Summary

It has been shown that *n*-butyllithium dimetalates 9-phenylcarbazole homonuclearly to give, subsequent to carbonation and hydrolysis, 9-(2′,6′-dicarboxyphenyl)-carbazole. The structure of this dibasic acid was established by cyclodehydration reactions. Some experiments concerned with resolution studies of cyclodehydration derivatives are described.

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[CONTRIBUTION FROM THE UNIVERSITY OF MINNESOTA AND FROM THE C. F. KETTERING FOUNDATION FOR THE STUDY OF CHLOROPHYLL AND PHOTOSYNTHESIS]

The Isolation of Photosensitizing Agents from Buckwheat[1]

BY SIMON H. WENDER,[2] ROSS A. GORTNER AND O. L. INMAN

Fagopyrism, commonly called buckwheat poisoning, was recognized as early as 1833 as being produced only in the presence of light.[3] Experimentally, Wedding[4] showed that following the ingestion of the buckwheat plant, certain white animals, such as white pigs, sheep, and cows become sensitized to the action of sunlight. These same animals, however, remain unharmed so long as they are kept out of sunlight. The general symptoms of fagopyrism consist of severe itchings, pruritus, erythema, edematous swellings of the face and ears, and, in many cases, convulsions, cerebral excitement, paralysis, and even death. The symptoms vary with the animal, and with the intensity and duration of sunlight. Blum[5] has recently presented an extensive and critical review on the reported phases of fagopyrism and related light diseases.

Oehmke,[6] Fischer,[7] Lutz and Schmid,[8] and other investigators attempted to isolate the active substances from buckwheat, but were only partially successful. Recently, Chick and Ellinger[9] found that the activating principle could be extracted from dried buckwheat flowers with mixtures of 10 parts of glacial acetic acid and 90 parts of methanol. These workers reported that studies on methods of further separation of the pigments were in progress. In this investigation, we have succeeded in adapting for use an experimental procedure for the isolation and purification of three substances from buckwheat. When each of the three substances thus isolated was fed to white guinea pigs, the animals became sensitized to sunlight.

Isolation and Studies of the Photosensitizing Agents.—The method of isolation involves extraction with proper solvents, chromatographic adsorption analysis, partition between immiscible solvents, and crystallization. Care was taken throughout the experimental work to avoid drastic chemical treatment and direct sunlight.

Five kilograms of air-dried, ground buckwheat (*Fagopyrum esculentum*) gathered when flowering, were extracted with 80% acetone, according to the method described for the preparation of chlorophyll from plants by Schertz.[10] The acetone solution was transferred to ether in a separatory funnel, and the ether washed carefully with water. The ether contained the substances desired. After removal of the ether by distillation on a steam-bath, a dark, waxy-like residue was obtained. This residue was dissolved in acetone which had been previously dried over anhydrous sodium sulfate. The acetone solution was chromatographed through a column containing talc (Merck, purified U. S. P.) tightly packed in the lower part of the column, and a smaller amount of anhydrous sodium sulfate (Merck, A. C. S. specifications) packed on top of the talc. The chromatogram was "developed" by washing with dried acetone. A blackish-purple material containing a substance termed "A" was on top of the anhydrous sodium sulfate. The talc was a light pinkish-purple color which gradually faded near the bottom into a pinkish-orange color.

Substance "A" was obtained by centrifuging with warm water the blackish-purple material from the top of the anhydrous sodium sulfate; then decanting the supernatant liquid, and repeating until the residue was freed of sodium sulfate. After drying *in vacuo*, the material was dissolved in hot, pure glacial acetic acid, filtered, and the filtrate con-

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(3) Hertwig. "Praktische Arneizmittellehre für Tierärzte." Berlin, 1833.

(4) Wedding, *Verh. d. Berl. Anthrop. Ges. Z. f. Ethnologie*, **19**, 67 (1887).

(5) Harold F. Blum, "Photodynamic Action and Diseases Caused by Light," Reinhold Publishing Corp., New York, N. Y., 1941.

(6) W. Oehmke, *Zentr. Physiol.*, **22**, 685 (1908).

(7) J. Fischer, Inaug. Dissert. Berlin, Köln-Ehrenfeld, 1909.

(8) H. E. W. Lutz and G. Schmid, *Biochem. Z.*, **226**, 67 (1930).

(9) H. Chick and P. Ellinger, *J. Physiol.*, **100**, 212 (1941).

(10) F. M. Schertz, *Plant Physiol.*, **3**, 487 (1928).

centrated to a small volume *in vacuo*. To this solution, a small amount of pure redistilled ether was added. On standing, precipitation occurred. The precipitate was filtered and washed with cold ether, then recrystallized.

The talc from the chromatogram was extracted continuously in a large thimble, using ethyl alcohol. The extraction removed substances "B" and "C." Another substance "D," as yet not tested for its photosensitizing ability, was left on the talc. The alcohol extract was filtered, transferred to ether, washed with distilled water, and the ether solution fractionated according to the method of Willstätter and Mieg.[11]

After repeated extractions with 15% hydrochloric acid (these extracts containing an impurity being finally discarded), substance "B" was removed from the ether by extractions with portions of 23% hydrochloric acid until the acid extractions were practically colorless. The remaining ether was washed with distilled water to neutrality and saved, for it still contained substance "C." The 23% hydrochloric acid extracts were transferred to fresh ether by dilution with distilled water, washed to neutrality, and again fractionated with hydrochloric acid. This procedure was continued until only the spectral bands attributed to "B" could be seen. The solution was red and quite fluorescent. It was dried over anhydrous sodium sulfate, filtered, and then the ether removed by distillation. The red residue was dissolved in hot methyl alcohol, filtered, and concentrated. A few ml. of hot water were added, and crystallization occurred on cooling. "B" was recrystallized and dried to constant weight *in vacuo*.

The ether solutions containing substance "C" were extracted with 5% sodium carbonate solution. Substance "C," removed in the extraction, was transferred to fresh ether by acidification with dilute hydrochloric acid, and washed to neutrality with distilled water. This process was repeated until an ether solution was obtained free of all spectral bands except those attributed to "C." This solution was washed to neutrality, dried over anhydrous sodium sulfate, filtered, and all of the ether removed by distillation. The residue was taken up in a large amount of methyl alcohol and filtered. Most of the methyl alcohol was removed *in vacuo*, until on sufficient concentration crystallization occurred. The crystals were filtered and washed with a few drops of cold methyl alcohol.

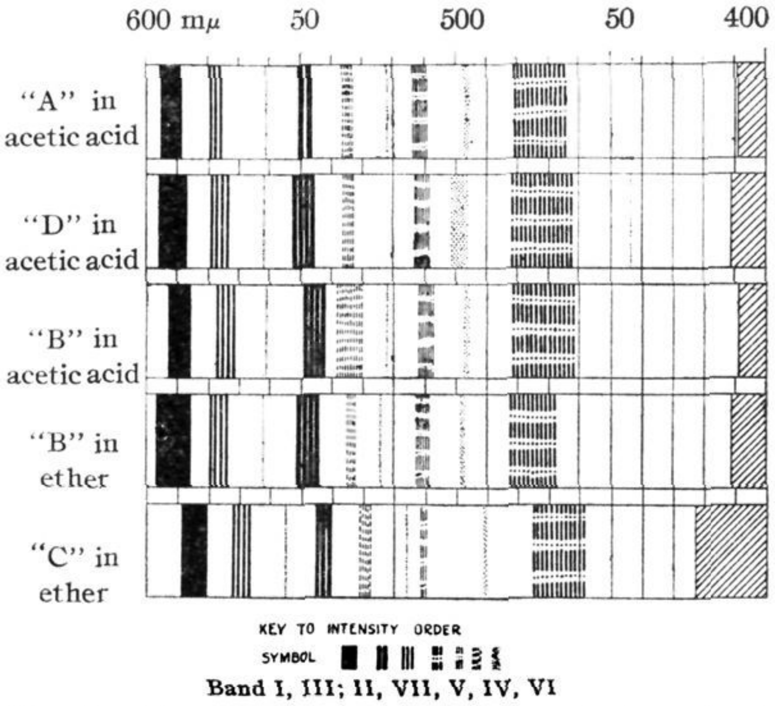

Fig. 1.—Absorption spectra of compounds from buckwheat.

Properties of the Pigments.—The substance "A" is purplish-black and quite insoluble in neutral ether, in petroleum ether, in methyl alcohol, ethyl alcohol, benzene, or acetone. It is slightly soluble in chloroform and in pyridine, and soluble in hot glacial acetic acid. When a small amount of "A" was finally dissolved in a considerable quantity of ether, dilute hydrochloric acid failed to remove it; 16% or higher percentages of hydrochloric acid, however, extracted it from the ether. When heated on a block to 375°, crystals of "A" were not observed to melt.

Substance "B" is insoluble in water, somewhat soluble in ether, methyl alcohol, benzene, ethyl acetate, chloroform, or olive oil, and quite soluble in hot acetone, amyl alcohol, pyridine, and glacial acetic and formic acids. In every one of these, the color of the solution is some shade of red, and the solutions are strongly fluorescent. The substance "B" dissolved slightly in 10% sodium hydroxide solution to produce a bright-green colored solution. The characteristic spectrum of "B" could no longer be observed. Crystals of "B" did not melt sharply even when heated to 375° on a block.

Substance "C" is slightly soluble in methyl alcohol and ethyl alcohol, and very soluble in acetone, in ether, and in glacial acetic acid. It dissolves slightly in dilute sodium hydroxide to give a green solution.

Each of the three substances isolated from buckwheat yielded fluorescent solutions.

In this study, another pigment "D," with a spectrum very similar to those of "A," "B" and "C" has been isolated, but the yield was so small that no animal tests on its photosensitizing action were made. The possibility of still other active pigments being isolated from the buckwheat was indicated by this investigation.

The Absorption Spectrum.—The absorption spectrum of "A" in glacial acetic acid is as follows (determined by direct reading on a large Zeiss grating spectroscope): I 595.5-(592.1)-588.7; II 579.6-(577.7)-575.7; shadow 561; III 550.7-(548.4)-546.1; IV 536.7-(534.9)-533.1; shadow 522; V 513.8-(511.5)-509.2; VI 499.6; VII 480.9-(472.6)-464.3; E.A. 409. Intensities I, III; II, VII, V, IV, VI.

The absorption spectrum of "B" in neutral ether is: I 596.8-(591.2)-585.6; II 579.3-(576.5)-573.7; shadow 562; III 551.5-(547.8)-544.0; IV 535.1-(533.8)-532.5; shadow 524; V 512.5-(510.7)-508.8; VI 497.8; VII 482.3-(475.0)-467.7; E.A. 411. Intensities I, III; II, VII, V, IV, VI.

The absorption spectrum of "B" in glacial acetic acid is: I 592.2-(589.4)-585.9; II 577.0-(574.2)-571.4; shadow 561; III 549.1-(545.6)-542.1; IV 534.8-(532.5)-530.2; shadow 522; V 512.1-(509.7)-507.3; VI 496.5; VII 481.3-(471.4)-461.4; E.A. 409. Intensities I, III; II, VII, V, IV, VI.

The absorption spectrum of "C" in neutral ether is: I 588.9-(584.7)-580.5; II 572.1-(569.3)-566.5; shadow 555; III 545.2-(542.7)-540.1; IV 531.3-(529.4)-527.4; V

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[11] R. Willstätter and W. Mieg, *Ann.*, **350**, 1 (1906).

511.3–(505.6)–499.8; VI 490.7; VII 474.8–(466.6)–458.3; E.A. 423. Intensities I, III; II, VII, V, IV, VI.

The absorption spectrum of "D" in glacial acetic acid is: I 598....596–(591.5)–586.9; II 579.1–(576.1)–573.1; shadow 561.5; III 552.9–(549.2)–545.5; IV 536.6–(534.7)–532.8; V 513.2–(510.9)–508.6; VI 501.4–(498.8)–496.1; VII 482.5–(472.2)–461.9; shadow 443.5; E.A. 411. Intensities I, III; II, VII, V, IV, VI.

Discussion.—No definite statement has been ventured as yet as to the identity of the isolated substances. Recently, Pace and Mackinney[12] isolated hypericin, the photodynamic pigment from St. John'swort (*Hypericum perforatum*). St. John'swort causes a disease, hypericism, which is very similar to fagopyrism. These investigators found hypericin to consist of at least six fractions. The absorption spectra obtained for these substances are surprisingly similar to the absorption spectra of the substances isolated in the present study on buckwheat. Pace and Mackinney suggest tentatively that this photodynamic substance is a partially reduced polyhydroxy derivative of helianthrone.

The substances isolated from buckwheat apparently still contained traces of some impurities, probably from the adsorbent used. Micro-combustion analyses obtained were thus deemed unsatisfactory. Substances "B" and "C" contained very small amounts of calcium. Qualitative tests for halogens were negative.

Pace[13] has shown recently that the region of the visible spectrum responsible for experimental hypericism, mainly between 540 and 610 mμ, corresponds, within reasonable limits, to the principal absorption bands of the pigment hypericin in the visible region of the spectrum. He concluded that hypericin is the photosensitizer responsible for the production of the syndrome of hypericism in domestic animals. Chick and Ellinger[9] found that the most active rays in experimental fagopyrism are those of wave lengths between 540 and 610 mμ. Not only is this wave length region the same one as that which Pace found for hypericism, but it agrees fairly well with the region of maximum absorption of the substances isolated from buckwheat in this study. Experimentally, each of the substances "A," "B" and "C" isolated from buckwheat proved to be a photosensitizing agent when fed to white guinea pigs, even when the rays of sunlight passed through ordinary window glass before reaching the test animals.

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Summary

A procedure has been described for the isolation and crystallization from buckwheat of three substances. Each of these substances has been found to produce photosensitization when fed to white guinea pigs. The presence of at least one more substance with a spectrum very similar to those of the three isolated substances has been indicated. The fluorescence of the isolated substances has been noted. The absorption spectrum of each of the substances has been recorded, and certain physical and chemical properties of the isolated substances have been described and compared with those of the photodynamic substance hypericin.

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(12) N. Pace and G. Mackinney, THIS JOURNAL, **63**, 2570 (1941).

(13) N. Pace, *Am. J. Physiol.*, **136**, 650 (1942).