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AN ANTIMALARIAL ALKALOID FROM HYDRANGEA. II. ISOLATION

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Ch'ang Shan roots, long used as a febrifuge in China, became of interest as a source of antimalarial drugs. In September, 1945, there became available to us approximately 2.0 kg. of root material purported to be Ch'ang Shan of Chinese origin. In testing acid aqueous extracts of the powdered roots in malaria-infected ducklings, antimalarial activity was observed (1). Concentration of the antimalarial component indicated the activity to be due to a substance having alkaloid properties. It was observed, that hot-water-insoluble phosphotungstates of the unknown substance(s) could be formed, and that the free base could be regenerated by decomposition of the phosphotungstate complex with barium hydroxide in 50% aqueous acetone. The activity could be extracted from an alkaline aqueous solution saturated with sodium chloride by chloroform or ethyl acetate, and could be extracted from the organic solvent by acidic solutions. Water-insoluble gold salts could be formed although not quantitatively and the free base could be regenerated by disassociation of the gold salt with silver.

With this limited supply of root, 13 mg. of a faint yellow oil was isolated as the hydrochloride, which crystallized on standing. After recrystallization from methanol and petroleum ether, 6 mg. of shiny needles in the form of rosettes were obtained. They were contaminated, however, by the presence of an occasional prismatic plate. The wide melting point range of 161° to 194° implied a mixture.

At this same time, other sources of antimalarial activity were investigated. Since the origin of the 2.0 kg. of Ch'ang Shan received was doubtful, and because it was suspected (2) that Ch'ang Shan might be botanically classified as a Saxifragacae, approximately 100 gms. of dried leaves of *Dichroa febrifuga* Lour. originating in the Bronx Botanical Gardens was made available to us. On the basis of isolation procedures developed for Ch'ang Shan, we were able to isolate 3.5 mg. of white needles crystallizing from methanol-petroleum ether as rosettes. The wide melting point of 165–190° as well as the presence of an occasional platelet again indicated the preparation to be a mixture. Although the small amounts of both the crude root of Ch'ang Shan and the leaves of *Dichroa febrifuga* Lour. available precluded the possibility of getting enough pure crystalline material for comparative data such as the melting points, analysis, crystal form, etc., the general chemical properties indicated the possibility that both antimalarials were similar or identical.

In testing other related plant sources (2) it was observed that traces of antimalarial activity could be demonstrated in an acid aqueous extract of hydrangea leaves picked from the front lawn of one of us.[1] Partial purification of the active compound(s) in these leaves (although no crystalline material was obtained due

[1] The late Dr. Y. SubbaRow deserves much credit for the initiation and successful culmination of this work.

to the low concentrations of activity and to the scarcity of material) indicated the activity to be due to an alkaloid-like substance.

Subsequently many samples of the Easter variety hydrangea obtained from various local greenhouses were tested. Extracts of plants from certain greenhouses contained significant amounts of a substance exhibiting antimalarial activity. The impetus gained by the availability of this plant as starting material led us to devote all our efforts towards the isolation of the active principle(s) as well as degradation studies (3) and synthesis (4) of the hydrangea alkaloid.

By use of procedures briefly mentioned above and given in detail below, 23 mg. of crystalline needles in the form of rosettes were isolated from 1.0 kg. of dried leaves and stems of hydrangea. After four re-crystallizations from methanol-acetone, the compound melted at 223–225° with decomposition. The compound produced suppressive antimalarial activity in ducklings at 0.1 mg./1 kg. of body weight. The free base, m.p. 137–138°, was prepared by neutralizing an aqueous solution of the dihydrochloride followed by extraction into chloroform and crystallization from acetone-petroleum ether.

The specific rotation of the dihydrochloride in water was $[\alpha]_D^{31}$ +12.8°. (c, 0.85). Analyses for OCH_3 and NCH_3 were negative. Millon's test for phenols was negative. The Bratton and Marshall test for aromatic amines was negative. The general isolation procedures, and the behavior of the material (stability in acid, instability in alkali) and the similarity of activity range in ducklings implied that the alkaloid originating from Ch'ang Shan, *Dichroa febrifuga* Lour., and the greenhouse variety of hydrangea might be similar if not identical although direct comparisons by analysis, infrared adsorption spectra, etc. were not possible at this time due to the unavailability of Ch'ang Shan root material and *Dichroa febrifuga* Lour. plant material.

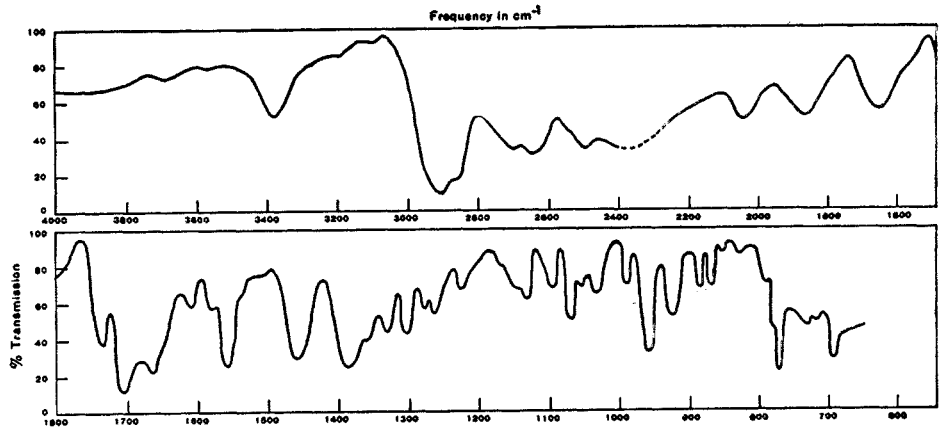
Search of the literature for possible references to the botanical identity of Ch'ang Shan and to related plants that could serve as possible sources of substances with antimalarial properties indicated the appearance of two papers by Lui and co-workers, (5, 6) referring to the febrifugal properties of extracts of leaves of the Chunine tree.[2] Jang and co-workers were greatly instrumental in arousing interest in the Ch'ang Shan plant by their work in attempting to classify it (7) as well as their report of having isolated four substances (8), two of them alkaloids, from this plant. Of the two alkaloids designated as Dichroine A and Dichroine B, the latter, melting at 237–238°, was reported to be active in avian malaria.

The suggestive implication from our data that the antimalarial alkaloid from *Dichroa febrifuga* Lour. (Ch'ang Shan) and hydrangea were the same was seriously questioned by the reported melting point of Dichroine B.

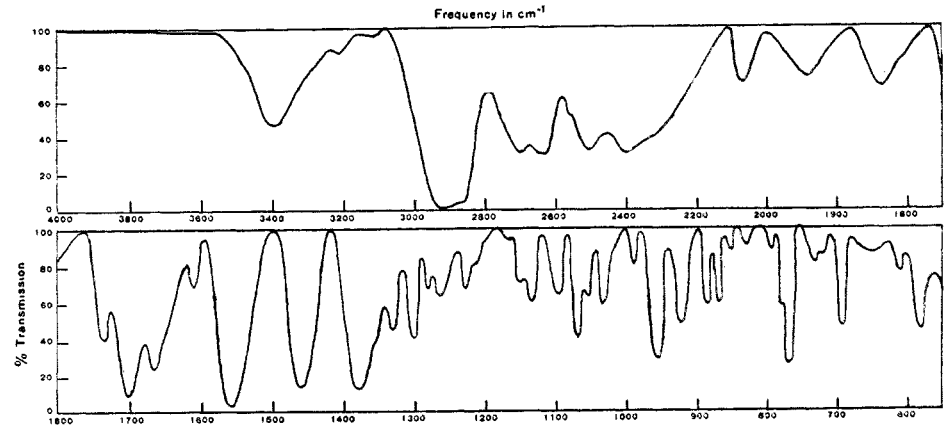
Further attempts were then directed to the isolation of sufficient alkaloid from an authentic sample of *Dichroa febrifuga* Lour. (Ch'ang Shan) for direct comparison. A crystalline compound was isolated which proved to be identical with the hydrangea alkaloid. Mixed melting points, ultraviolet and infrared

[2] *Dichroa febrifuga* Lour. is at times referred to by the proprietary names of Chunine Tree or Ch'ang Shan.

adsorption spectra, elemental analysis, and antimalarial activity were identical. The infrared adsorption spectrum of a sample of alkaloid isolated from hydrangea and from Ch'ang Shan is included below.



INFRA-RED ABSORBTION SPECTRA OF ALKALOID ISOLATED FROM CHANG SHAN



INFRA-RED ABSORBTION SPECTRA OF ALKALOID ISOLATED FROM HYDRANGEA

FIG. 1.

As experience developed, simpler methods of isolation were found. Chronologically, different methods of obtaining crystalline material were developed. Illustrative of the procedures followed is the method described below for obtaining the active alkaloid from hydrangea as well as Ch'ang Shan.

EXPERIMENTAL

Method I. Air- or oven-dried green leaves of hydrangea or the pulverized roots of Ch'ang Shan (200 g.) were suspended in 20 to 40 liters of water and the mixture was adjusted to *p*H 1.5 to 2.0 with concentrated HCl. The mixture was refluxed for three hours and the insoluble debris was removed by filtration through canvas cloth. The insolubles were re-extracted two additional times in a similar manner. The acid aqueous extracts were combined and con-

centrated *in vacuo* to approximately one liter. The solution was adjusted to *p*H 8.5 and then saturated with ammonium sulfate. Five volumes of acetone were added and after shaking, the supernatant aqueous acetone phase was removed and the procedure was repeated twice. During the extractions the liquid was maintained at *p*H 8.5. The combined extracts were concentrated *in vacuo* to remove the acetone. The residual aqueous solution was saturated with sodium chloride and was adjusted to *p*H 8.5 with 0.5 *N* NaOH or $NaHCO_3$. The solution was extracted four times with equal volumes of chloroform. The combined chloroform extracts, which contained the active compound, were washed once with approximately 500 cc. of a saturated aqueous solution of sodium chloride and the active component was then removed by extraction with approximately one liter of 1 *N* sulfuric acid. The acidic water extract was adjusted to 1 *N* with sulfuric acid and an excess of a 20% solution of phosphotungstate acid in 1 *N* sulfuric acid was then added. The precipitate was collected and washed with approximately 200-cc. portions of boiling water until the water-soluble phosphotungstates had been removed. (Three washings were usually sufficient.) The insoluble phosphotungstates from the hydrangea are quite gummy while those from the Ch'ang Shan plant can be well dispersed during the hot water extraction.

The hot-water-insoluble phosphotungstates were dissolved in acetone water (1:1) and the complex was decomposed by the addition of $Ba(OH_2)$. The solution reached *p*H 10.5 at this point and the solution had to be kept *cold* (+2°). After quick centrifugation to remove the insoluble barium phosphotungstate, the supernatant was adjusted to *p*H 6.0 with 0.1 *N* H_2SO_4 which removed the Ba as the sulfate, and the acetone was removed *in vacuo*. The aqueous residue in the flask (approximately 300 cc.) was adjusted to *p*H 8.5, saturated with sodium chloride, and then extracted three times with equal volumes of chloroform. The combined chloroform extracts were dried with potassium carbonate. After drying, two or three cc. of absolute acetone saturated with dry HCl gas was added. On cooling, micro crystals usually deposited immediately, but occasionally crystal formation required standing overnight at +2°. The precipitate was collected and after crystallization from methanol-acetone melted at 200°. Further crystallizations yielded material melting at 223–225°. The elemental analyses were as follows: C, 50.83; H, 5.89; N, 11.33; ionic Cl, 18.54. The analytical values for the free base were: C, 63.76; H, 7.02; N, 14.3. The theoretical analytical values for the empirical formula $C_{16}H_{19}N_3O_3$ are C, 63.76; H, 6.34; N, 13.9. The yield of crystalline material varied considerably from batch to batch depending on whether the starting material was hydrangea or Ch'ang Shan. The mean yields were between 0.005% and 0.03%, by this procedure.

Method II. Oven-dried and ground hydrangea leaves (2 kg.) were extracted with 16 liters of methanol containing 160 cc. of concentrated HCl. After refluxing 8 hours, the insolubles were removed and the leaves were again extracted with 16 liters of methanol containing 2 or 3 cc. of concentrated HCl. The pooled methanol extracts were concentrated *in vacuo* and the tarry residue was slurried with approximately 500 cc. of water. The water extract was adjusted to *p*H 1.0 and extracted with $CHCl_3$ until no further pigmented material was removed. The $CHCl_3$ solution was washed once with 500 cc. of 0.1 *N* HCl, and this acid wash was combined with the main acid-aqueous phase. The amber solution was saturated with NaCl, adjusted to *p*H 8.5, and then extracted four times with equal volumes of ethyl acetate. The combined ethyl-acetate phases were extracted with 500 cc. of 0.1 *N* HCl, and then with an additional 100 cc. of 0.1 *N* HCl. The combined acid extract was saturated with NaCl, adjusted to *p*H 8.5, and then extracted three times with 100 cc. of $CHCl_3$. The combined $CHCl_3$ extracts were dried with K_2CO_3 and then a few cc. of absolute acetone containing dry hydrogen chloride was added. Crystals were usually obtained on cooling overnight. The yield of crystalline alkaloid varied between 0.005% and 0.03% depending on the lot of hydrangea used.

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SUMMARY

A crystalline antimalarial substance having the empirical formula $C_{16}H_{19}N_3O_3 \cdot 2HCl$ has been isolated from the greenhouse variety of hydrangea. The compound is acid stable but decomposes in alkali. The alkaloid has been found to be identical with an alkaloid present in Ch'ang Shan as indicated by identical elemental analysis, ultraviolet adsorption spectra, infrared adsorption spectra, and antimalarial activity in the malaria-infected duckling.

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