyl groups together with the free acid present. The sodium methylate reacts with the carboxyl groups to give a salt and an ester, and it also reacts with the free acid. The anhydride present is measured by the difference between the equivalents of total acidity and the equivalents of anhydride plus free acid. The two reactions involved are represented by the following equations:



Direct Titration

The first attempt was the direct titration of one pair of samples. The procedure was the one used by Smith and Bryant with slight modifications as to weight of sample

and concentration of reagent. A result of 990.8 mgm was obtained which is quite low compared to 1000 mgm, the theoretical value. The detection of the end-point was uncertain. This was the main reason for doing the rest of the determinations by the residual technique.

Residual Titration

The pair of samples analyzed in Determination No. 1 gave a value of 1000.62 mgm, which was very good. Unfortunately, it was not possible to duplicate this result. The same procedure was used in Determination No. 2, except that 10 ml of sodium methylate was used. In this case the results deviated by as much as 103.4 mgm. The results are shown in Table XXVb.

In Determinations No. 3 and No. 4, ten ml of pyridine was used in the determination of the total acidity and in both cases a precipitate was obtained upon addition of the sodium hydroxide solution. The results were very poor as shown in Tables XXVIb and XXVIIb.

Better results were obtained in Determination No. 5 where the samples for titration were obtained from aliquots. The best values were obtained in Determination No. 6 where aliquots were used and the titration was carried out under an atmosphere of nitrogen. Here the deviation varied from -0.1 mgm to -1.3 mgm, as represented in Table XXIXb.

In Determination No. 7 the cantharidin recovered from a column was analyzed by this method and the result was 12.1 mgm low.

Analytical Determinations on Cantharidin Obtained from Extract of Cantharides

Although the results obtained on pure cantharidin were not satisfactory, it was desired to run a few preliminary determinations on cantharidin obtained from the extract.

From four columns, two analyses were made which gave results of 0.540% and 0.535%, as shown in Table XXXIb. The Cantharides used assayed 0.581% by the N.F. method.

In Determination No. 2 a weighed amount of cantharidin was added to the extracts before placing in the columns. Eight columns were run from which four analyses were made. The results shown in Table XXXIIb do not agree closely enough to be reliable.

Conclusions

The results of the attempted determination of cantharidin by a sodium methylate titration indicate that the method is not sufficiently reliable to be adapted to the assay of Cantharides.

In the experiments on pure cantharidin, the conditions may probably be controlled to produce a minimum error, as suggested by the results obtained for the titration under nitrogen. However, this does not seem to offer any advantage over the method already devised whereby the benzene eluates are washed and titrated with sodium hydroxide in the presence of pyridine.

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EXPERIMENTAL WORK

PART ONE

ISOLATION OF CANTHARIDIN FROM EXTRACT OF CANTHARIDES BY PARTITION CHROMATOGRAPHY

A. General Methods

1. Packing the Columns

All the columns were prepared in the same manner using 2 cm x 35 cm glass tubes. These glass tubes were fitted with a wad of glass wool in the bottom to hold the supporting phase, which was Mallinckrodt silicic acid (100 mesh). A weighed amount of the silicic acid was placed in a mortar with a definite volume of the non-mobile phase, and after thorough mixing, enough of the mobile phase was added to form a slurry which was charged in the chromatographic tube and packed by means of a glass plunger, care being taken to exclude air bubbles. Enough force was exerted to make the mobile phase flow at the rate of five to eight drops per minute. When the silicic acid mixture was well packed and excess mobile phase had drained off, a solution of the substance to be analyzed was applied to the column by means of a pipet. As this solution passed into the column, eluent was introduced and the elution begun.

2. Location of the Components

The effectiveness of a chromatographic separation can be conveniently determined by using the aluminum dish method, which has distinct advantages over other methods

previously used: (a) the aluminum dishes* are easily identified by numbering them; (b) they do not absorb moisture from the air; (c) and their weight remains fairly constant. But possibly the main advantage is that the components are collected in a relatively pure state and are easily recovered.

The following procedure was used: the dishes were first washed in warm chloroform followed by warm alcohol, and then placed in cardboard boxes of appropriate size to accommodate twenty dishes in a single layer; the wash liquid was allowed to evaporate at room temperature. When dry, they were weighed in an projection balance. Their weight was between 1.2 and 1.5 grams.

The eluate from the column was collected in ten ml fraction in test tubes using an automatic fraction cutter. Each portion was poured into one of these aluminum dishes and the test tube was then rinsed a few times into the dish to ensure complete removal of all the eluate from it. After the cuts were collected, the dishes were allowed to stand in a warm place until the solvent had evaporated completely; then they were weighed again. The difference between this weight and the weight of the clean dishes represented the amount of component removed. By plotting this weight against the number of the fraction, a graph representing the location of the components was obtained.

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*Aluminum foil moisture dishes obtained from E. H. Sargent & Co.

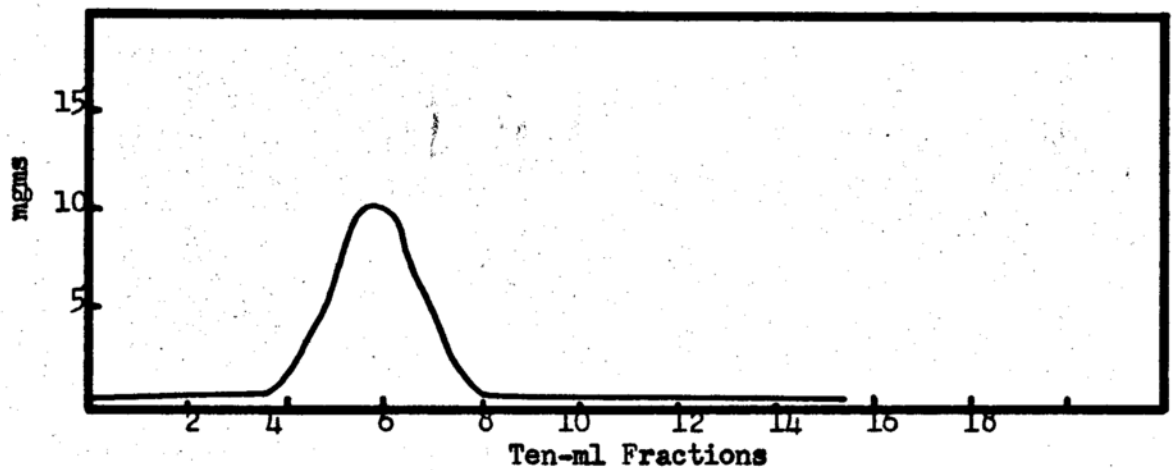

Fig. 1a. Partition chromatogram of cantharidin using 25 ml of 70% aqueous methanol as internal phase and Skelly C as external phase. The weight of the sample used was 14.1 mgms and the weight of sample recovered was 10.1 mgms in fractions 4 to 8. The sample was dissolved in 20 ml of Skelly C and 8 ml of chloroform.

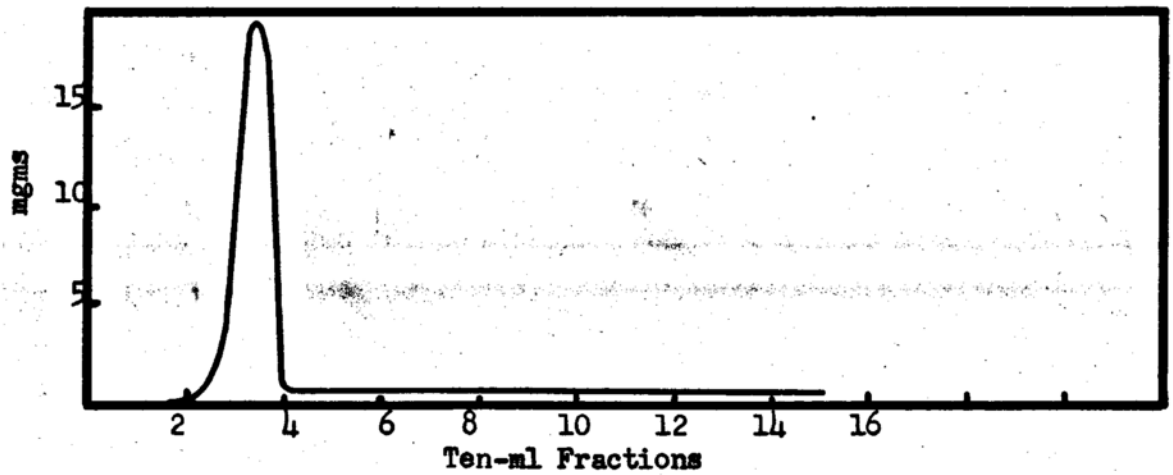

Fig. 1b. Partition chromatogram of cantharidin using 25 ml of 70% aqueous methanol as internal phase and Skelly C as external phase. The weight of the sample was 26.7 mgms and the weight recovered was 19.1 mgms in fractions 3 and 4. The solvent for the sample was 3 ml of chloroform.

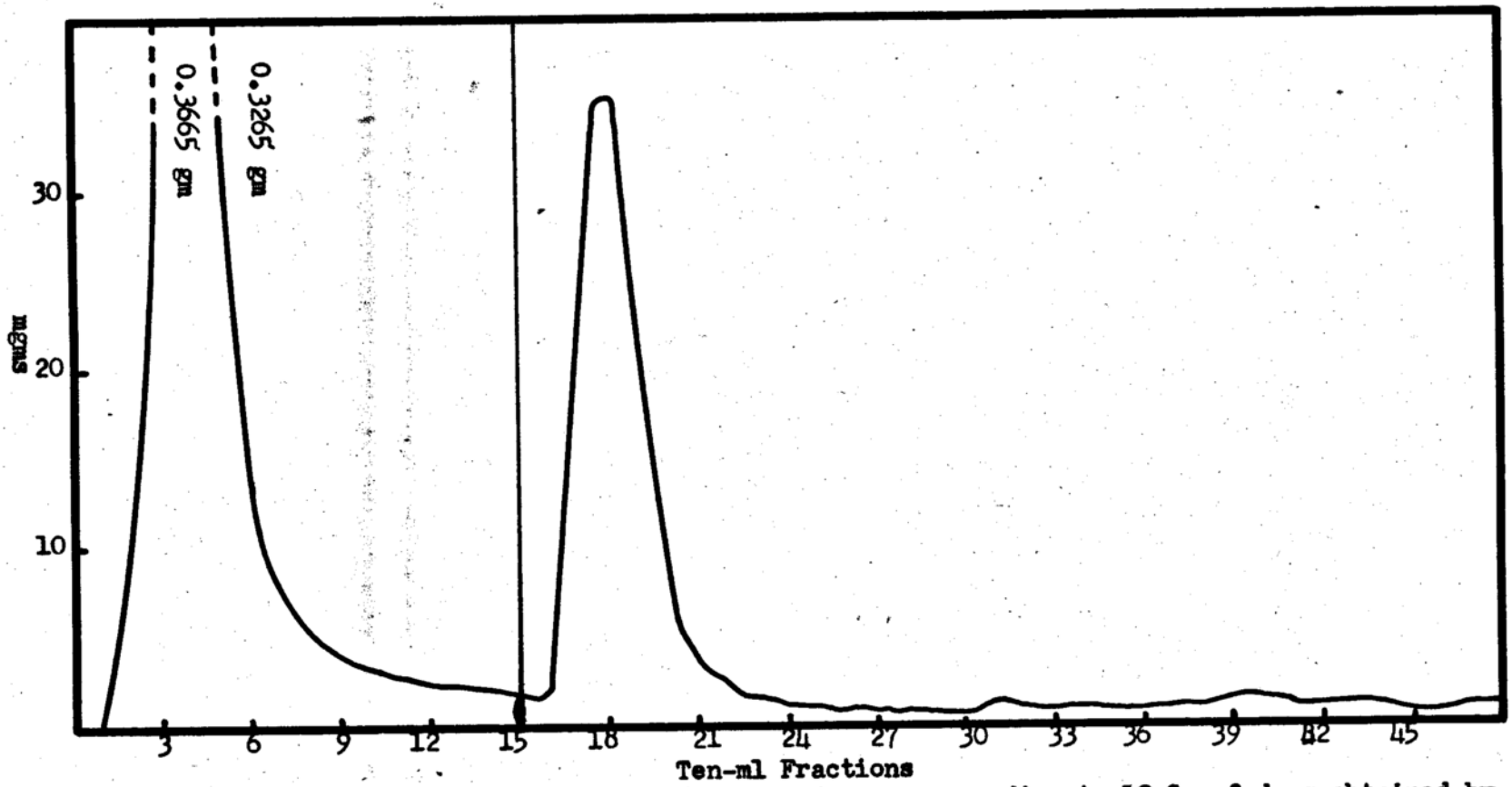

Fig. 2 Partition chromatogram of extract of Cantharides corresponding to 10 Gm of drug obtained by the N. F. procedure. Twenty ml of methanol was used as the internal phase. Petroleum ether (b.p. 30°-75°) was used for fractions 1 to 15 and chloroform for fractions 16 to 48. Total amount of material in fractions 17 to 21 is 84.4 mgms or 0.844%,

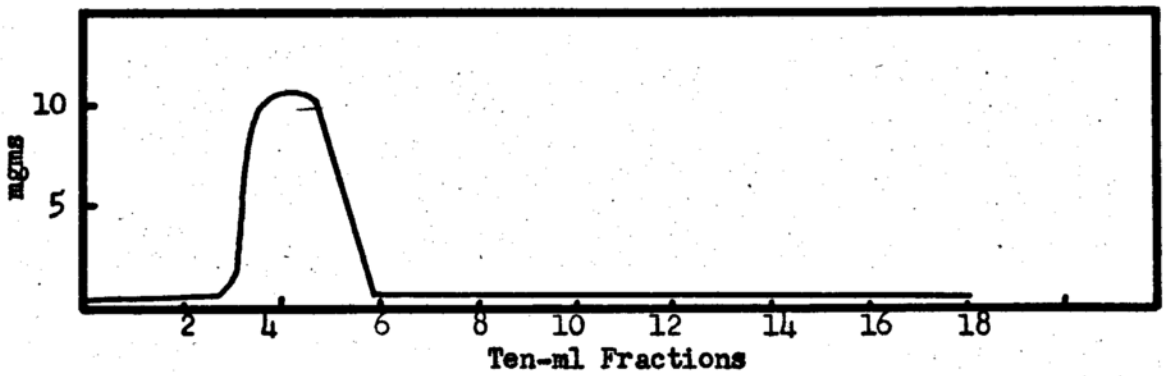

Fig. 3a. Partition chromatogram of cantharidin using 20 ml of 66% sulfuric acid as internal phase and 50% benzene in chloroform as external phase. Five ml of a mixture of equal volumes of chloroform and benzene was used to dissolve 19.6 mgms of sample. 10.7 mgms of sample was recovered in fractions 3 to 6.

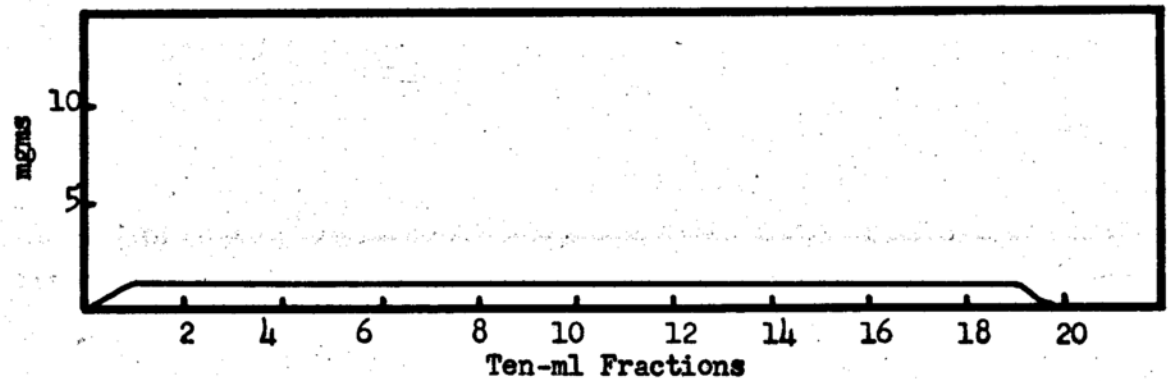

Fig. 3b. Partition chromatogram of cantharidin using 30 ml of 66% sulfuric acid as internal phase and 80% benzene in chloroform as external phase. A few ml of chloroform was used to dissolve 14.7 mgms of sample, of which 12.9 mgms was recovered in fractions 2 to 19.

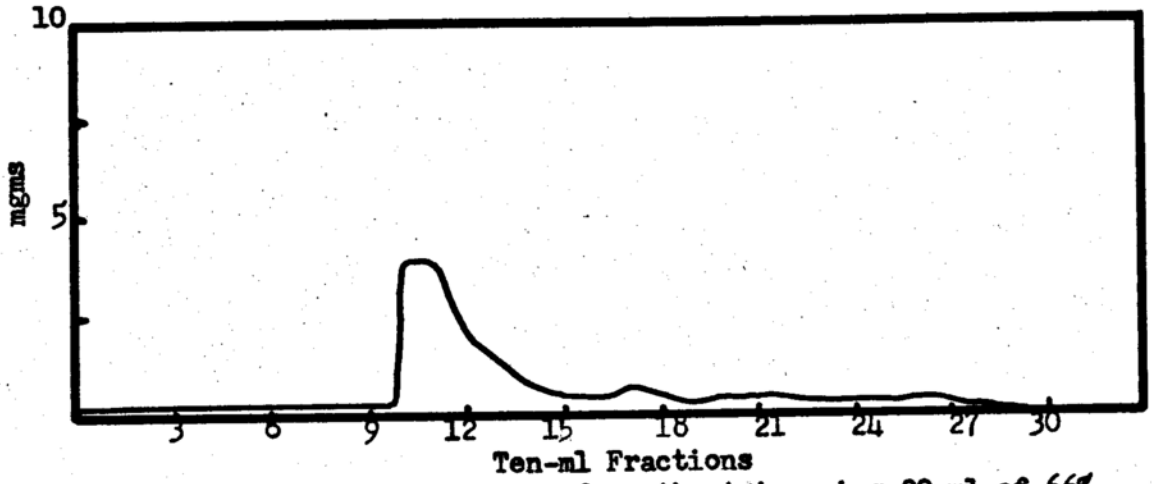

Fig. 4a. Partition chromatogram of cantharidin using 20 ml of 66% sulfuric acid as internal phase and 80% benzene in chloroform as external phase. A sample weighing 13.8 mgms was dissolved in 5 ml of the benzene-chloroform solution used as external phase. 14.8 mgms of material was recovered in fractions 10 to 21.

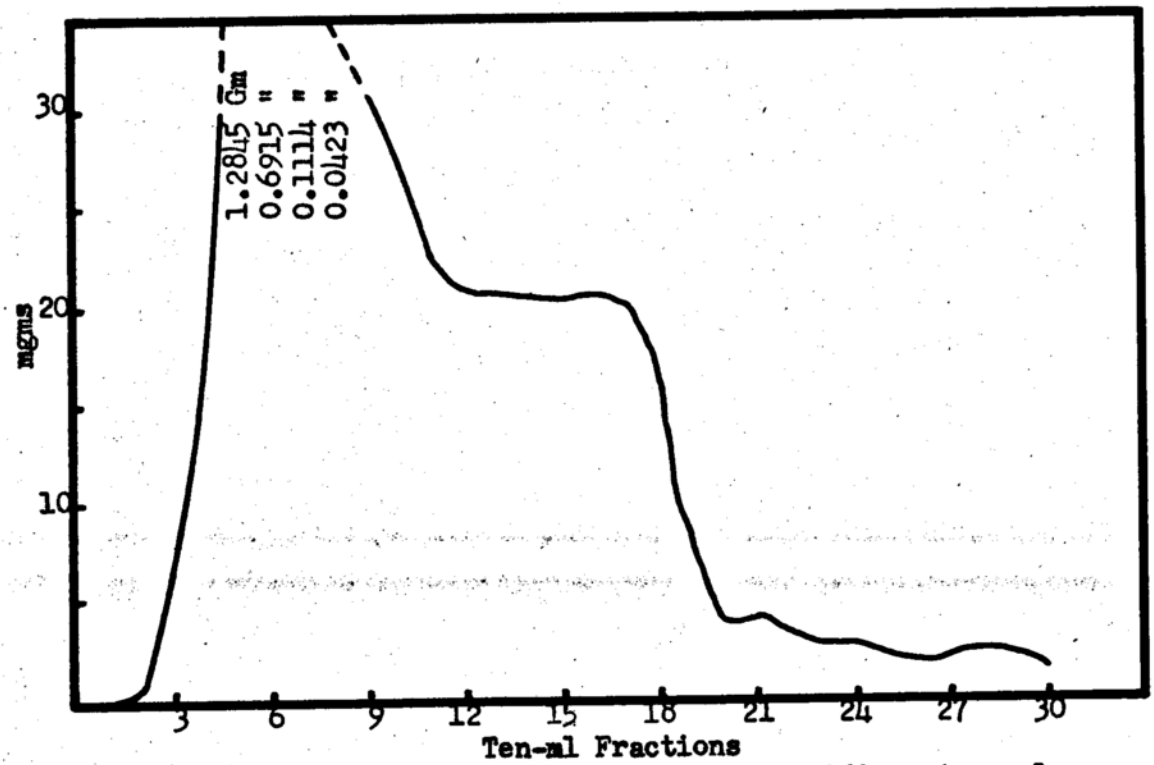

Fig. 4b. The Effect of Fat on Elution of Cantharidin. A sample weighing 19.3 mgms was dissolved in 2.5 Gm of olive oil. Twenty ml of 66% sulfuric acid was used as internal phase and 80% benzene in chloroform as external phase. A weight of 2.4024 Gm of oil mixed with cantharidin was recovered in fractions 5 to 20.

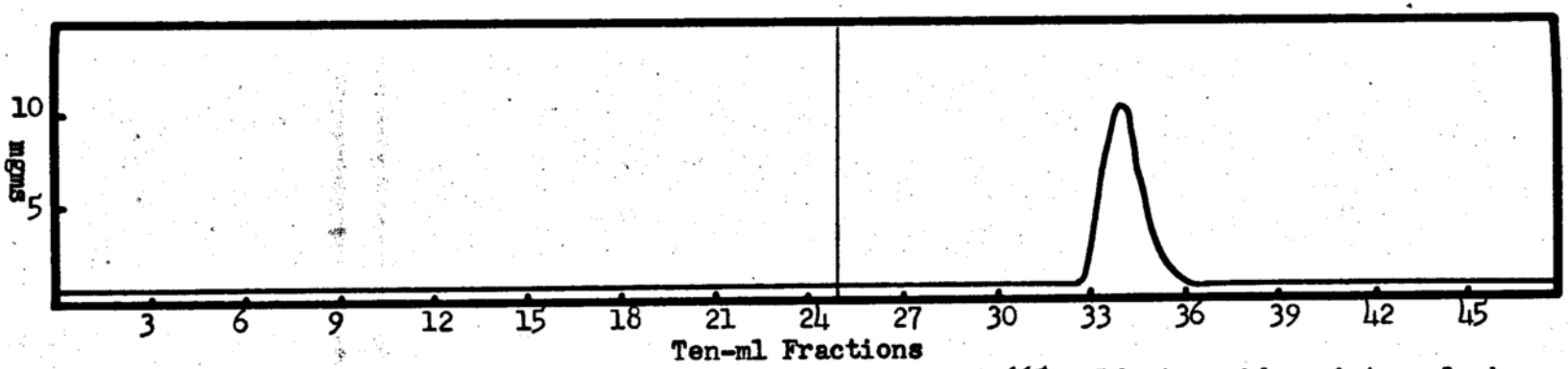

Fig. 5a. Partition chromatogram of cantharidin using 20 ml of 66% sulfuric acid as internal phase. A sample weighing 10.1 mgms was dissolved in a few ml of the benzene-chloroform. Skelly C was used for fractions 1 to 25 and 80% benzene-chloroform for fractions 26 to 48. Fractions 33 to 35 represent a weight of 12.8 mgms.

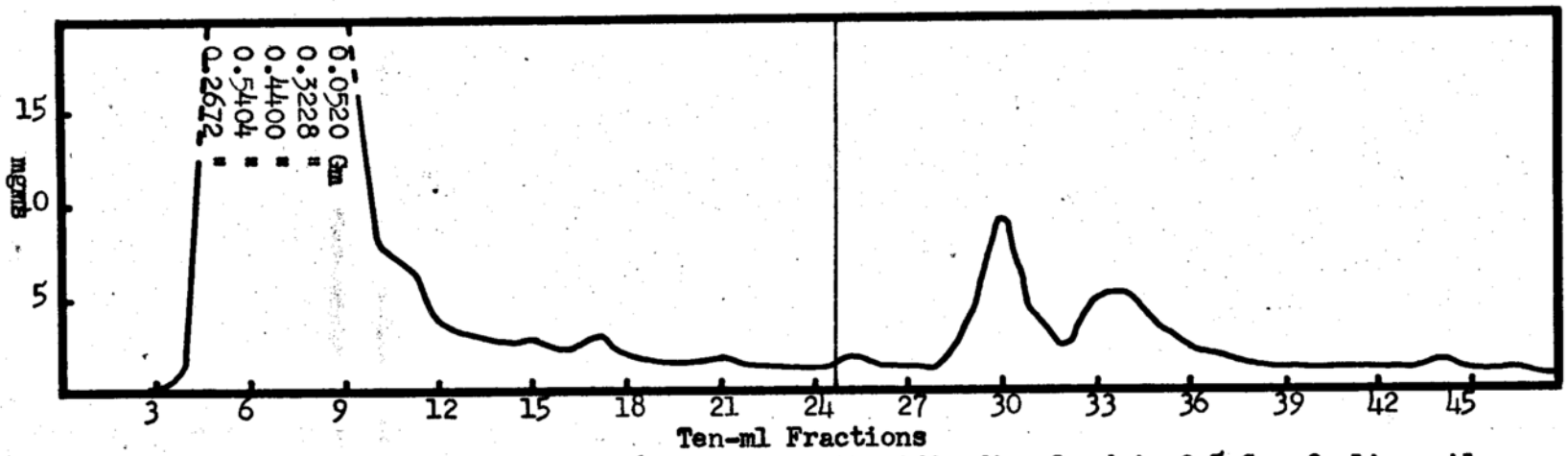

Fig. 5b. Partition chromatogram of 10.6 mgms of cantharidin dissolved in 2.5 Gm of olive oil, using 20 ml of 66% sulfuric acid as internal phase. Fractions 5 to 9 contained most of the oil and traces of crystalline material (cantharidin), Fractions 29 to 32 and Fractions 33 to 35 were a mixture of crystalline and oily material. Skelly C was used for fractions 1 to 25 and 80% benzene-chloroform for fractions 26 to 48.

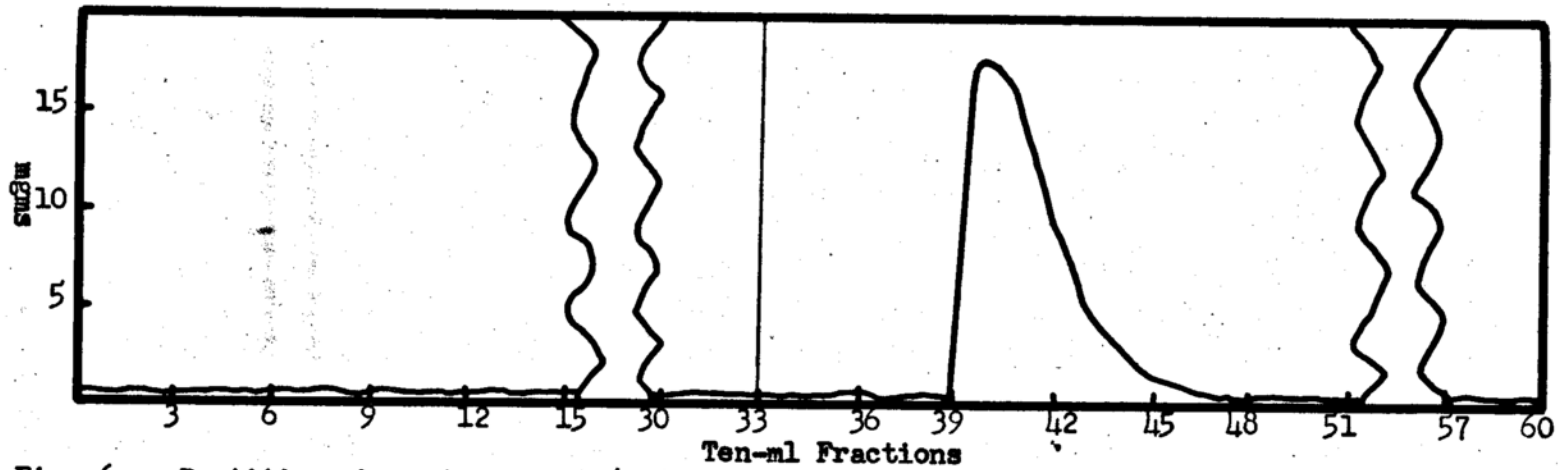

Fig. 6a. Partition chromatogram of 50.7 mgms of cantharidin dissolved in a few ml of benzene. Twenty ml of 66% sulfuric acid was the internal phase. Petroleum ether (b.p.30-75) was used for fractions 1 to 33 and benzene (thiophene0free) for fractions 34 to 60. A total weight of 50.8 mgms of material was recovered in fractions 40 to 43.

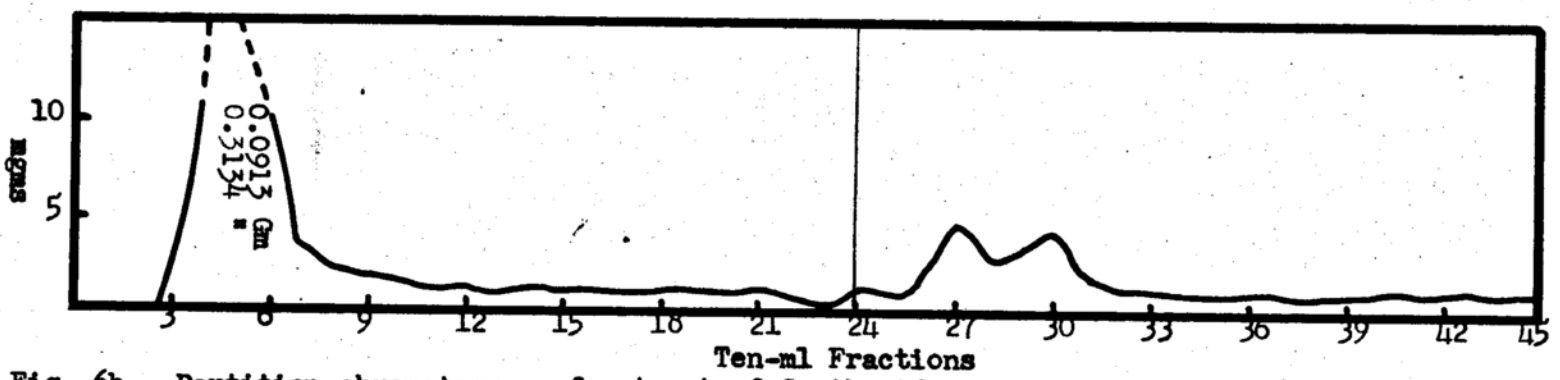

Fig. 6b. Partition chromatogram of extract of Cantharides corresponding to 15 Gm of drug. (See p.27for preparation of the extract.) Twenty ml of 66% sulfuric acid was used as internal phase. Skelly C was used for fractions 1 to 24 and 80% benzene-chloroform for fractions 25 to 48. Fractions 4 to 7 represent most of the fatty material, fractions 26 to 28 consisted of greasy and crystalline material, while fractions 29 and 30 represent 9.9 mgms of cantharidin or 0.06%.

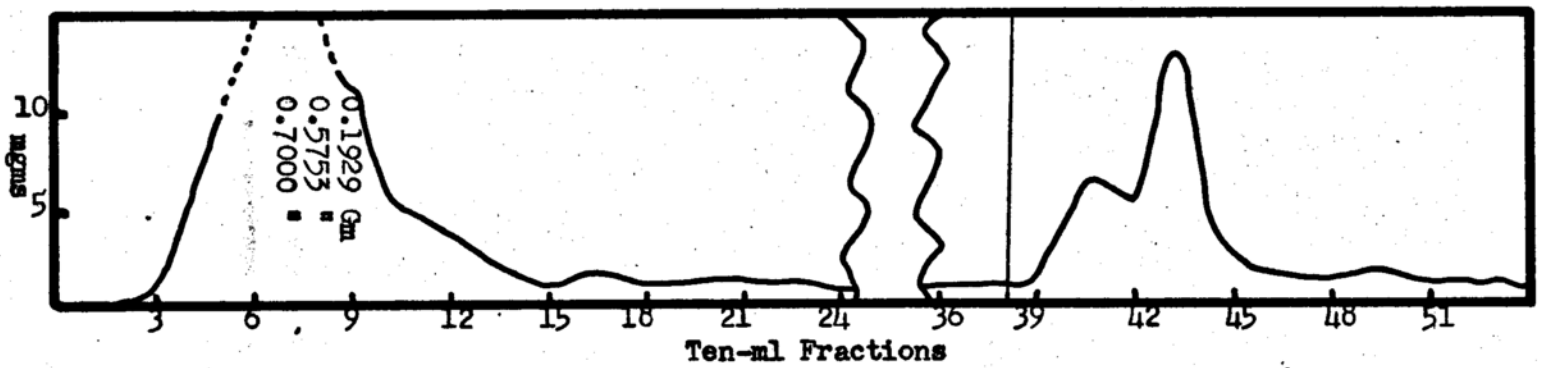

Fig. 7a. Partition chromatogram of extract of Cantharides corresponding to 15 Gm of drug. (See p.27 for preparation of the extract.) Twenty ml of 66% sulfuric acid was used as internal phase. Skelly C was used for Fractions 1 to 38 and 80% benzene-chloroform for Fractions 39 to 57. Fractions 5 to 12 included most of the fatty material, some of which was also present in Fractions 40 to 42. Fractions 43 to 45 represent a weight of 27.9 mgms of cantharidin or 0.18%.

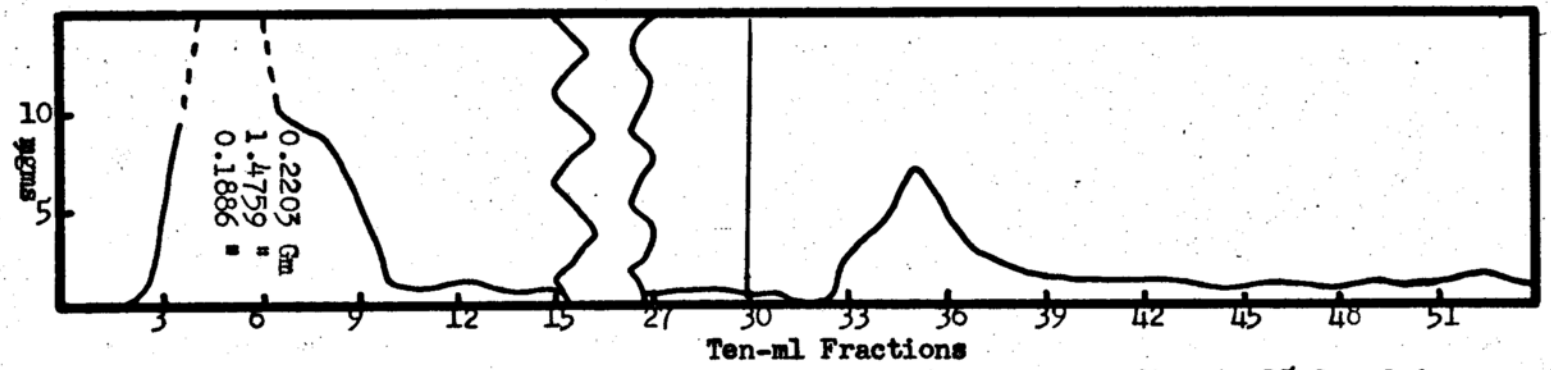

Fig. 7b. Partition chromatogram of extract of Cantharides corresponding to 15 Gm of drug (prepared as for 7a), to which was added 15.7 mgms of pure cantharidin. Twenty ml of 66% sulfuric acid was the internal phase. Skelly C was used for Fractions 1 to 30 and 80% benzene-chloroform for Fractions 31 to 55. Fractions 4 to 9 represent the fatty material of the extract, while 21.6 mgms of cantharidin was obtained in Fractions 34 to 37.

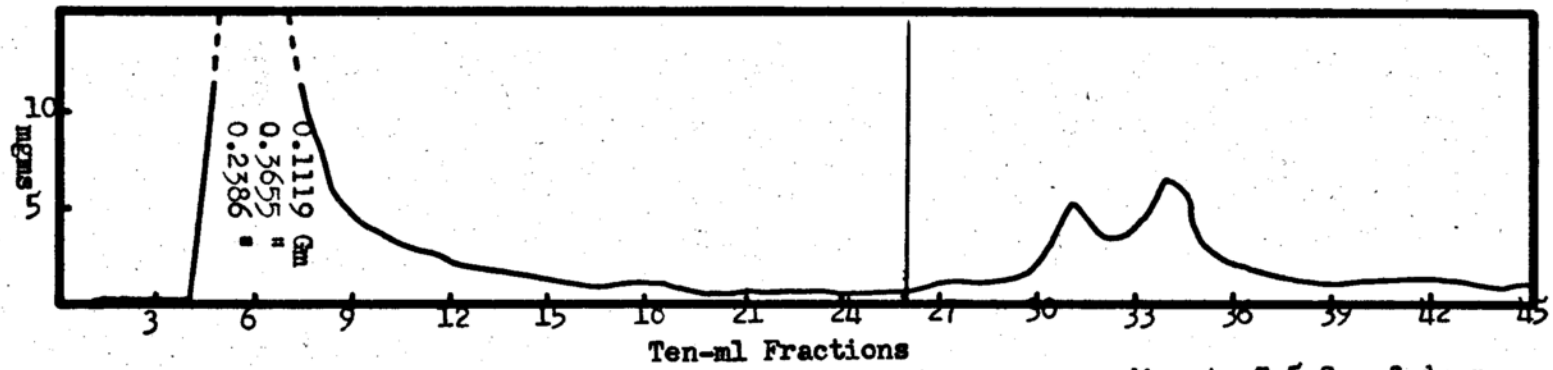

Fig. 8a. Partition chromatogram of extract of Cantharides corresponding to 7.5 Gm of drug, (see p.27 for preparation of extract), using 20 ml of 66% sulfuric acid as internal phase. Skelly B was used for fractions 1 to 26 and 80% benzene-chloroform for fractions 27 to 48. The fatty material of the extract was eluted in fractions 5 to 12. 13.5 mgms of cantharidin was recovered in fractions 33 to 36, preceded by more fatty material in fractions 30 to 32.

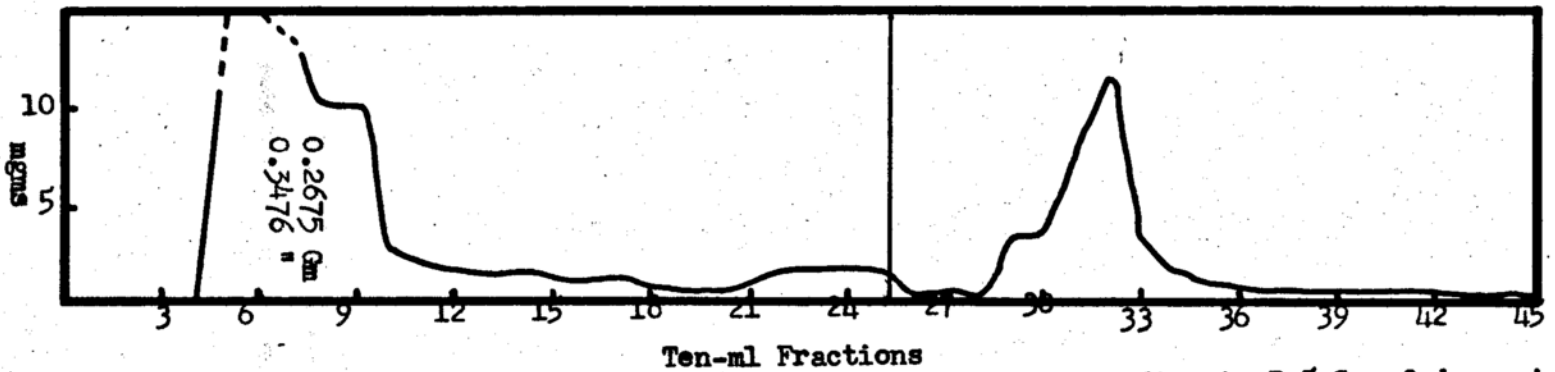

Fig. 8b. Partition chromatogram of extract of Cantharides corresponding to 7.5 Gm of drug, to which was added 12.7 mgms of pure cantharidin, using 20ml of 66% sulfuric acid as internal phase. Skelly B was used for fractions 1 to 25 and 80% benzene-chloroform for fractions 26 to 48. The fat was obtained in fractions 5 to 10 and 23.3 mgms of cantharidin in fractions 29 to 33.

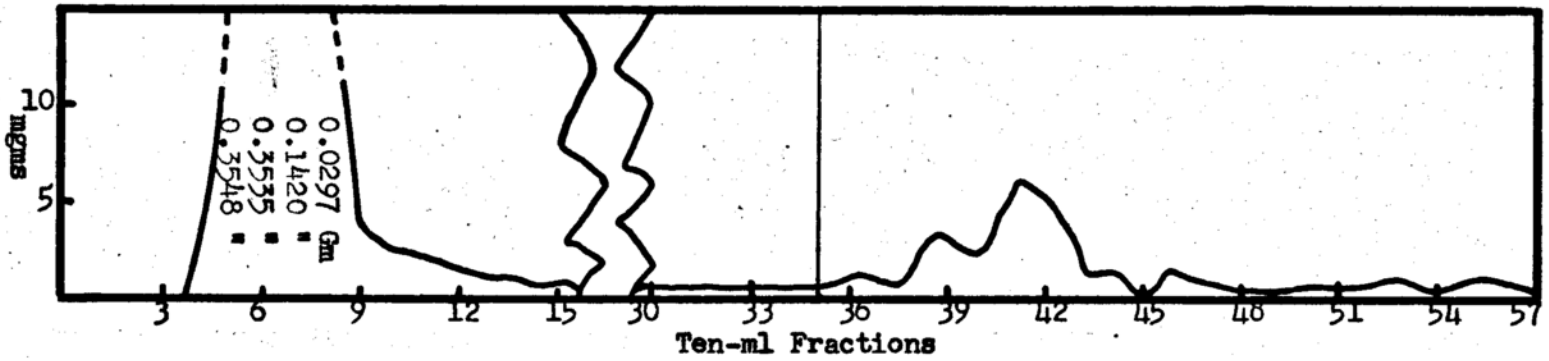

Fig. 9a. Partition chromatogram of extract of Cantharides corresponding to 9 Gm of drug (see p.27 for preparation of extract), using 20 ml of 66% sulfuric acid as internal phase. Skelly B was used for fractions 1 to 35 and 80% benzene-chloroform for fractions 36 to 57. Fractions 4 to 9 represent the fatty material, while 14.7 mgms or 0.16% of cantharidin was obtained in fractions 41 to 43, preceded by more fat in fractions 39 to 40.

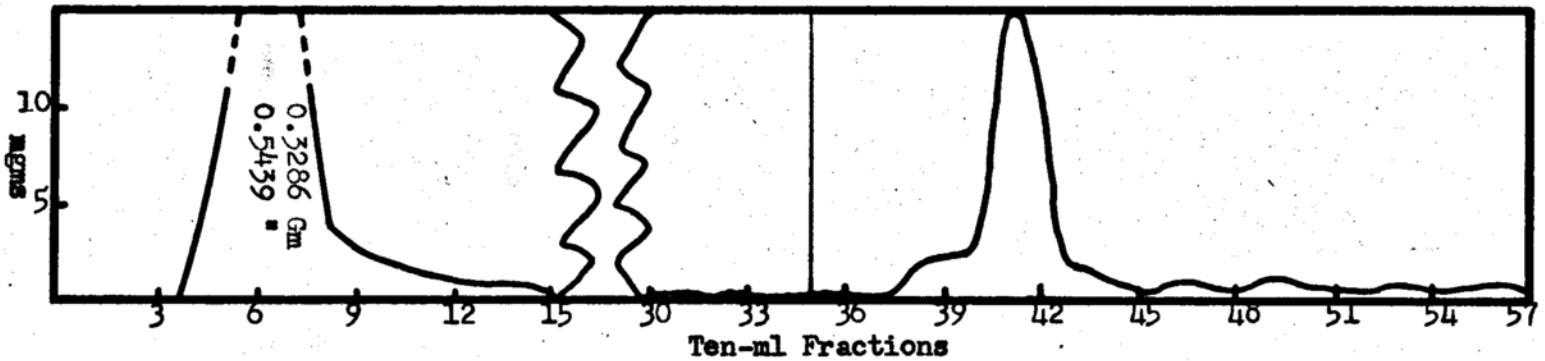

Fig. 9b. Partition chromatogram of extract of Cantharides corresponding to 9 Gm of drug, to which was added 17.8 mgms of pure cantharidin. Twenty ml of 66% sulfuric acid was the internal phase. Skelly B was used for fractions 1 to 35 and 80% benzene-chloroform for fractions 36 to 57. The fat was obtained in fractions 4 to 9 and 28.0 mgms of cantharidin in fractions 40 to 42.

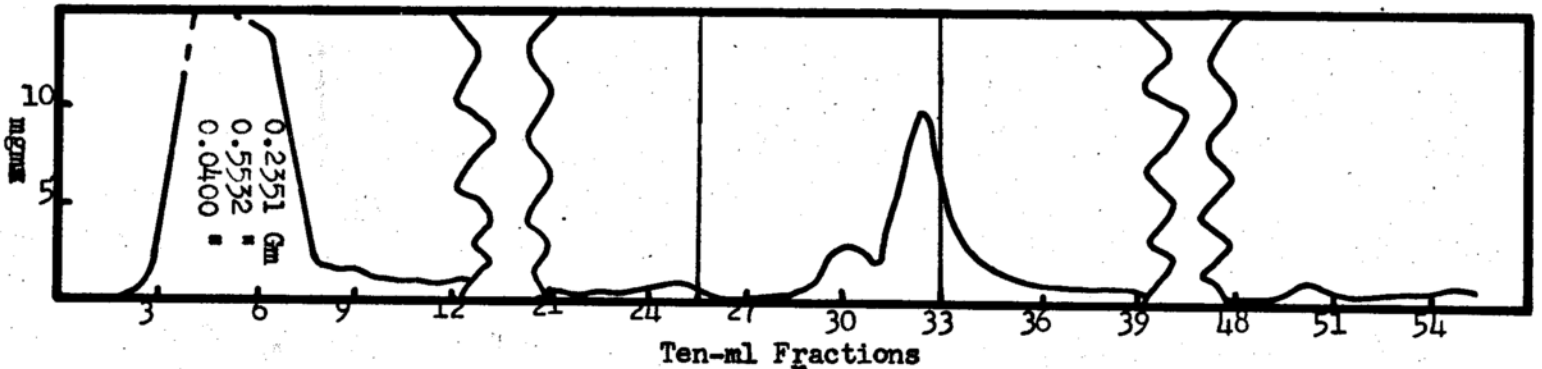

Fig. 10a. Partition chromatogram of extract of Cantharides corresponding to 7.5 Gm of drug, prepared according to the N.F. procedure. Twenty ml of 66% sulfuric acid was the internal phase. Skelly B was used for fractions 1 to 25, benzene for fractions 26 to 33, and 80% benzene-chloroform for fractions 34 to 55. Fractions 4 to 8 contained the fatty material, and 21.3 mgms or 0.284% of cantharidin was obtained in fractions 32 to 34, preceded by more traces of fat in fractions 30 and 31.

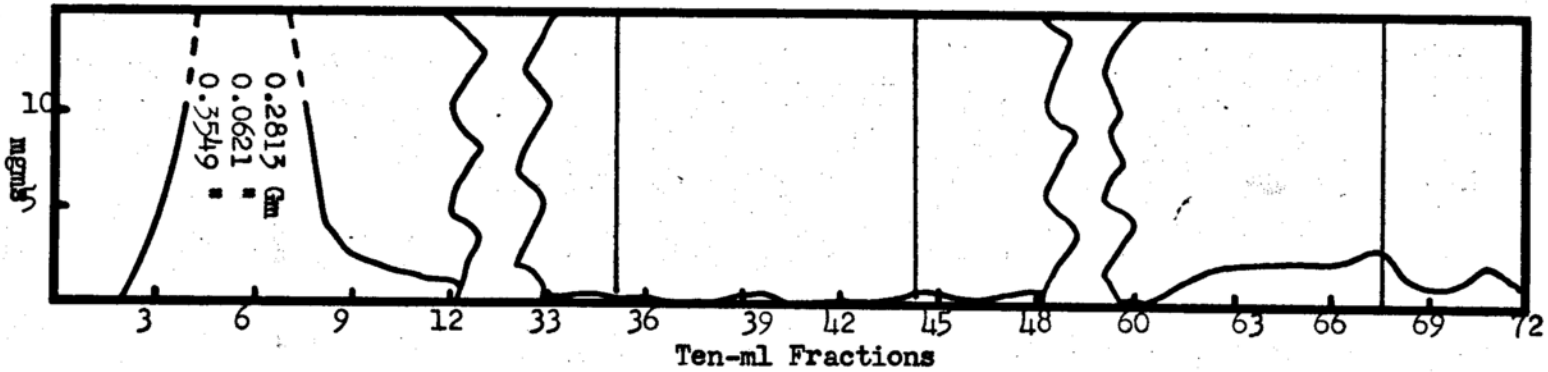

Fig. 10b. Partition chromatogram of extract of Cantharides corresponding to 7.5 Gm of drug, prepared according to the N. F. procedure. Twenty ml of 66% sulfuric acid was the internal phase. Skelly B was used for fractions 1 to 35, 20% benzene-Skelly B for fractions 36 to 44, 40% benzene-Skelly B for fractions 45 to 67, and 80% benzene-Skelly B for fractions 68 to 72. Fractions 4 to 9 contained the fat and fractions 63 to 72 represented 20.3 mgms of cantharidin with traces of impurities.

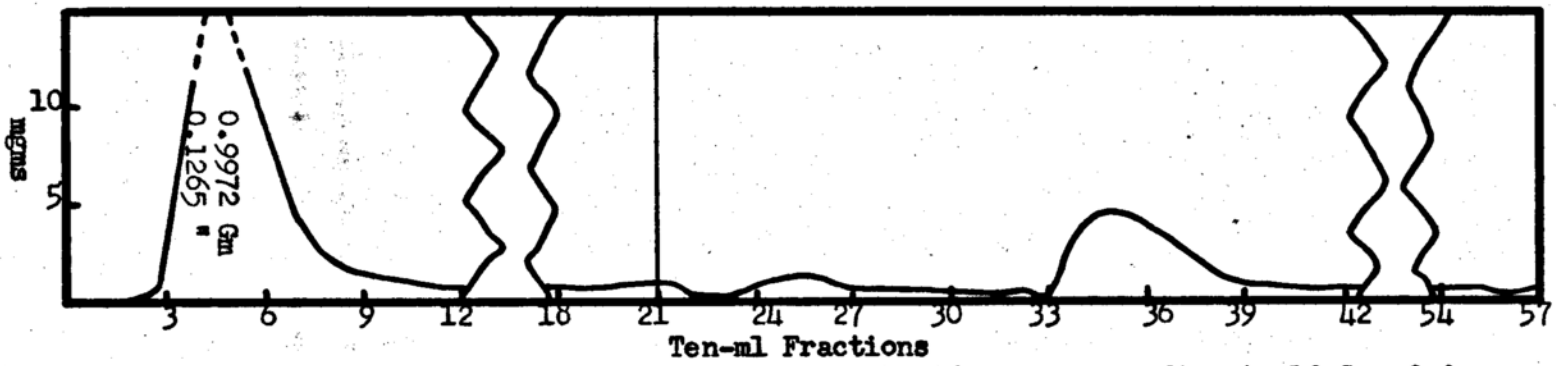

Fig. 11a. Partition chromatogram of extract of Cantharides corresponding to 10 Gm of drug, prepared following the N. F. procedure. Twenty ml of 66% sulfuric acid was used as the internal phase. Skelly B was used for fractions 1 to 21 and 50% benzene-Skelly B for fractions 22 to 56. The fat was obtained in fractions 4 to 7 and 21.3 mgms or 0.213% of material was obtained in fractions 34 to 39.

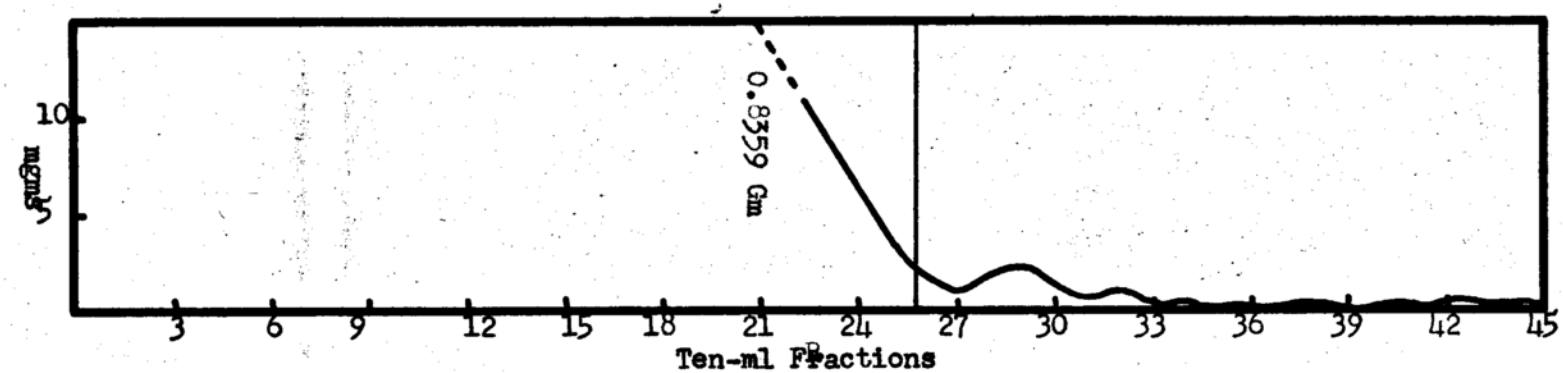

Fig. 11b. Partition chromatogram of the Skelly B eluate obtained from the column represented in Fig. 11a. Twenty ml of 66% sulfuric acid was the internal phase. Skelly B was used for fractions 1 to 25 and 50% benzene-Skelly B for fractions 26 to 45. The absence of cantharidin shows that none of this material was obtained with the fat from the column in Fig. 11a.

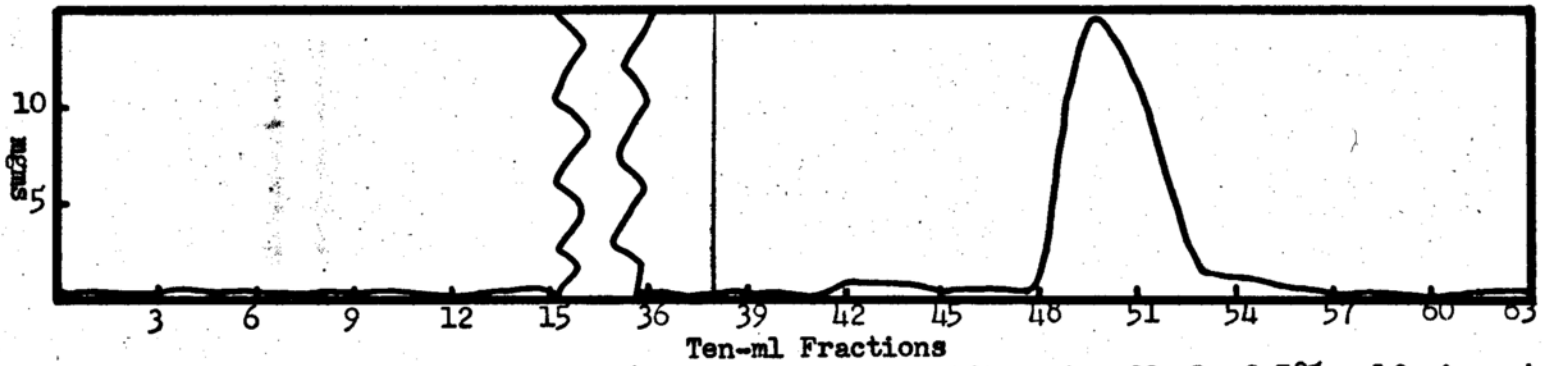

Fig. 12a. Partition chromatogram of 52.1 mgms of cantharidin using 20 ml of 78% sulfuric acid as internal phase. Petroleum ether (b.p. 30°-75°) was used for fractions 1 to 38 and benzene (thiophene-free) for fractions 39 to 61. The sample was dissolved in 5 ml of benzene, and 52.5 mgms of material was recovered in fractions 49 to 53.

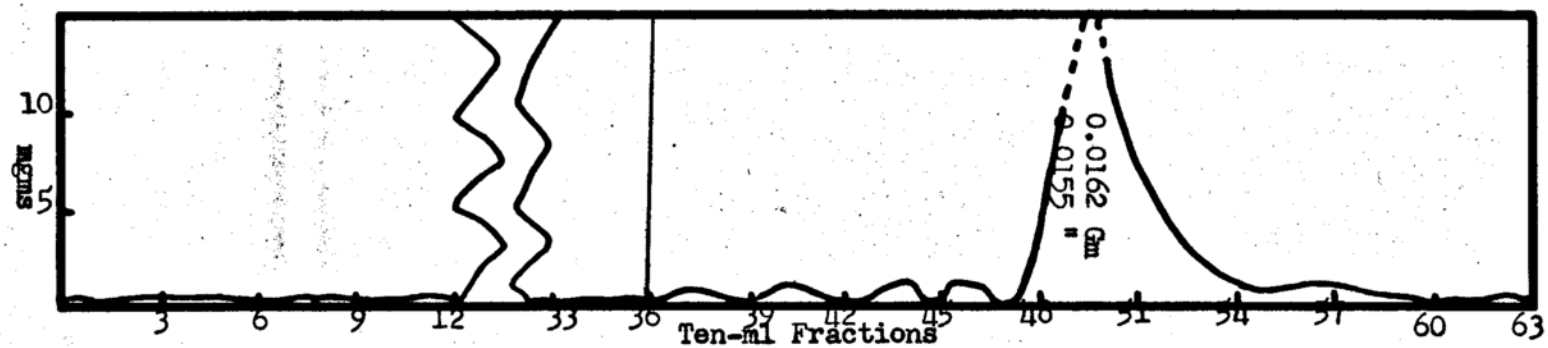

Fig. 12b. Partition chromatogram of 53.0 mgms of cantharidin using 20 ml of 78% sulfuric acid as internal phase. Petroleum ether (b.p.30°-75°) was used for fractions 1 to 36 and benzene (thiophene-free) for fractions 37 to 66. The sample was dissolved in 5 ml of benzene, and 53.0 mgms of material was recovered in fractions 49 to 51.

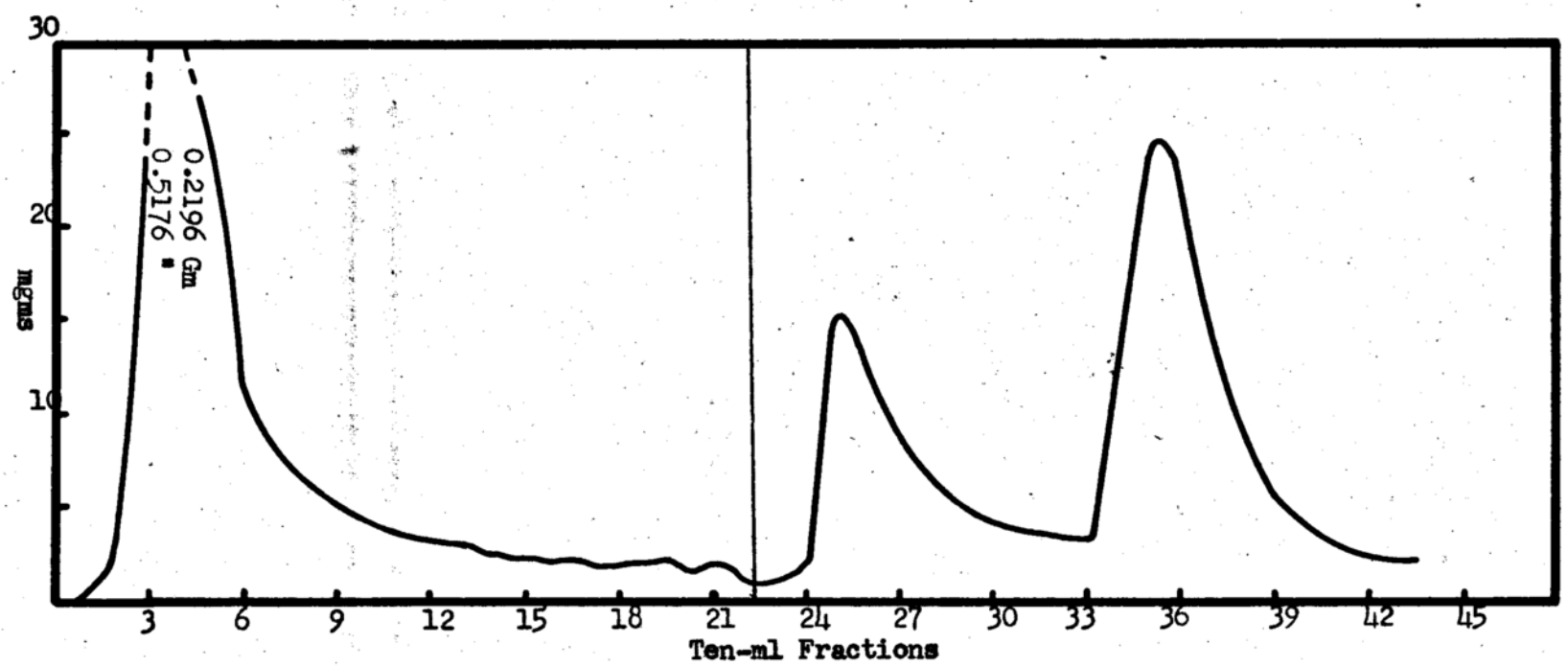

Fig. 13 Partition chromatogram of extract of Cantharides corresponding to 20 Gm of drug, using 20 ml of 78% sulfuric acid as internal phase. See p.33 for preparation of the extract. Petroleum ether (b.p. 30°-75°) was used for fractions 1 to 22 and benzene (thiophene-free) for fractions 23 to 44. Fractions 3 to 9 represent the elution of the fat. Fractions 34 to 39 weighed 88.6 mgms or 0.443%. Fractions 25 to 30 contained more fatty material.

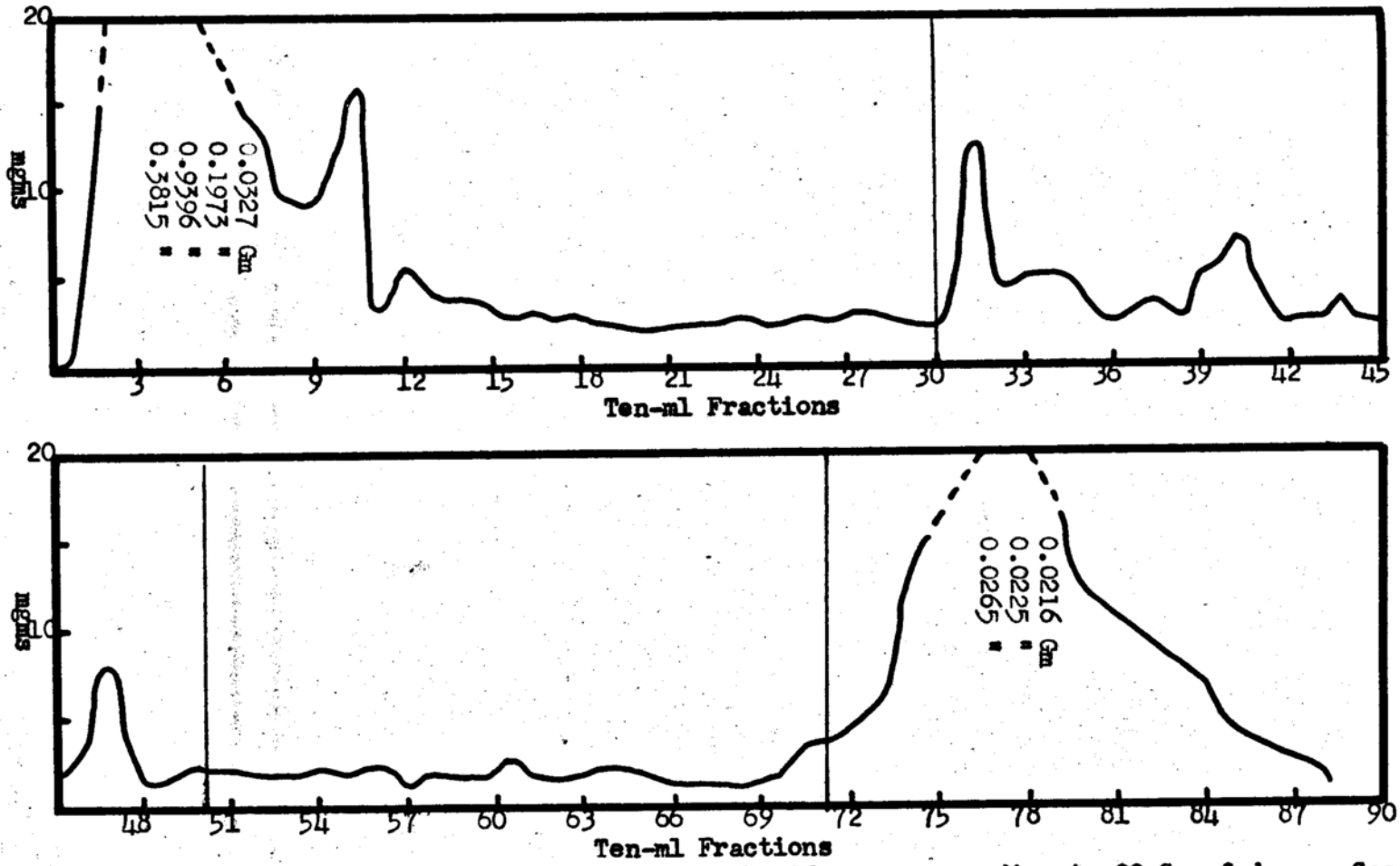

Fig. 14 Partition chromatogram of extract of Cantharides corresponding to 20 Gm of drug. See p33 for preparation of the extract. Twenty ml of 78% sulfuric acid was the internal phase. Petroleum ether (b.p. 30°-75°) was used for fractions 1 to 30, 20% benzene-petroleum ether for fractions 31 to 50, 40% benzene-petroleum ether for fractions 51 to 71, and benzene for fractions 72 to 88. Fractions 2 to 12 represent the fat, and fractions 73 to 87 weighed 169.6 mgms. The latter consisted of cantharidin with traces of fatty material.

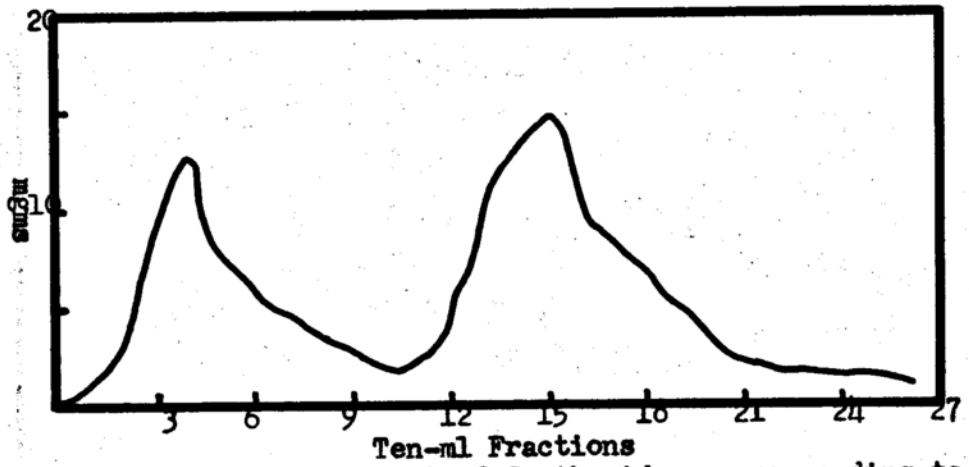

Fig. 15a. Partition chromatogram of extract of Cantharides corresponding to 10 Gm of drug. See p.33 for the preparation of the extract. Twenty ml of 78% sulfuric acid was the internal phase. The column was first eluted with petroleum ether (not represented in the figure) and then with benzene. Fractions 3 to 6 represent traces of fatty material. Fractions 12 to 19 weighed 90.1 mgms and contained traces of fatty material with the cantharidin.

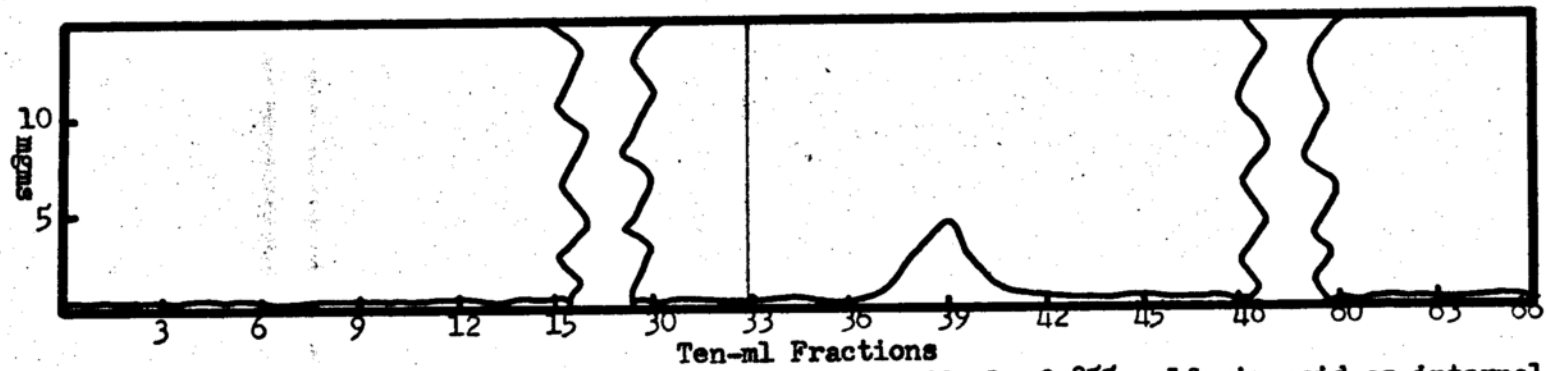

Fig. 15b. Partition chromatogram of cantharidin using 20 ml of 85% sulfuric acid as internal phase. Petroleum ether (b.p. 30-75°) was used for fractions 1 to 33 and benzene (thiophene-free) for fractions 34 to 63. Fractions 38 to 40 consisted of liquid material; the cantharidin was not recovered from the column. The sample had been dissolved in 5 ml of benzene.

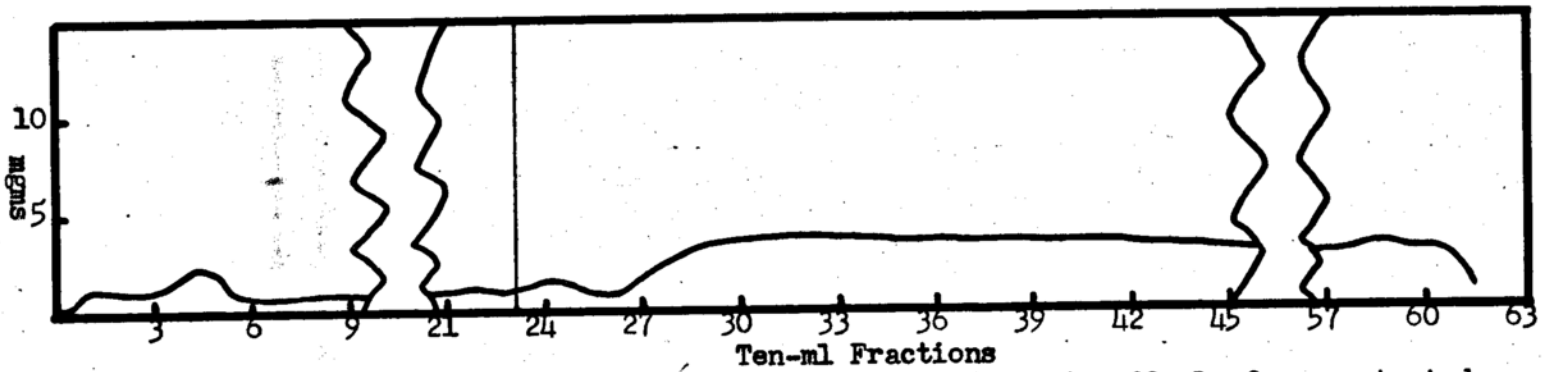

Fig. 16a. Partition chromatogram of 49.7 mgms of cantharidin using 20 ml of concentrated sulfuric acid as the internal phase. Petroleum ether (b.p. 30°-75°) was used for fractions 1 to 23 and benzene (thiophene-free) for fractions 24 to 61. The shape of the curve is due to the presence of benzene-insoluble material in the dishes. The sample was dissolved in 5 ml of benzene.

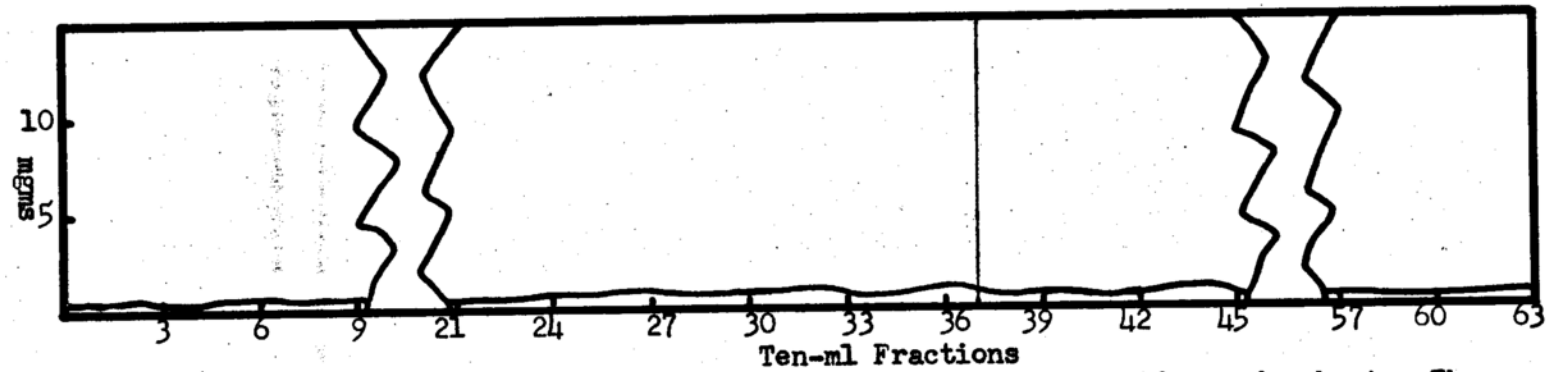

Fig. 16b. Chromatogram of 52.4 mgms of cantharidin using silicic acid as adsorbent. The sample was dissolved in 5 ml of benzene. Petroleum ether (b.p. 30°-75°) was used for fractions 1 to 36 and benzene (thiophene-free) for fractions 37 to 63. No material was obtained by elution.

PART TWO

TITRATION OF CANTHARIDIN

Determination of Cantharidin by a Pyridine-sodium Hydroxide Titration

1. Reagents
   a. Pyridine, C.P.
   b. Aqueous sodium hydroxide, c.1N
   c. Phenolphthalein in alcohol, 1%
   d. Hydrochloric acid, c.1N
2. Apparatus
   a. Microburettes, 10 ml.
   b. Pipettes, 5, 25, 50 ml.
   c. Erlenmeyer flasks, 125 ml.
3. General Procedures
   a. Direct Titration

The samples were weighed and dissolved in pyridine in 125 ml Erlenmeyer flasks and allowed to stand for one-half hour. After this time the solutions were titrated with the sodium hydroxide solution, using three drops of the phenolphthalein indicator, to the appearance of a faint pink color.

   b. Residual Titration

The samples were dissolved in the pyridine in

125 ml flasks and allowed to stand for one-half hour. The sodium hydroxide solution and ten ml of water was added and set aside for another one-half hour. Three drops of phenolphthalein indicator was added, and the excess alkali was titrated with 0.1N hydrochloric acid. A blank was run at the same time.

4. Analytical Determinations on Pure Cantharidin

The cantharidin used in these determinations was a white granular powder melting at 220.5°-221°.

Only the amounts of reagents used are given, since the details of the manipulations are given in the section of general procedures and the rest of the data appears in the tables.

a. Direct Titration

Determination No. 1

Five ml of pyridine was used to dissolve the samples. The end-point was difficult to detect because an intense red color was produced after addition of each drop of alkali which disappeared slowly. The data and results are given in Table I.

Table I

Wt. Sample Mgms.	Vol. NaOH .1462N (ml)	Wt. Found By By Titration (mgm)	Error in Mgms.
24.3	1.65	23.6	-0.6
25.4	1.75	25.1	-0.3
22.7	1.60	22.0	-0.2
24.7	1.77	25.3	+0.6
19.2	1.28	18.3	-0.9

The calculations for the first example are illustrated as follows:

Eq. wt. cantharidin = $\frac{\text{Molecular Weight}}{2}$

$= \frac{196.2}{2} = 98.1$

ml NaOH x N NaOH	= meq NaOH = meq Cantharidin
1.65 x 0.1462	= 0.2412
mgm Cantharidin	= 98.1 x 0.2412 = 23.6 mgms.

b. Residual Titration

Determination No. 1

The samples were dissolved in 5 ml of pyridine. Five ml of sodium hydroxide solution and ten ml of water was used. Table II shows the data and results.

Table II

Wt. Sample (Mgm)	Vol. HCl (Sample) ml	Vol. HCl (blank) ml	Normality HCl	Wt. Found By Titration	Error in Mgms
30.4	4.45	7.50	.0937	28.03	-2.37
37.2	3.55	7.50	.0937	36.3	-0.9
21.1	5.35	7.60	.0931	20.5	-0.6
22.2	5.15	7.60	.0931	22.4	+0.2
19.5	5.45	7.60	.0931	19.7	+0.2
22.1	5.30	7.70	.0931	22.2	+0.1
33.0	4.10	7.70	.0931	32.9	-0.1
25.1	4.90	7.70	.0931	25.9	+0.8
34.1	4.03	7.60	.0925	32.4	-1.7
20.5	5.50	7.60	.0925	19.1	-1.4
*126.1	1.75	15.25	.0931	124.2	-1.9

*Ten ml of pyridine and alkali was used.

The calculations are essentially the same as given for the direct titration, except that the equivalents of alkali consumed were obtained from the difference of the acid required by the blank and the acid required by the sample. An illustration for the first sample follows:

Volume acid (blank) 7.50
Volume acid (sample) 4.45
3.05

3.05 x 0.0937 = 0.2858 = meq. acid = meq base consumed.

0.2858 x 98.1 = 28.03 mgs C_{5}ntharidin

Determination No. 2

The material recovered in fractions 40 to 43 of the column represented in Fig. 6a (see page 73 for experimental details) was washed into a beaker with small portions of chloroform. The solvent was allowed to evaporate at room temperature then the titration was carried out using ten ml of pyridine and alkali. No water was used in this determination and the indicator used was 1% phenolphthalein in acetone. The results are given in Table III.

Table III

Fig. No.	Ml. HCl (Sample)	Ml. HCl. (Blank)	Mgm. Canth. Volumetric	Mgm. Canth. Gravimetric	Mgm Canth Added to Column
6a	5.94	10.75	50.8	50.8	50.7

HCl = 0.1078N

Determination No. 3

Two columns were packed using 20 ml of 78% sulfuric acid as internal phase, and the weighed samples, dissolved in 5 ml of benzene, were poured into them. Four hundred ml of petroleum ether (b.p. 30°-75°) was used as the first eluent and then the columns were eluted with 300 ml of benzene (thiophene-free). Most of the benzene was evaporated with the help of heat and the last traces were removed by a current of air. The dry white crystalline material which remained was dissolved in ten ml of pyridine and ten ml of alkali was added, the excess of which was titrated with 0.1N hydrochloric acid. Results are shown in Table IV.

Table IV

Column	Ml. HCl (Sample)	Ml. HCl (Blank)	Mgm. Canth. Volumetric	Mgm. Canth Added to Column	Error in Mgms.
1	5.09	10.54	57.6	57.3	40.3
2	5.55	10.53	52.7	53.4	-0.7

HCl = 0.1078N

5. Analytical Determinations on Cantharidin obtained from Extract of Cantharides

a. Direct Titration

Determination No. 1

The cantharidin fractions from several columns which were run in Part One of this investigation were

determined by titration. The aluminum dishes containing the cantharidin were rinsed with a minimum amount of chloroform, collecting the rinsings in 125 ml Erlenmeyer flasks. The solvent was removed completely with the aid of mild heat from a steam bath and a current of air. The dry residues which remained were weighed, then dissolved in 10 ml of pyridine and titrated according to the direct titration procedure. Table V gives the results and also a comparison of the gravimetric and volumetric determinations.

Table V

Fig. No.	Ml. NaOH	Mgm. Canth. Grav.	Mgm. Canth. Volumetric	Grav. Minus Vol. (Mgm)	Canth. Added to Columns
9a	0.38	14.7	6.4	8.3	0
9b	0.75	28.0	10.8	17.2	17.8
10a	0.65	21.3	9.3	12.0	0
10b	0.50	20.3	7.2	13.1	0
11a	1.35	21.3	19.4	1.9	0

NaOH = 0.1462N

Determination No. 2

The residue of cantharidin obtained from the MP assay was titrated. Ten ml of pyridine was used as solvent. The gravimetric and volumetric results are given in Table VI.

Table VI

% Canth. NF Assay	% Canth. Volum.	Deviation %
0.205%	0.179%	0.026%

b. Residual Titration

Determination No. 1

A column using 20 ml of 66% sulfuric acid as internal phase was packed as described on page 66 The extract from 10 Gm of Cantharides (assay 0.195) was prepared by extracting the drug, previously saturated with 2 ml of concentrated hydrochloric acid, with 100 ml of chloroform for about 3 hours in a Soxhlet apparatus. The solution was concentrated to about 5 ml by mild heat and a current of air, and then it was poured into the column. Two hundred and fifty ml of Skelly B was passed through, collecting the total amount in a beaker. This was followed by the same volume of thiophene-free benzene, collecting the eluate in another beaker. The benzene was removed by warming gently while applying a current of air. The residue was dried in a vacuum desicator and titrated following the residual method. Five ml of pyridine and five ml of alkali was used, followed by 10 ml of water. Results are shown in Table VII.

Table VII

Column No.	Ml. HCl Sample	Ml. HCl Blank	Mg. Canth. Volum.	% Canth.
1	4.65	7.60	26.75	0.267

HCl @ 0.0931N

Determination No. 2

A fresh batch of Cantharides was assayed according to the National Formulary method. The residues were dissolved in five ml of pyridine and 5 ml of alkali was added. The excess alkali was titrated with 0.1N hydrochloric acid. Two 15 Gm samples from the same batch of drug were saturated with 2 ml of concentrated hydrochloric acid and extracted with 150 ml of chloroform in a Soxhlet for about three hours. One hundred ml aliquots of each extract were poured into beakers and the solvent removed by heating gently. The residues obtained were purified as directed in the National Formulary, weighed, and titrated. Five ml of pyridine was added as solvent. Five ml of alkali was added and the excess was titrated with 0.1N hydrochloric acid. Tables VIIIa and VIIIb give the results.

Table VIIIa

N.F. Assay

Sample No.	Ml. HCl Sample	Ml. HCl Blank	Mgm. Canth. Volum.	Mgm. C.nth. Grav.	Diff. Mgm.
1	1.16	7.60	58.4	59.4	1.0
2	"	"	"	60.2	"

HCl = 0.0925N

Table VIIIb

Chloroform Extract

Sample No.	Ml. HCl Sample	Ml. HCl Blank	Mgm. Canth. Volum.	Mgm. Canth. Grav.	Diff. Mg.
1	1.16	7.60	58.5	62.1	3.6
2	1.14	7.60	58.9	"	

HCl = 0.0925N

Determination No. 3

Four 10 Gm portions of Cantharides (assay 0.597%) were extracted each with 100 ml of chloroform in a Soxhlet apparatus for three hours. Weighed amounts of pure cantharidin were added to two of the extracts. The chloroform was removed by mild heat and a current of air, and the residues were purified as directed in the N.F. and weighed. Each of purified residues obtained was dissolved in ten ml of pyridine, an equal volume of alkali was added, followed by ten ml of water. The excess alkali was titrated with N/10 hydrochloric acid.

Table IX

Sample No.	Ml HCl Sample	Ml HCl Blank	Total Canth Volum Mgm	Total Canth Grav Mgm	Added Canth Mgm	Total Minus Added Volum	% Canth Volum	Total Minus Added Grav	% Grav
1	4.60	11.12	60.3	65.2	-	60.3	0.603	65.2	0.652
2	4.47	11.12	59.2	65.7	-	59.2	0.592	65.7	0.657
3	2.00	11.12	82.8	90.0	25.0	57.8	0.578	65.0	0.650
4	2.33	11.12	79.8	87.2	22.3	57.5	0.575	64.9	0.649

HCl = 0.0925N

Determination No. 4

Four 10 Gm samples of Cantharides (assay 0.581%) were extracted according to the N.F. procedure. A weighed amount of pure cantharidin was added to each of two extracts. The solvent was removed and the residues were purified as directed in the N.F., and weighed. The purified residue obtained was dissolved

in 10 ml of pyridine and ten ml of alkali and water was added. The excess alkali was titrated with 0.1N hydrochloric acid. Results are shown in Table X.

Table X

Sample No.	Ml HCl Sample	Ml HCl Blank	Total C.nth Volum	Total Canth Grav	Added Canth	Total Minus Added Volum	% Volum	Total Minus Added Grav	% Grav.
1	5.31	11.12	52.7	56.3	*	52.7	0.527	56.3	0.563
2	4.95	11.12	55.9	60.0	*	55.9	0.559	60.0	0.600
3	1.45	11.12	89.5	96.9	54.3	35.2	0.352	42.6	0.426
4	2.77	11.12	75.8	80.5	32.8	43.0	0.430	47.7	0.477

HCl = 0.0925N

Determination No. 5

The extract from 10 Gm of Cantharides (assay 0.581%) was obtained as described on page 33. Eight such extracts were used in the determination. The extracts were concentrated to about 5 ml and poured into columns containing 20 ml of 66% sulfuric acid as the internal phase. The columns were eluted first with 300 ml of petroleum ether (b.p. 30°-75°) and then with 250 ml of benzene (thiophene-free), collecting the two eluates in separate beakers. The benzene was evaporated off by mild heat and a current of air. Two of the residues were purified by washing with a mixture of equal volumes of petroleum ether and absolute alcohol saturated with cantharidin, as directed in the N. F. They were weighed and titrated. The residues from the other six columns were titrated in the beakers in which

the eluates had been collected. Ten ml of pyridine, alkali and water was used. Results are shown in Table XI.

Table XI

Sample No.	Ml HCO_3 Sample	Ml HCl Blank	Mgm Canth Volum	% Canth Volum	% Canth Grav
1	6.27	14.22	72.1	0.721	•
2	6.97	"	65.8	0.658	•
3	7.62	"	61.1	0.611	•
4	6.13	"	74.1	0.741	•
5	6.15	"	73.2	0.732	•
6	6.65	"	68.7	0.687	•
*7	8.37	"	53.5	0.535	0.587
*8	8.87	"	48.5	0.485	0.540

HCl = 0.0925N
*Purified Residues

Determination No. 6

Six aliquots, each representing 10 Gm of Cantharides (assay 0.581%), were obtained as described on page 33 Weighed amounts of cantharidin were added to three of the extracts. The chloroform was evaporated by mild heat and a current of air to a volume of about 5 ml. Six columns were packed using 20 ml of 66% sulfuric acid as internal phase and the extracts were poured into them. They were eluted first with 300 ml of petroleum ether (b.p. 30°-75°) followed by 250 ml of thiophene-free benzene, collecting each eluate in a separate beaker. The benzene was evaporated on a steam bath and with a current of air. The residues, apparently impure, were washed with a mixture of

petroleum ether and absolute alcohol saturated with cantharidin. The crystals obtained were dried in the oven at 60°C for one-half hour, and then were titrated. Ten ml of pyridine, alkali and water was used. Results are shown in Table XII.

Table XII

Sample No.	Ml HCl Sample	Ml HCl Blank	Total C$_a$nth Volum	Total C$_a$nth Grav	Added Canth	Total Minus Added Volum	% Volum	Total Minus Added Grav	% Grav
1	5.42	14.85	85.6	98.0	26.4	59.2	0.592	71.6	0.716
2	6.85	14.85	72.59	61.4	"	72.6	0.726	61.4	0.614
3	4.62	11.12	59.0	60.2	"	59.0	0.590	60.2	0.602
4	4.60	11.12	59.2	65.6	"	59.2	0.592	65.6	0.656
5	3.02	11.12	73.5	81.0	26.4	47.1	0.471	54.6	0.546
6	2.70	11.12	76.41	84.2	25.5	50.9	0.509	58.7	0.587

HCl = 0.0925N

Determination No. 7

Four 10 Gm samples of a fresh batch of powdered Cantharides were assayed according to the N.F. procedure. The cantharidin residues, after having been weighed, were titrated. Ten ml of pyridine and alkali was used. No water was employed in the process.* Results are shown in Table XIII.

Table XIII

Sample No.	Ml HCl Sample	Ml HCl Blank	Mgm Canth Volum	% Canth Volum	% Canth Grav
1	4.08	8.79	49.8	0.498	0.776
2	3.80	8.79	52.8	0.528	0.752
3	3.51	8.79	50.8	0.508	0.648
4	4.49	8.79	45.5	0.455	0.707

HCl = 0.1078N

*From here on no water was used and 1% phenolphthalein in acetone was used as indicator for the titration.

Determination No. 8

The cantharidin recovered from the columns represented in Figs. 14 and 15a was transferred from the aluminum dishes to beakers by washing with a minimum amount of chloroform. The solvent was removed and the dry residues were titrated. For the column in Fig. 14, 20 ml of pyridine and alkali was used. For the other column, 10 ml of each reagent was employed. No water was added to either one. Results are shown in Table XIV.

Table XIV

Fig.	Ml HCl Sample	Ml HCl Blank	Mgm Canth Volum	% Canth Volum	Mgm Canth Grav	% Canth Grav
14	11.21	21.35	107.2	0.536	169.9	.848
15a	4.56	10.53	63.1	0.631	90.1	.901

HCl = 0.1078N

Determination No. 9

Two aliquots each representing 10 Gm of Cantharides assay 0.721) were prepared as described on page 33 and poured into two columns containing 20 ml of 78% sulfuric acid as internal phase. The columns were first eluted with 400 ml petroleum ether (b.p. 30°-75°) to remove the fat, collecting the total volume in a beaker. Ninety ml of benzene were then used, and collected separately. Finally, the columns were eluted with 200 ml of benzene (thiophene-free). The latter eluates, after removal of the solvent with the

aid of a current of air and mild heat, yielded apparently impure residues. These were titrated. Ten ml of pyridine and alkali was employed. Inspection of the 90 ml benzene fraction showed no cantharidin present. Results are shown in Table XV.

Table XV

Sample No.	Ml HCl Sample	Ml HCl Blank	Mgm Canth Volum	% Canth Volum
1	3.35	10.53	75.9	0.759
2	2.76	10.53	81.2	0.812

HCl = 0.1078N

Determination No. 10

Four columns were packed using 20 ml of 78% sulfuric acid as the internal phase. The extract corresponding to 10 Gm of Cantharides (see page 33 for preparation), was placed in each column and eluted with the following solvents in succession, collecting each in a seperate beaker: 400 ml petroleum ether (b.p. 30°-75°), 90 ml of benzene, (thiophene-free) and 200 ml of benzene. Three of the 200 ml portions of benzene were washed with distilled water until the washings were no longer acidic to litmus. (About three times with 75 ml portions.) The benzene layers were then dried overnight with anhydrous sodium sulfate. The drying agent was filtered off and rinsed with fresh benzene, adding the rinsings to the filtrates. The benzene solutions were evaporated to dryness on a

hot plate at low heat, and with the help of a current of air. The residues were placed in the oven at 60°C for fifteen minutes. The residual titration was carried out. Ten ml of pyridine and sodium hydroxide was employed but no water.* The unwashed residue was also titrated in the same manner. Results are shown in Table XVI.

Table XVI

Sample No.	Ml HCl Sample	Ml HCl Blank	Mgm Canth Volum	% Canth Volum
1	4.89	10.61	61.3	0.613
2	4.79	"	61.6	0.616
3	4.80	"	61.4	0.614
**4	2.43	"	86.5	0.865

HCl = 0.1078N
**Residue obtained from unwashed benzene eluate.

Determination No. 11

The extract corresponding to 50 Gm of Cantharides (.721%) was prepared (page 33) and four 25 ml aliquots of this extract, representing 5 Gms of drug, were used. A weighed amount of cantharidin was added to two of the extracts, all of them were allowed to evaporate at room temperature to about 5 ml and poured into columns containing 20 ml of 78% sulfuric acid as the internal phase. Four hundred ml of petroleum ether (b.p. 30°-75°) was used as first eluent, 90 ml of thiophene-free

*The remainder of the elutions, treatment of eluates and titrations were carried out in this manner.

benzene as the second, and another 200 ml volume of the latter was used for the final elution. The 200 portions of benzene eluates were treated as described in determination No. 10. Results are shown in Table XVII.

Table XVII

Sample No.	Ml HCl Sample	Ml HCl Blank	Mgm Total Canth	Mgm Canth Added to Extract	Total Minus Added Canth	% Canth
1	4.27	10.25	63.2	32.6	30.6	0.612
2	4.24	"	63.6	32.9	30.7	0.614
3	7.26	"	31.6	"	31.6	0.632
4	7.24	"	31.8	"	31.8	0.636

HCl = 0.1078N

Determination No. 12

The columns were run using extract and in the same manner as described in Determination No. 11. In this case, however, instead of adding pure cantharidin to the extracts, it was added to the 200 ml portions of benzene eluates before washing them with water. From then on the process was identical to the above determination. Results are shown in Table XVIII.

Table XVIII

Sample No.	Ml HCl Sample	Ml HCl Blank	Mgm Total Canth	Mgm Canth Added to Eluate	Total Minus Added Canth	% Canth Volum	Mgm Canth Grav	% Canth Grav
1	3.75	10.27	69.0	34.3	34.7	0.694	*	
2	4.29	"	63.2	30.0	33.2	0.664	*	
3	7.05	"	34.1	-	34.1	0.682	*	
4	7.00	"	34.6	-	34.6	0.692	*	
*5	6.78	"	36.5	-	36.5	0.730	42.0	0.840

HCl = 0.1078N
*Residue obtained from unwashed benzene eluate.

Determination No. 13

Fifty grams of powdered Cantharides was moistened with 2 ml of concentrated hydrochloric acid and extracted for ten hours in a Soxhlet apparatus with 150 ml of a mixture of two parts of benzene and one part of petroleum ether (N.F. solvent of extraction). The extract was made up to a volume of 250 ml with the same solvent in a volumetric flask and 50 ml aliquots, corresponding to 10 Gm of drug were used. Three of the aliquots were concentrated to about 5 ml and poured into partition columns containing 20 ml of 78% sulfuric acid as the internal phase. The elution and treatment of the eluates was as in Determinations 10 to 12. The residue from the fourth aliquot was purified and dried as directed in the National Formulary, and weighed. Results are shown in Table XIX.

Table XIX

Sample No.	Ml HCl Sample	Ml HCl Blank	Mgm Canth Volum	% Canth Volum	Mgm Canth Grav	% Canth Grav
1	4.72	10.23	58.27	0.583	•	•
2	4.69	"	58.59	0.586	•	•
3	4.75	"	57.95	0.580	•	•
*4	4.72	"	58.27	0.583	70.0	0.700

HCl * 0.1078N
*Residue purified according to the N. F.

Determination No. 14

Four 15 Gm samples of Cantharides were extracted as directed in the National Formulary. One hundred ml of the extracts were concentrated to about five ml by evaporating at room temperature, at first, and later applying heat and a current of air. Three of the residues obtained were purified by passing them through columns containing 20 ml of 78% sulfuric acid as the internal phase. The elution of the columns and treatment of the eluates, was carried out as in Determination 10 to 13. One of the residues was purified according to the N.F. Results are shown in Table XX.

Table XX

Sample No.	Ml HCl Sample	Ml HCl Blank	Mgm Canth Volum	% Canth Volum	Mgm Canth Grav	% Canth Grav
1	4.80	10.23	57.43	0.574	•	•
2	4.78	"	57.63	0.576	•	•
3	4.83	"	57.10	0.571	•	•
*4	4.76	"	57.84	0.578	79.9	0.799

HCl * 0.1078N
*Residue purified according to the N. F.

Determination No. 15

Four 15 Gm portions of Cantharides were moistened with 2 ml of concentrated hydrochloric acid and extracted with 150 ml of chloroform in a Soxhlet apparatus for six hours. Three one-hundred ml aliquots were evaporated to dryness at room temperature and then dissolved in five ml of benzene. Three columns were packed and eluted as in Determinations 10 to 14. The treatment of the eluate was also as indicated in the determination mentioned. One of the residues was purified following the N. F. procedure. Results are shown in Table XXI.

Table XXI

Sample No.	Ml HCl Sample	Ml HCl Blank	Mgm Canth Volum	% C_{a}nth Volum	Mgm Canth Grav	% C_{a}nth Grav
1	3.93	10.23	66.62	0.666	"	"
2	4.22	"	63.6	0.636	"	"
3	4.00	"	65.9	0.659	"	"
*4	3.96	"	66.31	0.663	73.2	0.732

HCl = 0.1078N
*Residue purified according to N. F.

Determination No. 16

Four 10 Gm portions of Cantharides were saturated with 2 ml of concentrated hydrochloric acid and extracted for 6 hours with 150 ml of chloroform in a Soxhlet apparatus. Each extract was evaporated to dryness by applying mild heat and a current of air. Three of them were purified by dissolving in 5 ml of benzene and chromatographing as in Determinations 10

to 15. The treatment of the eluates and of the cantharidin obtained from them, was also as in the previous determinations, except that twenty ml of alkali was used instead of ten ml. One residue was purified according to the N. F. Results are shown in Table XXII.

Table XXII

Sample No.	Ml HCl Sample	Ml HCl Blank	Mgm Canth Volum	% Canth Volum	Mgm Canth Grav	% Canth Grav
1	14.45	20.45	63.5	0.635		
2	14.37	"	64.3	0.643		
3	14.33	"	64.8	0.648		
*4	14.40	"	64.0	0.640	68.7	0.687

HCl = 0.1078N
*Residue purified by N.F.

Determination of Cantharidin by a Sodium Methylate Titration Together with a Pyridine-Sodium Hydroxide Titration

1. Reagents

   a. Dry methanol

      Preparation: Five grams of dry magnesium turnings and 5 g of resublimed iodine were placed in a 2 liter round bottom pyrex flask and the latter was fitted with a reflux condenser. Fifty to seventy-five ml of methyl alcohol was added through the condenser and the mixture was warmed on the water bath until the iodine disappeared. Nine hundred ml of methyl alcohol was then added and the mixture was boiled for 30 minutes under reflux. The product was distilled, taking precautions to exclude moisture.*

   b. Sodium methylate solution, 0.1N

      Preparation: About 2.3 Gm of metallic sodium was dissolved in one liter of dry methanol, C.P. The solution was kept in a glass bottle fitted with an Ascarite tube.

   c. Phenolphthalein in acetone, 1%

   d. Aqueous sodium hydroxide, 0.1N

   e. Hydrochloric acid, 0.1N

   f. Pyridine, C.P.

2. Apparatus

   a. microburettes, 10 ml

   b. pipettes, 10 ml, 25, 50 ml

   c. Erlenmeyer flasks, 125 ml

3. General Procedures

   a. Direct Titration

      Determination of total acidity. The samples were

*Vogel, A. I., A Textbook of Practical Organic Chemistry, Longmans, Green, and Co., Inc., New York, 1948.

dissolved in the pyridine and after being set aside for one-half hour, the solution was titrated with the standard sodium hydroxide from a microburette, using three drops of the phenolphthalein indicator.

Determination of anhydride plus free acid. The weighed samples of cantharidin were dissolved in dry methanol, warming gently to effect dissolution. When cool the solution was titrated with the standard methylate solution from a microburette, using three drops of the phenolphthalein indicator.

b. Residual Titration

Determination of total acidity. The samples were dissolved in the pyridine and set aside for one-half hour. The sodium hydroxide reagent was then added, using a pipette, followed by ten ml of distilled water, and allowed to stand for another one-half hour. The excess sodium hydroxide was titrated with standard hydrochloric acid, from a ten ml microburette, using three drops of phenolphthalein indicator. A blank was run at the same time.

Determination of Anhydride plus Free Acid. The samples were dissolved in methanol by gentle warming. The sodium methylate was added by means of a pipette. After half an hour, the excess methylate was titrated with the 0.1N hydrochloric acid, using three drops of the phenolphthalein indicator.

4. Analytical Determinations on Pure Cantharidin

Only the amount of reagents will be given, since the manipulations are described in the section of general procedures. The cantharidin used consisted of a white granular solid, m.p. 220.5°-221°.

a. Direct Titration

Determination No. 1

A sample weighing 52.5 mgms was titrated directly to determine the total acidity. Twenty-five ml of pyridine was used. The detection of the end-point was uncertain.

Another sample weighing 63.9 mgms was used to determine the anhydride plus the free acid. Twenty-five ml of dry methanol was used as a solvent for the sample. In these determinations each pair of samples constitutes one analysis. Data and results are shown in Tables XXIIIa and XXIIIb, respectively.

Table XXIIIa

	Total Acidity		Anhydride plus free acid	
Prs of Sample	Wt. Sample	ml NaOH	Wt. Sample	ml methylate
1	52.5 mgm	4.37	63.9 mgm	2.12

NaOH = 0.1209N
CH_3ONa = 0.1509N

Calculations:

Total Acidity:

Vol NaOH x N NaOH = meq. NaOH = meqs total acidity

$$\frac{4.37 \times 0.1209}{.0525} = 10.06 \text{ per 1 Gm sample}$$

Anhydride plus free acid:

Vol. methylate x N methylate = meq. methylate
= meq. anhydride
= plus free acid.

$\frac{2.12 \times 0.1509}{.0525}$ = 5.01 per 1 Gm Sample

meq. anhydride = meq. total acidity - (meq. anhydride plus free acid)

10.06 - 5.01 = 5.05

mgm cantharidin = M. W. x meq.

= 196.2 x 5.05 = 990.08

Table XXIII$_b$

Prs	Millieq. per Gm sample NaOH (a)	$NaOCH_3$ (b)	-CO-O-CO- (a-b)	mgm canth. (196.2 (a-b)	error in mgm
1	10.06	5.01	5.05	990.08	9.92

b. Residual Titration

Determination No. 1

Total acidity: The samples were dissolved in 25 ml of pyridine and ten ml of 0.1 N sodium hydroxide was used.

Anhydride plus free acid: Twenty-five ml of dry methanol was used as solvent for the samples. Seven and one-half ml of sodium methylate solution was used. The data are given in Table XXIV

Table XXIV

	Total Acidity	Anhydride plus free acid
Wt. Sample	48.4 mgms	55.7 mgms
Vol HCl	6.30 ml	7.47 ml
Vol HCl (blank)	10.89 ml	10.20 ml
Normality HCl	0.1112	0.1112

Calculations and Results:

Total Acidity:

Vol HCl (blank) : 10.89
- Vol HCl (sample : 6.30
4.59

4.59 x 0.1112 = 0.1112 = meq. total acidity

$\frac{0.5104}{0.0484}$ = 10.55 meq. total acidity per 1 Gm sample

Anhydride plus free acid:

Vol HCl (blank) : 10.20
- Vol HCl (sample) : 7.47
2.73

2.73 x 0.1112 = 0.3036 = meq. anhy. free acid

$\frac{0.3036}{0.0557}$ = 5.47 = meq. anhy. free acid per 1 Gm sample

10.55 - 5.45 = 5.10

5.10 x 196.2 = 1000.62 mgms.

Determination No. 2

The procedure for this determination was exactly as for determination No. 1, except that ten ml of sodium methylate was used in the determination of the anhydride plus the free acid. Data and results are shown in Tables XXVa and XXVb, respectively.

Table XXVa

	Total Acidity			Anhydride plus Free Acid		
Pairs of Samples	Wt. sample in mgms	ml HCl	Ml HCl blank	Wt. sample in mgms	ml HCl	Ml HCl blank
1	75.9	3.60	10.72	62.1	10.35	13.62
2	73.9	3.67	"	60.8	10.72	"
3	53.6	5.65	"	73.0	9.88	"
4	63.2	4.71	"	72.8	10.10	"

HCl = 0.1112N

Table XXVb

Prs. of Samples	Millieq per Gm Sample NaOH (a)	$NaOCH_3$ (b)	-CO-O-CO- (a-b)	mgm Canth 196.2 (a-b)	Error in mgm
1	10.43	5.86	4.57	896.6	-103.4
2	10.61	5.30	5.31	1041.8	+ 41.8
3	10.52	5.69	4.83	947.5	- 52.5
4	10.59	5.38	5.21	1032.8	+ 32.8

Determination No. 3

Total acidity: The samples were dissolved in ten ml of pyridine and ten ml of aqueous sodium hydroxide was added, followed by ten ml of water. A precipitate was formed which did not dissolve even on addition of more water.

Anhydride plus free acid: (Direct Titration) The samples were dissolved in fifty ml of dry methanol. and titrated directly with the sodium methylate. Data and results are shown in Tables XXVIa and XXVIb, respectively.

Table XXVIa

Pairs of Samples	Total Acidity Wt. sample mgms	Ml HCl (Sample)	Ml HCl (Blank)	Anhydride Plus Free Acid Wt sample mgms	Ml Methylate
1	70.3	4.06	10.40	61.9	2.17
2	71.1	4.04	10.40	58.0	2.06

HCl = 0.1112N, CH_3ONa = 0.1481N

Table XXVIb

Pairs of Samples	Milliequivalents per Gm Sample NaOH (a)	$NaOCH_3$ (b)	-CO-O-CO- (a-b)	mgm Canth. 196.2 (a-b)	Error in mgm
1	10.02	5.19	4.83	947.75	-52.25
2	9.95	5.26	4.69	920.18	-19.82

Determination No. 4

Total acidity: The samples were dissolved in ten ml of pyridine and after the time indicated in the general procedure, ten ml of the sodium hydroxide solution was added followed by the same volume of water. Here again, a precipitate was obtained.

Anhydride plus free acid: The samples were dissolved in 25 ml of dry methanol. Ten ml of sodium methylate reagent was added and the procedure for the residual titration was carried out. Data and results are shown in Tables XXVIIa and XXVIIb, respectively.

Table XXVIIa

Pairs of Samples	Total Acidity Wt Sample mgms	Ml HCl Sample	Ml HCl Blank	Anhydride Plus Free Acid Wt Sample mgms	Ml HCl Sample	Ml HCl Blank
1	65.8	4.77	10.71	58.5	10.75	13.35
2	53.2	5.90	10.71	54.8	10.95	13.35
3	59.0	5.75	10.54	61.8	8.95	11.75
4	63.0	4.93	10.54	64.2	8.75	11.75

HCl = 0.1077N

Table XXVIIb

Pairs of Samples	Milliequivalents per Gm Sample NaOH (a)	$NaOCH_3$ (b)	-CO-O-CO- (a-b)	Mgm Canth 196.2 (a-b)	Error in mgms
1	10.13	4.94	5.19	1018.2	+18.2
2	10.05	4.87	5.18	1016.3	+16.3
3	8.74	4.87	3.87	759.29	-240.71
4	9.59	5.03	4.56	894.67	-105.33

Determination No. 5

The cantharidin was recrystallized from chloroform. Its melting point was 216.5°. A sample weighing 0.2452 Gm was dissolved in 100 ml of chloroform. Four 20 ml aliquots, each corresponding to 50.8 mgms of cantharidin, were poured into dry 125 ml Erlenmeyer flasks. The solvent was removed using mild heat from the steam bath and a current of air. The residues were dried in the oven at 60°C and titrated by the residual method, using ten ml of pyridine, ten ml of sodium hydroxide, and ten ml of water solution for the determination of the total acidity. Twenty-five ml of methanol and ten ml of sodium methylate solution was used for the determination of the anhydride plus the free acid. Data and results are shown in Tables XXVIIIa and XXVIIIb, respectively.

Table XXVIIIa

Pairs of Samples	Total Acidity: Mgm Canth in Aliquot	Total Acidity: Ml HCl (Sample)	Total Acidity: Ml HCl (Blank)	Anhydride Plus Free Acid: Mgm Canth in Aliquot	Anhydride Plus Free Acid: Ml HCl (Sample)	Anhydride Plus Free Acid: Ml HCl (Blank)
1	50.8	6.30	10.64	50.8	9.91	11.77
2	50.8	6.00	10.64	50.8	9.91	11.77
*3	53.32	6.89	10.68	53.32	9.40	11.80

HCl ≡ 0.1077N

*Pair No. 3 was obtained from a different aliquot and in the determination of the total acidity, 25 ml of pyridine was used.

Table XXVIIIb

Pairs of Samples	Milliequivalents per Gm Sample NaOH (a)	$NaOCH_3$ (b)	-CO-O-CO (a-b)	Mgm Canth 196.2 (a-b)	Error in mgms
1	0.4674	0.2003	0.2671	52.4	+1.6
2	0.4987	0.2003	0.2994	58.7	-7.9
3	0.5759	0.2585	0.2574	50.50	-2.8

Determination No. 6

Recrystallised cantharidin, m.p. 216.5°, was used. The titration was carried out as in determination No. 5, except that nitrogen was used to exclude air during the procedure. Data and results are shown in Tables XXIXa and XXIXb, respectively.

Table XXIXa

Pairs of Samples	Total Acidity: Mgm Canth in Aliquot	Ml HCl (sample)	Ml HCl (blank)	Anhydride Plus Free Acid: Mgm Canth in Aliquot	Ml HCl Sample	Ml HCl Blank
1	50.6	6.04	10.70	50.6	6.84	9.17
2	50.6	6.04	10.70	50.6	6.84	9.17
3	50.4	6.04	10.75	50.4	6.88	9.21
4	50.4	6.06	10.75	50.4	6.88	9.21

HCl = 0.1078N

Table XXIXb

Pairs of Samples	Milliequivalents per Wt. Sample Used NaOH (a)	$NaOCH_3$ (b)	-CO-O-CO (a-b)	Mgm Canth 196.2 (a-b)	Error in mgms
1	0.5023	0.2512	0.2511	49.27	-1.3
2	0.5023	0.2512	0.2511	49.27	-1.3
3	0.5077	0.2512	0.2565	50.3	-0.1
4	0.5056	0.2512	0.2544	49.9	-0.5

Determination No. 7

A column was prepared using 20 ml of 66% sulfuric acid as internal phase. A 133.1 mgm sample of cantharidin was dissolved in a few ml of benzene and placed in the column, which was eluted first with 300 ml of petroleum ether (b.p. 30°-75°) and then with 250 ml of benzene (thiophene-free). The benzene eluate was collected in a 250 ml volumetric flask and divided in two 125 ml portions. Each portion was concentrated in vacuo to about 15 ml, poured into tared Erlenmeyer flasks and the rest of the solvent was removed by a current of air. The residues which remained were dried in the oven at 60C for half an hour and weighed. One of the residues was titrated to determine the total acidity. Ten ml of pyridine and ten ml of alkali was used. The material in the other flask was used to determine the anhydride plus the free acid. Twenty-five ml of methanol and ten ml of sodium methylate was used. Data and results are shown in Tables XXXa and XXXb, respectively.

Table XXXa

Pairs of Samples	Total Acidity Mgm Canth in each Fraction	Ml HCl Sample	Ml HCl Blank	Anhydride Plus Free Acid Ml HCl Sample	Ml HCl Blank
1	66.6	5.20	10.79	8.74	11.75

HCl = 0.1077N

Table XXXb

Pairs of Samples	Milliequivalents per Wt. Sample Used NaOH (a)	$NaOCH_3$ (b)	-CO-O-CO- (a-b)	Mgm Canth 196.4 (a-b)	Error in mgm	Mgm Canth Grav
1	0.6020	.3242	0.2778	54.5	12.1	63.2 70.5

5. Analytical Determinations on Cantharidin Obtained from Extract of Cantharides

All the titrations in this section were done by the residual technique.

Determination No. 1

The chloroform extract from 50 Gm of Cantharides (assay 0.581%) was prepared as described on page 33 Four aliquots corresponding to 10 Gm of drug were concentrated to a volume of about 5 ml and poured into columns containing 20 ml of 66% sulfuric acid and the internal phase. The columns were first eluted with 400 ml of petroleum ether (b.p. 30°-75°), followed by 250 ml of benzene (thiophene-free), collecting the eluates in separate beakers. The benzene eluates were concentrated in vacuo to about 15 ml and poured into tared Erlenmeyer flasks. The remainder of the solvent was removed with the aid of an air current and low heat from a heating mantle. The residues remaining in the flasks were obviously impure, consisting of a yellowish crystalline material. They were dried in the oven at 60°C for one-half hour and weighed. The anhydride plus the

free acid was determined in two of the residues. Twenty-five ml of methanol was used to dissolve the samples. Ten ml of sodium methylate was added and the excess alkali was titrated with 0.1N hydrochloric acid. The material in the other flasks was titrated to determine the total acidity. Ten ml of pyridine was used as a solvent and ten ml of 0.1N sodium hydroxide was added. The excess alkali was titrated with 0.1N hydrochloric acid. An amorphous solid was formed which would not dissolve even after the addition of 20 ml of water. The mixture in all the flasks was opalescent. Data and results are shown in Tables XXXIa and XXXIb, respectively.

Table XXXIa

Pairs of Samples	Total Acidity		Anhydride Plus Free Acid	
	Ml HCl Sample	Ml HCl Blank	Ml HCl Sample	Ml HCl Blank
1	4.39	10.73	7.92	11.72
2	4.44	10.73	7.99	11.72

HCl = 0.1077N

Table XXIb

Pairs of Samples	Milliequivalents per Wt. of Sample Used				
	NaOH (a)	$NaOCH_3$ (b)	-CO-O-CO- (a-b)	Mgm Canth 196.2 (a-b)	% Canth
1	0.6821	0.4093	0.2729	53.97	0.540
2	0.6768	0.4017	0.2751	53.54	0.535

Determination No. 2

Extract of Cantharides corresponding to 20 Gm of drug was prepared as described on page 33. A sample of pure cantharidin weighing 138.8 mgm was added to it, and after concentrating to about 5 ml, was poured into a column containing 20 ml of 66% sulfuric acid as the internal phase. The first eluant was 250 ml of petroleum ether (b.p. 30°-75°) followed by an equal volume of benzene (thiophene-free). The latter eluate was divided in two equal portions and poured into tared Erlenmeyer flasks. The solvent was removed almost completely in vacuo and the last traces were removed by a current of air. The material which remained in each flask was obviously impure. They were dried in the oven at 60°C for half an hour and weighed. Finally one portion was titrated for total acidity and the other was used to determine the anhydride plus the free acid. Two other columns (No. 2 and 4) were run in the same manner but no cantharidin was added to the extract. A weight of 121.2 mgm of cantharidin was added to another portion of extract and used for another column (No. 3). Data and results are shown in Tables XXXIIa and XXXIIb respectively.

Table XXXIIa

Pairs of Samples	Total Acidity		Anhydride Plus Free Acid	
	Ml HCl Sample	Ml HCl Blank	Ml HCl Sample	Ml HCl Blank
1	8.91	21.64	5.10	11.77
2	3.85	10.75	8.12	11.77
3	9.34	21.49	5.28	11.76
4	3.30	10.55	8.10	11.85

HCl @ 0.1077N

Table XXXIIb

Pairs of Samples	Milliequivalents Per Wt. Sample Used			Total Mgm Canth	Canth Added per Fraction	Total Canth Minus Added	% Canth
	NaOH (a)	$NaOCH_3$ (b)	-CO-O-CO (a-b)	Canth 196.2 (a-b)			
1	1.3710	0.7184	.6526	128.04	69.4	58.6	0.586
2	0.7431	0.3931	.3500	68.67	-	68.7	0.687
3	1.3086	0.6979	.6107	119.8	60.6	59.2	0.592
4	0.7808	0.4039	.3769	73.94	-	73.9	0.739

Proposed Assay for Cantharides

As a result of the observations made and conclusions reached during the present investigation, the following assay is proposed for the cantharidin content of Cantharides.

Preparation of the extract. Mix thoroughly a sample of powdered Cantharides weighing approximately 50 Gm with 2 ml of concentrated hydrochloric acid, moistened with chloroform, place in an extraction thimble, and extract for 6 hours in a Soxhlet extractor using about 150 ml of chloroform. Transfer the chloroform extract to a 250 ml volumetric flask, rinse the extraction flask with chloroform, add the rinsings to the flask, and make up to 250 ml with chloroform. Transfer four fifty ml aliquots to 100 ml beakers and allow to evaporate to dryness at room temperature. Mild heat from a steam bath and a current of air may be applied to hasten the evaporation. Just before pouring into the columns, dissolve each of the dry extracts in 5 ml of benzene.

Packing the column. Place 30 Gm of silicic acid in a mortar with 20 ml of 78% sulfuric acid. After thorough mixing, add enough petroleum ether (b.p. 30-75) to form a slurry and pack this into a chromatographic tube (2 cm x 35 cm fitted with a wad of glass wool at its lower end), by means of a glass plunger, taking care to exclude air bubbles. Enough force should be exerted to make the petroleum ether flow at the rate of five to eight drops per minute.

Elution of the column. When the silicic acid mixture is well packed, and the excess petroleum ether has drained off, add the benzene solution of the extract carefully, avoiding disturbance of the surface of the column. Use a rubber policeman to transfer all the material from the beaker, and then rinse it with small (a few ml) portions of petroleum ether (b.p. 30-75°). After all the liquid has passed into the column, begin the elution with petroleum ether. To maintain a continuous "head" of eluent, a flask with a glass tube attached to it is used in an inverted position in the column. Continue the elution with petroleum ether until 400 ml of eluate has been collected. Then use benzene (thiophene-free) as eluant, discarding the first 90 ml of benzene eluate and collecting the 200 ml in a beaker. Wash this benzene eluate three times with 75 ml portions of distilled water (or until the washings are no longer acidic to litmus paper). Allow the washed benzene layer to dry overnight over anhydrous sodium sulfate, filter, collect the filtrate in a 250 ml beaker and evaporate to dryness, applying low heat and a current of air. The cantharidin residue which remains is titrated.

Titration of Cantharidin. To the beaker containing the residue add 10 ml of pyridine, allow to stand for half an hour, then add 10.00 ml of 0.1N aqueous sodium hydroxide and allow to stand for another half an hour. Titrate the excess alkali

with 0.1 N hydrochloric acid using a 1% solution of phenolphthalein in acetone as indicator. Run a blank with each set of samples. The results are calculated from the following formula:

(Volume HCl required for blank - volume HCl for sample)
(N HCl) (98.1) = mgm Cantharidin in 10 Gm

$$\% \text{ Cantharidin} = \frac{\text{mgm cantharidin}}{10} \times 100$$