

[CONTRIBUTION FROM THE DEPARTMENT OF AGRICULTURAL CHEMISTRY, PURDUE UNIVERSITY AGRICULTURAL EXPERIMENT STATION, IN COÖPERATION WITH U. S. REGIONAL SOYBEAN INDUSTRIAL PRODUCTS LABORATORY]<sup>1</sup>

## Genistin (an Isoflavone Glucoside) and its Aglucone, Genistein, from Soybeans

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Genistein, which is now known to occur in soybeans as the aglucone of genistin, was isolated from Dyer's Broom (*Genista tinctoria*) in 1899 by Perkin and Newbury.<sup>2</sup> The isoflavone nucleus was established for genistein by Baker and Robinson<sup>3</sup> in 1926, when they found it to be identical with prunetol, and in 1928 they synthesized genistein. The constitution of genistein was thereby established as 5,7,4'-trihydroxyisoflavone.

In 1931 Walz<sup>4</sup> isolated genistin from a 90% methanol extract of soybean meal. He found that hydrolysis of genistin with hydrochloric acid gave one mole of genistein and one mole of glucose. The glucose was identified by optical rotation and by a Bertrand determination of the reducing sugar after its separation from the aglucone. From degradative experiments he found that the glucose was bound to genistein at position seven and that the constitution of genistin was 5,7,4'-trihydroxyisoflavone-7-glucoside.

In 1939 Okano and Beppu<sup>5</sup> reported four isoflavones from soybeans. One of these was named isogenistein, but apparently none of them had the same physical properties shown by genistin or genistein.

During the course of a study of the carbohydrates of soybeans, an attempt was made to isolate certain sugars from a methanol extract of oil-free soybean meal. After the removal of some of the phosphatides from a concentrated methanol solution by the addition of acetone, the solution was concentrated and upon the addition of water, white, granular aggregates crystallized. These crystals, when dissolved in hot ethanol, gave a reddish-violet coloration with an ethanol solution of ferric chloride. The properties of the purified material agreed with those reported by Walz for genistin.

This paper confirms some of Walz's work and

(1) The U. S. Regional Soybean Industrial Products Laboratory is a cooperative organization participated in by the Bureaus of Agricultural Chemistry and Engineering and Plant Industry of the United States Department of Agriculture and the Agricultural Experiment Stations of the North Central States of Illinois, Indiana, Iowa, Kansas, Michigan, Minnesota, Missouri, Nebraska, North Dakota, Ohio, South Dakota and Wisconsin.

(2) A. G. Perkin and F. G. Newbury, *J. Chem. Soc.*, **75**, 830 (1899).

(3) W. Baker and R. Robinson, *ibid.*, 2713 (1926); 3115 (1928).

(4) E. Walz, *Ann.*, **489**, 118 (1931).

(5) K. Okano and I. Beppu, *Bull. Agr. Chem. Soc. Japan*, **15**, 110 (1939).

presents further evidence that the sugar hydrolyzed from genistin is *D*-glucose. Additional physical and chemical properties of genistin and its aglucone, genistein, are also presented. The melting points, optical rotation and carbon and hydrogen analyses of the compounds presented are in agreement with the data presented by Walz for the corresponding substances.

A small amount of some other crystalline material was obtained from the mother liquors, which gave qualitative evidence for another flavone. Work is in progress in this Laboratory on the isolation and identification of this material.

### Experimental

**Isolation and Properties of Genistin.**—Commercial soybean flakes, which had been extracted with hexane, were placed in a cloth bag in 10-kg. quantities and put into the extraction chamber of a Lloyds extractor. Sufficient methanol (about 24 liters) was added to cover the bag of flakes. The valves in the apparatus were so adjusted that the extractor would operate as a reflux. Steam was passed through the coil surrounding the extraction chamber and the mixture was refluxed for twenty-four hours. The extract was drained off, a fresh supply of methanol was added to the flakes and the mixture was again refluxed for twenty-four hours. The extracts were combined and concentrated to a volume of about 1.5 liters. Acetone was added to the concentrate until precipitation ceased. The acetone precipitated some of the phosphatides, which carried with them carbohydrates, saponin and other impurities. After the supernatant liquid became clear, it was decanted and concentrated on the steam-bath to a thin sirup. About two volumes of water was added to this sirup and on standing for a day at room temperature the genistin crystallized in yellowish-white granular aggregates.

The crude genistin was removed from the mother liquor by centrifuging. It was then dissolved in approximately 80% hot ethanol, treated with Nuchar and filtered. Crystallization was effected by concentrating the filtrate and cooling it to room temperature. At this point the crystals were contaminated with saponin. This was detected readily by adding a few crystals to concentrated sulfuric acid. When saponin is present the citron-yellow color due to genistin soon changes to red and later to purple. With pure genistin, however, the yellow color with sulfuric acid is permanent. After several recrystallizations from 80% ethanol the genistin was obtained in pale-yellow thin rectangular plates of m. p. 256° (Walz recorded 254–256°). Further treatment of the mother liquors with Nuchar, followed by concentration, yielded additional genistin. The total yield was 0.10% of the dry hexane-extracted soybean flakes.

When a few drops of ferric chloride was added to an ethanol solution of the substance, a reddish-violet coloration was obtained. The substance was insoluble in cold water, but slightly soluble in hot water, hot ethanol and in hot methanol. It was soluble in hot 80% ethanol, hot 80% methanol and in hot acetone. It was quite soluble in pyridine. When dissolved in alkali it gave a yellow color.

Genistin dried in an Abderhalden drier at 110° gave the following analytical data—calcd. for  $C_{21}H_{20}O_{10}$ : C, 58.31; H, 4.66. Found: C, 58.05; H, 4.70. *Optical rotation*. 0.3 g. in 50 ml. of *N*/50 NaOH at 21° rotated  $-0.336^\circ$ ;  $l = 2.0$  dm.;  $[\alpha]^{21}_D -28^\circ$ . Walz recorded for genistin  $[\alpha]^{21}_D -27.7^\circ$ .

Genistin (1.0 g.) was acetylated by refluxing for one hour with acetic anhydride (20 ml.) and one drop of concentrated sulfuric acid. The product was separated from the reaction mixture by the addition of water and filtration of the precipitate. The substance crystallized from ethanol in long prisms with a tendency to twist into spirals; yield 1.5 g., m. p. 188°. Walz reported for genistin hexaacetate a melting point of 188°.

*Anal.* Calcd. for  $C_{21}H_{14}O_{10}(CH_3CO)_6$ : C, 57.87; H, 4.71;  $CH_3CO$ , 37.73; mol. wt., 684. Found: C, 57.78; H, 4.92;  $CH_3CO$ , 37.63; mol. wt. (Rast), 686.

The acetyl determinations reported herein were made by the Kunz<sup>6</sup> method as follows. To 100 mg. of the substance 25 ml. of acetone was added. The mixture was cooled in an ice-salt-bath and 25 ml. of 0.1 *N* NaOH was added dropwise. The solution was then kept below 0° for one hour and back-titrated with 0.1 *N* hydrochloric acid. A blank was run on the acetone used.

**Isolation of the Aglucone, Genistein.**—Genistin (4 g.) was treated with 90 ml. of methanol and 20 ml. of concentrated hydrochloric acid. After about three hours of refluxing the substance was all in solution, but hydrolysis was continued for another three hours. Water was then added to the cooled solution to precipitate the aglucone. Ether was added to dissolve the precipitate and the two layers were separated by means of a separatory funnel. The ether was evaporated and the substance was crystallized from hot 60% ethanol, as white, rectangular and six-sided rods; yield 2.3 g., m. p. 296° (dec.). Walz recorded a melting point of 296–298° for genistein. The substance was water-insoluble, but was soluble in the usual organic solvents. It gave a reddish-violet coloration with ferric chloride. For analysis the substance was dried in an Abderhalden drier at 110°. The water solution from the above reaction mixture was saved for the identification of the sugar.

*Anal.* Calcd. for  $C_{15}H_{10}O_5$ : C, 66.6; H, 3.73; mol. wt., 270. Found: C, 66.66; H, 3.60; mol. wt. (Rast), 282. *Optical rotation*. 0.1029 g. in 25 ml. of acetone gave no rotation.

Genistein (1 g.) was acetylated by refluxing for one hour with 20 ml. of acetic anhydride and a drop of concentrated sulfuric acid. The mixture was poured into ice water and the precipitate was filtered off. The product crystallized from ethanol in white, needle clusters and an occasional boat-shaped form; yield 1.05 g., m. p. 200°. Walz

recorded a melting point of 200–202° for genistein triacetate. It was soluble in organic solvents.

*Anal.* Calcd. for  $C_{15}H_7O_5(CH_3CO)_3$ : C, 63.63; H, 4.07;  $CH_3CO$ , 32.58; mol. wt., 296. Found: C, 63.16; H, 4.18;  $CH_3CO$ , 32.17; mol. wt. (Rast), 293.

**Color Tests.**—The reduction color tests described by Asahina and co-workers<sup>7</sup> as characteristic of flavones were also given by genistein and genistin.

The reactions were performed according to the directions of Wolfrom and co-workers,<sup>8</sup> who applied them to osage orange pigments. Reduction with magnesium and hydrochloric acid gave no coloration with either genistin or genistein. Reduction with sodium amalgam, however, produced orange-red solutions forming dark red precipitates on acidification with an excess of concentrated hydrochloric acid.

Both genistin and genistein gave a yellow coloration with the boric acid test, which was carried out as described by Wilson.<sup>9</sup>

**Identification of the Sugar as *D*-Glucose.**—The aqueous hydrolyzate from genistin was heated on the steam-bath to evaporate most of the methanol, then refluxed for three hours to hydrolyze the methyl glucoside. The mixture was then neutralized with silver carbonate and clarified with Nuchar. The clear liquid was concentrated under reduced pressure to about 4 ml. The sirup was dissolved in 20 ml. of methanol and used to prepare the potassium aldonate by the method described by Moore and Link.<sup>10</sup> A quantitative reduction on an aliquot of the liquid previous to neutralization showed a total of 0.796 g. of sugar calculated as glucose. This amount (less the aliquot used, 19.9 mg.) gave 0.81 g. of potassium aldonate, m. p. 180° (dec.), the melting point reported for potassium gluconate by Moore and Link. Corresponding amounts of reagents were used on this to prepare the benzimidazole derivative as described by Moore and Link.<sup>10</sup> After treatment of the reaction product three times with Nuchar, the crystalline product melted at 215° (dec.). A mixture of this with known *D*-glucobenzimidazole also melted at 215° (dec.).

**Glucobenzimidazole Picrate.**—The above *D*-glucobenzimidazole was used to make the picrate as described by Moore and Link.<sup>10</sup> The product melted at 207° (dec.). When mixed with known glucobenzimidazole picrate, the melting point was unchanged.

#### Crystallographic Optical Properties

**Genistin.**—Crystallized from 80% ethanol genistin appears, under the microscope, as thin rectangular plates (Fig. 1). In parallel polarized light (crossed nicols): the birefringence is strong. The extinction is parallel and the elongation is positive. Refractive indices:  $n_\alpha = 1.580$  found across the elongated plates;  $n_\beta = \text{indet.}$ ;  $n_\gamma = 1.650$  found along the elongation, all  $\pm 0.003$ . No interference figures were observed in convergent polarized light (crossed nicols), which were suitable for establishing the  $\beta$ -value.

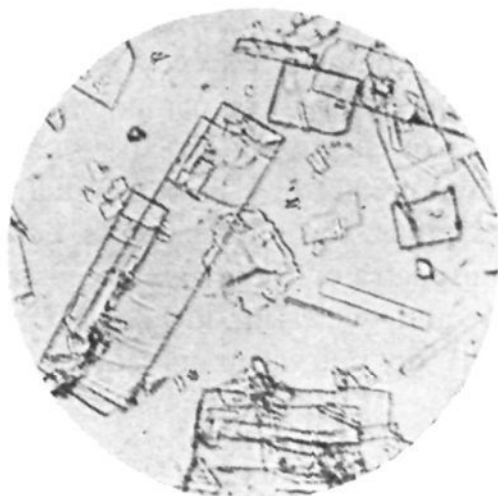
(7) Y. Asahina and M. Inubuse, *Ber.*, **61**, 1646 (1928); **64**, 1256 (1931); Y. Asahina, G. Nakagome and M. Inubuse, *ibid.*, **62**, 3016 (1929).

(8) M. L. Wolfrom, P. W. Morgan and F. L. Benton, *THIS JOURNAL*, **62**, 1484 (1940).

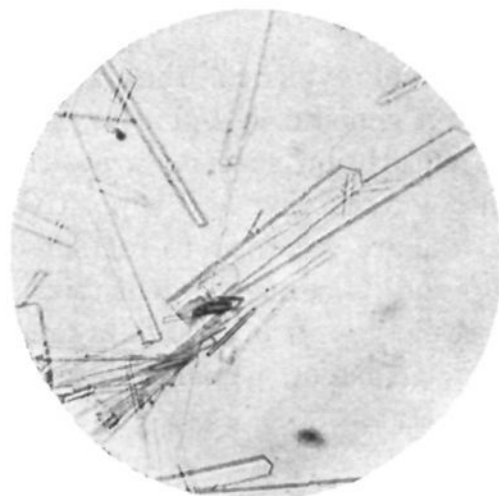
(9) C. W. Wilson, *ibid.*, **61**, 2303 (1939).

(10) Stanford Moore and Karl P. Link, *J. Biol. Chem.*, **133**, 293 (1940).

(6) A. Kunz and C. S. Hudson, *THIS JOURNAL*, **48**, 1982 (1926).

Fig. 1.—Genistin ( $\times 75$ ).

**Genistein.**<sup>11</sup>—*In ordinary light*, under the microscope, this substance shows broad, rectangular and six-sided rods, and hemimorph forms, pentagonal in outline (Fig. 2).

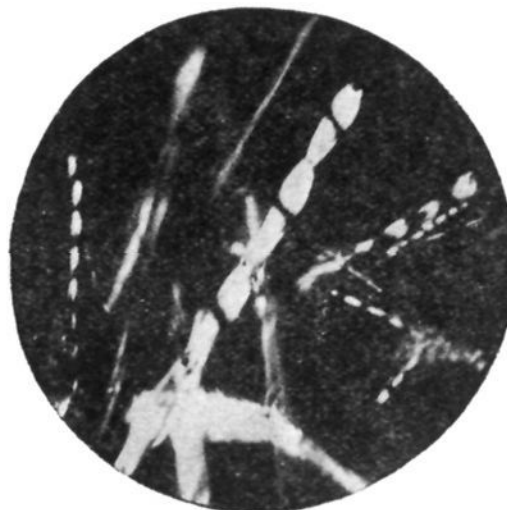
Fig. 2.—Genistein ( $\times 75$ ).

Refractive indices (determined by the immersion method in organic liquids):  $n_\alpha = 1.530$ ;  $n_\beta = \text{indet.}$ ;  $n_i = 1.645$  (probably approximating the  $\beta$ -value);  $n_\gamma = >1.733$  (methylene iodide), all  $\pm 0.002$ ,  $n_\gamma - n_\alpha = 0.203$ .  $n_\alpha$  is frequently found lengthwise on rods, and on irregular fragments. No interference figures were observed in convergent polarized light (crossed nicols), therefore an intermediate index,  $n_i = 1.645$  (commonly found), could not be positively confirmed as the  $\beta$ -value.  $n_\gamma$  is usually found on rods crosswise. *In parallel polarized light (crossed nicols)*: the birefringence is strong and many of the rods show peculiar and characteristic blues or purples of the type frequently referred to as anomalous interference colors. The extinction is parallel and the elongation is negative.

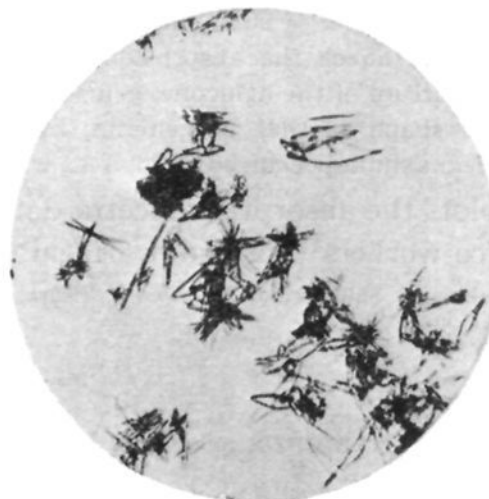
**Genistin Hexaacetate.**—Crystallized from ethanol this substance appears in ordinary light as fine, long prisms. *In parallel polarized light (crossed nicols)* a peculiar barber-pole appearance is observed (Fig. 3). This is apparently due to the tendency of the slender crystals to twist. On the larger untwisted crystals the extinction is parallel and the elongation is positive. An occasional face shows no extinction and gives an optic axis interference figure in convergent polarized light (crossed nicols). On these

(11) The writer is indebted to George L. Keenan, of The Federal Security Agency, Food and Drug Administration, Washington, D. C., for the optical crystallographic data for genistein reported here.

$n_\beta = 1.556$ . Across slender prisms showing red color between crossed nicols,  $n_\alpha = 1.520$ ;  $n_\gamma = 1.577$  found lengthwise, all  $\pm 0.003$ .

Fig. 3.—Genistin hexaacetate under polarized light ( $\times 540$ ) (crossed nicols).

**Genistein Triacetate.**—This substance, when crystallized from ethanol, in ordinary light appears in clusters of needles and boat shaped forms (Fig. 4). *In parallel*

Fig. 4.—Genistein triacetate ( $\times 75$ ).

*polarized light (crossed nicols)*: the birefringence is strong and many of the boat shaped crystals show characteristic blue and purple colors. The extinction is parallel and the elongation is negative. Refractive indices:  $n_\alpha = 1.508$  is found lengthwise on the boat shaped forms;  $n_\beta = 1.556$  is found on occasional fragments showing an optic eye (in convergent polarized light, crossed nicols);  $n_\gamma > 1.733$  (methylene iodide) found crosswise on crystals showing blue color between crossed nicols, all  $\pm 0.003$ .

**Absorption Spectra of Genistin and Genistein.**<sup>12</sup>—Figure 5 shows the ultraviolet absorption spectra of genistin and genistein in 85% ethanol. The ordinate is the specific absorption coefficient  $\alpha$ , from the relation  $\log I_0/I = \alpha cl$ , with  $c$  the concentration in grams per liter and  $l$  the length of the cell in centimeters. The spectra were determined on a photoelectric spectrophotometer similar in principle to that described by Hogness and co-workers.<sup>13</sup>

(12) The absorption spectra were determined by Jonathan W. White, Jr., of the Department of Agricultural Chemistry, Purdue University.

(13) T. R. Hogness, F. P. Zscheile, Jr., and A. E. Sidwell, Jr., *J. Phys. Chem.*, **41**, 379 (1937).

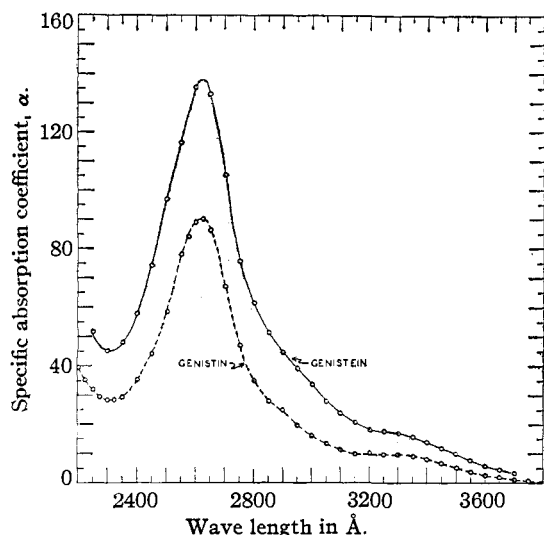


Fig. 5.—Absorption spectra of genistin and genistein.

A large Hilger double monochromator with quartz optics was employed to isolate spectral regions which varied between 6 and 13 Å. For genistin a single maximum was found at 2625 Å., with  $\alpha = 90.5$ . Recrystallization of the genistin did not affect the absorption spectrum. The absorption spectrum of the aglucone, genistein, was of the same general shape as that of genistin, but somewhat higher. The maximum occurred at 2625 Å., with  $\alpha = 138$ .

If one plots the absorption spectra data of Asahina and co-workers<sup>14</sup> for iridin, irigenin, tectori-

(14) Y. Asahina, B. Shibata and Z. Ogawa, *J. Pharm. Soc. Japan*, **48**, 150, 1093 (1928).

din and tectorigenin in the same terms used in Fig. 5, a maximum is observed in the region of 2680 Å. for each of these compounds. A minimum occurs in the region of 2400 Å. and a shelf in the region of 3200 Å. Thus a similarity in the general shape of the curves exists between the absorption spectra of these compounds and those of genistin and genistein, although the maxima and minima do not occur at exactly the same wave lengths.

### Summary

1. A crystalline pale yellow substance has been isolated from oil-free soybean meal. The data on this substance and its derivatives are in agreement with the data for genistin and its derivatives reported by Walz, who also isolated it from soybeans.

2. Additional evidence, that the sugar hydrolyzed from genistin is *d*-glucose, is presented.

3. The absorption spectra, a positive Wilson boric acid color reaction and a positive Asahina reduction test are recorded to further characterize genistin and its aglucone, genistein.

4. Crystallographic optical data are presented for genistin, genistein, genistin hexaacetate and genistin triacetate.

LAFAYETTE, INDIANA

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## Preparation of the Dinitrochlorobenzenes from the Corresponding Dinitroanilines\*

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In connection with clinical studies of the specificity of response of persons eczematously sensitized to aromatic compounds, the need arose for specimens of the dinitrochlorobenzenes. Synthesis of five of the six possible isomers was required since only 2,4-dinitrochlorobenzene was commercially available.

The 3,4,<sup>1</sup> 2,5-<sup>2</sup> and 2,6-<sup>3</sup> isomers are formed directly by the action of nitric acid on benzene derivatives. The last substance cited has also been prepared from tosyl chloride and 2,6-dinitrophenol.<sup>4</sup> Chlorination of *m*-dinitrobenzene

has been reported to give 3,5-dinitrochlorobenzene.<sup>5</sup> The Sandmeyer reaction has been used in the preparation of 3,5-<sup>6</sup> 2,5-<sup>7</sup> 2,3-<sup>7a</sup> 2,4-<sup>8</sup> and 2,6-dinitrochlorobenzene<sup>9</sup> from amino compounds.

For purposes of clinical study, the quantitative separation of the isomers was of more fundamental importance than the removal of unrelated impurities, and it was thought that the chances of satisfying this requirement would be increased by using syntheses which involved a plurality of steps between the isolation of the final product and the reaction which fixed the relationship of the substit-

\* Not copyrighted.

(1) Laubenheimer, *Ber.*, **9**, 760 (1876); Mangini and Deliddo, *Gazz. chim. ital.*, **63**, 612 (1933).

(2) Kehrman and Grab, *Ann.*, **303**, 1 (1898).

(3) Borsche and Rantscheff, *ibid.*, **379**, 152 (1911).

(4) Kubota, *J. Chem. Soc. Japan*, **53**, 404 (1932); *C. A.*, **27**, 274 (1933).

(5) German Patent 108,165; *Chem. Zentr.*, **71**, I, 1115 (1900).

(6) Bader, *Ber.*, **24**, 1653 (1891); de Kock, *Rec. trav. chim.*, **20**, 111 (1901).

(7) (a) Hollemann and ter Weel, *ibid.*, **35**, 46-47 (1915); (b) Körner and Contardi, *Atti accad. Lincei*, **23**, I, 281 (1914).

(8) Hodgson and Walker, *J. Chem. Soc.*, 1620 (1933).

(9) Körner and Contardi, *Atti accad. Lincei*, **23**, II, 464 (1914).