761. *The Chemistry of Subterranean Clover. Part I. Isolation of Formononetin and Genistein.*

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Two *iso*flavones, formononetin (7-hydroxy-4′-methoxy*iso*flavone) and genistein (5 : 7 : 4′-trihydroxy*iso*flavone) have been isolated from the " chloroplast fraction " of subterranean clover, and genistein has been shown to be mainly responsible for its œstrogenic activity.

The œstrogenic properties of the Dwalganup strain of subterranean clover (*Trifolium subterraneum* L.) are of great importance to sheep breeding in the drier areas of Western Australia, and have already been the subject of considerable investigation (Bennetts, Underwood, and Shier, *Austral. Vet. J.*, 1946, **22**, 2; Bennetts, *ibid.*, p. 70; 1947, **23**, 10; Curnow, Robinson, and Underwood, *Austral. J. Exp. Biol. Med. Sci.*, 1948, **26**, 171; East, Underwood, and Bennetts, *ibid.*, 1949, **27**, 105; Robinson, *ibid.*, p. 297). Concentration of the œstrogen into the " chloroplast fraction " of plants was effected by Curnow, Legg, and Simpson (*Biochem. J.*, 1950, **46**, xix), and examination of concentrated extracts prepared from this material (Curnow, Thesis, Univ. London, 1950; Beck and Braden, in the press) has shown that the œstrogen is phenolic, but its infra-red absorption spectrum (Curnow, private communication) and comparison of its partition coefficients with those of known steroid œstrogens failed to identify it.

In the present investigation, the " chloroplast fraction " prepared from a large quantity of clover was extracted with alcohol and, after removal of the fats from this extract with light petroleum, crystals separated from a concentrated alcoholic solution. These were identified as formononetin (7-hydroxy-4′-methoxy*iso*flavone) from the analyses and melting points of the compound and some of its derivatives, and by comparison of the degradation product, 2 : 4-dihydroxyphenyl 4′-methoxybenzyl ketone, with a synthetic specimen (Baker and Eastwood, *J.*, 1929, 2897).

Formononetin was previously obtained from *Ononis spinosa* L. where it occurs as the glycoside ononin (Hlasiwetz, *J. prakt. Chem.*, 1855, **65**, 419; Wessely and Lechner, *Sitzungsber. Akad. Wiss. Wien*, 1930, **139**, II*b*, 1061; Wessely, Lechner, and Dinjaski, *ibid.*, 1933, **142**, II*b*, 411).

From the material in the mother-liquor, chromatography on alumina, followed by crystallisation from aqueous alcohol, yielded, in addition to a further quantity of formononetin, a considerable quantity of genistein (5 : 7 : 4′-trihydroxy*iso*flavone) which was identified in a similar manner, and also by comparison with a synthetic specimen. This was prepared by condensing 2-hydroxy-4 : 6-dimethoxyphenyl 4′-methoxybenzyl ketone (Badcock, Cavill, Robertson, and Whalley, *J.*, 1950, 2965) with ethyl formate to give 5 : 7 : 4′-trimethoxy*iso*flavone, which was demethylated to genistein.

Genistein has been reported as glycosides in *Genista tinctoria* L. (Perkin and Newbury, *J.*, 1899, **75**, 830), *Soja hispida* L. (Walz, *Annalen*, 1931, **489**, 118), and *Sophora japonica* L. (Charaux and Rabaté, *J. Pharm. Chim.*, 1935, [viii], **21**, 546) and was synthesised by Baker and Robinson (*J.*, 1928, 3115).

Biological tests on natural and synthetic genistein have demonstrated its œstrogenic activity and, although this is low (about 10^{-5} that of œstrone), the amount present (2 mg. per 100 g. of fresh clover) indicates that it must be the principal œstrogen in the clover.

EXPERIMENTAL.

M. p.s are corrected. Analyses are by Drs. Weiler and Strauss, Oxford, and Dr. W. Zimmermann, Melbourne.

Preparation of "Chloroplast Fraction."—Freshly mown subterranean clover (Dwalganup strain; 4020 kg.), collected in 1950 at the period of maximum growth (October), which previous workers had found to be the time of maximum œstrogenic activity, was macerated in 700-kg. batches in a hammer mill and pressed in a hydraulic press. In this way 1500 l. of juice were obtained (corresponding to 43% of the total water content of the clover); they were heated to 90° as rapidly as possible (90-l. batches, with four 2-kw. immersion heaters). After being left for 12 hours to cool, the supernatant liquid was siphoned off and the sludge filtered on Whatman No. 4 filter papers in Büchner funnels, dried at 100° to 7—10% moisture content, and powdered (15 kg.).

Extraction of "Chloroplast Fraction."—Extraction of the "chloroplast fraction" (4·4 kg.) by percolation with alcohol (24 l.) at room temperature gave, on evaporation of the solvent, 473 g. of extract. Exhaustive extraction of this with boiling light petroleum (b. p. 40—60°) removed 118 g. of material with negligible œstrogenic activity, and the residue was dissolved in alcohol (320 ml.) and poured slowly into ether (2880 ml.), which was cooled in ice and stirred constantly. After being kept at room temperature overnight, the insoluble residue, which showed negligible œstrogenic acitivty, was separated and the solution evaporated to dryness (100 g.).

Isolation of Formononetin.—The above material (100 g.) was extracted five times with light petroleum (100 ml.), and the residue (89·5 g.) dissolved in the minimum amount of boiling alcohol, treated with charcoal, and filtered. On cooling, formononetin separated (5·2 g.). It was readily purified by solution in cold, very dilute aqueous sodium hydroxide, precipitation with acid, and crystallisation, or by chromatography on alumina. It separated from alcohol in long, colourless needles, curved when impure, m. p. 258° (Found: C, 71·8, 71·6; H, 4·8, 4·6; OMe, 10·6%; *M*, 291. Calc. for $C_{16}H_{12}O_4$: C, 71·6; H, 4·5; OMe, 11·6%; *M*, 268). Light absorption in ethanol: $\varepsilon_{max.}$ 27,440 at 2500 Å; $\varepsilon_{max.}$ 11,240 at 3000 Å. Wessely, Lechner, and Dinjaski (*loc. cit.*) record m. p. 259°.

Acetylformononetin. Formononetin (1·0 g.) and acetic anhydride (5 ml.) were refluxed for 15 minutes and the product isolated and washed with water and crystallised from alcohol. It formed colourless needles, m. p. 171—172° (0·8 g.) (Found: C, 69·2; H, 4·7; $CH_3{\cdot}CO$, 27·7. Calc. for $C_{18}H_{14}O_5$: C, 69·7; H, 4·5; $CH_3{\cdot}CO$ and $H{\cdot}CO_2H$ calc. as $CH_3{\cdot}CO$, 27·7%). Wessely, Lechner, and Dinjaski (*loc. cit.*) and Mahal, Rai, and Venkataraman (*J.*, 1934, 1769) record m. p. 170°.

Propionylformononetin. Formononetin (1·0 g.) and propionic anhydride (5 ml.) were refluxed for 15 minutes and the product isolated and washed with water. *Propionylformononetin* formed long, colourless needles, m. p. 132°, from alcohol (Found: C, 70·1; H, 4·8. $C_{19}H_{16}O_5$ requires C, 70·4; H, 5·0%).

7 : 4′-*Dimethoxy*iso*flavone.*—Addition of excess of diazomethane in ether to a solution of formononetin in alcohol formed 7 : 4′-dimethoxy*iso*flavone, rosettes of needles, m. p. 162—163°, from alcohol or benzene (Found: C, 72·8; H, 4·9; OMe, 19·7, 19·9%; *M*, 256. Calc. for $C_{17}H_{14}O_4$: C, 72·3; H, 5·0; OMe, 22·0%; *M*, 282). Wessely, Lechner, and Dinjaski (*loc. cit.*) record m. p. 163°.

Daidzein.—Formononetin (0·5 g.) was refluxed with hydriodic acid (*d* 1·7; 10 ml.) for 2 hours, and the mixture poured into water and decolorised with sulphur dioxide. The daidzein obtained (0·44 g.) formed colourless needles, m. p. 325° (decomp.), from alcohol. It gave no colour with ferric chloride Wessely and Lechner (*Ber.*, 1933, **66**, 685) record m. p. 323°.

2 : 4-*Dihydroxyphenyl* 4′-*Methoxybenzyl Ketone.*—Formononetin (0·35 g.) was dissolved in sodium hydroxide solution (0·4N.; 30 ml.) and heated for 15 minutes on the water-bath. After acidification, the product was filtered off (0·32 g.) and formed colourless prisms, m. p. 158°, from aqueous alcohol (Found: C, 69·8; H, 5·5. Calc. for $C_{15}H_{14}O_4$: C, 69·7; H, 5·5%). Its m. p. showed no depression on admixture with a synthetic specimen prepared by the method of Baker and Eastwood (*loc. cit.*).

This ketone (0·5 g.), acetic anhydride (5 ml.), and pyridine (2 drops) were refluxed for 15 minutes and the product (0·2 g.) was isolated and washed with water. 2 : 4-*Diacetoxyphenyl* 4′-*methoxybenzyl ketone* formed colourless prisms, m. p. 123—124°, from alcohol (Found: C, 67·2; H, 5·4. $C_{19}H_{18}O_6$ requires C, 66·7; H, 5·3%).

The hydroxy-ketone (0·2 g.) and propionic anhydride (3 ml.) were refluxed for 15 minutes and the product (0·1 g.) was isolated and washed with water. 2 : 4-*Dipropionoxyphenyl* 4′-*methoxybenzyl ketone* separated from aqueous alcohol as colourless needles, m. p. 79—80° (Found: C, 68·0; H, 6·1; OMe, 8·3. $C_{21}H_{22}O_6$ requires C, 68·1; H, 6·0; OMe, 8·4%).

2-*Hydroxy*-4-*methoxyphenyl* 4′-*Methoxybenzyl Ketone.*—This compound, prepared by the action of diazomethane on 2 : 4-dihydroxyphenyl 4′-methoxybenzyl ketone or by alkaline hydrolysis of 7 : 4′-dimethoxy*iso*flavone, crystallised from alcohol in colourless needles, m. p. 102° (Found: C, 70·4; H, 5·75; OMe, 21·7. Calc. for $C_{16}H_{16}O_4$: C, 70·6; H, 5·9; OMe, 22·8%). Wessely and Lechner (*Sitzungsber. Akad. Wiss. Wien*, 1930, **139**, II*b*, 1061) record m. p.s 92—93° and 104°.

Isolation of Genistein.—The filtrate from the separation of formononetin was concentrated to 150 ml. and, while hot, poured into cold, stirred ether (2850 ml.). This solution was chromatographed on acid-washed alumina (500 g.), and the column eluted with alcohol–ether containing increasing amounts of alcohol. From the initial 5% alcohol–ether filtrates and the first 10% alcohol–ether eluate a pale yellow solid (28·4 g.) was obtained, and on crystallisation from aqueous alcohol this yielded formononetin (0·97 g.) and genistein (17·8 g.). From the column, cut into 5 sections each of which was extracted with 4 × 200 ml. of hot 5% hydrochloric acid in alcohol, a further quantity of genistein (6·6 g.) was obtained by evaporation to dryness, addition of water, and extraction with ether. Genistein formed colourless needles, m. p. 300—301° (decomp.), undepressed by a synthetic sample, on crystallisation from aqueous alcohol (Found : C, 66·6; H, 3·65%; *M*, 237. Calc. for $C_{15}H_{10}O_5$: C, 66·7; H, 3·7%; *M*, 270). Light absorption in ethanol : $\varepsilon_{max.}$ 42,700 at 2625 Å. Walter (*J. Amer. Chem. Soc.*, 1941, **63**, 3273) reported a somewhat lower value for $\varepsilon_{max.}$ in 85% ethanol at the same wave-length.

Genistein (1 g.) and acetic anhydride (5 ml.), refluxed for 15 minutes and poured into water, formed triacetylgenistein (0·7 g.), colourless needles, m. p. 203—204°, from alcohol.

Prepared in a similar manner *tripropionylgenistein* formed colourless needles, m. p. 192°, from alcohol (Found : C, 66·1; H, 4·85. $C_{24}H_{22}O_8$ requires C, 65·7; H, 5·1%).

5 : 7 : 4′-*Trimethoxy*iso*flavone*.—(*a*) Reaction of genistein with excess of diazomethane in ether formed 5 : 7 : 4′-trimethoxy*iso*flavone, which crystallised from alcohol in colourless prisms, m. p. 162—163°, undepressed on admixture with a specimen prepared by method (*b*) (Found : C, 68·7; H, 4·9%; *M*, 296. Calc. for $C_{18}H_{16}O_5$: C, 69·2; H, 5·2%; *M*, 312).

(*b*) A solution of 2-hydroxy-4 : 6-dimethoxyphenyl 4′-methoxybenzyl ketone (Badcock, Cavill, Robertson, and Whalley, *loc. cit.*; 3·27 g.) in ethyl formate (50 ml.) was added dropwise to powdered sodium (3·2 g.) at 0°. After 24 hours at 0°, ice was added and the product extracted with ether. It crystallised from alcohol in colourless prisms, m. p. 162—163° (1·74 g.).

2-*Hydroxy*-4 : 6-*dimethoxyphenyl* 4′-*Methoxybenzyl Ketone*.—Hydrolysis of 5 : 7 : 4′-trimethoxy*iso*flavone (0·2 g.) with N-sodium hydroxide (10 ml.) and alcohol (5 ml.) for 2 hours on the water-bath formed 2-hydroxy-4 : 6-dimethoxyphenyl 4′-methoxybenzyl ketone, prisms (from benzene–light petroleum), m. p. 88—89°, undepressed by a synthetic specimen (Badcock, Cavill, Robertson, and Whalley, *loc. cit.*) (Found : C, 67·3; H, 5·95. Calc. for $C_{17}H_{18}O_5$: C, 67·5; H, 6·0%).

Œstrogenic Activity of Genistein.—The biological assays, which were carried out by Dr. D. H. Curnow, Mr. A. B. Beck, and Mr. J. D. Biggers will be reported in detail elsewhere. However, it may be stated that preliminary assays using the vaginal cornification and uterine weight methods, have shown that genistein is œstrogenically active at a total dose per mouse of 1 mg., and is probably a pro-œstrogen.

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