I and II. STUDIES ON *DICHROA FEBRIFUGA*

III. A METHOD FOR ESTIMATING CHLORIDE

IV. STUDIES ON *CAMBARUS CLARKII*

Thesis by

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Summary

Two crystalline, optically active alkaloids, named febrifugine and isofebrifugine, have been isolated from extracts of the roots of *Dichroa febrifuga*. The method of isolation involves selective adsorption, and is capable of showing the presence of a few milligrams of alkaloid. One of these alkaloids, febrifugine, is a powerful antimalarial, having more than 100 times the activity of quinine against bird malaria. Studies on the structures of these alkaloids indicate them to be 3-substituted-4-quinazolones of empirical formula $C_{16}H_{19}O_3N_3$.

A method is described for determining the chloride concentration in an aqueous solution by measuring the potential of a cell composed of a reference half cell, and a silver, silver chloride, chloride ion half cell. The determination takes *ca.* 2 to 3 minutes, is made with 10 ml. of solution, and is reproducible to *ca.* 4% for chloride concentrations above 10^{-4} formal.

Experiments on the fast closer system of *Cambarus clarkii* are described, and show that magnesium ion blocks the muscle contraction without blocking the muscle action potential. Other experiments are described, indicating that the twitch height of the muscle contraction is dependent on the rate of perfusion, when the perfusing solution is allowed to flow through the propodite.

Acknowledgements

My deepest thanks go to the many people and institutions which made possible the researches described in this thesis. The investigations of Parts I and II were carried out in part under a contract recommended by the Committee on Medical Research between the Office of Scientific Research and Development and the California Institute of Technology, and in part under a Research Grant from the United States Public Health Service. Most of the D. febrifuga root material and technical assistance was furnished by Eli Lilly and Company. Botanically identified D. febrifuga roots and leaves were furnished by the Abbott Laboratories. The pharmacological results reported here were obtained from Dr. K.K. Chen of Eli Lilly and Co., Dr. A.P. Richardson of E.R. Squibb and Sons, Dr. E.K. Marshall, Jr. of Johns Hopkins University School of Medicine, and Dr. L.H. Schmidt of Christ Hospital, Cincinati.

My especial thanks go to Dr. J.B. Koepfli who introduced me to a very exciting branch of chemistry and who guided the largest part of the work described in this thesis.

I would like to express my deep appreciation to Prof. Howard J. Lucas who has been a true friend and inspiration to me throughout my studies at the Institute. I regret that the recent war brought to a sudden close the researches we had only begun.

To Prof. C.A. Wiersma of the Division of Biology I would express my regrets that I was not able to spend more time in his laboratories, studying a branch of science which I found most fascinating.

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PART I.- ISOLATION OF POTENTIAL ANTIMALARIALS FROM DICHROA FEBRIFUGA.

Section 1. Introduction

In the search for new antimalarials carried out in the United States during the recent war, interest in indigenous Chinese drugs was stimulated by the appearance of two papers in 1941 by S. K. Liu[1,2]. In these papers Liu gives considerable details regarding the isolation of a crystalline alkaloid, sinine, from Fraxinus malacophylla Hemsley, and gives pharmacological data indicating good antimalarial activity (presumably from the alkaloid) in extracts of the plant.

In the closing paragraph of each of his papers Liu mentions another anti-malarial herb also found in Yunnan and called "Chunine tree", the leaves of which contain an effective ingredient named "Chunine". He says "the fever relieving action seems stronger than (that of) the sinine leaf".

Reputable workers in China[3], Great Britain[4,5], and the United States[6] were unable to duplicate Liu's work with Fraxinus. No trace of alkaloids could be found, and no anti-plasmodial activity could be demonstrated. This of course threw considerable doubt on the veracity of

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1. Liu, Shao-Kwang, Chang, Yao-teh, Ch'un, Tz'-e-kwang, and Tan, Shih-chieh, Chinese Medical Jour. 59, 575-77 (June 1941).
2. Liu, Shao-Kwang, National Medical Jour. of China, 27, 327 (1941).
3. Letters from Dr. Needham to Dr. Work in England.
4. Tomkin and Work, Nature, 156, 630 (1945).
5. Mukerji, Nature, 158, 170 (1946).
6. Koepfli and Mead, Interim Report to Board for the Coordination of Malarial Studies, June 1945.

any claims made by Liu.

However, samples of Chunine obtained from Liu were found to possess considerable antimalarial activity. Chunine is the proprietary name given by him to the ground leaves of a plant identified as "from Saxifragaceae". Further botanical identity is lacking although some Chinese workers (see for example Chang-Shaw Jang,[8]) think it is the leaves of some Ch'ang Shan, probably D. Febrifuga. The material was tested by Dr. Richardson for the Abbott Laboratories and found to have in the F-1 test a Q of 0.1 to 0.25*. They report (see 6) obtaining a fraction from Chunine with a Q of 5 to 10 in F-1. Koepfli and Mead[6] working independently also were able to obtain a fraction of Q 4 to 8. Both groups agreed that the active fraction did not contain alkaloids. A scarcity of material and the lack of botanical identity made a thorough investigation of Chunine impossible.

During the first half of 1943 Dr. Joseph Needham, on a British scientific mission in China, reported [3] that the Chinese workers were quite interested in another indigeneous drug called "Ch'ang Shan". This appeared to be a mixture of plants, including Dichroa febrifuga

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* Q is the "quinine equivalent" which indicates the activity of the material relative to the activity of quinine. The letter and number of the test (F-1 in this case) indicate the laboratories in which the test was carried out, and the conditions employed (species of Plasmodium, test animal, method of administering the drug, etc). Most of the testing for the isolation work described later in this thesis was carried out under the supervision of Dr. K.K. Chen at Eli Lilly and Company. The test animal was the duck, infected with P. lophurae; and, in the main, samples were injected intravenously, t. i. d. For a discussion of the quinine equivalent and the methods for testing see Wiselogle, Survey of Antimalarial Drugs, 1941-1945, Edwards (1947).

and Clerodendron yunnanense (a detailed discussion will be given later) and was actually in use in the Chinese Army. It is said to be common in Szechuan and Kweichow. According to Dr. Yang Shih-Hsien, plant biochemist at the National United Southwestern University (formed from Peiping, Ching-hya, and Nankai Universities), this drug contains only glucosides. Other Chinese workers, Dr. Tang Tang-Han of the Pharmacological Institute, West China Union University, Chengtu, and Dr. Yeh Chiao of the Organic Chemistry Department, Wuhan University, Chiating, confirmed the absence of any alkaloids in Ch'ang Shan.

Ch'ang Shan was relatively available, the Chinese workers had considerable hope for it, Chunine might be the leaves of the same plant, and tests indicated Q' s of 0.05 to 0.15 for the crude ground roots. These facts made an investigation of Ch'ang Shan in this country appear to be a promising field of research.

Since Ch'ang Shan is a generic name, and more than six different plants are known by this name in different parts of China, the question of botanical identity was a serious one during the early work on Ch'ang Shan. The supply of material was limited to such an extent that the isolation and especially the structure determination of the active principle might not be complete before additional plant material would be needed. With no certainty regarding the botanical identity, it might be very difficult to get additional samples of the same material. Furthermore, even if the isolation and structure determination could be completed, the value of the work as a contribution to the permanent literature would be considerably lessened if the identity of the plant was unknown.

Fortunately, by the middle of 1945 the botanical identity of Chungking Ch'ang Shan had been quite well established as Dichroa febrifuga, both by comparisons made at the Smithsonian by Dr. Walker, and by inference from reports received from China. A brief summary of two communications from China, one by Pei Chien[7] and the other by Chang-Shaw Jang[8], is given below, to indicate the salient facts concerning Ch'ang Shan.

"The antimalarial virtue of Ch'ang Shan was discovered about four thousand years ago by Emperor Sheng Nung, the King of Herbs". The root is recommended, but the leaves and shoots (called Shu Chi) are also effective against malaria. On account of its unpleasant smell and stronger irritant action to the stomach, Shu Chi is not so often used. Most authorities in China believe this Ch'ang Shan to be a Saxifragaceae, Dichroa febrifuga Lour. Because of the appearance of the roots it is also known as Huang (yellow) Ch'ang Shan and Chi Chi Kuo (chicken-bone) Ch'ang Shan. (Other Chinese names include Hengshan, Hytsao, Chi-shih-tsao, Ya-shih-tsao.) This Ch'ang Shan is a perennial semi-evergreen shrub, something more than 9 feet high with many branches. The leaves are "simple, opposite, with or without fine hairs on the vein above and beneath the leaf, oblong-lanceolate, about

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7. Chien, The Chinese Anti-Malarial Drug--Changshan, sent to Abbott Laboratories by A.G. Saunders from Sino-British Science Cooperation Office, Chungking (June 16, 1945).
8. Jang, Chinese Medical Jour. April-June, 1944.

8 inches long, acute on the base up to the petiole, with teeth along the margin". The plant bears a small, light blue flower and a blue, ellipsoidal berry. It grows mostly in the southern provinces of China along the Yangtze river, and is also found in India, Java, and the Phillipine Islands. It is abundant in Szechuan Province, but "it is said that those grown in Kweichow are more precious".

In addition to this yellow or chicken-bone Ch'ang Shan there are several others. Pei (white) Ch'ang Shan is identified as Mussaenda divaricata Hutchinson. This Ch'ang Shan and the Cinchona tree belong to the same family, Rubiaceae, and it is not too surprizing to find antimalarial activity reported.

Hou (Japanese) Ch'ang Shan has been identified as Orixa japonica Thunberg. It belongs to the family Rutaceae, and grows in Korea, Japan, and in China south of the Yangtze and along the sea coast. "Several Chinese botanists misused Orixa japonica as the scientific name of yellow Ch'ang Shan."

Haichow (name of a city) Ch'ang Shan has been identified as Clerodendron trichotomum Thunberg. This plant belongs to the family Vergenaceae, and is a small tree, about 30 feet high. It is very ordinary in Kiangsu and Chekiang, but rare in the southwest provinces. There is a variety in Szechuan Province.

Reported from Kunming in addition to yellow Ch'ang Shan, called there Shu or T'u Ch'ang Shan, are Chi Gu Ch'ang Shan, an Alstonia from the family Apocyanaceae; and Dien (the old name for Yunnan) Ch'ang Shan, Clerodendron yunnanense from the Verbenaceae family.

Still another, Tou (local) Ch'ang Shan, is reported as Hydrangea thunbergii.

The literal translation of Ch'ang Shan is "mountain herb". The above discussion indicates just how apropos this translation is. A sample of Ch'ang Shan obtained from one part of China may be very different from one obtained in another part. Furthermore, it is by no means certain that a given sample of Ch'ang Shan is botanically homogeneous.

The isolation experiments described in this thesis were carried out for the most part with Ch'ang Shan obtained from Chungking, and fairly well identified as D. febrifuga. It is to be emphasized, however, that the samples were not identified at the time of collection, but were in the main bought in the open market in Chungking from Chinese herbalists, either as chopped roots or as whole roots. Furthermore, any chopped root material may have been treated with hot water or steam in order to facilitate chopping. Such a treatment may very well alter the active principle present in the roots.

The amount of Ch'ang Shan available in this country has been strictly limited. For the sake of a complete record it seems desirable to list here the material available for the work reported in this thesis:

Lot A:- five pounds of chopped roots sent to the N.R.C. by Dr. P.Z. King of the National Health Administration, Chungking. This material was labelled Ch'ang Shan, D. febrifuga.

Lot B:- about 300 pounds consisting of six pooled samples which Eli Lilly and Company obtained from Chinese drug stores in various parts of this country and abroad. This material was mostly in the form of chopped roots, and although bought as Ch'ang Shan, there is no knowledge concerning the original source or its botanical iden-

tity. This material is referred to as San Francisco Ch'ang Shan and had a Q of 0.08 in I-5 test.

Lot C:- about 500 pounds of whole roots purchased in Chungking by an agent of Eli Lilly and Company. A sample of the powdered roots had Q 0.16 in I-5. Most of the work reported in this thesis was done with this material.

Lot D:- approximately 100 pounds of whole root material labelled Ch'ang Shan, D. febrifuga sent to the N.R.C. by Dr. P.Z. King of the National Health Administration of Chungking. Presumably this material was bought in the open market in Chungking.

In summary then, at the beginning of 1945 indigenous Chinese herbs appeared to offer at least two fruitful avenues of research aimed at finding new antimalarials. The first was Chunine, the leaves of a plant of obscure botanical identity (possibly D. febrifuga) containing an active principle probably non-alkaloidal in nature, and being available in quantities far too small for a satisfactory investigation. The second was a Ch'ang Shan fairly well identified as the roots of D. febrifuga, available in sufficient quantity for a significant amount of research to be carried out, and containing an active principle probably non-alkaloidal in nature.

The work of this thesis has further indicated that Chungking Ch'ang Shan is indeed D. febrifuga, and that the active principle is in fact an alkaloid. Furthermore, both Chunine and D. febrifuga leaf material have been shown to contain this same alkaloid, so presumably Chunine is at least in part the leaves of D. febrifuga.

A recent publication by Tsu[9] indicates the potentialities of

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9. Tsu, Cheng Fang, Jour. Tropical Medicine and Hygiene, 50, 75 (1947)

Ch'ang Shan as an antimalarial. Tsu gives clinical data on the use of water extracts of Ch'ang Shan for treatment of 587 out-patients and 81 hospitalized patients suffering from malaria. Of 322 out-patients with vivax malaria, 67% were cured, and of 253 with falciparum malaria, 63% were cured. There were only six relapses out of 67 hospitalized cases of tertian vivax malaria, and 11 of 14 cases of falciparum malaria were cured.

Pharmacological studies on the pure, active principle are reported in Section 12.

Section 2

Early Investigations of Ch'ang Shan and D. Febrifuga.

In 1945, since sufficient material was available, Drs. Koepfli and Mead at this institution began an investigation of Chungking Ch'ang Shan (D. febrifuga). It was my pleasure to join them in this work in November of that year. Before giving a detailed account of the work which followed, it would be desirable to examine the small amount of existing chemical literature on Dichroa febrifuga Lour.

The first chemical investigations on the plant were made by Hartwich[10] who in 1897 reported the isolation of a glucoside "Dichroin". No details are available to this writer. In 1945 Tomkin and Work[4] reported that an acid extract of Ch'ang Shan (reputed to be D. febrifuga) gave no sign of alkaloids, but gave considerable activity against P. galinaceum. A few months later in 1946, Hooper[11] reported that some fifty years earlier he had examined D. febrifuga and found no alkaloids. At about the same time Jang, Fu, Wang, Huang, and Chou[12] reported the isolation from Ch'ang Shan (D. febrifuga) of four substances, two neutral principles, Dichroin A (m. 228 to 230°) and Dichroin B (m. 179 to 181°), and two alkaloids, Dichroine A (m. 230° dec.) and Dichroine B (m. 237 to 238° dec.). Of the four, Dichroine B was the only one active against P. galinaceum in chicks. This is the extent of the literature concerning the chemical investigations of the plant, except for a brief communication by Koepfli, Mead, and

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10. Hartwich, Neue Arzneidrog, 1897, 125
11. Hooper, D., Nature, 157, 106 (1946).
12. Jang, Fu, Wang, Huang, Chou, Science, 103, 59 (1946).

Brockman[13] which reports in brief some of the work reported in this thesis.

The work carried out by Mead prior to November 1945 and the conclusions drawn from it may be summarized as follows.

1. There were two approaches to this problem; either attempts could be made to isolate pure compounds from the plant and test such compounds for activity, or the various crude fractions obtained in different steps of a schematic isolation could be tested and attempts made to isolate pure compounds from those fractions which had a high concentration of activity. The latter approach was chosen because it is the more direct, because Eli Lilly and Company kindly offered to carry out the necessary testing, and because preliminary experiment had indicated that crystalline material of any sort was difficult to obtain. The bio-assays were of course time consuming and tended to delay the progress of the work.

2. The ground root material is difficult to exhaust of activity by extractions with various solvents such as methanol, ether, water, and mixtures of these. The order of 10 to 20% of the activity was extracted by repeated extractions with alcohol and water. There was the definite possibility that activity was being destroyed during these extractions.

3. Working with these alcohol and water extracts, it was possible to obtain a concentrate with a Q of 40. (The procedure for obtaining the concentrate will not be given here, since it was, with minor variations,

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13. Koepfli, Mead, and Brockman, Jour. Amer. Chem. Soc., 69, 1837 (1947).

the same as those given in Section 3.) This fraction gave a strong test for alkaloids with Mayer's Reagent*. From this fraction a crystalline substance was obtained, melting crude at 150 to 152°. When recrystallized from hot acetone the melting behavior changed, the sample now partially melting at 136 to 137° and completely melting at 150 to 151°. This material was not tested as an antimalarial, because further work indicated that it was an artifact produced by heat (see 4 below).

4. Working with a similar extract it was possible to isolate a base melting at 128°, and from a crude mixture a hydrochloride which melted at 210 to 212° (dec.). The 128°-melting base was tested and found to be inactive ($\underline{Q}$ < 1.2i). (This base was later shown to have some antimalarial activity; see Section 12.) Furthermore, when this base was heated for a short time above its melting point, and the melt recrystallized, a new substance melting at ca. 150° was obtained. During this heating a loss of ca. 1% of the weight was observed. This new material gave a hydrochloride which melted at ca. 212° (dec.). The 128°-base formed a very hygroscopic hydrochloride which could not be characterized. The ultraviolet adsorption spectra of the two bases were essentially the same. Attempts to recrystallize the 128°-base were unsuccessful, the material was slowly soluble in hot benzene, alcohol, and acetone, but would not crystallize out on cooling. When the solvent was evaporated from such solutions, a gummy residue was obtained. This gum would go particulate when triturated with acetone

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* A solution of 13.55 g. of mercuric chloride and 50 g. of potassium iodide in one liter of water.

in the cold. After such treatment, the melting point behavior might change, the compound partially melting at 128° then apparently resolidifying and finally melting at 150°.

5. The general behavior of the compounds isolated and the fact that only a small fraction of the activity expected from the Q of the original material is isolated in these active concentrates indicate that the active principle is a very labile molecule, that the isolation procedure is a poor one, or both.

6. Since the 128°-base was inactive, and since the 137°-and 151°-bases are artifacts of the 128°-base and were presumed to be inactive (it was later shown that this is not the case) there were several possibilities regarding the active principle: the 128°-base might be an artifact of the still unisolated active base (the implication being that the active principle is an extremely labile substance); the active principle might not be an alkaloid at all; the active principle might be a base, but present in such small quantities that its isolation is difficult.

Section 3

Isolation of Inactive Isofebrifugine and of a Crude Active Fraction

When I began working on this investigation, Dr. Mead was leaving for Occidental College, so in order to avoid unnecessary duplication it was decided that he would work with the leaf material and I would work with the root material of the plant. Dr. Mead's experiments had indicated that the active principle present in the roots probably has basic properties and probably is quite labile. It seemed pertinent then to duplicate, more or less, his isolation procedure, in order to become acquainted with some of the properties of the material to be worked with in a new attempt to find the active principle.

Eli Lilly and Co. was kind enough to carry out large scale extractions of the crude root material with various solvents, and after evaporating to dryness send us the residues for further work. Such dried extracts were used for the isolation indicated schematically in Fig. 1. Emulsion formation was very troublesome during these operations. The butanol extractions in steps 3 and 6 were recommended by Mead because they seemed to remove undesirable amorphous material and make the final basic fraction cleaner. The particulate base obtained in step 8 was washed with butanol and then acetone. Its melting behavior was as follows: darkened and melted at 126 to 127° but not to a clear liquid, rather to a viscous cloudy gel which seemed to clear and flow at ca. 147°.

This 127° - base was apparently the same one isolated by Mead, and found to be inactive as an antimalarial. About 168 mg. of this

Figure 1. The isolation of isofebrifugine (exp. 2).

1. Methanol-water extracts of Lot C Ch'ang Shan: Shake with water and HCl to make pH 2 to 3. Filter.	
2. Aqueous solution, pH 2 to 3: Extract with $CHCl_3$.	Solid: Discard.
3. Aqueous phase, pH 2 to 3: Extract with BuOH.	$CHCl_3$ Phase: Discard.
4. Aqueous phase, pH 2 to 3: Make basic (pH ca. 8) with Na_2CO_3 and extract with $CHCl_3$.	BuOH phase: Discard.
5. $CHCl_3$ phase: Extract with 0.1 N HCl.	Aqueous phase: Discard.
6. Aqueous phase, pH 1: Extract with BuOH.	$CHCl_3$ phase: Discard.
7. Aqueous phase, pH 1: Make basic with Na_2CO_3 and extract with $CHCl_3$.	BuOH phase: Discard.
8. $CHCl_3$ phase: Dry with Na_2SO_4 and evaporate until ca. 1 ml. of dark oil remains. Triturate with acetone to obtain particulate base, m. 127° (see text).	Aqueous phase: Discard.

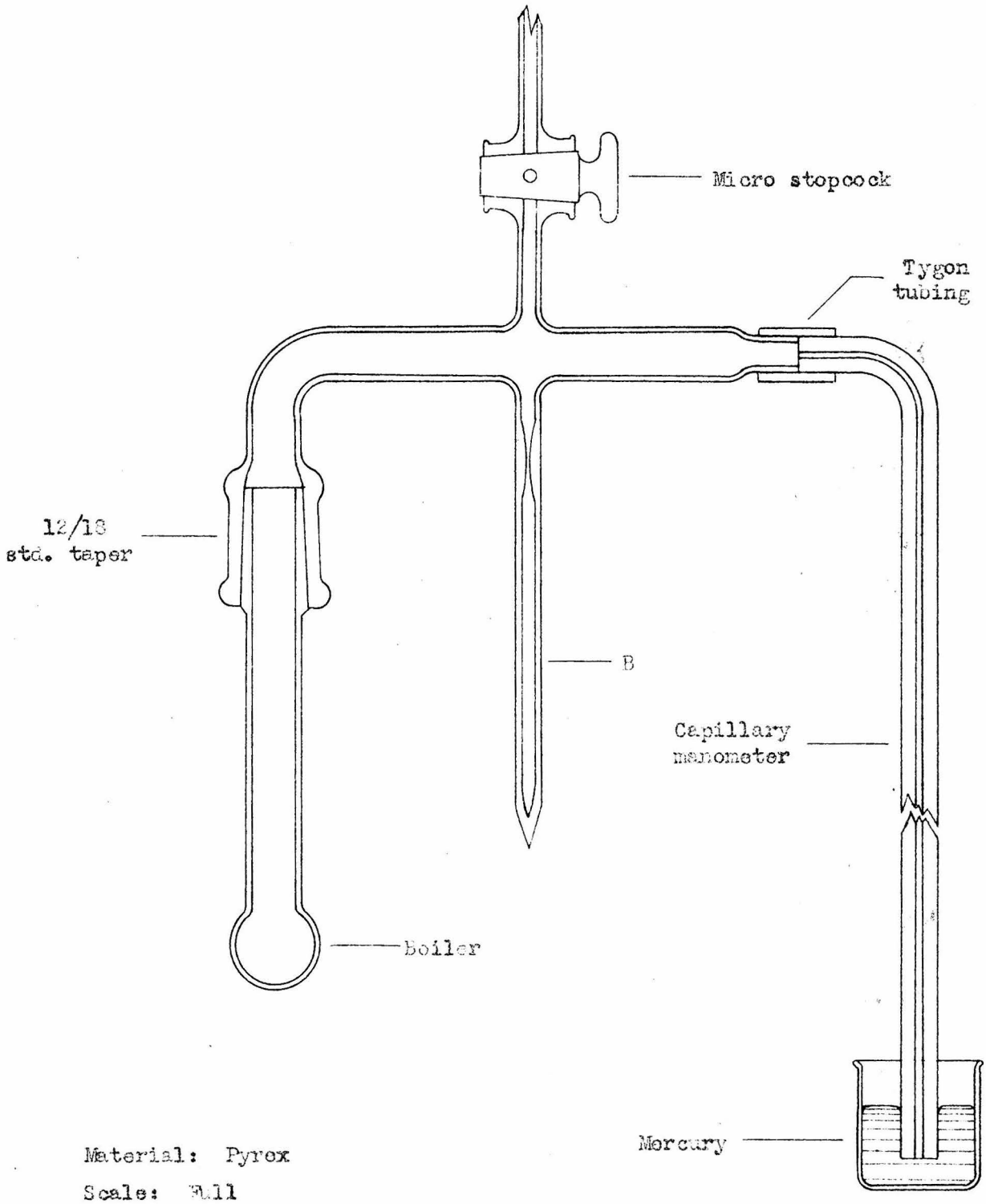
crude base was obtained, and although it was not the active principle being sought, it seemed desirable to investigate its properties to a limited extent.

Some solubilities were determined roughly on 1-mg. samples. At room temperature the compound was not very soluble in acetone, butanol, isopropyl alcohol, ethanol, benzene, dioxane, or ligroin. At 100° it was quite soluble in benzene, dioxane, and slowly in acetone, but it would not crystallize out on cooling and agitating.

A little of the free base was dissolved in absolute alcohol with the aid of heat, and dry HCl was passed into the solution until it was saturated. No precipitate formed, and the addition of benzene followed by evaporation to a small volume gave a very hygroscopic jelly, which could not be crystallized.

A 150-mg. sample of this 127°-base was decomposed in the apparatus diagrammed in Fig.2. The sample was heated for twenty-five minutes at 135 to 140°, after the apparatus had been evacuated to 0.8 mm. pressure. The pressure increased to ca. 40 mm. during the heating, but when a cooling bath (dry ice in isopropyl ether) was placed around receiver B, the pressure decreased again to ca. 2 mm., and a white crystalline solid collected in the cooled tube. This receiver was sealed off and allowed to warm to room temperature. The solid melted to a clear liquid. A freezing point determination was made, and the material was found to melt at -15 to -14°. The amount of liquid appeared to be ca. 1 to 2 microliters, which corresponded to the loss in weight of the sample in the boiler (1.92 mg. loss, 1.2%). The boiling point of the liquid was taken by the Emich method, and found to be 83° the first time, and 87° the second time on the same sample. The

Figure 2. Apparatus for the decomposition of isofebrifugine.



Material: Pyrex

Scale: Full

liquid gave an iodoform test rapidly in the cold, and is believed to have been a mixture of acetone and water.

The residue in the boiler was taken up in hot acetone and allowed to crystallize. The crystalline sample obtained gelled at 134° and melted and darkened at 151°. When this material was recrystallized from hot acetone it melted completely at 134 to 136°.

The mother liquors from these crystallizations were taken to dryness and heated again, this time for ca. 30 min. at 140 to 150°. It was possible to isolate from the residue a small amount of light colored solid melting at ca. 192°. On evaporation, the mother liquors gave a very dark oil which could not be crystallized.

The results of the isolation and heat treatment described above were, in the main, in agreement with the earlier results obtained by Dr. Mead. First, a base melting at ca. 127° had been isolated. This base was known to be inactive as an antimalarial. It has since been isolated in a pure state and has been named "isofebrifugine" (m. 129 to 130° cor.). For convenience this name will be used in the following discussions. Second, isofebrifugine is labile to heat, being converted to other compounds (presumably inactive too) melting at ca. 136, 151, and 192°. Third, no satisfactory method for recrystallization of isofebrifugine had been found.

Since isofebrifugine isolated by this above procedure was not the active principle, and since it was assumed that artifacts formed from it by heat were likewise not the desired principle, it remained to examine the mother liquors, to determine whether the activity was still there, or whether it had been destroyed. The acetone soluble

portion of the mother liquors was taken to dryness, and then taken up in dilute hydrochloric acid. The filtered acid solution was made basic with sodium carbonate and extracted with chloroform. The chloroform extract was dried with sodium sulfate, taken to dryness, and sent for testing. This crude material was found to have a Q of 10. The amount of material obtained indicated that only a small fraction of the activity of the original extract had been concentrated in this fraction, but at least all of the activity had not been destroyed. Since, these mother liquors had stood around for several days before being worked up, it was easily possible that considerable decomposition had taken place if the active principle was as labile as it was believed to be.

Attempts to obtain a crystalline base from this active fraction isolated from the mother liquors were unsuccessful. Amorphous precipitates were obtained with chloroplatinic acid, picric acid, and mercuric chloride. The material was dissolved in acetone and fractionally precipitated with ether, but this too led to amorphous precipitates.

The amount of crude active fraction available above was the order of a few hundred milligrams, and it was soon necessary to isolate another batch. The method is schematically indicated in Fig. 3. Because of the suspected labile nature of the active principle, all operations were carried out as rapidly as possible in order to keep the material in solution a minimum length of time; and where convenient (always when the solutions were basic) the operations were carried out in a cold room at ca. 4°.

Again isofebrifugine, the 127°-base, was isolated along with its heat artifacts, melting approximately at 134 and 150°; and again the activity was found to remain in the mother liquors from these bases (step 9).

Figure 3. The isolation of isofebrifugine and a crude active fraction (exp. 18).

1. Water extracts of Lot C Ch'ang Shan: Shake with water and HCl to make pH 2 to 3. Filter off and discard solid. Extract first with $CHCl_3$, then with BuOH and discard extracts. Make aqueous phase basic with Na_2CO_3 and extract with $CHCl_3$.

2. H_2O phase, pH 8 to 9: Extract with BuOH.

3. BuOH phase: Extract with 0.2 N HCl. | H_2O phase: Discard.

4. H_2O phase: Make basic with Na_2CO_3 and extract with BuOH. | BuOH phase: Discard.

5. BuOH phase: Dry with Na_2SO_4 and evaporate to dryness (41 mg.) as 18-43. Q >30. | H_2O phase: Discard.

6. $CHCl_3$ phase: Extract with 0.2 N HCl.

7. H_2O phase: Make basic with Na_2CO_3 and extract with $CHCl_3$. | $CHCl_3$ phase: Discard.

8. $CHCl_3$ phase: Dry with Na_2SO_4 and evaporate to brown-orange oil. When triturated with acetone this gave 246 mg. of base m. 126 to 151°, and a second crop of 80 mg. m. 134°. | H_2O phase: Discard.

9. The mother liquors were taken to dryness, taken up in dilute HCl, made basic with Na_2CO_3, extracted with $CHCl_3$. This was taken to dryness, triturated with acetone to get 38 mg., m. 127°. The acetone mother liquors were taken to dryness (124 mg.), taken up in HCl (pH 3), and sent for testing. A Q of 25 to 40 was found.

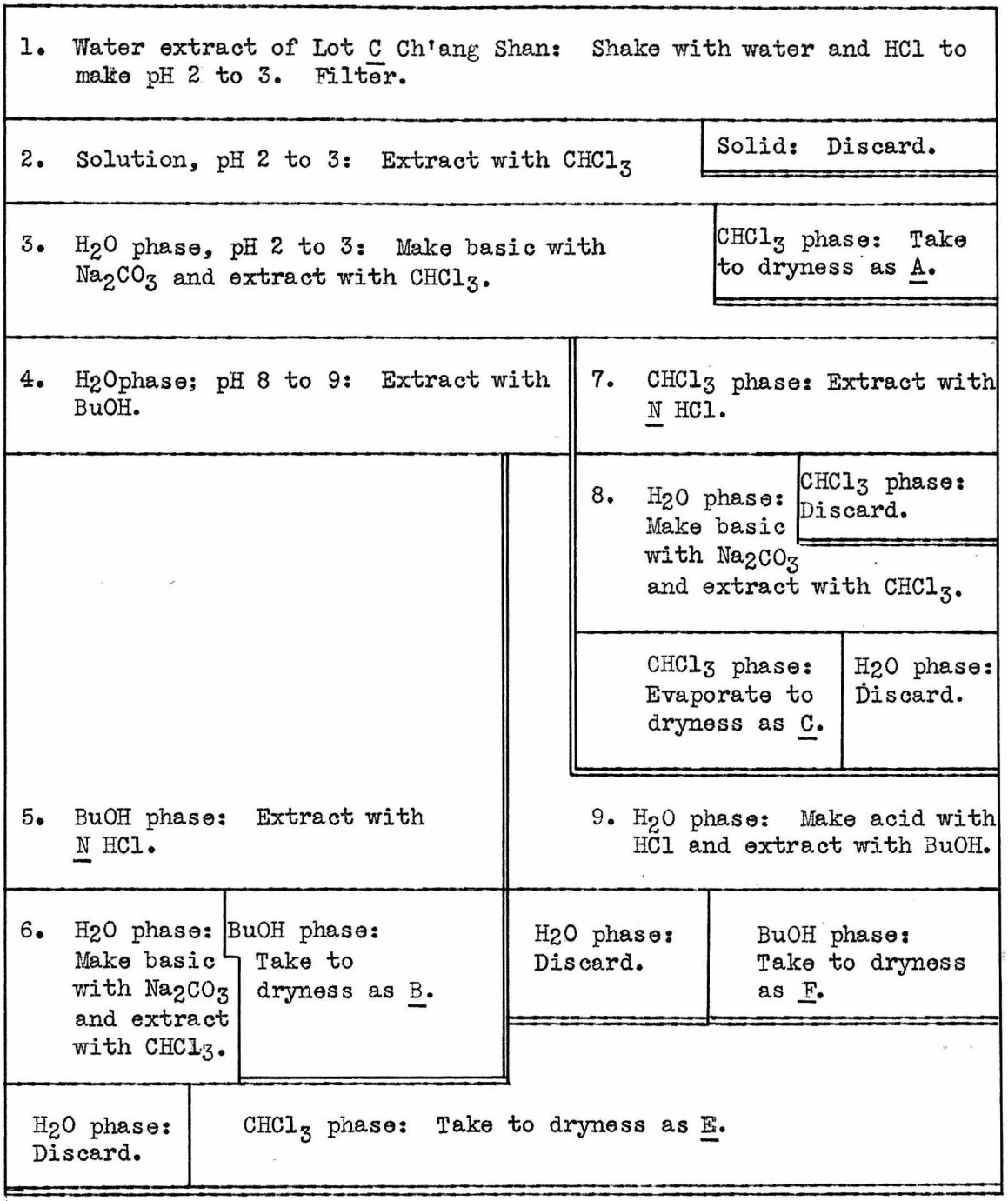
Another very significant fact was observed during this experiment, namely, All the activity was not extracted from the basic aqueous solution by chloroform (end of step 1) for additional activity, highly concentrated ($\underline{Q} > 30$), was extracted later by butanol (steps 2 to 5). The small amount of active concentrates again did not account for more than a small fraction of the original activity.

Since a large portion of the above concentrates were used for the testing it was necessary to carry out another extraction. This is outlined in Fig. 4. Fraction C was triturated with acetone and yielded ca. 70% of isofebrifugine.

In this experiment the total recovered activity (based on Q values) was ca. 36% (45.7/128). Since the Q of a sample may be in error by as much as a factor of 2, this recovery is fairly satisfactory. Although the activity was spread throughout the various fractions, evidently a considerable concentration had been effected in E which had a Q of 100 (one of the highest values of Q every observed for a naturally occuring substance). However, this fraction amounted to only 153 mg. of crude material. Root material was strictly limited; and if any work were to be done on structure determination after the isolation was completed, it seemed undesirable to sacrifice any more root material than necessary to complete the isolation of a sample of the active principle. If even a small sample of the pure active principle could be isolated, then enough of its properties probably could be determined to work out a better scheme for its isolation.

The most powerful method known for the separation of small

Figure 4. The isolation of isofebrifugine and crude active fractions, showing how the activity is spread throughout the various fractions (exp. 24).



Sample	Weight (g.)	Q	Q x Weight	Totals
Starting material	400	0.32	128	128
A	3.4	2	6.8	
B	1.9	8	16	
C	0.761	15	7.6	
E	0.153	100	15.3	45.7
F	11.4	toxic		

quantities of organic compounds with a high degree of purity is chromatography. This method appeared to have the greatest possibility of success for the amount of material available, and after many disappointing experiments, it finally led to the isolation of the pure active principle.

Section 4

Introduction to the Chromatography* of the Alkaloids of D. Febrifuga.

The development of a satisfactory chromatographic method for the separation and isolation of the active principle required considerable time and effort. In the first place, the technique was entirely new to me, and although I was quite aware of its potentialities, I was not fully cognizant of its pitfalls and obstacles. In the second place, the very nature of the method, which requires an empirical search for a suitable adsorbent, developer, and eluent, is such that considerable time may be spent more or less fruitlessly. In the third place, this particular problem was complicated by the fact that the compounds being sought were colorless, and essentially non-fluorescent, i.e., the fluorescence in concentrated solution was so weak that it did not show at all when the substance was spread out on a column. Moreover, no specific color tests could be found which would be suitable for locating a chromatographic zone. In the fourth place, the substances were very insoluble in ligroin and benzene, two of the most desirable solvents for chromatography. This meant that the more polar solvent, chloroform, had to be used, with the possible disadvantage that com-

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* I would like to express here my appreciation both to Dr. W.A.Schroeder who kindly introduced me to the techniques of chromatography and who guided my early experiments in that field, and to Prof. L. Zechmeister who was always willing to discuss the problems which arose during the course of the chromatographic investigation and who offered many timely suggestions which helped lead these experiments to a successful conclusion.

pounds might be too weakly adsorbed from such solutions.

On the other hand, a method of analysis for the alkaloids seemed available, viz., the ultraviolet absorption spectra. Although at this time it was not certain that isofebrifugine was pure, it did have a very characteristic absorption spectrum (see Part II), and furthermore, the most active crude fraction, E, Fig. 4 (@ 100) although non-crystalline had an absorption spectrum very similar to that of isofebrifugine. Thus, unless the active principle composed only say 10% of the fraction and did not have a very strong absorption in the ultraviolet, it seemed reasonable to associate the characteristic spectrum of isofebrifugine with the unknown active principle.

Because the methods for locating zones and analyzing eluates were common to all the chromatographic experiments, a brief discussion of these methods will be given here to avoid unnecessary repitition later. Possible zones were located by three different methods:

1. The columns were examined in ultraviolet light for possible fluorescence. The crude material being chromatographed fluoresced brightly under ultraviolet light (a G.E. Mazda Purple X bulb with a Corning 5840 filter was used as a light source), and some fluorescence could be seen on the columns. However, it came to light during the investigation that the active principle had practically no fluorescence.

2. The columns were streaked with alkaline permanganate; 0.5% NaOH and 0.1% $KMnO_4$ were the concentrations chosen after a series of different concentrations were tried. Isofebrifugine rapidly gives a green color with this strength of the reagent. The columns were post-washed with ligroin or chloroform to remove from the column the developer

which usually contained enough alcohol or acetone to interfere with the streak. This streak was not particularly good, but no other color test could be found. The test was frequently indistinct, and when small amounts were being looked for, they often did not show up at all by streak.

3. The columns were arbitrarily sectioned and eluted into separate flasks. The eluates were evaporated to dryness at room temperature (to avoid possible decomposition from heat), and the residues were taken up in ethanol, methanol, or N/20 HCl and examined spectrophotometrically, in a Beckman, Model 1 D U, Quartz Spectrophotometer. This method was always used as a check on the first two methods, as a means of quantitative analysis, and as a means of partial identification. In general, the entire spectrum of a fraction was taken to detect any changes which might take place on chromatographing. In the main, no changes were observed, and in later experiments only a few points of the spectrum were taken near the important maxima and minima, so that the ratios of the extinctions at these various extremes could be compared. If these ratios were not significantly different from those of the spectrum of isofebrifugine, it was assumed that the spectrum of the fraction being tested was also not significantly different from that of isofebrifugine. Where it seemed advisable, blank columns were prepared, i.e., duplicate columns with the omission of the sample to be chromatographed, and corrections for the spectra obtained were applied. Knowing the 1% extinction of the starting material at a given wavelength, and assuming Beer's law to hold, it was possible to determine the recovery of material in a roughly quantitative way from the extinctions

and volumes of the various eluates.

Using these methods, more than 100 columns were prepared and examined before the crystalline active principle was finally isolated. A detailed account of these experiments seems unwarranted here, and only a brief resume of the work will be given to indicate the nature of the experiments and the results. Several adsorbents were tried, and they will be considered separately in the following sections.

A careful characterization of isofebrifugine seemed desirable since it was one of the substances known to contaminate the active principle, and since it was probably structurally related to the active principle and any facts concerning it might help later in the structure determination. Also, since there was available more isofebrifugine than crude active fraction, preliminary experiments were usually carried out with isofebrifugine.

The aim of the following chromatographic experiments was first to find the conditions under which one or more well defined zones could be developed. Then, if any such zone could be eluted, evaporated, taken up again in chloroform, and rechromatographed under the same conditions to give a similar zone, it would be assumed that a pure compound had been isolated.

Section 5

Silicic Acid as a Chromatographic Adsorbent for the Alkaloids

The adsorbent used in these experiments was Merck Reagent Silicic Acid mixed with one-half part of Celite. Number 2 columns, 15 cm. long were used, and were prewashed with one of two standard prewashes to give a reproducible adsorbent. The strong prewash consisted of 10 ml. of anhydrous ethyl ether, 30 ml. of 1:1 acetone-ether mixture, 25 ml. of ether, 30 to 60 ml. of ligroin, and 2 ml. of chloroform. This prewash gives a reproducible, strong adsorbent. The weak prewash consisted of 30 ml. of ether, 30 ml. of absolute ethanol, 60 ml. of ligroin, and 2 ml. of chloroform. This prewash gives a much weaker adsorbent than the strong prewash. This method for obtaining a standard adsorbent has been thoroughly tested[14], and was recommended to me by Dr. W.A. Schroeder.

Approximately 5-mg. samples were introduced to the column from 5 ml. of chloroform. The sample was washed on with 2 ml. of chloroform, and this was followed by the desired developer. After the desired amount of developer had been drawn through the column, it was postwashed with 60 ml. of ligroin (60-70).

The results obtained using silicic acid as an adsorbent were in general not encouraging. The main difficulty was simply that this adsorbent was too strong, even when prewashed to be weak. A large percent-

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14. (a) Le Rosen, A.L., Jour. Amer. Chem. Soc., 67, 1683 (1945) and 69, 87 (1947). (b) Schroeder, W.A., "Annals of the New York Academy of Sciences", (in press). (c) Trueblood, K.N., Thesis, Calif. Inst. of Tech.

age of the material was adsorbed at the very top of the column, and was very difficult to move. Solvents which are normally strong eluents were found to be weak developers. Some typical results indicating the types of developers and eluents tried are given in Table I and Table II.

None of the solvents tried as developers showed any great promise. Even pure acetone moved the top zone only very slowly. The material which appeared in the zone between 50 and 70 mm. on the strong adsorbent with 3:1 chloroform-ligroin developer fluoresced blue under ultraviolet light, had a slight, non-characteristic adsorption in the ultraviolet, and amounted to less than 5% of the isofebrifugine used as starting material. These chromatograms indicated, then, that the isofebrifugine isolated by the methods of Section 3 was not pure, but contained at least traces of another compound.

Two eluents, a mixture of ethanol, chloroform, and concentrated hydrochloric acid, and a mixture of diethylamine and benzene, appeared to have some promise, but both had drawbacks. The mixture of ethanol, chloroform, and concentrated hydrochloric acid was found to elute as high as 60% (see TableII) of the material. Evaporation of the resulting solutions, which were apparently colorless, gave colored residues, which were of course hydrochlorides if the material was indeed an alkaloidal base. Before this material could be rechromatographed it would be necessary to convert it back to the free base. The quantitative aspects of this step were unknown. Furthermore, the appearance of color in the residue seemed to be an artifact and not something eluted from the column; therefore, it seemed wise to look for a simpler method which had less chance of altering the active principle.

TABLE I

Effect of Different Developers on the Movement of Zones from 5- to 6-mg. Samples of Crude Isofebrifugine Adsorbed from Chloroform on a Number 2, 15 cm. Column of Silicic Acid.

Prewash	Developer	Vol. (ml.)	Zone positions (mm. from top)	
strong	3:1 $CHCl_3$-ligroin	30	0-2	25-40
strong	3:1 $CHCl_3$-ligroin	50	0-2	50-70
strong	3:1 $CHCl_3$-ligroin	50	0-2	50-70*
weak	3:1 $CHCl_3$-ligroin	30	0-5	
weak	3:1 $CHCl_3$-ligroin	50	0-4	
weak	1:4 EtOAc-benzene	100	0-4	
weak	acetone	45	0-10	
weak	acetone	250	10-15	

* This 50-70 zone was rechromatographed under duplicate conditions, and was found to appear in the same place on the column.

TABLE II

Effectiveness of Different Eluents for Isofebrifugine Adsorbed on Silicic Acid

Prewash	Eluent	Sample mg.	Eluted mg.	percent
strong	1:9 EtOH-Et_2O	5.7	0.0	0
	1:4 EtOH-$CHCl_3$	5.7	0.5	9
	EtOAc	5.2	0.5	10
	1:4:0.007 EtOH-$CHCl_3$-12N HCl	4.8	2.0	42
	1:4:0.015 EtOH-$CHCl_3$-12N HCl	5.0	3.0	60
weak	1:4 EtOH-Et_2O	5.0	0.5	10
	acetone	6.1	1.9	31
	acetone (let stand 1 hr.)	5.8	1.1	19
	1:1 Et_2NH-$CHCl_3$	5.0	3.3	66
	1:1 Et_2NH-benzene	5.9	4.1	70

The mixture of diethylamine and benzene eluted 70% of the material, but again evidence of artifact formation was found. A colored substance was formed in sufficient quantity to obscure the spectrum of moderate quantities of the original material. This artifact was found to arise from the interaction of the diethylamine and the benzene. Diethylamine, carefully fractionated through a 60 cm. column of glass rings, left no visible residue on evaporation, and alcohol used to rinse the flask gave a very slight absorption in the ultraviolet. Benzene similarly fractionated likewise gave no residue on evaporation, and alcohol used to rinse the flask gave very slight absorption. When a mixture of these two was evaporated, however, a brown oily residue was left in the flask, and this material in alcohol had a very considerable absorption both in the ultraviolet and in the visible. The use of this eluent, then, would contaminate any product isolated, and render uncertain the results of any spectrophotometric analysis.

Most of the experiments discussed above were done with inactive, non-recrystallized isofebrifugine. A trial with a very active crude fraction indicated that similar results would be forthcoming. The scarcity of material made further experimentation with silicic acid seem undesirable, at least until other adsorbents had been given a trial.

Section 6

Calcium Hydroxide, Talc, and Calcium Carbonate as Chromatographic Adsorbents for the Alkaloids and the Purification of Isofebrifugine

The adsorbents used for these experiments were Shell calcium hydroxide, talc from an unidentified source, and Merck Heavy Powder Calcium Carbonate, each mixed with one-half part of Celite. A brief examination of these adsorbents indicated that the compounds being worked with are weakly adsorbed from chloroform. Calcium hydroxide and talc were not investigated beyond a preliminary trial.

Calcium carbonate was investigated briefly, and a few useful facts were indicated. The material is quantitatively eluted from calcium carbonate by 1:1 ethanol-chloroform mixture. Several diffuse fluorescent zones were found to be poorly developed by chloroform. These zones on rechromatographing did not appear in corresponding positions, but in the main were found to spread throughout the column. Some fluorescent material was strongly adsorbed and stayed at the top of the column when developed with chloroform; some fluorescent material was not adsorbed and ran through into the filtrate when sufficient chloroform had been added to wet the entire column. It was also found that isofebrifugine was rapidly washed into the filtrate by chloroform.

A 100-mg. sample of impure isofebrifugine was dissolved in chloroform, and this solution passed through a number 2 column of calcium carbonate and washed through with an additional volume of chloroform. The column retained 3 mg. of fluorescent material. The filtrate was

recrystallized by dissolving the material in chloroform, adding 1½ volumes of ethanol, and evaporating the solution slowly with nitrogen until the volume was reduced to about one-quarter of the original. This solution on standing in the ice box gave colorless crystals, which after two recrystallizations amounted to 40 mg. and melted sharply to a clear, colorless liquid at 128.7 to 129.5° (cor.). This was the first time a satisfactory method for recrystallizing isofebrifugine had been found. The base was inadvertently lost, but was believed to be pure isofebrifugine. Evidence obtained later (see Section 7) substantiated this belief. A chloroform solution of this compound had practically no fluorescence under the ultraviolet light.

Certain anomalous results were obtained when the crude active fraction was chromatographed on calcium carbonate, and developed with chloroform. Three fractions with the absorption spectrum of isofebrifugine were apparent: one adsorbed at the top, one adsorbed about half way down the column, and one appearing in the filtrate (presumably isofebrifugine). When either the top zone or the middle zone was rechromatographed under similar conditions, the same three fractions were observed, with a large percentage of the material appearing in the top and middle zones on the column. Such an effect might be observed on an overloaded column, but subsequent work with alumina (see Section 7) indicated that this was very likely a "double zone" effect. (For a discussion of the double zone effect on silicic acid see reference 14 b.)

About 10 mg. of crude active fraction was dissolved in chloroform and passed through a number 2, 15 cm. column of calcium carbonate and the column developed with 60 ml. of chloroform. The eluate from the column was found to contain ca. 5 mg. of material having a Q of 100, and the

filtrate, ca. 5 mg. of material with a Q of 20. Evidently the active principle was adsorbed preferentially.

Although in these preliminary trials calcium carbonate showed some promise as an adsorbent, it had its drawbacks, and it was considered desirable to try some preliminary experiments with alumina before making a thorough investigation of calcium carbonate. As indicated in the next section, alumina turned out to be a very fine adsorbent for the separation desired; and as a result, calcium carbonate was not further investigated.

Section 7

Alumina as a Chromatographic Adsorbent for the Alkaloids and the Isolation of Pure Febrifugine

The discussion of this adsorbent will be somewhat more detailed than those of the other adsorbents, because alumina was found to be by far the best adsorbent for the problem at hand. The adsorbent used was ALORCO, Grade F, minus 80 mesh alumina, reground to pass 200 mesh, and mixed with one-half part of Celite. At the beginning of this investigation there was no alumina in stock, and a very limited quantity of used adsorbent was kindly made available to me by Dr. Zechmeister's group. It was necessary to clean up and activate this material before use. This was carried out by a procedure recommended by Dr. John Sease. The material was washed thoroughly with methanol, partially dried, placed in a porcelain dish, and set afire. After the methanol was burned out, the dish was heated with a Mecker burner until the powder no longer bubbled, then for an additional 30 to 60 minutes. The body of the powder was at ca. 300 to 350°, and the bottom of the dish was heated to a dull-red color. This ignited alumina was then partially deactivated by adding to it the desired amount of water and shaking it for several hours in a sealed bottle on a mechanical shaker.

Some preliminary experiments indicated that the best adsorbent was one containing 4 g. of water per 100 g. of alumina. The alkaloids were reasonably strongly adsorbed by this adsorbent from chloroform, and could be more than 70 to 75% recovered by elution with 1:1 methanol-

chloroform mixture. Most of the colored material in the crude fractions of alkaloid was adsorbed at the very top of the column and could easily be removed by shaving off the top 1 to 2 mm. of the column.

The general method of the investigation of alumina was very nearly the same as that described for silicic acid. Somewhat smaller samples (2 to 3 mg.) were used on number 2 columns to avoid any possibility of overloading the column. Examination under the ultraviolet was of little aid, since the fluorescence either stayed at the very top or moved through the column at the solvent rate into the filtrate. The alkaline permanganate streak, although not particularly distinct or sensitive, was the best method available for locating zones on the column. Any zones indicated were always eluted, and the eluates were examined spectrophotometrically. Spectra were taken in methanol so that the samples could be easily recovered by evaporation, and could be rechromatographed if desired.

After some experimentation using different developers, appropriate conditions were found which separated the crude active fraction into three main divisions: a zone at the top of the column, a zone about half way down the column, and the filtrate. Each of these three divisions had an absorption spectrum very similar to that of isofebrifugine (see Part II).

The conditions and results for a typical chromatogram are outlined in Fig. 5. The three divisions which resulted from chromatographing the crude material looked most encouraging, but as these fractions were rechromatographed a most distressing phenomenon was observed. A chromatogram of zone A (Fig. 5) under the same conditions as the original chromatogram gave the original three divisions, a top zone, a middle zone, and a small amount of material in the filtrate. Likewise, a chromatogram of

Figure 5. A typical chromatogram of the crude active fraction on recovered alumina (exp. 70).

Column: #2, 15 cm., recov. Al_2O_3 (4 g. H_2O/100 g.)-Celite, 2:1.

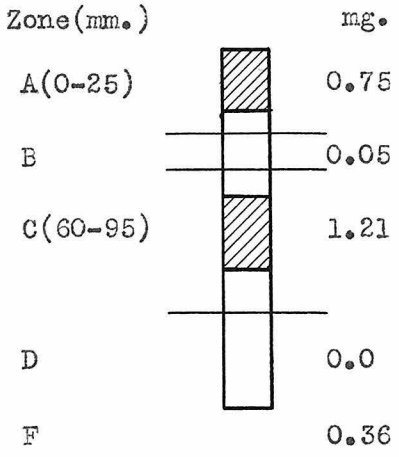
Prewash: 30 ml. $CHCl_3$.

Sample: 3.0 mg. crude active fraction in 2 ml. $CHCl_3$.

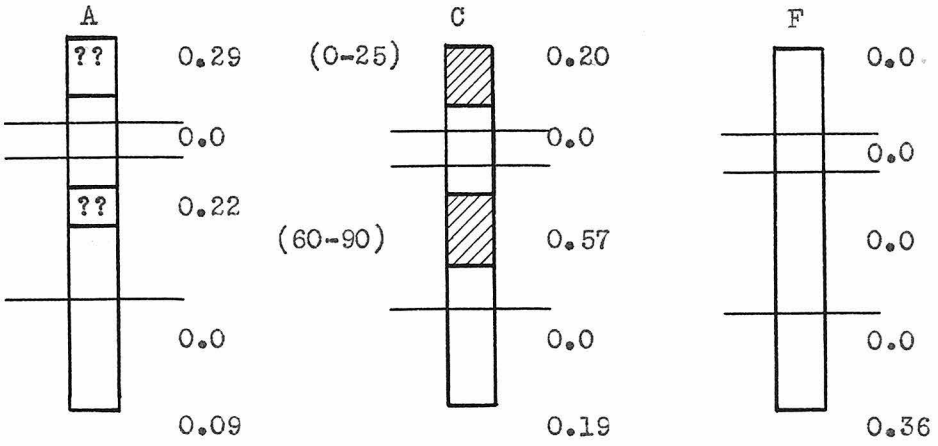
Development: Wash on with 10 ml. $CHCl_3$, dev. with 90 ml. 2% methanol in $CHCl_3$.

Postwash: 30 ml. $CHCl_3$.

Results: Permanganate streak indicated zones shown by shading; eluates were analyzed spectrophotometrically.



Eluates from sections A, C, and F were chromatographed again under identical conditions and gave the following results.



C, the original middle zone, gave rise to divisions corresponding to the original three. A chromatogram of F, the material in the filtrate, showed that this material again appeared in the filtrate.

Table III gives the results of a few experiments which showed quite clearly this "double zone" effect. It will be noticed that the total of the material in the two zones and the filtrate does not equal the total sample started with. This is accounted for by four principal factors: 1) some colored material is strongly adsorbed at the very top of the column, and this is cut off and discarded; 2) after the column is streaked with alkaline permanganate, the streaked portion of the column is cut out and rejected, the amount discarded may amount to 5 to 10% of the column; 3) the alkaloids may not be quantitatively eluted from this adsorbent; 4) no attempt was made to keep the inaccuracies of the analytical method below ca. 5%.

In experiment 2 (Table III) after the material in the two zones and in the filtrate was determined gravimetrically it was investigated spectrophotometrically. After corrections for a blank column were applied to the results, it was found that the spectra were indeed so similar (see Table IV) that any differences could be accounted for by experimental difficulties. The maxima and minima for each of the three fractions occurred at the same wavelengths, and even the 1% extinctions were very nearly the same, particularly for T and B. The ratio of the 1% extinction of a maximum to that of another maximum or minimum is a quantity independent of concentration, and hence void of errors in weighing. Such ratios are given in Table IV, and they show how nearly the same the spectra of these three fractions are.

TABLE III

Summary of Some Experiments Showing the Double Zone Effect

Sample	Top zone (T)	Bottom zone (B)	Filtrate (F)	Ratio T/B
Crude:				
1. 3.0 mg.	0.75	1.21	0.36	0.62
2. 10.2 mg.	2.0*	3.8*	1.2*	0.53
3. 3.1 mg.	0.52	(1.4[a])	0.49	0.37
Rechromatographed:				
4. T from 1	0.29	0.22	0.09	1.31
5. B from 1	0.20	0.57	0.19	0.35
6. T from 2	0.56	0.79	0.20	0.71
7. B from 2	0.52	2.2	0.23	0.24
8. B[a] from 3	0.21	1.23	0.07	0.17

* Quantities marked by an asterisk (*) were determined gravimetrically, all others were determined spectrophotometrically.

a) During elution and evaporation this fraction was kept cooled to 0° in an ice bath.

TABLE IV

Spectral Data for Fractions Obtained in Experiment 2 (Table III)

Extremes		1% Extinction		
mμ	type	T	B	F
225	maximum	740	765	800
250	minimum	131	123	147
266	maximum	211	213	232
302	maximum	104	103	119
Ratios of 1% Extinctions				
	225/266	3.50	3.60	3.45
	266/250	1.61	1.73	1.58
	266/302	2.04	2.06	1.95

A possible explanation for this double zone effect is the following. T and B represent a compound which exists in solution in two readily interconvertible forms, for example, lactam-lactim, keto-enol, or cis-trans, and furthermore these forms can be separated on the alumina column. When either form is eluted, however, the solution quickly becomes a mixture of the two forms so that a rechromatogram gives two zones. The material appearing in the filtrate, F, might represent a decomposition product or more probably a more stable isomer of the compound represented by T and B.

If this theory is correct, it is possible that a change in temperature would change the ratio of T to B in solution. An attempt was made to carry out chromatograms in a cold room kept at ca. 4°, but it was found that under the conditions indicated in Fig. 5 no zone appeared on the column and most of the material appeared in the filtrate. In experiments 3 and 8, Table III, the eluate from the original chromatogram was kept cold during the evaporation to note the effect on the ratio of T to B, in the rechromatogram. The ratio was quite small, but the significance of this is not too clear considering the wide variation observed in the experiments carried out at room temperature. The room temperature experiments were carried out for the most part in August, in a laboratory on the west end of the building, so that the changes in temperature throughout the day were often as large as 7°. No record of this factor was kept, so that no correlation can be attempted.

Since so little was known of the nature of the mixture being chromatographed, it was considered unwise to spend more time investigating such factors extensively. It did seem pertinent to prepare one

large chromatogram and attempt to isolate crystalline material from these three fractions if possible. Such an experiment was carried out with 78 mg. of crude starting material. This gave a column on which permanganate indicated a single zone corresponding to B (Table III). Elution of the column and evaporation gave 11 mg. of a dirty oily residue corresponding to T, and 40 mg. of slightly colored solid corresponding to B, and a spectrum of the filtrate indicated 15 mg. of material with the characteristic spectrum of isofebrifugine. From the 40 mg. of colored solid it was possible to obtain 20 mg. of colorless needles by taking the material up in chloroform, adding benzene and evaporating (at room temperature with nitrogen) to about three-fourths of the original volume and letting the solution stand in the ice box over night. This material sintered at ca. 120° and finally melted at ca. 145°. Since there was no guarantee that this material was the active principle, a sample was sent to Eli Lilly and Co. for a duck test. The results were most gratifying, Q 100 to 150.

It was now certain that the active principle is an alkaloid, and the name "febrifugine" was given to the substance. Although the melting behavior indicated that the material might not be pure, a definite step forward had been made.

As pointed out earlier, a very limited amount of alumina was available for these experiments, and any large scale operations would be made very tedious by the necessity of recovering the adsorbent after each chromatogram. Fortunately, at about this time, a back order of alumina arrived and solved this problem of supply. It was necessary, of course, to test the new adsorbent to ascertain if its properties were the same as those of the recovered adsorbent which had been

used up to this time.

It was very soon discovered that the new adsorbent was not identical with the old recovered adsorbent. Figure 6 shows in outline a typical chromatogram using the new alumina as an adsorbent. The streak gave no indication whatever that the double zone effect was being observed. On the contrary, a single zone was evident, and remained fairly constant on rechromatographing. Several experiments to change the activity of the new alumina were tried involving various heat treatments and water deactivations similar to the recovery process for the old alumina, but none of them gave an adsorbent which gave the double zone effect described above for the old recovered alumina.

A 100-mg. sample of crude material was chromatographed on the new alumina and yielded 43 mg. of needle-like crystals (from chloroform and benzene as described above) softening and sintering at ca. 120, and finally melting at 135°. It was noticed in this experiment that considerable oil was eluted from the column (probably machine oil from the mill used to grind the alumina). It was thought that this might be responsible in part for the poor melting range. With the material available at this time several distribution coefficients and solubilities were determined, in order to work out a method for freeing the active principle of extraneous material from the column, and also in order to get a more satisfactory method for the isolation of the crude material from the plant. The details of these determinations will be given later (Section 8). Suffice it to say here, that these determinations led to a better isolation method which will be described below.

Figure 6. A typical chromatogram of the crude active fraction on new alumina (exp. 91).

Column: #2, 15 cm., new Al_2O_3-Celite, 2:1.

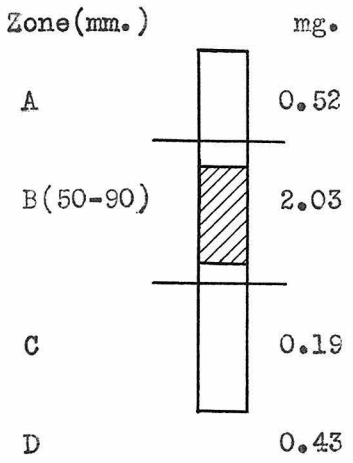
Prewash: 10 ml. $CHCl_3$.

Sample: 4.1 mg. crude active fraction in 2 ml. $CHCl_3$.

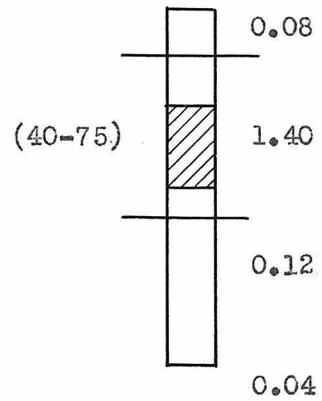
Development: Wash on with 5 ml. $CHCl_3$, dev. with 60 ml. 2% MeOH in $CHCl_3$.

Postwash: 30 ml. $CHCl_3$.

Results: Permanganate streak indicated single zone (shaded).



Eluate B was rechromatographed under duplicate conditions to give the following results.



The problem of the poor melting behavior of febrifugine was also cleared up by several recrystallization experiments. An outline of these recrystallizations is given in Fig. 7. All crystallizations were carried out at room temperature and below. The general procedure was to dissolve the material in some solvent at room temperature and evaporate with frequent stirring until the volume was considerably reduced and crystals had begun to form. Then the mixture was placed in an ice box for a few hours to allow the material to crystallize. When desired, a second solvent in which the material was not very soluble was added before the material was placed in the ice box. The crystals were collected by centrifugation, washed with the same solvent (sometimes finally with ether), and dried at reduced pressure and room temperature. The melting points were not changed when the samples were dried in an Abderhalden at ca. 80°, 1mm., over P_2O_5. It can be seen from Fig. 7 that the material crystallizes from ethanol, acetone-chloroform mixture, and ethyl acetate in a form which melts quite sharply at ca. 137°. From chloroform, the material melts at ca. 152°. The evidence from these experiments seems to indicate that the compound is dimorphic. It was later demonstrated by analysis that the compound was not solvated. Furthermore, material melting sharply at ca. 138° was obtained from chloroform when the solution was seeded with the 138° material obtained from ethanol. It has also since been found that when the calculated amount of sodium hydroxide is added to a saturated solution of febrifugine dihydrochloride the free base crystallizes out in the form melting at 152° unless the solution is seeded

Figure 7. Recrystallizations of febrifugine indicating the dimorphic nature of the compound.

(All melting points are uncorrected.)

<table>
<tr><td colspan="4">65 mg. crystalline febrifugine: Stir with $CHCl_3$.</td></tr>
<tr><td colspan="2">Soln: Evap. to small vol. Add benzene.</td><td colspan="2">Solid: Dissolve in $CHCl_3$. Evap. to small vol. Add Et_2O.</td></tr>
<tr><td colspan="2">Hairlike needles: 30 mg. sinter 120, m. 149-52. Stir with acetone.</td><td colspan="2">Hairlike needles: 10 mg. sinter 120, m. 152-5. Dissolve in $CHCl_3$. Evap. until crystals form.</td></tr>
<tr><td rowspan="2">Soln.: Evap. to dryness. Dissolve in $CHCl_3$. Evap. to small vol. Add Et_2O.</td><td rowspan="2">Solid: 5 mg. m. 137-8 Dissolve in $CHCl_3$. Evap. to small vol. Add Et_2O.</td><td>Soln.: Evap. to dryness. Dissolve in EtOAc. Evap. until crystals form.</td><td>Solid: soft 145, m. 151-3</td></tr>
<tr><td colspan="2">Crystals: m. 134-36. Dissolve in $CHCl_3$. Evap. until crystals form.</td></tr>
<tr><td>Crystals: 20 mg. Sinter 120, m. 132-45. Dissolve in const. boiling $CHCl_3$-acetone (66:34). Evap. to small vol.</td><td>Crystals: sinter 120, m. 145-7.</td><td colspan="2">Crystals: soft 144, m. 148-50.</td></tr>
<tr><td colspan="2">Crystals: 9 mg. m. 136-7. Dissolve in $CHCl_3$. Evap. until crystals form.</td><td colspan="2">Mo. liq.: Evap. to small vol.
Crystals: 5 mg. m. 135-6.</td></tr>
<tr><td colspan="4">Crystals: m. 150-52.</td></tr>
<tr><td colspan="4">Combine all crystalline fractions: Dissolve in EtOH. Evap. to dryness. Dissolve in EtOH. Evap. to small vol. Add Et_2O.</td></tr>
<tr><td colspan="4">Crystals: m. 137-8.</td></tr>
</table>

freely with the lower melting modification. Both forms give the same dihydro compound in the same yield when reduced in ethanol in the presence of platinum. Both forms give the same zone when chromatographed under the same conditions. This additional evidence confirms the conclusion that febrifugine is dimorphic.

The recrystallizations outlined in Fig. 7 also give some evidence that the material being worked with is homogeneous. The first step was to stir the material with chloroform, and then separate the saturated solution from the solid. The solution gave the same crystalline material that the solid gave on recrystallization. This is reasonable evidence that the starting material is a single substance.

As confirmatory evidence for the purity of the material the chromatogram outlined in Fig. 8 was prepared. It can be seen from the figure that a single zone was indicated by alkaline permanganate, and that this zone was cut into three parts and each worked up separately to yield the same product. This would seem to indicate quite conclusively that the material isolated from the column is indeed a single, pure compound.

The following method for isolating material from the column was used because it freed the material from machine oil which was present in the eluates. The elution was carried out with 1:1 chloroform-methanol mixture. The eluates were evaporated to dryness at room temperature and at reduced pressure. The residues were taken up in 10 ml. of N/20 HCl, and the aqueous solution washed once with 10 ml. of ligroin and once with 10 ml. of chloroform. The ligroin and chloroform contained the

Figure 8. Chromatogram indicating the homogeneity of febrifugine.

Column: #6, 24 cm., new 2:1 Al_2O_3-Celite.

Prewash: 100 ml. $CHCl_3$.

Sample: 167 mg. crude active fraction in 40 ml. $CHCl_3$.

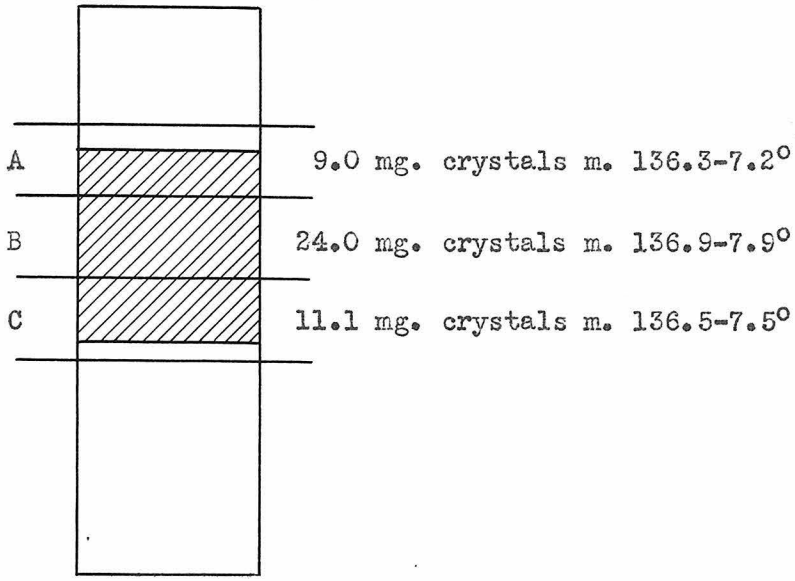
Development: Wash on with 75 ml. $CHCl_3$, dev. with 1000 ml. 2% MeOH in $CHCl_3$.

Postwash: 500 ml. $CHCl_3$.

Streak: 0.1% $KMnO_4$ - 1% NaOH.

Elution: 250 ml. of 1:1 MeOH-$CHCl_3$ per section.

Results: (m. p. 's uncorrected.)



Crystals from A, B, and C were combined and recrystallized from ethanol to give 38.9 mg. of crystals m. 139 to 140° (cor.).

oil and were discarded. The acid solution was next made basic with sodium carbonate and extracted three times with 5-ml. portions of butanol. The butanol phases were combined and taken to dryness at reduced pressure at room temperature. The crystalline residues were next dissolved in a small amount of absolute ethanol. A small amount of insoluble sodium carbonate was left. The alcohol was evaporated to a small volume with frequent stirring, and allowed to stand in the ice box for several hours. The crystalline solid was then collected by centrifugation and washed with ethanol, ethanol-ether, and ether.

At the time this method for recrystallization was used it was known that the activity of febrifugine was decreased significantly by heat (see Section 8). Material at this time was very scarce, and it did not seem wise to take the chance of destroying material by looking for a more suitable method for crystallization. It has since been found that small quantities of the alkaloid may be crystallized satisfactorily from hot ethanol provided the operations are carried out with reasonable dispatch. Analytical samples have been prepared both ways (analytical results are given in Part II), but the recrystallization from hot ethanol is superior because it facilitates the removal of ash from the sample.

Under the conditions of the chromatogram indicated in Fig. 8, isofebrifugine appears in the filtrate. Pure isofebrifugine can be obtained from this filtrate in the following way. The filtrate is extracted with dilute hydrochloric acid. The aqueous phase is made basic with sodium carbonate and extracted with chloroform. The chloroform is dried with sodium sulfate and evaporated to dryness at room temperature. The

residue can be recrystallized by dissolving it in chloroform, adding two volumes of ethanol, evaporating at room temperature with frequent stirring until the volume is reduced to ca. one-fourth, and letting the mixture stand in the ice box to crystallize.

Isofebrifugine can also be crystallized from methanol by slow evaporation at room temperature. A 120-mg. sample of isofebrifugine (m.* 126.5 to 127.5°) was dissolved in methanol. The solution was evaporated with nitrogen, with frequent stirring until crystals formed. After cooling in the ice box for several hours, a first crop was obtained, amounting to 33 mg. melting at 127.0 to 128.0°. The mother liquors were further evaporated, and a second crop was obtained, amount to 31 mg., melting at 127.0 to 128.0°. These two crops were combined and crystallized from chloroform-ethanol mixture as described above. The yield was 57 mg., melting at 127.2 to 128.2°. This material was again crystallized from chloroform-ethanol mixture and yielded 43 mg. of material melting at 127.1 to 128.1°. This behavior seems sufficient evidence that the material is pure. (Analytical results are given in Part II.)

It has recently been found that isofebrifugine can be satisfactorily crystallized from hot methanol if the operations are carried out rapidly.

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* Melting points given in this paragraph are uncorrected.

Section 8

Effect of Heat on Febrifugine and some Distribution Coefficients of Febrifugine and Isofebrifugine

As soon as ca. 20 mg. of febrifugine was available in crystalline form, several experiments were carried out to determine enough of its properties to aid in devising a better isolation scheme. From recrystallization experiments it was known that febrifugine is quite insoluble in ligroin, benzene, and ether. It is somewhat soluble in water, ethanol, ethyl acetate, acetone, methanol, and chloroform. It is very much more soluble in a mixture of chloroform and methanol than it is in either solvent alone, and likewise much more soluble in a mixture of water and ethanol than in either alone.

Up to the time that crystalline febrifugine was first isolated, extractions of the root material had been made with hot solvents, e. g., alcohol and water; therefore it seemed desirable to investigate the effect of heat on febrifugine in solution. Two milligram samples were sealed in glass tubes with the desired solvents and heated in a bath of boiling ethanol for six hours. The tubes were opened, evaporated to dryness with nitrogen at 40 to 50°, and the residues taken up in N/50 HCl. There was no change in the adsorption spectrum in any of the trials. The samples were sent to Eli Lilly and Co. who tested the antimalarial activity in ducks. The results are shown in Table V.

It is quite apparent from the results, that at 80° the activity is markedly decreased in six hours. This fact showed in a very clear manner that a different method was needed for the isolation,

TABLE V

Quinine Coefficients of Febrifugine after Heating in Solution at 80° for Six Hours

Solvent	Q
Abs. ethanol	25
Abs. ethanol 1 M in NH_3	25
6 N HCl	62.5
Control (no heating)	189

and led to the preliminary experiments with fuller's earth (see Section 9) which ultimately developed into a very satisfactory isolation procedure.

The other information which seemed desirable in working out an isolation procedure was the distribution coefficients of the alkaloids between various solvents and water. This information could be obtained with very little expenditure of material, for small quantities of the alkaloids could be determined by means of the spectrophotometer.

The procedure for determining the distribution coefficients was the following. A stock solution of the alkaloid was made up in chloroform at a concentration of ca. 0.2 mg. per ml. Aliquots of this solution containing ca. 1 mg. were pipetted into mixing cylinders. If it was desired to carry out the experiment using some solvent other than chloroform, the chloroform was evaporated and 10 ml. of the desired solvent added. Otherwise, the volume of chloroform was brought up to 10 ml. Ten milliliters of the desired aqueous phase was next added, and the mixture was shaken on on a mechanical shaker for 30 to 60 min. The phases were then separated

and analyzed spectrophotometrically.

The extinctions (at the 266-mμ -maximum) of the aqueous phases were taken in acid solution. The organic phases were analyzed differently depending on their constitution. Chloroform solutions were evaporated to dryness, and the residues taken up in methanol before the extinctions were measured. Butanol solutions were diluted with methanol before the extinctions were taken, and the 1% extinction was assumed to be the same in this solvent as it is in methanol. Since the amount of starting material was known the analyses could be checked by comparing the total alkaloid found with the amount started with. The agreement was usually quite good, and was always within ca. 10%. Suitable blanks were prepared, of course, and a correction applied to the extinction where necessary.

The results of these determinations are given in Table VI. It can be seen there that febrifugine is best extracted from aqueous basic solution by butanol. In practice it has been found convenient to use 1:4 butanol-chloroform because less inorganic matter contaminates the product, although the distribution is not quite so favorable.

For the results for aqueous solutions at different pH's and chloroform, it is possible to calculate a value for pK_B for febrifugine, assuming that at pH' s higher than 8.5 the compound exists as the free base, and that the salt form is quantitatively in the aqueous phase at lower pH's. The value obtained for the data at pH 5.87 is <6.9, and at pH 7.13 is 6.25. Dr. Mead had previously carried out a potentiometric titration of isofebrifugine, and found two basic groups, pK 6.3 and 12.

TABLE VI

Distribution Coefficients for Isofebrifugine and Febrifugine

Solvents		Coefficients, (C_1/C_2)	
1	2	Febrifugine	Isofebrifugine
N/20 HCl	$CHCl_3$	50	68
N/20 HCl	BuOH	4	4
N/20 HCl	1:4 BuOH-$CHCl_3$	47	-
N/20 HCl	2:1 BuOH-ligroin	30	-
$CHCl_3$	N/20 NaOH	-	10
$CHCl_3$	Na_2CO_3, pH 9.8	-	10
BuOH	N/20 NaOH	-	6
1:19 BuOH-$CHCl_3$	N/20 Na_2CO_3	2.4	-
1:9 BuOH-$CHCl_3$	N/20 Na_2CO_3	5.1	-
1:4 BuOH-$CHCl_3$	N/20 Na_2CO_3	11	-
BuOH	Na_2CO_3, pH 10.1	31	-
EtOAc	Na_2CO_3, pH 9.47	1.2	-
pH 5.87 *	$CHCl_3$	28	-
pH 7.13 *		7.6	-
pH 8.51 **		1.6	-
pH 10.1 **		1.3	-
pH 11.4 ***		1.6	-
pH 11.5 ***		1.5	-

* Citrate buffer. ** Sodium carbonate-bicarbonate buffer.
*** Sodium hydroxide.

Section 9

Fuller's Earth as an Adsorbent of the Alkaloids

The experiments described in the previous section on the effect of heat on febrifugine made clear the need for a new isolation method which does not require heat. The obvious choice of solvent to extract basic compounds from plant material is dilute mineral acid, provided decomposition is not promoted. It had not been used in the early isolation method because: (1) it was not known that the active principle is an alkaloid; and (2) fairly large volumes of solvent are employed in the original extraction of the plant material, and some sort of concentration is necessary. Evaporation, the usual method of concentration, leads to the formation of a strongly acidic solution which may destroy the substance being isolated, or if the acid is neutralized, large quantities of inorganic salt must be contended with. There is again, however, a method of obvious choice for concentration a small amount of material dissolved in a large amount of solvent, namely, adsorption. The choice of adsorbent is perhaps not quite so obvious, although it has been known for some time that charcoal, Lloyd's Reagent, and clays strongly adsorb many alkaloids from aqueous solution[15].

A few preliminary experiments with fuller's earth indicated that febrifugine is strongly adsorbed from acid solution and can be removed by shaking with aqueous sodium carbonate and butanol. In

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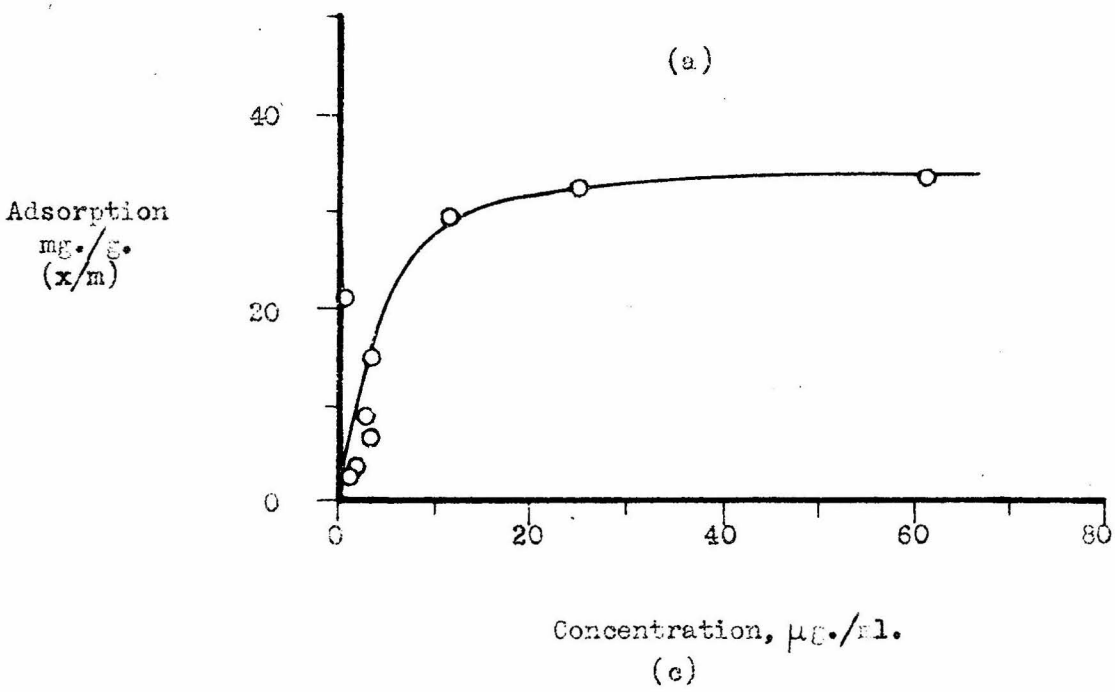
15. Weiser, Colloid. Chemistry, p. 72, New York: Wiley (1939).

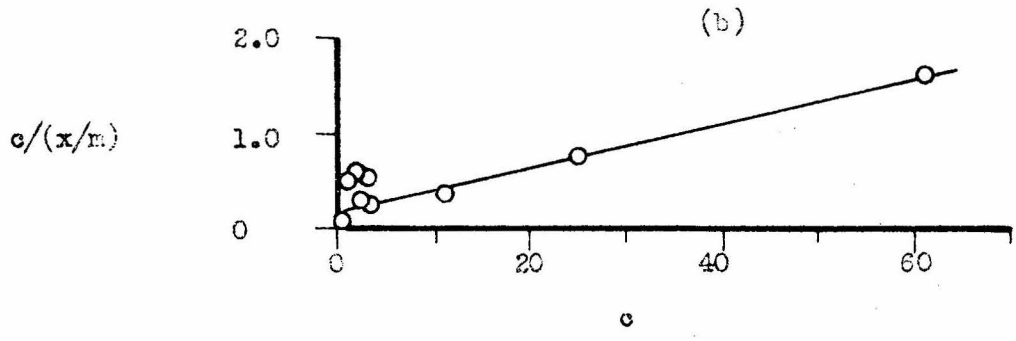
order to have some basis for estimating the amount of adsorbent needed for a given amount of alkaloid, an approximate adsorption isotherm for the adsorption of a mixture of the crude alkaloids on fuller's earth was determined. So that the acidity could be controlled, acid washed fuller's earth was prepared by treating Mefford COSO Fuller's Earth with hydrochloric acid until the water remained acidic to Congo Red Paper. The solid was then filtered off, washed with distilled water, and dried at 100°. The loss in weight during this treatment was ca. 36.5%. Samples of this adsorbent were weighed into conical flasks, a stock solution of the alkaloids in N/20 HCl was pipetted into each, and after 30 to 60 min. of intermittent shaking the solutions were filtered through a pad of Celite, and the extinctions of the solutions at the 266-mμ maximum were taken with a Beckman spectrophotometer. Suitable blanks were prepared, and the extinctions corrected for adsorption caused by material from the adsorbent. A preliminary experiment in which similar samples were treated in the same way except for the time of shaking indicated that the adsorption is complete in less than 10 min.

From the extinctions it was possible to calculate the amount of alkaloid still in the solution, and by difference the amount adsorbed on the fuller's earth. The results were calculated in terms of the weight of the original fuller's earth before the acid wash and are shown graphically in Fig. 9.

The values of concentration in the region 0 to 5 μg. per ml. are not very accurate because the extinctions at these concentrations are small, and the blanks were relatively large, so that the corrected extinctions might be in considerable error. For the purpose at hand,

Figure 9. Adsorption isotherm for the crude alkaloids adsorbed on fuller's earth from N/20 hydrochloric acid.





however, these inaccuracies are not serious.

The plot of c/(x/m) versus c, given in Fig. 9 b, indicates that the adsorption isotherm is probably of the type to which the Langmuir equation, $c/(x/m) = 1/ab + c/b$, is applicable. This shows the adsorbent to be saturated with respect to the alkaloids at ca. 43 mg. per gram, so that the least amount of fuller's earth which could adsorb one gram of alkaloids is ca. 23 g.

In Fig. 10 a more useful plot of the curve in Fig. 9 is given in terms of the amount of adsorbent needed to adsorb from a solution of given concentration one gram of alkaloid with a loss (material remaining in solution) of 1, 5, or 10%. It can be seen from this graph that at initial concentrations greater than ca. 100 μg./ml. the adsorption is quite efficient. If 90% adsorption is considered sufficient, the initial concentration may be as low as 10 μg./ml. and still not require undue amounts of adsorbent.

In order to make use of the graph in Fig. 10, it is necessary to know the concentration of alkaloid in the solution being work with. A convenient and satisfactory analysis can be made with Dragendorff's Reagent*. In 0.1 N HCl, 3 drops of Dragendorff's Reagent added to 1 ml. of solution will give an immediate cloudiness with 12 μg. of alkaloid, a barely perceptible cloudiness in 1/2 to 1 min. with 6 μg., and a clear solution with 3 μg. (If allowed to stand for several minutes the solution may become cloudy.) An approximate value of the concentration of alkaloid in a solution can be obtained by serially diluting a sample

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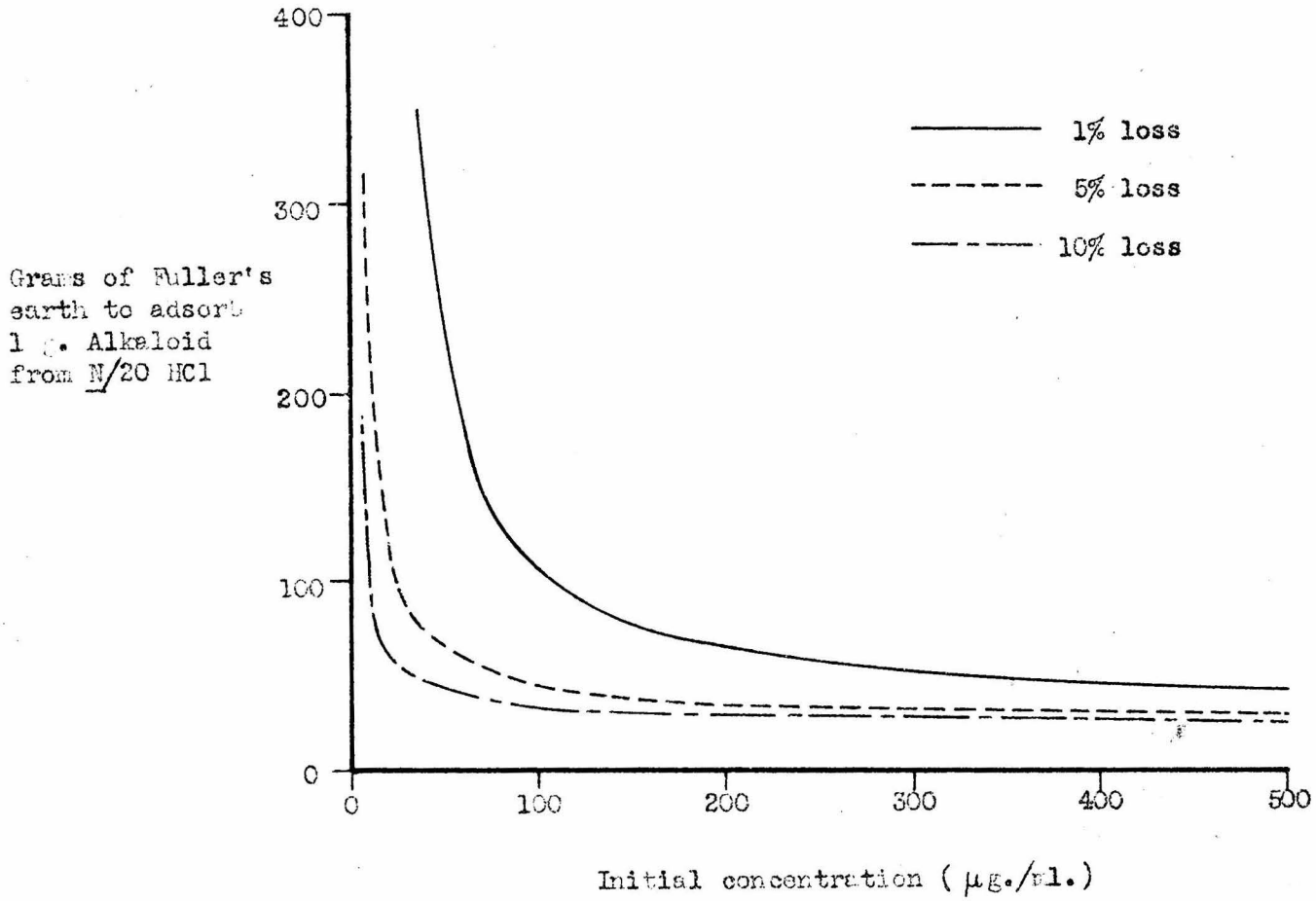
* This reagent consists essentially of an acidic solution of potassium and bismuth iodides. See The Merck Index, p. 696, 5th ed. (1940).

Figure 10. Curves indicating the amount of fuller's earth needed to adsorb one gram of crude alkaloids at different efficiencies.



with 0.1 N HCl until no test is obtained in 1/2 to 1 min., assuming the concentration to be 5 μg./ml., and multiplying by the dilution factor. The plant extract sometimes gives a pronounced deepening in color with Dragendorff's Reagent, but this is easily distinguished from the cloudiness, which is the alkaloid test. The test is not critically dependent on the acid concentration, but the sensitivity decreases with increasing acidity. The solution must be acidic to prevent the precipitation of basic salts of bismuth.

From the data accumulated at this point it was possible to devise an isolation procedure. The procedure is given in some detail in the following section. It was found to be quite satisfactory, and is the method used to isolate the material being used for the structure determination of the alkaloids (discussed in Part II).

Section 10

Final Procedure for Isolating the Alkaloids

The crude alkaloids are obtained by extracting the ground plant material with dilute hydrochloric acid, adsorbing the alkaloids on fuller's earth, removing them from the fuller's earth with butanol under alkaline conditions, concentrating them by distribution between immiscible solvents, and finally evaporating to dryness. Febrifugine and isofebrifugine are separated by means of the dihydrochlorides or by chromatography.

Extraction of the Roots.- The roots are ground to 8 to 10 mesh and packed in a percolator. They are next soaked in 0.1 N HCl for 48 hours or longer, the HCl is drained off, and fresh acid is added to the roots. This process is repeated until the roots are essentially exhausted of alkaloid, as indicated by the Dragendorff test (Section 9). Extraction of the root material should be complete before the concentration of alkaloid in the final solution is less that ca. 75 to 100 μg./ml.

The combined extracts are next filtered through a pad of Celite to clarify the solution. The concentration (and total amount) of alkaloid is determined by serial dilution and Dragendorff's Reagent as described in Section 9. From this data and the graph given in Fig. 10, the amount of fuller's earth necessary for 99% adsorption is found, and this amount of adsorbent plus a 50% excess (to allow for errors in the determinations involved) is added to the clear acid extract. Sufficient 12 N HCl is added to make the mixture strongly acidic to Congo Red. The equivalent weight

of fuller's earth (reacting as a base) was found to be ca. 100 to 150, so the amount of acid needed could be calculated roughly. The mixture is stirred for several hours, and the pH is tested occasionally with Congo Red Paper, additional HCl being added if necessary. Although it is known that the adsorption takes place rapidly, the mixture is stirred for several hours because it is desirable both to insure good contact between the relatively small amount of adsorbent and large amount of liquid, and to dissolve out the acid-soluble compounds in the fuller's earth.

At the beginning of the last hour of stirring, enough Celite to equal one-third the weight of fuller's earth is added. When the stirring is complete, the mixture is filtered through a pad of Celite weighing about one-sixth the weight of fuller's earth. The filtrate is tested with Dragendorff's Reagent, and if found to contain very little alkaloid is discarded.

Elution with Butanol.- The filter cake is now mixed with enough water to make a thin paste. It was found that too much water leads to undesirable fuller's earth-butanol emulsions later, and it was felt that too little water might make the extraction unduly slow because of poor contact between solid and butanol. An amount of water approximately equal in weight to the fuller's earth and Celite has been found to be satisfactory. Next, 6 g. of sodium carbonate per 100 ml. of water is added. This should make the mixture decidedly basic. In the event that the pH is less than 8.5, more sodium carbonate is added. To the resulting basic paste is added an amount of butanol equal to three times the amount of water added. This mixture is vigorously stirred or

shaken for about two hours.

On standing, if the proper proportions of solid, water, and butanol were attained, the mixture separates into a top butanol phase and a bottom solid-aqueous phase. The butanol is siphoned off, and more (one-third the original amount) is added for a second extraction.

If the proper proportions of solid, water, and butanol were not attained, a solid-butanol emulsion may form the bottom layer. It is then necessary to filter the mixture and separate the water and butanol phases of the filtrate. Water-butanol emulsions may be encountered, but they are easily broken by centrifugation. The aqueous phase is returned to the solid, and additional butanol is added for a second extraction.

A total of two or three extractions with butanol elutes most of the alkaloids from the fuller's earth. The solid can then be discarded, and the combined butanol extracts treated as indicated below.

Concentration by Distribution Between Immiscible Solvents.- To the combined butanol extracts are added 1/2 its volume of ligroin (60-70) and 1/2% of its volume of 6 N HCl. That is, to one liter of butanol extract is added 500 ml. of ligroin and 5 ml. of HCl. After shaking, the mixture is allowed to stand until the phases can be separated. The butanol-ligroin phase is extracted three more times with 0.1 N HCl, using portions equal to 8% of the original butanol volume.

The combined acid extracts, which contain the alkaloids, are made basic with sodium carbonate and extracted three times with 1:4 butanol-chloroform mixture, using portions equal to one-third the volume of the aqueous phase.

The extraction into acid and back into butanol-chloroform

mixture from basic solution is repeated. The butanol-chloroform solution is then dried with sodium sulfate, and evaporated almost to dryness at room temperature. A light colored solid is left as a residue, and it is mixed with a little ether and filtered off. Evaporation of the filtrate gives a second crop. This combined material is the crude mixture of alkaloids from the plant.

Separation of Febrifugine and Isofebrifugine.- To separate isofebrifugine and febrifugine from this mixture, use is made of the fact that the dihydrochloride of febrifugine is easily crystallizable from 90 to 95% ethanol, whereas the corresponding salt of isofebrifugine is very hygroscopic, and is soluble in this solvent.

The crude alkaloids are taken up in hot ethanol and 10% excess of 12 N HCl is added to convert the material to dihydrochlorides. The mixture is seeded with febrifugine dihydrochloride and allowed to crystallize over night in the ice box. The mother liquors are concentrated for a second crop of crystals. The febrifugine dihydrochloride which is obtained from this step is recrystallized by dissolving in a minimum of hot 1:1 water-ethanol mixture, and adding sufficient absolute alcohol to make the final concentration of alcohol 90%.

The mother liquors from the original preparation of febrifugine dihydrochloride are next taken almost to dryness at room temperature, the residue is taken up in a small amount of water, sufficient sodium carbonate is added to make the solution basic, and it is extracted with chloroform. The chloroform is evaporated at room temperature until the mixture becomes semisolid. The solid is then filtered off and washed with ethanol and ether. This solid recrystallized quickly from hot methanol gives pure isofebrifugine.

The combined mother liquors from the above several crystallizations are best worked up for additional material by chromatographing them from chloroform on alumina as described earlier in Section 7.

To obtain the free base from febrifugine dihydrochloride the material is dissolved in a minimum amount of water, and the calculated amount of 2.5 N NaOH is added in the cold. The free base slowly crystallizes out on standing a few hours in the ice box. It may be recrystallized from hot ethanol.

Section 11

Application of the Isolation Method to Various Plant Materials

Essentially the method outlined in the previous section was applied to several batches of root material and to one small sample of Chunine (see Section 1). The results obtained are given in Table VII. The following significant results have been found:

1. The yield of alkaloids is much higher by this fuller's earth method than it was by the original, hot alcohol or hot water extraction method. (Compare 3 with 5, Table VII.)

2. A sample of botanically identified Dichroa febrifuga has been shown to contain the same alkaloids that Ch'ang Shan (Chungking) contains. (See 2, Table VII.) This is convincing evidence that the botanical identity of the Ch'ang Shan being worked with is in fact D. febrifuga.

3. A sample of Chunine (Section 1) has been found to contain febrifugine. The fact that 25 g. of plant material is all that was necessary to demonstrate conclusively the presence of febrifugine indicates how powerful the isolation method is. The fact that Chunine is admittedly leaf material from Saxifragacea combined with the fact that it contains febrifugine seems quite conclusively to indicate that Chunine is at least in part the leaves of D. febrifuga. Using this same isolation method, Dr. Mead has found that the leaves of botanically identified D. febrifuga also contain febrifugine.

4. The alkaloidal content of the root material varies between 0.05 and 0.1%.

TABLE VII

Febrifugine and Isofebrifugine from Different Plant Material by the Fuller's Earth Method (Section 10)

Run	Material		Alkaloids[a]		
	wt. kg.	Description	crude g.	febrifugine g.	isofebrifugine g.
1.	2.9	Chungking Ch'ang Shan, Lot D.	2.54	1.27	0.17
2.	2.9	Dichroa Febrifuga, root material from India[b].	1.50	0.95	0.03
3[c].	23	Ch'ang Shan, Lot C (Lilly P-44899)	17.6	4.9[d](6.9)	4.6[d](5.4)
4.	0.025	Chunine	0.017	0.008	-
5[e].	23	Ch'ang Shan, Lot C	4	0.54	0.75

a. As the free base.

b. Material botanically identified at the time of collection, and kindly furnished by the Abbott Laboratories.

c. The first steps of this large scale isolation were carried out at Eli Lilly and Co.

d. Recrystallized. The figure in parentheses is the yield once crystallized.

e. This material was worked up by the method of Section 3. The original extraction was made with hot alcohol at Eli Lilly and Co. The quality of the crude base is much poorer than that of the crude base from the fuller's earth method.

5. Runs 1 and 2 (Table VII) indicate that febrifugine makes up by far the largest part of the alkaloids present in the plant. Runs 3 and 5, in which more heat was used, indicate the alkaloids to be about half febrifugine and half isofebrifugine. Since it is known that these two alkaloids are interconvertible (see Part II) when heated in solution, it would appear quite possible that the plant actually does not contain isofebrifugine, and that this alkaloid is formed as an artifact during the isolation.

6. In addition to febrifugine and isofebrifugine, the fraction of crude alkaloids contains small amounts of other basic substances, some dark oils and some high melting solids (m. p.'s ca. 200 and > 240). It is not known whether these are artifacts or if they actually represent bases present in the original plant material. No extensive attempt to characterise them has been made.

Section 12

Summary of the Properties of Febrifugine and Isofebrifugine

Febrifugine crystallizes from ethanol in colorless needles, m. 139 to 140° (cor.). A second form can be obtained from chloroform or water, m. 152 to 154° (cor.). The specific rotation, $[\alpha]_D^{25}$, is + 6° (c 0.5, chloroform) and + 28° (c 0.5, ethanol).

This base is approximately 1 to 3% soluble at room temperature in water, ethanol, acetone, and chloroform; very soluble in methanol-chloroform mixtures and in water-ethanol mixtures; insoluble in ether, benzene, and petroleum ether.

The base is very active as an antimalarial. Dr. Richardson reports a Q of 100 in test G-5 (P. lophurae in ducks) both by intravenous injection and orally. Dr. Coatney reports a Q of 64 in test A-1 (P. galinaceum in chicks) both intravenously and orally.

The base is very toxic. Dr. Schmidt reports an acute oral toxicity for mice as 2.0 mg. of febrifugine per kg. body weight. Single oral doses of 2 to 8 mg. per kg. kill mice within 24 to 72 hours. It is notable that even at such large doses as 1600 mg. per kg. death rarely occurs before 12 hours. The only noteworthy finding in autopsies of mice at any doses is the presence of erosion in the pyloric region of the stomach. A monkey given 0.75 mg. per kg. daily lost weight to a marked extent, and was definitely ill, although the symptoms were not well-defined except for the loss in appetite.

Isofebrifugine crystallizes from methanol in colorless chunky

prisms, m. 129 to 130° (cor.), $[\alpha]_D^{23}$ +131° (c 0.35, chloroform).

At room temperature this base is approximately 0.5% soluble in water, 3 to 4% in methanol, 7% in chloroform, very soluble in methanol-chloroform mixtures, and insoluble in ether, acetone, benzene, and petroleum ether.

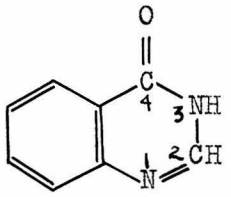
This base has given different results as an antimalarial in different laboratories. Dr. Marshall found it to be inactive at the maximum tolerated dose in test D-1 (P. lophurae in ducks) when given intravenously two times a day. The Q is reported then as less than 1, toxic. Dr. Chen reports a Q of 1 in the I-2 test (P. lophurae in ducks) when the drug is given intravenously, t.i.d. Dr. Richardson reports Q 1 in test G-5 (P. lophurae in ducks) intravenously, t.i.d.

PART II. STUDIES ON THE STRUCTURES OF FEBRIFUGINE AND ISOFEBRIFUGINE

The structures of febrifugine and isofebrifugine are unknown. Studies beyond those given in this thesis are being continued, and it is hoped they will lead to a thorough elucidation of the structures. The work is being done in co-operation with Dr. Mead at Occidental College, and to avoid unnecessary duplication the studies have been divided so that Dr. Mead is studying oxidative degradation, and I am studying the functional groups and hydrolytic degradation.

It is to be emphasized that any conclusions indicated in this part of this thesis are tentative. Any interpretation of the facts offered at this time may require revision later, because of newly discovered information. Because of this state of flux, the experimental facts will be presented in the first two sections with little or no interpretation. In the last section a summary of the facts and a possible interpretation will be given.

Early in the structure determination Dr. Mead found that both alkaloids when oxidized with basic permanganate at room temperature give good yields of 4-quinazolone (I).



I

Section 1

Empirical Formula and Functional Group Tests

In this section are given the results of many micro-analyses and many micro-tests, e.g., spot tests, hydrolytic tests on 1-mg. samples, and classification tests done on 1-mg. samples. In various places spot tests will be mentioned briefly. The detailed procedures which were followed in carrying out these spot tests are given in Feigl, Spot Tests, 3rd. Ed., New York: Elsevier (1946). The micro-analyses were carried out by Wm. Saschek, G. Oppenheimer, and G.A. Swinehardt.

The micro-analysis of febrifugine and isofebrifugine proved to be somewhat difficult in that carbon and hydrogen values fluctuated considerably. This difficulty was traced at least in part to the method of drying. Samples dried to constant weight at room temperature over P_2O_5 at reduced pressure gave good analytical results, but samples dried at 78° were more likely to give variable results, and a sample dried at 110° was found to lose weight continually, and a sublimate could be seen collecting on surrounding vessels. This sublimate had been observed earlier when a sample of isofebrifugine was heated at ca. 135 to 140° for some time in vacuo in a sublimation apparatus. The sublimate formed in ca. 24% yield (based on weight of original material), melted at ca. 212°, and is now believed to have been 4-quinazolone.

Some typical analyses for the alkaloids are given in Table VIII. The results for both free bases and for febrifugine dihydrochloride, coupled with the fact that a potentiometric titration of isofebrifugine

TABLE VIII

Results of Micro-analyses of the Alkaloids

Compound	Drying temp.	C	H	N	Cl	Anal. Lab.
febrifugine	80°	63.51*	6.07*	13.94*	--	a
	80°	63.15*	5.97*	13.76*	--	a
	80°	63.3	5.6	14.0	--	b
	110°	--	--	13.86*	--	a
	110°	--	--	13.82*	--	a
	25°	63.30*	6.22*	--	--	a
	25°	63.47*	6.34*	--	--	a
	25°	64.2	6.6	--	--	b
	25°	64.14	6.34	--	--	b
isofebrifugine	110°	--	--	14.04	--	a
	80°	63.20	5.82	14.07	--	a
	80°	63.3	6.4	14.2	0.0[c]	b
	25°	63.50	5.96	--	--	a
febrifugine dihydrochloride	80°	51.17	5.63	10.87	18.40[d]	a
	80°	--	--	10.97	18.62[d]	a
	25°	51.39	5.85	11.30	18.57[e]	a
	25°	51.26	5.82	11.15	18.64[e]	a
	25°	--	--	11.16	18.91[d]	a
	25°	--	--	11.16	18.90[d]	a
$C_{16}H_{17}O_3N_3$		64.24	5.72	14.02	--	
$C_{16}H_{19}O_3N_3$		63.78	6.36	13.95	--	
$C_{16}H_{21}O_3N_3$		63.33	6.98	13.85	--	
$C_{16}H_{19}O_3N_3Cl_2$		51.63	5.14	11.29	19.05	
$C_{16}H_{21}O_3N_3Cl_2$		51.34	5.66	11.23	18.95	
$C_{16}H_{23}O_3N_3Cl_2$		51.11	6.16	11.16	18.85	

a. Wm. Saschek, College of Physicians and Surgeons, Columbia University.

b. G. Oppenheimer and G.A. Swinehardt, California Institute of Technology.

c. Pregl dry combustion. d. Ionic. e. Carius.

* Values marked with an asterisk were corrected for ash.

had indicated an equivalent weight of ca. 300, leave little doubt that the correct empirical formula for both febrifugine and isofebrifugine is $C_{16}H_{19}O_3N_3$. The possibility that the number of hydrogen atoms is incorrect by 2 is not ruled out, but analyses of several derivatives made subsequently are consistent with the choice of 19.

The Hertzig-Meyer N-methyl determination indicated that no methoxy groups are present, but gave values for N-methyl of 0.75% for febrifugine and 5.3% for isofebrifugine, determined gravimetrically. The theoretical value for one N-methyl group is 5.0%. The value for isofebrifugine was redetermined by the volumetric procedure, and this time was found to give 1.1%. The determination has since been carried out on the dihydro-alkaloids with negative results. It seems quite definite that there is no N-methyl group, as such, present in either febrifugine or isofebrifugine, but perhaps some other grouping which on heating with hydriodic acid gives some methyl iodide. For example, the grouping $\equiv C\text{-}O\text{-}CH_2\text{-}N =$ has been postulated to account for the formation of methyl iodide in methyl-imino determinations of vomicine[16], and varying amounts of methyl iodide have been observed in N-methyl determinations of 4-quinazolone-3-acetic acid[17].

Micro-catalytic hydrogenations using platinum oxide (Adam's catalyst) and hydrogen at one atmosphere and room temperature showed that febrifugine takes up one mole of hydrogen in ethanol or glacial acetic acid; isofebrifugine takes up one mole of hydrogen in ethanol,

- -

16. Henry, Plant Alkaloids, p. 539, 3rd. Ed., Philadelphia: Blakiston.
17. Späth and Nikawitz, Ber., 67, 45 (1934).

and 2 moles (or more) in glacial acetic acid; 4-quinazolone takes up no hydrogen (in 6 hrs.) in ethanol, and one-half mole in glacial acetic acid. Preparation of crystalline dihydro-compounds in ethanol has been successfully carried out on a larger scale, but no crystalline product could be obtained from isofebrifugine reduced in glacial acetic acid (2 moles of hydrogen). Yields of the crystalline dihydro-compounds are low (ca. 70% for febrifugine and less than 50% for isofebrifugine), and it seems indicated that the reduction leads to the formation of more than one compound in each case.

Both febrifugine and isofebrifugine form crystalline dibenzenesulfonyl derivatives when treated with benzenesulfonyl chloride in pyridine. These derivatives are non-basic (insoluble in 0.1, 1.0, and 6.0 N HCl). The acetyl and benzoyl derivatives are oils. As yet no pure, crystalline derivatives of the dihydro-compounds have been prepared. Attempts with benzenesulfonyl chloride and tosyl chloride gave compounds (presumably low melting) which could not be satisfactorily crystallized. A derivative with 3, 5-dinitrobenzoyl chloride has so far not been obtained pure.

The Zerwitinoff active hydrogen micro-determination has not given satisfactory results.

Febrifugine and isofebrifugine form crystalline compounds when treated with hydrochloric acid and sodium nitrite in aqueous solution. The compounds are difficult to purify and sufficient quantities for analysis have not been prepared. The compounds formed give a Libermann test for the nitroso group, and are presumed to be N-nitroso derivatives of a secondary aliphatic amine. The derivative

from febrifugine melts at 161 to 169° with the slow evolution of a gas; the derivative from isofebrifugine melts at 178.5 to 180° with the slow evolution of a gas.

The alkaloids do not react at room temperature in alcoholic solution with carbon disulfide to form dithiocarbamates (spot test for primary and secondary aliphatic amines). A solution of sodium nitroprusside and acetaldehyde gives no color with febrifugine when sodium carbonate is added, but isofebrifugine under these conditions gives a weak purple color (spot test for secondary amine). With a saturated solution of chloranil in dioxane, isofebrifugine gives a violet color in ca. 15 sec. and febrifugine a violet color in ca. 60 sec. (most amines give red, blue, or green colorations).

Febrifugine forms a crystalline oxime and semicarbazone, but isofebrifugine and dihydrofebrifugine under the same conditions do not. A spot test involving the formation of a bisulfite addition compound is negative for febrifugine and isofebrifugine. No color is formed with febrifugine and isofebrifugine in a saturated solution of o-dianisidine in glacial acetic acid (negative color test for aldehydes). Both alkaloids reduce Tollen's Reagent in 1 to 5 min., but the dihydro-compounds do not, even on standing 3 hours.

The solubility of febrifugine and isofebrifugine in water and in 0.1 N NaOH were determined by stirring a few mg. of each alkaloid with a few drops of solvent for 5 min., centrifuging, drawing out a sample in a weighed capillary, weighing the sample of solution, and determining the amount of alkaloid spectrophotometrically. The values obtained were

the following: febrifugine 21 mg./ml. of water, and 21 mg./ml. of 0.1 N NaOH; isofebrifugine 5.2 mg./ml. of water, and 13 mg./ml. of 0.1 N NaOH. 4-Quinazolone's unsubstituted in the three position are in general quite soluble in dilute sodium hydroxide whereas these alkaloids are not.

When a few milligrams of febrifugine or isofebrifugine is heated with strong sodium hydroxide solution (30%) a blob of resin appears very quickly, and there is given off a basic gas which was shown to consist of ammonia and some strong smelling secondary amine. On heating in more dilute (2.5 N) sodium hydroxide, a dark color forms but no resin separates. The resulting hydrolyzates were shown to contain formic acid (spot test using chromatropic acid) and a diazotizable aromatic amine (diazotization was carried out in HCl solution with sodium nitrite, and the presence of a diazonium salt was demonstrated by adding the solution to a basic solution of β-naphthol whereupon a bright orange color developed). This amine has since been shown to be anthranilic acid. When dihydrofebrifugine is treated with strong base no resin forms and no basic gas is liberated. The hydrolyzate gives the tests for anthranilic acid and formic acid. When the alkaloids are heated in concentrated hydrochloric acid at 100° for 30 min. no anthranilic acid can be demonstrated.

4-Quinazolone's in general are stable toward acid hydrolysis. 4-Quinazolone and 2-substituted-4-quinazolone's are also quite stable toward basic hydrolysis. However, 4-quinazolone's substituted in the 3-position are evidently more easily hydrolyzed. A 1-mg. sample of 3-allyl-4-quinazolone heated with 10 μl. of 2.5 N NaOH in a bath of boiling water gave a strong test for anthranilic acid after heating 5 min.,

even though most of the sample did not dissolve in the aqueous base. A 1-mg. sample of 2-methyl-4-quinazolone under the same conditions is completely soluble, and heated for 30 min. gave a very weak test for anthranilic acid. A sample of 4-quinazolone under these conditions gave no test for anthranilic acid after heating for 30 min.

The absorption spectra of febrifugine in ethanol and in N/20 HCl are given in Fig. 11 and in Fig. 12, resp. The spectra of isofebrifugine, dihydrofebrifugine, dihydroisofebrifugine, 2-methyl-4-quinazolone, and 3-allyl-4-quinazolone are remarkably similar to that of febrifugine. Separate curves for all these compounds are not given because the shape and general characteristics are the same, however, in Tables IX and X the molar extinctions at the wavelengths of principal maxima and minima are given. It can be seen in these tables that the 3-substituted-4-quinazolone has maxima and minima within 1 mμ of those of the alkaloids. The 2-substituted-4-quinazolone has maxima and minima shifted in general a few mμ toward the shorter wavelengths, compared with the alkaloids. The molecular extinctions of all these compounds are remarkably constant, varying generally less than 10% from the average at any given extreme.

These spectra were taken at concentrations of ca. 10^{-4} to 10^{-5} molal which gave extinctions between ca. 0.3 and 0.9 as read on the Beckman spectrophotometer. To get values for the 225-mμ maxima it was necessary to use more dilute solutions than the ones used for the other extremes. There has been some evidence that solutions of these compounds do not obey Beer's Law. However, this evidence has not been convincing, and for extinctions in the range 0.3 to 0.9 the variance

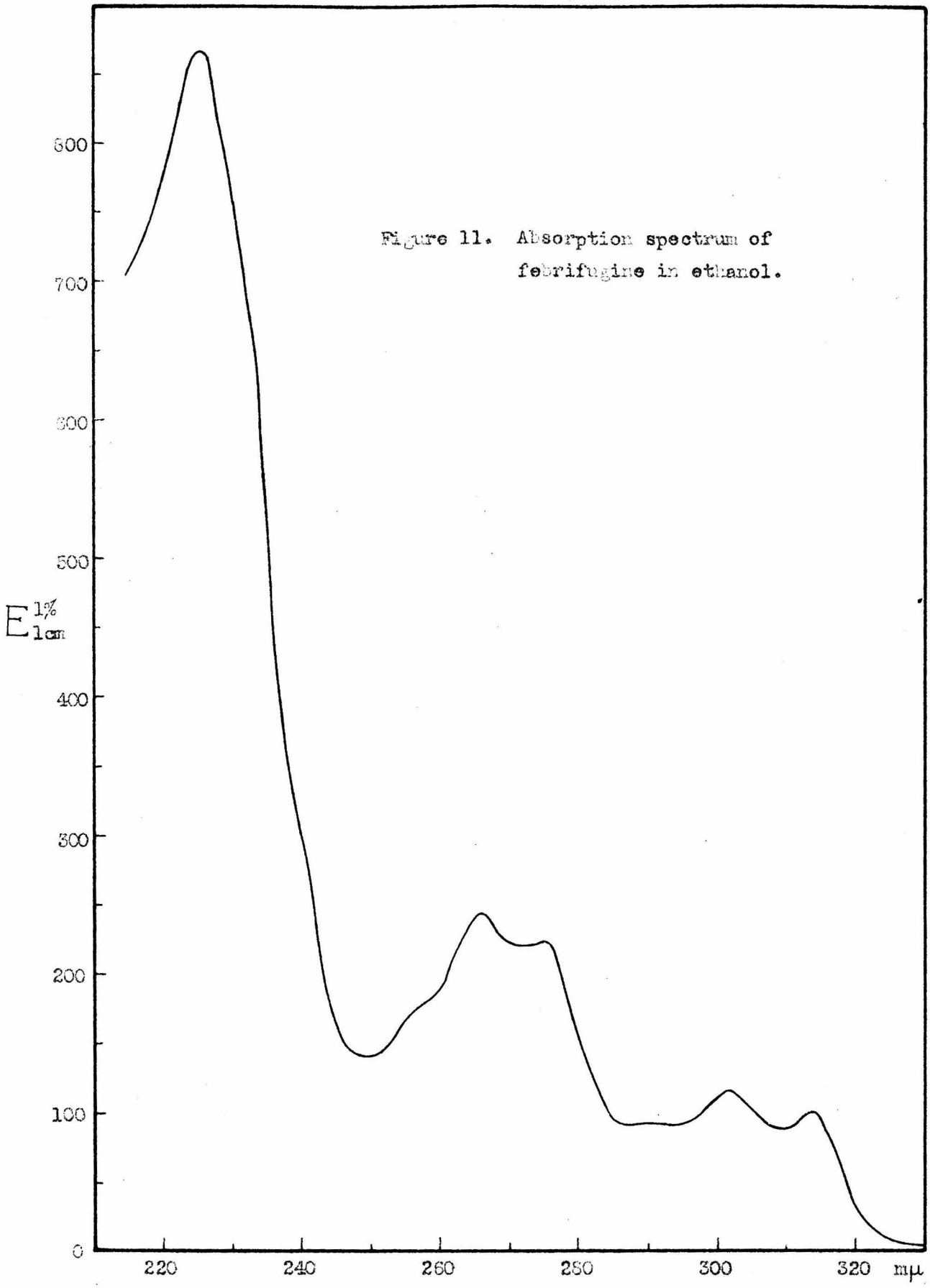


Figure 11. Absorption spectrum of febrifugine in ethanol.

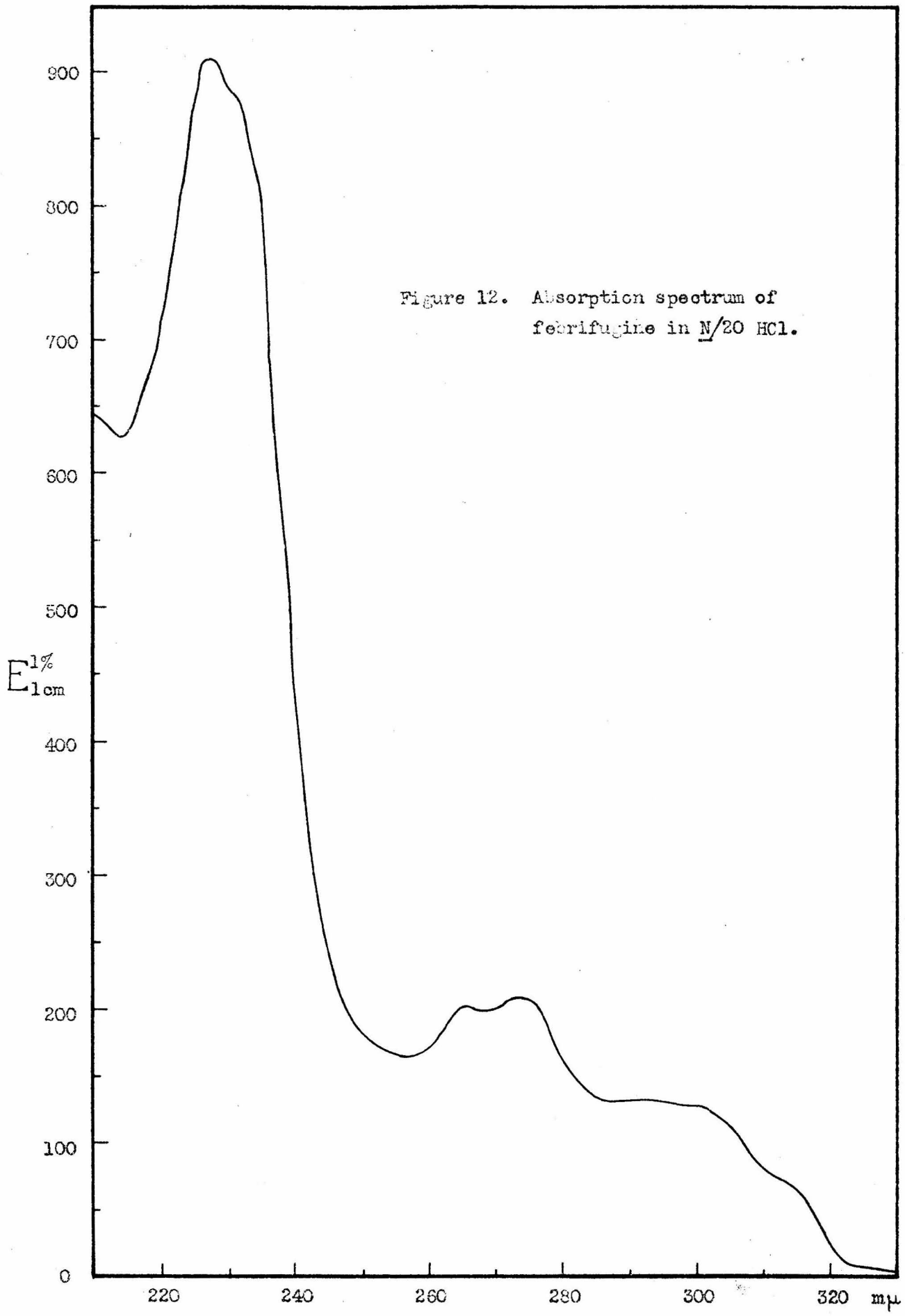


Figure 12. Absorption spectrum of febrifugine in N/20 HCl.

TABLE IX

Molal Extinctions for Spectra Taken in Ethanol

(molal extinction x 10^{-3})

Compound	Maximum		minimum		maximum		maximum		maximum		maximum	
	mμ	E_m	mμ	E_m	mμ	E_m	mμ	E_m	mμ	E_m	mμ	E_m
febrifugine	225	26.1	250	4.21	266	7.34	275	6.68	302	3.49	314	3.04
isofebrifugine	225	24.6	249	4.09	266.5	7.10	275	6.62	303	3.46	314	2.77
dihydro-febrifugine	226	26.3	249	4.58	267	8.33	275	7.75	302	3.91	314	3.12
dihydro-isofebrifugine	226	26.1	249	4.02	267	7.91	275	7.41	302	3.65	314	2.92
3-allyl-4-quinazolone	225	24.2	249	3.78	267	7.44	275-6	6.88	301-2	3.61	313	2.94
2-methyl-4-quinazolone	225	26.2	247	4.84	263-4	7.60	-	-	303-4	4.06	315	3.30

TABLE X

Molal Extinctions for Spectra Taken in N/20 HCl

(molal extinction x 10^{-3})

Compound	minimum		maximum		minimum		maximum		maximum	
	mμ	E_m	mμ	E_m	mμ	E_m	mμ	E_m	mμ	E_m
febrifugine	214	18.9	227	27.4	257	4.94	266	6.04	274	6.26
isofebrifugine	215	16.9	229	24.8	257	4.69	266	5.78	274	6.08
dihydro-febrifugine	216	18.3	229 232	25.6	257	4.60	-	-	274	6.48
dihydroiso-febrifugine	217	16.7	229 232	24.7	256	4.64	-	-	275	6.63
2-methyl-4-quinazolone	-	-	232	28.4	256	4.47	-	-	270	5.45

has not been greater than a few percent. It is believed that the values of molar extinctions reported in Tables IX and X are not in error by more than ca. 5% from the combined inaccuracies of weighing, dilution, instrumental difficulties, and any possible deviation from Beer's Law.

Isofebrifugine and febrifugine are interconvertible. If isofebrifugine is refluxed in ethanol for about 2 hours, a yield of ca. 50% of febrifugine can be isolated by chromatography or through the dihydrochloride. Samples of isofebrifugine heated neat above the melting point for several minutes appear to give higher yields of febrifugine dihydrochloride (ca. 80%). A sample of febrifugine was refluxed for 3 hours in chloroform and the solution then chromatographed. A 43% yield of isofebrifugine was isolated. A solution of febrifugine in chloroform containing a small amount of iodine was illuminated with an arc lamp for 1 hour, and then chromatographed. Small quantities of isofebrifugine were found (ca. 2 or 3%).

Dr. Mead has recently found that in basic solution the alkaloids take up four oxidizing equivalents of periodate, and a crystalline product can be isolated in good yield. This material appears to have the empirical formula $C_{11}H_{12}O_2N_2$. Dihydrofebrifugine does not take up any periodate under these conditions.

This oxidation product has some remarkable properties. Its absorption spectrum in dilute hydrochloric acid is very nearly the same as that of the original alkaloids. In ethanol or in alkaline aqueous solution the absorption spectra are quite different, having a strong absorption with a maximum between ca. 280 mμ and 315 mμ depending on the pH. If

this colorless oxidation product is dissolved in a small amount of dilute hydrochloric the solution soon turns a bright yellow, and when sodium bicarbonate is added a yellow amorphous precipitate is obtained. If the solution is made more strongly basic (sodium carbonate or sodium hydroxide) a very strong amine smell is noticed. If a very dilute solution of the compound in water is made acidic with hydrochloric acid no yellow color develops.

This oxidation product takes up hydrogen in ethanol in the presence of platinum (Adam's catalyst). The rate of hydrogen uptake is irregular and slow, but the amount of hydrogen taken up becomes constant after about six hours, and corresponds to an uptake of one mole for a compound with a molecular weight of ca. 200. Evaporation of the alcohol leaves a colorless oil which no longer gives a bright yellow solution in acid, and which has an absorption spectrum similar to that of a 4-quinazolone with regard to the positions of the extremes, but differing considerably in relative extinctions. Since the spectrum was taken on a sample of unpurified material a useful interpretation can not be given. It does seem certain that the absorption spectrum of the reduced compound is different from that of the oxidation product.

Alkaline hydrolysis of this oxidation product on a micro-scale closely parallels the hydrolysis of febrifugine; anthranilic acid, formic acid, and ammonia are observed, and the solution darkens considerably. Oxidation of the compound with alkaline permanganate gives 4-quinazolone.

The preparations and analyses of the derivatives discussed above

are given below.

Preparation of Dihydrofebrifugine.- 409 mg. (1.36 millimoles) of febrifugine was dissolved in 50 ml. of absolute ethanol, 50 mg. of platinum oxide (Adam's catalyst) was added, and the solution was shaken in an atmosphere of hydrogen (at one atm. pressure) until no more hydrogen was taken up. About 86% of the theoretical amount of hydrogen (1.36 millimoles) was taken up. A sample of maleic acid hydrogenated under the same conditions took up 87% of the theoretical amount of hydrogen, so it is believed that actually the theoretical amount is taken up, but the method for measuring the uptake is inexact. The reduction took ca. one hour. The catalyst was filtered off, and the solution evaporated to dryness at reduced pressure. The crystalline residue was recrystallized from hot ethanol. Yield was 263 mg. (64%) of colorless crystals melting at 187 to 192°. A second crop of 34 mg. was obtained, melting at 163 to 184°. The mother liquors taken to dryness amounted to 107 mg. of oil.

The dihydrofebrifugine was recrystallized two times from hot ethanol to constant melting point of 192 to 193°.

Anal. Calc. for $C_{16}H_{21}O_3N_3$: C, 63.33; H, 6.98; N, 13.85. Found: C, 63.34; H, 6.99; N, 14.07.

A second reduction of a larger sample gave similar results. 1.023 g. of febrifugine in 75 ml. absolute ethanol in the presence of 102 mg. catalyst gave a yield of 565 mg. (55%) first crop melting at 191.5 to 192.5°. A second crop amounted to 161 mg. melting at 175 to 182°.

Preparation of Dihydroisofebrifugine.- The same method was used here as that given under the preparation of dihydrofebrifugine. 400 mg.

(1.33 millimoles) of isofebrifugine in 50 ml. of absolute ethanol in the presence of 50 mg. of Adam's catalyst took up essentially the theoretical amount of hydrogen (1.33 mol.) in ca. three hours. The catalyst was filtered off, and the solution taken to dryness at reduced pressure. The residue was an oil which could be crystallized from acetone or acetone-benzene mixture. The first crop from acetone amounted to 182 mg. (45%) melting at 151 to 154°. A second crop from acetone-benzene amounted to 97 mg. melting at 86 to 124°. The mother liquors evaporated to dryness amounted to 127 mg. of oil.

The dihydroisofebrifugine was recrystallized from acetone to constant melting point. Three recrystallizations were required, the pure compound melting at 156.5 to 157.5°.

Anal. Calc. for $C_{16}H_{21}O_3N_3$: C, 63.33; H, 6.98; N, 13.85. Found: C, 63.41; H, 6.93; N, 13.77.

Preparation of the Benzenesulfonyl Derivatives of Febrifugine and Isofebrifugine.- 52 mg. (0.17 millimole) of isofebrifugine, 104 mg. (0.59 millimole) of benzenesulfonyl chloride, and 114 mg. (1.9 millimoles) of pyridine were mixed in a centrifuge tube and allowed to stand overnight.

The mixture was now stirred with 0.25 ml. 6 N HCl. A thick oil separated and was washed with water. The oil was crystallized from aqueous alcohol. Samples recrystallized from chloroform-ethanol mixture and from ethanol-water mixture had identical sharp melting points of 182.5 to 183.5° (dec.). The compound is not very soluble in aqueous alcohol even when hot. The yield was 48 mg. of colorless crystals.

Anal. Calc. for $C_{28}H_{27}O_7S_2N_3$: C, 57.85; H, 4.68; N, 7.23. Found: C, 57.91; H, 4.64; N, 7.29.

The same method was applied to 49 mg. (0.16 millimole) febrifugine 101 mg. (0.57 millimole) benzenesulfonyl chloride, and 115 mg. (1.9 millimoles) pyridine. The resulting compound was recrystallized three times from aqueous alcohol to constant melting point. The yield was 41 mg. of colorless crystals, melting at 148.0 to 148.5°. This compound was much more soluble in hot aqueous alcohol than the isofebrifugine derivative.

Anal. Calc. for $C_{28}H_{27}O_7S_2N_3$: C, 57.85; H, 4.68; N, 7.23. Found: C, 57.80; H, 4.98; N, 7.31.

Preparation of Febrifugine Semicarbazone.- 98 mg. (0.26 millimole) of febrifugine dihydrochloride, 100 mg. (0.90 millimole) semi-carbazide hydrochloride, and 255 mg. (3.80 millimoles) sodium acetate (anhydrous) were weighed into a small centrifuge tube. About 1 ml. of water was added, and the mixture stirred until a clear solution resulted. This was allowed to stand over night at room temperature.

The solution was now made just basic with sodium hydroxide solution. A colorless compound soon began to crystallize. After several hours in the ice box the solid was collected, washed with water, and recrystallized from aqueous alcohol. The compound partially melted at 110-115° and finally at ca. 185 to 190°.

When recrystallized from ethyl acetate or absolute alcohol, the compound melted sharply at 187 to 188° with the evolution of a gas. The yield was 26 mg.

Anal. Calc. for $C_{17}H_{23}O_3N_6$: C, 56.90; H, 6.43; N, 23.40. Found: C, 57.07; H, 6.48; N, 23.14.

Preparation of Febrifugine Oxime.- 96 mg. (0.26 millimole) of febrifugine dihydrochloride, 104 mg. (1.51 millimoles) of hydroxylamine hydrochloride, and 299 mg. (4.45 millimoles) of sodium acetate (anhydrous) were weighed into a small centrifuge tube. About 1 ml. of water was added, and the mixture stirred until a clear solution resulted. This was allowed to stand over night at room temperature.

The solution was now made just basic with sodium hydroxide solution. On cooling and scratching, a white crystalline compound separated. This was collected, washed with water, and recrystallized twice from aqueous alcohol.

The yield was 45 mg. of colorless crystals, darkening slightly above 210° and melting with decomposition at 224 to 225°.

Anal. Calc. for $C_{16}H_{20}O_3N_4$: C, 60.80; H, 6.31; N, 17.69. Found: C, 60.85; H, 6.22; N, 17.45.

Section 2

Hydrolytic Degradation

The preliminary micro-tests indicated that the alkaloids are cleaved by aqueous base to anthranilic acid and ammonia. Larger experiments were set up using *ca.* 100-mg. samples of the alkaloids, 2.0 ml. of 2.5 *N* NaOH, and allowing the mixtures to stand one to two days at room temperature.

In two experiments nitrogen was passed through the mixture and the effluent gas was passed through a known amount of standard hydrochloric acid. Back titration of this acid indicated that from isofebrifugine a relatively small amount of ammonia is formed, *viz.*, 0.044 milli-equivalent of base from 0.30 millimole of alkaloid. From febrifugine 0.015 milli-equivalent of base was obtained from 0.27 millimole of alkaloid. When the reaction mixture of febrifugine was heated at 100° for two hours, an additional 0.031 milli-equivalent of base was found, and the hydrolyzate darkened considerably. After the back titration to determine the amount of volatile base collected, the titration solutions were evaporated to dryness on a steam bath. A sample of the residue was placed in the depression of a micro-culture slide, a drop of strong sodium hydroxide solution added, and a hanging drop of platinum chloride solution was placed above the solution. In a few moments crystals formed in the hanging drop, and microscopic examination showed them to be the characteristic octahedra of the ammonium salt. That the volatile base

was not entirely ammonia was evident from a strong characteristic odor above the basic solution, suggesting a combination of piperidine and impure acetamide (mousy).

The alkaline hydrolyzates were next brought to pH 8 to 9 by adding hydrochloric acid, and the mixtures extracted with 1:4 butanol-chloroform. These extracts on drying and evaporating to dryness yielded about 20% of the original weight of alkaloid as a dark brown oil, soluble in dilute hydrochloric acid, and precipitated from the acid solution by base. Attempts to prepare the acetyl derivative, the picrate, and the chloroplatinate led to amorphous, colored precipitates. When this material was heated with 20% aqueous sodium hydroxide, a globe of resin collected rapidly. When this resin was heated in a dry test tube, the vapors above it gave a pine splinter test for pyrrole. The characteristic amine smell was present during this treatment.

The hydrolyzate was next acidified with hydrochloric acid until the pH was between 3 and 4, and the mixture was extracted with chloroform. This chloroform extract when taken to dryness gave a light colored crystalline solid amounting to ca. 40% of the original weight of alkaloid. This material was sublimed, and then recrystallized from aqueous alcohol, and it proved to be anthranilic acid (m. 145 to 146°, no depression in mixed melting point with an authentic sample). On the basis of one mole of anthranilic acid per mole of alkaloid, the yield is ca. 88% of theoretical.

The aqueous phase from these extractions was next taken to dryness or to a small volume in a vacuum desiccator over sodium hydroxide. This residue if warmed with 20% aqueous sodium hydroxide gave very rapidly a blob of resin which gave a pine splinter test when heated with

zinc dust. The material given off during this heating with zinc could be collected in dilute HCl, and the resulting solution gave a positive test with Ehrlich's Reagent (test for pyrroles and indoles) and a positive test for secondary amine with sodium nitro-prusside and acetaldehyde (Simon test). On other experiments attempts were made to prepare an acetyl derivative, a picrate, a chloroplatinate, an oxime, and a phenacyl ester from this residue, but in every case the only isolable material was a dark oil or resin.

Briefly then, alkaline hydrolysis of febrifugine and isofebrifugine gives a good yield (88%) of anthranilic acid, a poor yield (5 to 10%) of ammonia (plus some other volatile base), an undetermined amount of formic acid, and unidentified resins and oils. Since the supply of alkaloids is strictly limited, it seemed unwise to expend more material on such hydrolyses, at least until more information is available on the structure of the "other half".

Preliminary experiments had indicated that alkaline hydrolysis of the dihydro-alkaloids was not accompanied to any great extent by the resin formation or the liberation of a volatile base. Because of this apparent increase in stability of the dihydro-compounds, hydrolytic experiments were started with dihydrofebrifugine. These experiments have not been completed, but encouraging results have been obtained.

The hydrolysis of dihydrofebrifugine was carried out in a manner very similar to that described above for febrifugine. The compound was mixed with 2.5 N NaOH (100 mg. of dihydrofebrifugine to 2 ml. NaOH) and allowed to stand at room temperature. The dihydrofebrifugine slowly

dissolved, taking 12 to 16 hours for complete solution. After complete solution had taken place the mixture was allowed to stand for an additional 12 to 16 hours. At no time during the hydrolysis would a piece of moist neutral litmus turn blue when held in the space above the mixture, and no color developed in the alkaline solution.

After the hydrolysis, the solution was diluted and extracted with chloroform. Evaporation of the chloroform gave essentially no residue. The solution was then acidified to pH 3 to 4, and again extracted with chloroform. Evaporation of the chloroform gave a good yield of anthranilic acid.

The aqueous phase was treated in two different ways in an attempt to isolate a derivative of the hydrolyzed fragment. An attempt to prepare an acetyl derivative led to the isolation of a basic, hygroscopic glass, while an attempt to prepare a benzenesulfonyl derivative led to the isolation of a crystalline compound. The methods employed are indicated briefly below.

1. The aqueous phase was made basic with NaOH, and shaken in the cold with a large excess of acetic anhydride. No crystalline derivative separated on cooling and scratching so the mixture was made acidic with hydrochloric acid and taken to dryness in a vacuum desiccator over NaOH. The solid (mostly sodium chloride) was extracted with absolute ethanol. The ethanol on evaporating left a very hygroscopic oil, which tested very acidic on moist pH paper. This was stirred with freshly prepared silver oxide in water, and the water removed in a vacuum desiccator over NaOH. The residue was an oil, very soluble in water, ethanol, and methanol, quite

insoluble in diethyl ether and benzene, and soluble in hot acetone and ethyl acetate, separating as an oil on cooling. No derivative could be obtained with ethanolic picric acid, aqueous chloro-platinic acid, Reinecke salt in dilute hydrochloric acid, or on heating with methyl iodide.

When the material (ca. 30 mg.) was distilled in a micro-sublimation apparatus, it darkened on heating but about one-third of the material distilled at ca. 150° and 0.6 mm. The distillate was a hygroscopic glass, which gave a pH of 8 to 9 on moist pH paper. It gave no spot tests for primary and secondary amine, gave a positive test for ester (or amide) by forming a hydroxamic acid with hydroxylamine in basic solution, gave a positive test for nitrogen by sodium fusion, and gave no characteristic absorption spectrum in ethanol in the region 220 to 300 mμ and essentially no absorption at all in the region 240 to 300 mμ at a concentration of ca. 0.25 mg. per ml.

2. A crystalline derivative was obtained from the alkaline hydrolyzate in the following manner. To the solution was added NaOH until the concentration was ca. 15%, then excess benzenesulfonyl chloride was added and the mixture shaken vigorously. Soon a pasty solid separated. This solid was insoluble in water, and in dilute hydrochloric acid, and could be recrystallized from acetonitrile to yield fine crystals, melting and decomposing at 211 to 213° (cor.). The analyses for this compound were C, 54.29; H, 5.09; S, 16.73; N, 5.16 and 5.06. These analyses are in good agreement with the formula $(C_8H_{13}ON_2)(C_6H_5SO_2)_3$, calc.: C, 54.2; H, 4.89; S, 16.7; N, 4.86.

When this compound was heated for one-half hour with alcoholic KOH, a new compound was obtained and could be crystallized from ethanol. It melted at 220 to 222° (cor.) and contained 6.63% N. Sufficient material was not available for other analyses. The difference in nitrogen content corresponds to the loss of one benzenesulfonyl group on hydrolysis.

Section 3

Summary and Conclusions

It is to be emphasized again that any conclusions drawn from the experimental work of the previous sections are tentative and very likely to change as additional information is obtained. However, a summary of the pertinent facts and a possible interpretation seems in order here.

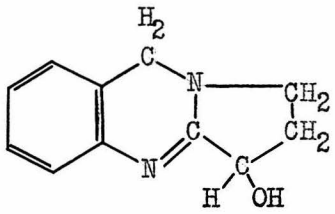
1. The empirical formula.- Both febrifugine and isofebrifugine have the same empirical formula, $C_{16}H_{19}O_3N_3$. Furthermore, the alkaloids are easily interconvertible on warming in solution, a property believed to be unique among the known alkaloids.

2. Presence of a Secondary Aliphatic Amine.- Evidence that both alkaloids contain a secondary aliphatic amine is quite strong. Both have a basic group of pK_B 6.3. Both form dibenzenesulfonyl derivatives which are insoluble in dilute hydrochloric acid and dilute sodium hydroxide. Both give nitroso compounds with sodium nitrite and hydrochloric acid. The Simon color test for secondary amines is positive but weak with isofebrifugine and negative with febrifugine. Both alkaloids give a color with chloranil in dioxane, but febrifugine responds more slowly. This might mean that the febrifugine is being converted to isofebrifugine under the conditions of the test. On the other hand, it might mean that febrifugine (and perhaps isofebrifugine) is actually a tertiary amine which has a labile carbon-nitrogen bond which is broken under the conditions of

these various tests.

3. The aromatic Portion of the Molecule is a 3-substituted-4-quinazolone.- There is excellent evidence that the alkaloids are 3-substituted-4-quinazolone's.

a) Both are oxidized by aqueous alkaline permanganate at room temperature to 4-quinazolone. When the alkaloids are heated above the melting point in vacuo for sometime, 4-quinazolone sublimes out. The alkaloid, vasicine (II), has been oxidized with permanganate to both 4-quinazolone[18] and to 4-quinazolone-



II

3-acetic acid[17].

b) The ultraviolet absorption spectra of the alkaloids and of 4-quinazolone's are very similar. The positions of maxima and minima agree within a few mμ , and the molal extinctions are very nearly the same. The absorption spectrum of a 3-substituted-4-quinazolone gives the best agreement, the position of the extremes agreeing within 1 mμ .

c) Alkaline hydrolysis of either alkaloid gives a good yield of anthranilic acid, a low yield of ammonia, an unknown yield of formic acid, and oils and resins

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18. Ghose, Krishma, Narang, and Ray, Jour. Chem. Soc., 2740 (1932).

of unknown constitution. Since at least one of the alkaloids, febrifugine, has been shown to contain a keto group, and since hydrolysis of a 3-substituted-4-quinazolone would give a primary amine, it is reasonable to interprete these oils and resins as condensation products of the primary hydrolysis products from the alkaloids. The fact that vapors from these resins (when heated) give a positive pine splinter test for pyrrole may indicate that an α-aminoketone is formed during the original hydrolysis and, among other condensation products, a small amount of some pyrrole derivative is formed. The appearance of small amounts of ammonia is also probably the result of some side reaction.

The presence of formic acid can be demonstrated in the alkaline hydrolyzates of not only febrifugine and isofebrifugine, but also each of the dihydro-alkaloids and the $C_{11}H_{12}O_2N_2$ oxidation product from periodate oxidation. Although formic acid might conceivably come from some other portion of the molecule, a very likely source is the 2-position of the 4-quinazolone. This implies of course thatthe 2-position is unsubstituted.

d) The alkaloids and the dihydro-alkaloids are not readily soluble in dilute sodium hydroxide. 4-Quinazolone's unsubstituted in the 3-position are commonly soluble in dilute base.

e) The alkaloids and the dihydro-alkaloids are quite easily hydrolyzed in 2.5 N NaOH. Hydrolysis of 4-quinazolone's unsubstituted in the 3-position is normally much slower.

f) An alkaloid with 14% nitrogen and 16% oxygen might be expected to melt much higher than febrifugine or isofebrifugine.

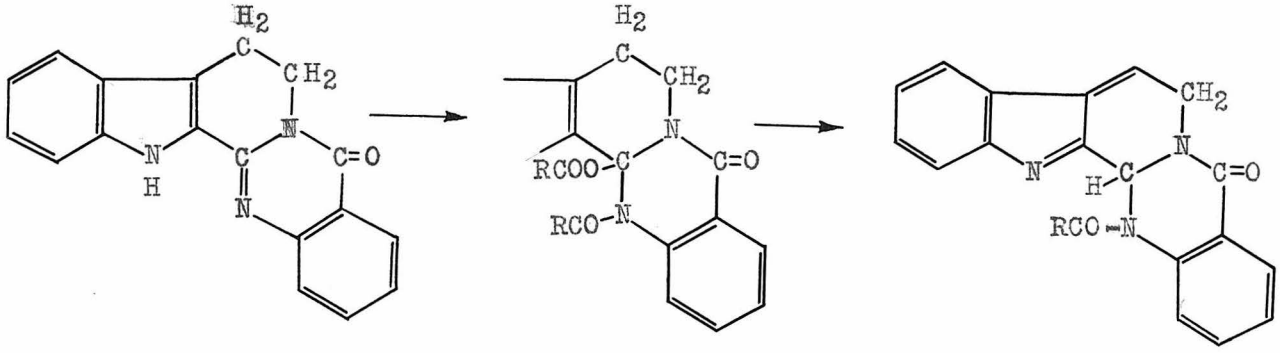
The melting points of 3-substituted-4-quinazolone's are consistently much lower than those of the 2-substituted isomers. For example, 3-phenyl-4-quinazolone melts at 139° while the 2-phenyl compound melts at 234°; the 3-propyl compound melts at 83° while the 2-propyl compound melts at 205°. The low melting points of the alkaloids would appear to indicate a 3-substituent on the quinazolone ring.

4. Oxygen Functional Groups.- The presence of a secondary amine and of a quinazolone ring system shows the function of all three nitrogens and one oxygen in the alkaloids. This leaves unexplained the function of two oxygen atoms.

Neither alkaloid gives a positive spot test for aldehyde or methyl ketone. Febrifugine shows a reactive carbonyl group by the formation of an oxime and a semicarbazone; isofebrifugine does not. Both compounds reduce Tollen's Reagent.

Both compounds give only dibenzenesulfonyl derivatives. Presumably one benzenesulfonyl group is attached to the secondary amine nitrogen, and the other to one of two atoms, either an oxygen which was originally a hydroxyl group or the 1-nitrogen of the quinazolone ring. This possibility that the 1-nitrogen is involved is indicated by the completely non-basic behavior of the dibenzenesulfonyl derivatives. They are insoluble in either dilute or strong hydrochloric acid. Quinazolone's are not strong bases (probable pK_B's ca. 11 to 13) but in general they are soluble in dilute acid. Acetyl and benzoyl derivatives involving the 1-nitrogen of a 4-quinazolone are known in the case of the alkaloid, rutacarpine (III), which is known to form a monoacetyl (and benzoyl) derivative (IV). The

postulated mechanism is[19] that the acid anhydride (or acid chloride and water) adds to the 1,2 double bond, and then a molecule of acid is split out with the indole hydrogen at the same time a hydrogen shifts to the 2-position, thus



III IV

If such a reaction is taking place between febrifugine or isofebrifugine and benzenesulfonyl chloride the most likely source for hydrogen would be a tertiary alcohol. A primary or secondary alcohol would probably just form the benzene-sulfonate.

In summary then, in addition to the quinazolone oxygen there is in febrifugine a keto-group and a hydroxyl-group. The oxygen functional groups in isofebrifugine are less clearly indicated, but a hydroxyl-group seems fairly certain. The keto-group present in febrifugine has not been demonstrated in isofebrifugine. This would seem to link the keto-group with the reversible isomerization of the two alkaloids.

5. Catalytic (Pt) Hydrogenation.- Both alkaloids in ethanol take up one mole of hydrogen, but crystalline dihydro-products are iso-

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19. Asahina, Chem. Zentr., 94 (III), 248 (1923).

lated in only 70% yield for febrifugine and 45% yield for isofebrifugine. Other reduction products appear to be formed. In glacial acetic acid febrifugine takes up one mole of hydrogen but isofebrifugine takes up 2 or more. No crystalline products have been isolated from this mixture.

The crystalline dihydro-compounds have absorption spectra very similar to the original alkaloids, so presumably the reduction has not taken place in the quinazolone portion of the molecule. Neither dihydro-compound reduces Tollen's Reagent, and dihydrofebrifugine does not give an oxime, a semicarbazone, nor reduce periodate as does febrifugine.

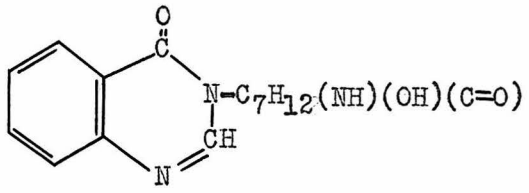
Although normally under these reducing conditions only ethylenic double bonds are reduced, it would appear that in the case of febrifugine a keto-group is being reduced. In the molecules of febrifugine and isofebrifugine there would seem to be a very labile center which is the point of alteration in the isomerization (interconversion), the oxidation by periodate or Tollen's Reagent, the reduction with hydrogen and platinum, and in the formation of resins on alkaline hydrolysis. Since these properties disappear on reduction (the possible interconversion of dihydrofebrifugine and dihydroisofebrifugine has not been studied) at the same time the keto-group can no longer be demonstrated, it seems logical to associate this labile center and consequently the point of reduction with the keto-group.

6. Hydrolysis of Dihydrofebrifugine.- Alkaline hydrolysis of dihydrofebrifugine gives anthranilic acid, formic acid, no ammonia or other volatile base, and a very water soluble fragment which gives a crystalline benzenesulfonyl derivative of the empirical formula $C_{26}H_{28}O_7S_3N_2$ (tentative). The identity of this compound is not known,

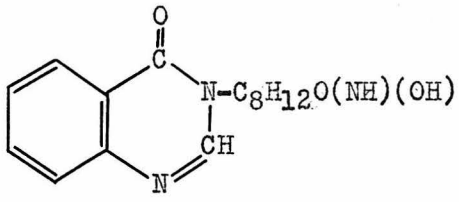
but it would appear to be a definite lead to the structure of the "other half".

7. Periodate Oxidation.- Oxidation of febrifugine or isofebrifugine with periodate in basic solution uses four equivalents of oxidizing agent, and yields a crystalline product which is tentatively assigned the formula $C_{11}H_{12}O_2N_2$. It appears to be a 3-substituted-4-quinazolone.

8. Best Formulae.- The best partial formulae which can be written for febrifugine and isofebrifugine from the data given above are the following:

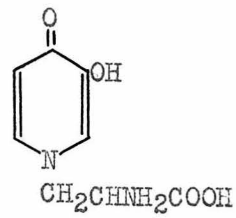


Febrifugine



Isofebrifugine

In conclusion, febrifugine and isofebrifugine occupy a singular position among the known alkaloids. They are unique in being readily interconvertible, optically active alkaloids. They are further distinguished, if the partial formulae given above are correct, by the fact that the known ring system is linked to the other portion of the molecule through a single bond to a nitrogen atom, and not by a typical fused ring system. Leucenol[20] (V) is the only other plant constituent known to the



V

writer which has this structural feature. There are of course N-methyl groups in several alkaloids, an N-ethyl group in aconitine, and a simple amide in piperine.

In addition to these interesting chemical features, febrifugine shows remarkable anti-plasmodial activity. Although numerous claims have been made for antimalarial activity of many alkaloidal compounds, no activity has been demonstrated for any naturally occuring alkaloids except those from Cinchona and the closely related Remijia. Very recently a slight activity has been claimed for alstonine in bird malaria.

Febrifugine, on the other hand, is extremely active against bird malaria, having a potency of at least 100 times that of quinine; and it is known that the crude drug, containing febrifugine, is active in human malaria. Although febrifugine is very toxic, and may not make a

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20. Adams, Jones, and Johnson, Jour. Amer. Chem. Soc., 69, 1810 (1947).

good therapeutic agent for malaria, still the structure determination may furnish a model which will lead to the syntheses of new and useful agents.

PART III[a]

THE ESTIMATION OF CHLORIDE BY MEASURING THE E.M.F. OF A SILVER, SILVER CHLORIDE, CHLORIDE ION HALF CELL*

John A. Brockman, Jr. and Thomas S. Lee**

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* This work was done under Contract OEM_{sr} 325 between the Office of Scientific Research and Development and the California Institute of Technology.

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A rapid procedure is described for the routine determination of small quantities of chloride. The measurements are made with 10 ml. of solution and are reproducible to ca. 4% for chloride concentrations above 10^{-4} formal and to ca. 4-8% for concentrations between 5×10^{-5} and 10^{-4} formal.

This method for determining chloride was developed to fill the need for a rapid, routine procedure for determining small quantities of bis(2-chloroethyl)sulfide and tris(2-chloroethyl)amine. Since the method is applicable to other compounds which readily give chloride ion in aqueous solutions, the results are reported here, although time was not available for a comprehensive study. In principle, the method is similar to a method used by Furman and Low (1) who employed a concentration cell to determine

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a. This part of the thesis is a copy of a manuscript which will be submitted to Analytical Chemistry. The work was done in cooperation with Thomas S. Lee.

small concentrations of chloride in the presence of other salts. However, for one half of the conventional concentration cell we have substituted a reference half cell consisting of a Beckman calomel electrode. The complete cell can be represented as follows:

$$Ag_{(s)}, AgCl_{(s)}, Cl^- \;//\; Cl^-, Hg_2Cl_{2(s)}, Hg_{(l)}.$$

The silver, silver chloride, chloride ion half cell did not contain the conventional silver, silver chloride electrode, but instead a silver electrode and a suspension of finely divided silver chloride were used. This modification appears to have been first used in measurements made in an undergraduate physical chemistry laboratory course given under the supervison of Prof. R. M. Badger and Dr. P. A. Shaffer, Jr. at this institution.

The two half cells were connected by a salt bridge of saturated ammonium nitrate solution. The potential of the cell was measured with a Beckman pH meter, and the concentration of chloride was found by comparing the potential obtained with a calibration curve.

Reagents

C.P. nitric acid, 10 formal and 2.5 formal.

A solution 0.01 formal in silver chloride and 0.2 formal in ammonium hydroxide, prepared by dissolving 0.07 g. of silver chloride in 1.7 ml. of 6 formal ammonium hydroxide and diluting with water to 50 ml.

Apparatus

The apparatus is shown in Figure 1. The sample solution was contained in a 20 ml. glass vessel with a convenient drain and plug, F, at the bottom. The solution was stirred by a motor driven glass propeller, C.

The reference half cell, A, was a No. 270 Beckman calomel electrode, and was connected to the lower jack of a Beckman Model G pH Meter. The ammonium nitrate salt bridge, B, was used to prevent chloride from the calomel electrode from leaking into the main cell.

The silver electrode, E, was a 5-7 cm. length of 12 gauge silver wire. It was held at the upper end by an alligator clip which was connected by a shielded cable to the top jack of the pH meter. The lower end of the wire dipped into the solution to a depth of ca. 2 cm.

Procedure

The silver wire is cleaned by dipping it into 10 formal nitric acid for 15 to 30 seconds and thoroughly rinsing it with distilled water. It was found that the potential of the cell is affected by failure to clean the silver electrode. Other methods, such as heating the electrode to glowing in a gas flame, washing with dilute nitric acid, or washing with strong ammonium hydroxide did not give satisfactory results. The recommended treatment with nitric acid was found to give reproducible cell potentials.

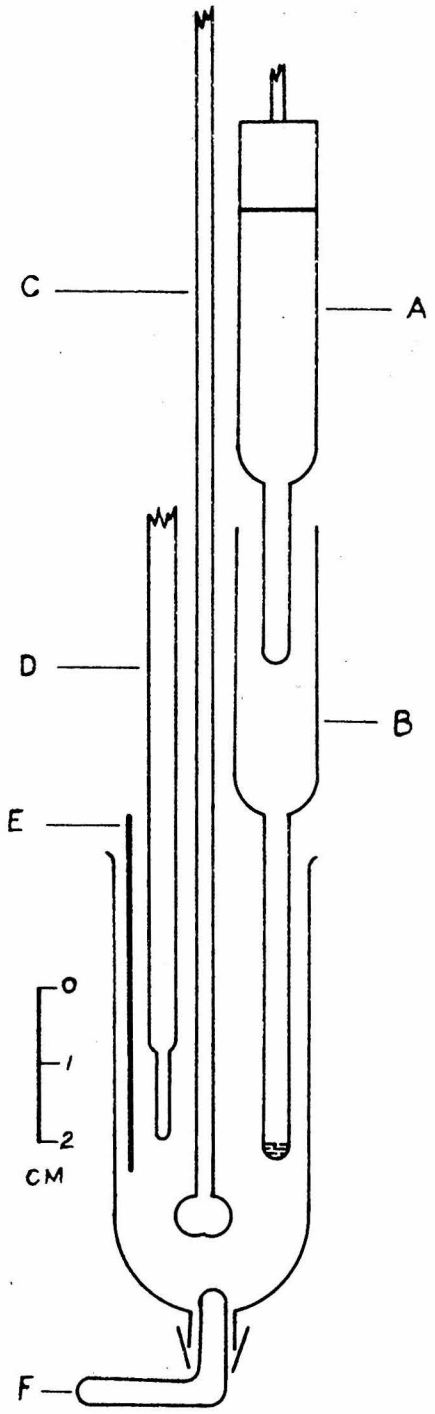


Figure 1. Apparatus.

A. Calomel reference half cell. B. Saturated ammonium nitrate salt bridge with sintered glass plug. C. Motor driven stirrer. D. Thermometer. E. Silver wire. F. Glass drain plug held in place by rubber tubing.

To the solution to be analyzed 1.0 ml. of 2.5 formal nitric acid is added, the solution is diluted to 10 ml. and poured into the cell, the stirrer is started, two drops of the silver chloride-ammonium hydroxide reagent are added to produce a finely divided suspension of silver chloride, and after 1 to 3 minutes the potential of the cell is measured with the pH meter. The amount of chloride is determined by reading from a calibration curve the value corresponding to the potential of the cell.

Calibration

From potentials determined for samples of known chloride concentrations the calibration curve is constructed by plotting the potential against the logarithm of the chloride concentration of the sample. Examples are shown in Figure 2. The calibration curve is a straight line for chloride concentrations above 10^{-4} formal. Below 10^{-4} the curve is not linear, and below 5×10^{-5} the potential changes very slowly with the concentration of chloride, because the amount of chloride from the dissolved silver chloride is comparable to the amount from the sample.

The potential observed when a certain sample of chloride was analyzed varied from day to day by as much as 3 millivolts even though the temperature at which the measurements were made was the same. Perhaps this was due to slow changes in the liquid junction potential of the ammonium nitrate bridge. The effect of this variation was nullified by constructing a new calibration curve each day. The new curve was made merely by measuring the potential of a known concentration of chloride, plotting the corresponding point on semilog coordinate paper, and draw-

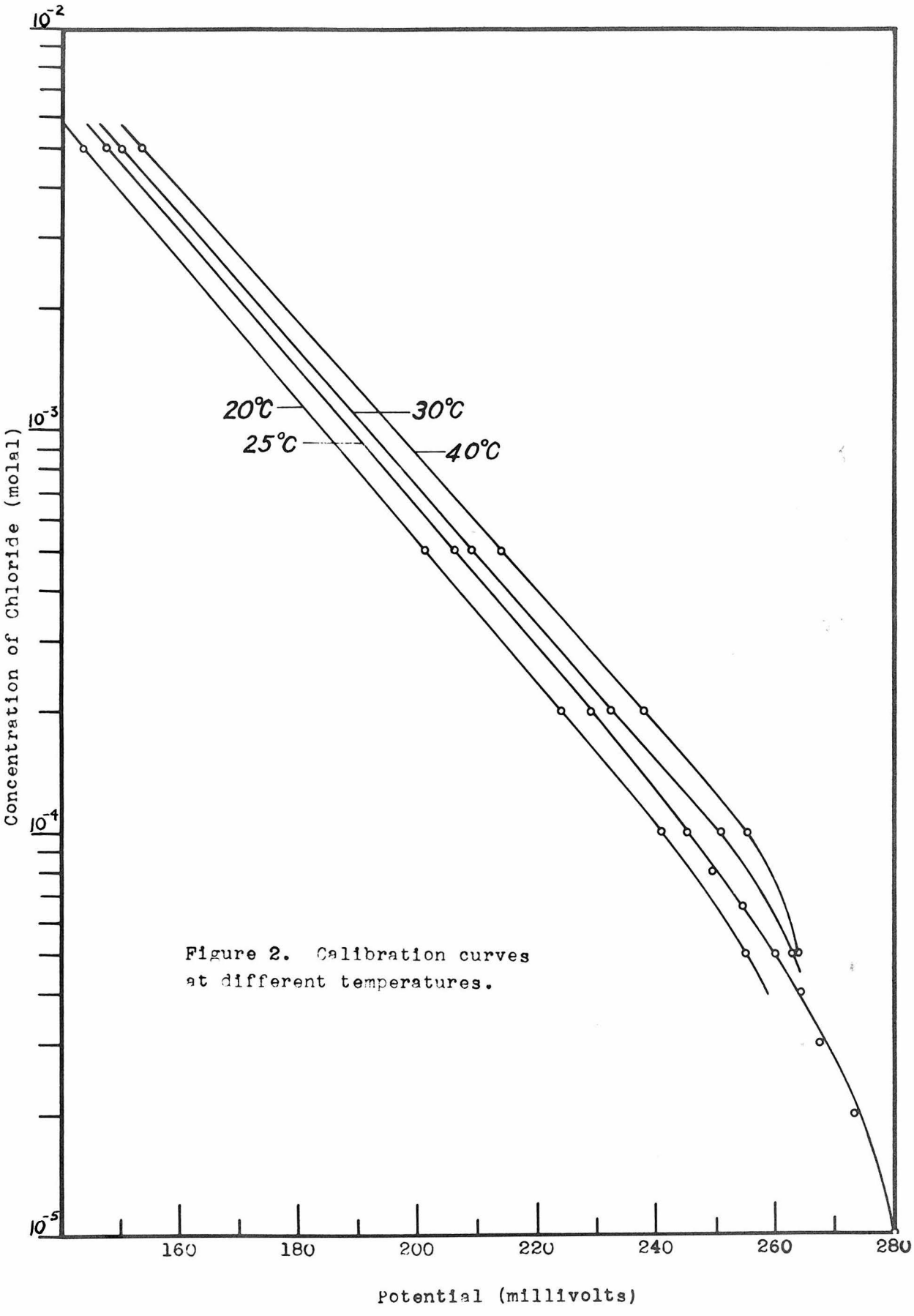


Figure 2. Calibration curves at different temperatures.

ing a curve parallel to a calibration curve already established. That is, the slope of the curve was found to be independent of day to day changes, and the intercept was determined by analyzing a chloride solution of known concentration. One standardization for each day was sufficient.

Factors Affecting the Determination

The sensitivity of the Beckman pH meter when used carefully is ca. 1 millivolt. This is the limiting factor in the accuracy of the method. In the linear portion of the calibration curve it corresponds to a relative error of 4% in the determination of chloride concentration. Between 10^{-4} and 5×10^{-5} formal it corresponds to an error of from 4 to 8%.

The ionic strength and the hydrogen ion concentration of the solution being analyzed should be the same as those of the solutions used for preparing the calibration curve. However, it was found that the potential of the cell is not critically dependent on the ionic strength in the range 0.25 to 1.00 molal, although there is a gradual shift of a few millivolts. Likewise, the hydrogen ion concentration is not critical between 0.05 and 0.25 molal. Certain ions or molecules when present in the solution interfere with the determination. For example, traces of iron salts or permanganate were found to affect the potential markedly. The determination could not be carried out in solutions of acetic acid at concentrations greater than ca. 3.5 formal because of anomalous effects. Amounts of thiodiglycol equivalent to the amount of chloride present, moderate amounts of manganous ion, and small amounts of oxalic acid did

not interfere.

Temperature affects the potential of the cell. (See Figure 2.) Both the slope and intercept of the calibration curve are changed. For a few degrees change in temperature the change in slope is not appreciable (less than 1 mv. per 10 fold change in chloride), but the intercept changes ca. 1 mv. per degree. If calibration curves are constructed for five degree intervals, interpolation gives sufficient accuracy for intermediate temperatures. If the temperature differences are small the method for constructing a daily standardization curve discussed under "calibration" may be used to construct a curve for the new temperature.

Data

This method was applied to the analysis of solutions of bis(2-chloroethyl)sulfide (I) and tris(2-chloroethyl)amine (II) in diethyl phthalate.

To obtain an aqueous solution of chloride from I, the diethyl phthalate solution was stirred with an excess of aqueous acidic permanganate, made basic with sodium hydroxide, heated 15 minutes in a bath of boiling water, made acidic with nitric acid, and the excess permanganate and manganese dioxide reduced with oxalic acid.

To obtain an aqueous solution of chloride from II, the diethyl phthalate solution was extracted with nitric acid, the resulting aqueous solution made basic with sodium hydroxide and heated on a bath of boiling water for 5 minutes. The cooled solution was acidified with nitric acid.

The chloride ion in the resulting solutions was then determined

in the manner described. Blanks were prepared by each method, and potentials corresponding to 20 micrograms of I and II were found. This correction has been applied to the results shown in Table I.

Acknowledgement

The OSRD project under which this work was carried out was under the joint supervision of Profs. Carl Niemann and E.H. Swift. Especial thanks goes to Prof. Swift for his timely suggestions during the course of the work and for his aid in preparing this manuscript.

References Cited

1) N.H. Furman and G.W. Low, Jr., J. Am. Chem. Soc. 57, 1585 (1935).

TABLE I

Substance	Amount present micrograms	Amount found micrograms	%
bis(2-chloroethyl)sulfide	39	50	128
	78	83	106
	156	158	101
	391	358	92
	1172	1157	99
	1953	2055	105
tris(2-chloroethyl)amine	27	37	137
	49	65	133
	54	69	128
	99	118	119
	157	167	106
	391	380	97
	782	770	99
	1173	1130	96
	3910	4140	106

PART IV. STUDIES ON CAMBARUS CLARKII

Section I

Effect of Magnesium on the Neuro-Muscular Junction

Introduction.- The narcotic action of magnesium on Crustacea is well known. The site of depression is, however, still a controversial point. There is general agreement that the site of action is not the nerve (4,6). Katz (4) claims a "curare effect" at the neuro-muscular junction, but he gives little evidence to support this claim. Furthermore, his preparations are to be critisized since he stimulated the whole nerve of crab walking legs with pulsating currents, a procedure which must at times lead to spurious results because of inhibition, and perhaps even because of heterofacilitation effects.

The present work was undertaken to reinvestigate this problem. Time was not available for a thorough study, so experiments were confined to the fast closer system of the crayfish, Cambarus clarkii. In this preparation it is not necessary to isolate the single nerve fibre for stimulation, since single shocks given to the whole nerve activate only the fast closer system.

Methods.- Non-regenerated claws were used. To study the effect of magnesium on the nerve, the method of Ellis, Thienes, and Wiersma (1) was employed. The whole nerve, isolated from the meropodite, was led through a small celluloid chamber and sealed in with vaseline. Various solutions were then introduced into the chamber, and the effect on the contraction of the muscle was noted by recording the movement of the dactylopodite, auxotonically on a smoked drum.

To study the effect of magnesium on the muscle and the neuromuscular junction, the method of Waterman (6) was used. Here the whole nerve was isolated in the meropodite according to the technique of van Harreveld and Wiersma (3). After the tip was cut off, the propodite was joined by a rubber tube to a funnel. Perfusion solutions were run in from Mariotte's bottles, allowed to flow through the claw, and finally to overflow from the meropodite. Perfusion rates depended on the size of the claw, and varied from 1 to 10 ml. per min. Movement of the dactylopodite was recorded auxotonically on a smoked drum.

The basic perfusion solution was that of van Harreveld (2). The magnesium concentration, normally 0.25 g. per liter, was varied in simple multiples and is recorded as 2X, 5X, etc. Thus, 0.50 g. per liter is recorded as 2X.

Stimulation was supplied at regular intervals in the form of single, break, induction shocks, at near-threshold strength. For a few experiments direct current stimulation was obtained with a Lucas pendulum, at roughly chronaxie values. Platinum stimulating electrodes were used throughout the work.

In recording muscle action potentials it was desirable to record isometrically at times, and perfusion was carried out by periodically injecting the desired solutions into the tip of the propodite with a syringe. The potentials were led off from the muscle by drilling small holes in the chitin of the propodite and inserting cotton moistened with van Harreveld's solution. These potentials were amplified and recorded photographically with a Mathews' oscillograph.

Results of Bathing the Nerve in Magnesium Rich Solutions.- No specific effect of magnesium on the nerve was observed. Even when the nerve was bathed in a solution containing 40X magnesium, there was no significant change in the muscle response. As would be expected, isotonic magnesium chloride (0.21 M) rapidly stopped the response. However, van Harreveld's solution to which was added enough magnesium chloride to make the concentration 0.21 M stopped the action only after about ten minutes, even though this solution is strongly hypertonic. Clearly then the effect of isotonic magnesium chloride was not a specific effect of magnesium, but rather the effect of the lack of other ions such as potassium, calcium, and sodium.

Results of Muscle Perfusion.- Increasing the magnesium concentration caused a reversible decrease in the amplitude of the muscle response. In many cases 5X magnesium would stop the muscle response, and 10X always rapidly stopped the mechanical action when recording was auxotonic. These results confirm the observations of Waterman (6).

Several attempts were made to record muscle action potentials, but the results were not altogether satisfactory. In those cases where monophasic action potentials were obtained it was not always possible to distinguish with certainty between the shock artifact and the action potential. On the other hand, when diphasic action potentials were obtained the interpretation is complicated by the fact that a relatively slight movement of the lead-off electrodes may change the shape of the potentials markedly (7).

However, the results seem to indicate quite clearly that the muscle action potential is not completely blocked by concentrations of magnesium which definitely block the muscle contraction. Plate I shows the results

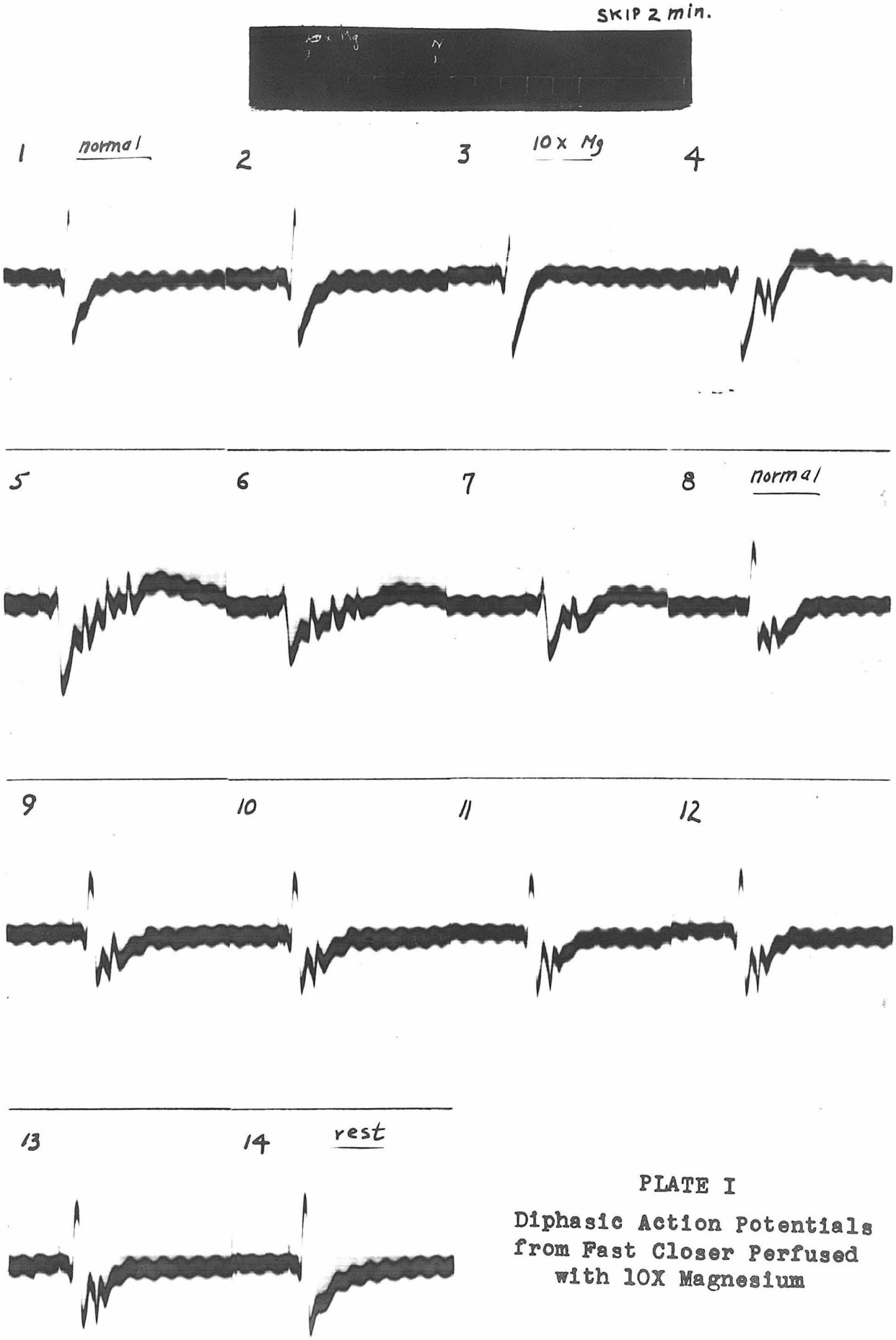


PLATE I

Diphasic Action Potentials from Fast Closer Perfused with 10X Magnesium

Section 2

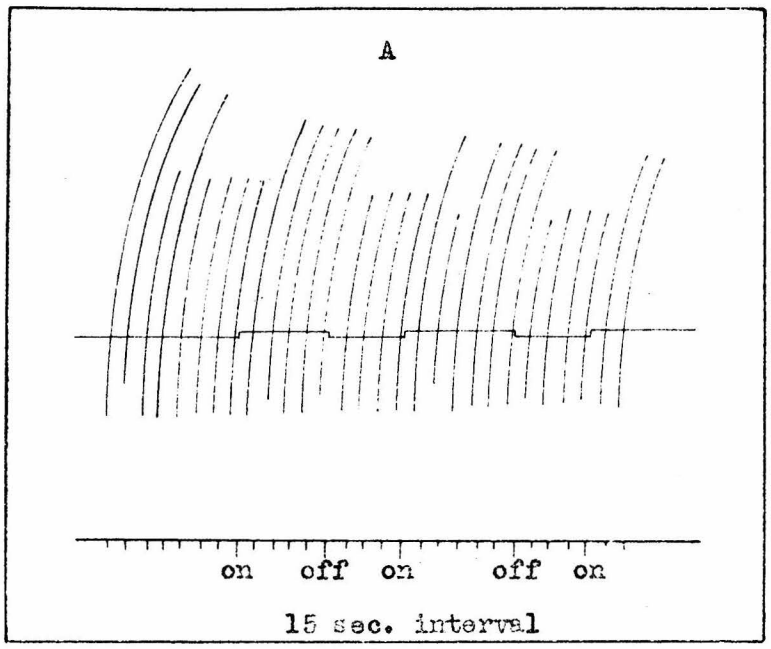
Effect of Perfusion on the Fast Closer Response

Introduction.- In the course of the investigation discussed in the previous section, a very interesting observation was made regarding the change in mechanical response when the rate of perfusion was changed. This effect was investigated briefly; and, although no definite conclusions can be drawn, there was a very clear indication that perfusion was washing away a "facilitating substance". There was also an effect which might be interpreted as the washing away of a "fatigue substance" but as indicated below, it is believed that this effect is really the result of multiple discharges.

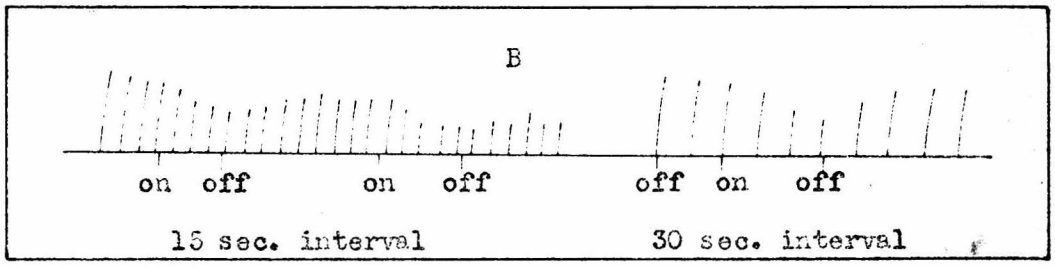
Method.- The same perfusion technique as described in Section 1 was used. The perfusion solution was that of van Harreveld. Stimulation was by induction shock or with a Lucas pendulum. Platinum electrodes were used throughout.

Results.- In many preparations the mechanical response was much greater when the perfusion was on, than it was when the perfusion was off. In other preparations just the opposite effect was observed. It was not possible to predict which effect would be observed, and indeed one preparation showed both effects at different times.

Plate II shows tracings made from two records which illustrate the effects described above. The record of A is typical of the change which appears to be the washing out of a fatigue substance. The magnitude of the change in response on changing the perfusion from on to off is so great and the rate of change is so rapid that an explanation based on



Stimulation by induction shock



Direct current stimulation for 0.96σ (Lucas pendulum)

PLATE II

Tracings of records which show the effect of perfusion

multiple discharges seems most tenable. It is very unlikely that a chemical substance could be washed away so quickly and completely.

In B (Plate II), however, direct current stimulation was applied for 0.96σ with a Lucas pendulum. This is a condition under which multiple discharge is very unlikely. It is also clear from the record that the change in twitch height is gradual and not sudden. In this case, then, there appears to be a real effect of perfusion.

Conclusions.- Marmont and Wiersma (5) have suggested that step 2 (see previous section) of the neuro-muscular transmission process might be under the control of two chemical mediators, one to account for facilitation and the other to account for inhibition. The present experiments indicate the presence of at least one such substance, a facilitating substance, which can be washed away by perfusion. The possibility that a fatigue or inhibiting substance is also present can not be substantiated by these experiments. It is believed that those records which could be explained as the washing out of a fatigue substance are better explained by multiple discharges which arise because of some increased excitability caused by the perfusion, perhaps because of mechanical irritation.

These experiments further indicate that this preparation, in which the perfusion solution is flowed through the propodite, is a rather poor one because of unpredictable responses to changes in the perfusion rate. Perfusion experiments for the muscles of the propodite would probably be more satisfactory if carried out by injecting the desired solutions.

References

1. Ellis, Thienes, and Wiersma, 1942, Biol. Bull. 83, 334-352.

2. van Harreveld, 1936, Proc. Soc. Exp. Biol. Med., 34, 428-432.

3. van Harreveld, and Wiersma, 1936, J. Physiol., 88, 78-99.

4. Katz, 1936, J. Physiol., 87, 199-221.

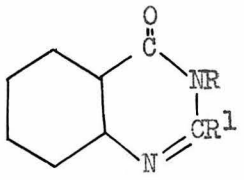
5. Marmont and Wiersma, 1938, J. Physiol., 93, 173-193.

6. Waterman, 1941, J. Cell. and Comp. Physiol., 18, 109-126.

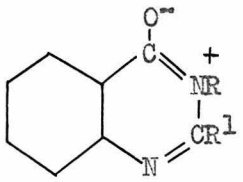
7. Wiersma and Wright, 1947, J. Exp. Biol., 23, 205-212.

PROPOSITIONS

1. The Structures of 4-quinazolones are analogous to those of the α- and γ-hydroxypyridines[a], being resonance-hybrids of structures of the following types:



and



R and R^1 = H or alkyl.

a. Taylor and Baker, pp. 530 to 533, Sidgwick's Organic Chemistry of Nitrogen, Oxford, 1942.

2. The stability of 4-quinazolones to acid hydrolysis, and the stability of 3-unsubstituted-4-quinazolones to alkaline hydrolysis compared with the 3-substituted compounds[b] can be accounted for by the increased resonance possible in the ions formed in acid or alkaline solution.

b. This thesis, pp. 77 and 78.

3. Febrifugine and isofebrifugine are 3-substituted-4-quinazolones[c].

c. This thesis, pp. 96 to 98.

4. The theoretically interesting compound, bromochloroiodomethane, might be prepared in the optically active state by the following steps: (1) successive iodination, bromination, and chlorination of 3-acetyl-2,4,6-tribromobenzoic acid; (2) resolution of the enantiomorphs resulting from (1); and (3) hydrolytic cleavage of the trihaloketone in basic solution.

5. Alkyl chlorosulfinates, formed from thionyl chloride and primary or secondary alcohols, could be reacted with hydroxy acids to give sulfites containing a carboxyl group. Such derivatives might be useful for the resolution of optically active alcohols.

6. The synthesis of alkyl thiolsulfinates from thionyl chloride proposed by Small, et. al.[d], was found to be unsuccessful. Better results would have been obtained using thionyl chloride in the following manner, similar to the known synthesis for the oxygen analog[e]:

$$SOCl_2 + RSH \longrightarrow RSSOCl + HCl$$

$$RSSOCl + R'MgX \longrightarrow RSSOR' + MgClX$$

d. Small, Bailey, and Cavallito, Jour. Amer. Chem. Soc., 69, 1710 (1947).
e. Carre and Libermann, Compt. rend., 200, 2086 (1935).

7. The theory for olfaction proposed by Dyson[f] is inadequate, and overlooks some significant physiological factors.

f. Dyson, Chem. and Ind., 57, 647-51 (1938).

8. Magnesium ion blocks the chain of events between motor impulse and muscle contraction in the fast closer of the crayfish at the same step that simple inhibition[g] does.

g. Marmont and Wiersma, J. Physiol., 93, 173-93 (1938).

9. Wiersma and van Harreveld[h] propose a chain of events connecting the nerve impulse with the muscle response in the slow and fast contractions in Crustacea. An additional common step between the muscle action potentials (of both the fast and slow systems) and the muscle contraction would help

explain some of the phenomena observed in inhibition[g] and heterofacilitation.

h. Wiersma and van Harreveld, Physiol. Zool., 12, 43, (1939).

10. Candidates for the degree of Master of Science are required to complete a course in the humanities. Candidates for the Degree of Doctor of Philosophy should not be immune to the humanistic. A few well chosen lecture courses requiring at most a few hours per week might form a much needed cultural supplement to a candidate's scientific training.