Oestrogenic Activity of Subterranean Clover

2. THE ISOLATION OF GENISTEIN FROM SUBTERRANEAN CLOVER AND METHODS OF QUANTITATIVE ESTIMATION

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The isolation of small amounts of genistein (5:7:4'-trihydroxy-isoflavone) from subterranean clover (*Trifolium subterraneum* var. *Dualganup*) and the demonstration of its weakly oestrogenic properties (Bradbury & White, 1951, and Biggers & Curnow, 1954), together with the difficulties in the synthesis of this compound, made it important that a more efficient method be found for the isolation of this isoflavone from the clover. Also, the amount of genistein isolated by Bradbury & White (2 mg./100 g. of fresh clover) and its low oestrogenic activity (Biggers & Curnow, 1954) were not nearly sufficient to account for the activity of the fresh plant (Robinson, 1949) and left considerable doubt as to whether this compound was responsible for the oestrogenic activity of the clover.

The present study has shown, however, that the clover contains much more genistein than found by these workers. The amount now found indicates that genistein could indeed be responsible for the activity of the plant and therefore for the infertility in sheep grazing on subterranean-clover pastures. This finding called for rapid and efficient methods of estimation of this compound in the plant. For the determination of the genistein content of the clover at various stages of growth, under various nutritional conditions, in different seasons and in various strains, an accurate method of estimation was required, whereas for the purposes of the plant geneticist a rapid method was desired for use with small amounts of the material (parts of a single plant), in which great accuracy is a secondary consideration. Methods fulfilling these two sets of requirements have been developed and are presented here.

EXPERIMENTAL

Materials

'Chloroplast fraction'. The 'chloroplast fraction' was prepared as described by Bradbury & White (1951) from the *Dualganup* strain of subterranean clover. From 4020 kg. of fresh plant 15 kg. of 'chloroplast' was produced.

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Fresh clover. Clover was cut and weighed in the field and placed immediately in ethanol.

Silica gel. Davison Chemical Corporation '200 mesh' silica gel was used.

Bioassay procedure. The methods described in the previous paper (Biggers & Curnow, 1954) were used for the assay of the oestrogenic activity of extracts.

Isolation of genistein from 'chloroplast'

Extraction. An amount of 100 g. of 'chloroplast' was extracted with 1 l. ethanol for 30 min. while being stirred under reflux. The residue was extracted with a further 500 ml. ethanol, under reflux for 15 min. The combined extract contained 7.2 g. of solid. Extraction of the chloroplast residue with ethanol and with ethanolic NaOH failed to yield any further oestrogenic material.

Purification. The combined ethanol extracts were evaporated *in vacuo* to a volume of 600 ml. Water was added to bring the volume to 1000 ml. and the mixture extracted 4 times with 200 ml. benzene. The benzene extracts were washed in countercurrent fashion 4 times with 200 ml. 60% (v/v) ethanol. This treatment removed from the aqueous ethanol all the chlorophyll and fats, and left a clear pale-yellow solution. The dark-green benzene phase contained 2.78 g. of solid which showed no oestrogenic activity on injection in ovariectomized mice either before or after saponification.

The aqueous ethanol phase was evaporated *in vacuo* to a volume of 200 ml. and extracted 4 times with 200 ml. of ether. The aqueous phase after concentration showed no oestrogenic activity. The ether phase was extracted 4 times with 20 ml. saturated aqueous NaHCO₃ and then twice with 20 ml. water. (The dark brown aqueous extract was acidified with H₂SO₄ and extraction with ether yielded 0.74 g. of material with no oestrogenic activity.) The ether phase contained 3.50 g. of light yellow solid with oestrogenic activity equal to that of approx. 6 µg. oestradiol. Chromatography of 250 mg. of this material on 50 g. silica gel, by using light petroleum-ether mixtures and determining the ultraviolet absorption curves of the eluates to locate the genistein fractions, as in the macro-method below, yielded 30 mg. of genistein, which after recrystallization from aqueous ethanol gave m.p. 300–301° (decomp.) (corr.) not depressed by a synthetic sample. Thus 100 g. of 'chloroplast' yielded 420 mg. of genistein. Related to the original fresh clover this is equivalent to about 2 mg./100 g. (12 mg./100 g. dry matter), which is the figure given by Bradbury & White (1951).

Isolation of genistein from fresh clover

From 100 g. fresh clover (16 g. dry matter), treated precisely as for the 'chloroplast', 59 mg. pure genistein were isolated. This is equivalent to 369 mg. genistein/100 g. dry matter of clover.

Macro-method of estimation of genistein in clover

Sampling. Samples of 30 g. fresh clover were taken from the field and placed immediately in 200 ml. ethanol. A separate sample was taken for drying and estimation of dry-matter content.

Extraction. The fresh clover was refluxed with the 200 ml. ethanol for 15 min., cooled and filtered. The residue was macerated with a further 300 ml. ethanol in a Townson and Mercer (Croydon, Surrey) top-drive macerator and refluxed for 30 min., cooled and filtered. The macerated residue was washed with 50 ml. ethanol and all the ethanol extracts were combined.

Extraction of the residue, either with ethanol or with ethanolic NaOH, failed to yield any extract with oestrogenic activity.

Aqueous ethanol-benzene partition. The combined ethanol extracts were evaporated *in vacuo* to 150 ml. and water was added to bring the volume to 250 ml. The resulting 60% ethanol solution was extracted with three 50 ml. portions of benzene, the separating funnel being gently rotated to avoid the formation of troublesome emulsions. The three benzene extracts were kept apart and extracted in counter-current fashion with five 50 ml. portions of 60% ethanol.

The benzene extract was evaporated to dryness, hydrolysed under reflux with 100 ml. ethanolic N-NaOH and the 'phenol' fraction isolated in the usual way. Injection in ovariectomized mice showed that it contained less than 5% of the total oestrogenic activity of the original extract.

Thus the fats and chlorophylls were retained in the benzene phase whereas the clear, faintly yellow aqueous-ethanol phase contained more than 95% of the oestrogenic material.

The partition coefficient of genistein between benzene and aqueous ethanol was estimated in the following manner. Samples of 0.5 mg. genistein were shaken with 50 ml. aqueous ethanol (50, 60 and 70%, v/v) and 50 ml. benzene and the phases allowed to separate. The temperature was 19°. The aqueous ethanol phases were evaporated to dryness *in vacuo* and the residues dissolved in 50 ml. ethanol. The extinction at 262.5 m μ . was determined with a Beckman spectrophotometer. Similar determinations were made for formononetin (7-hydroxy-4'-methoxy-isoflavone) by using the extinction at 249 m μ . The results are given in Table 1.

The absorption curves of genistein and formononetin in pure ethanol are shown in Fig. 1. Over the range 0.1 mg./100 ml. the optical density at 262.5 m μ . of genistein solutions was found to be directly proportional to the concentration.

With the partition ratio of 1.39 for genistein between 60% ethanol and benzene, the theoretical recovery of genistein in the purification procedure is 97.9%. For formononetin the theoretical recovery is 59.3%. Very little formononetin, however, is found in the purified clover extracts. Formononetin is relatively insoluble in ethanol (175 mg./100 ml. at 22°), whereas genistein is readily soluble.

Separation of phenols. The aqueous ethanol solution was evaporated *in vacuo* to a volume of 100 ml., saturated with

NaCl and extracted with four 100 ml. portions of ether. The combined ether solution was then extracted with six 25 ml. portions of 0.1 N-NaOH. CO₂ was passed into the combined NaOH extracts until they were saturated, and the phenols were extracted with four 100 ml. portions of ether. The combined ether solution was washed with three 20 ml. portions of water, and evaporated to dryness to yield the phenols.

Chromatography. An amount of silica gel 200 times the weight of the phenols was transferred with stirring into a water-jacketed column 15 mm. in diameter filled with light petroleum (40–60°) and the phenols were chromatographed on this column with 50 ml. portions of dry light petroleum-ether mixtures as eluting solvents, running from pure light petroleum to 10, 20, 30...100% (v/v) ether. Finally acetone and then methanol were run to elute the column completely. The pressure at the top of the column was increased by nitrogen at 50–150 mm. Hg. From 20 to 30 fractions were obtained in this way. These fractions were weighed and the recoveries from a number of columns varied from 100 to 110%, the excess being due presumably to some material eluted from the silica gel.

Table 1. Partition of genistein and formononetin between aqueous ethanol and benzene

The partition ratio (concentration in aqueous ethanol/concn. in benzene) was determined using 50 ml. volumes of the two phases and 0.5 mg. of the substance investigated. The concentrations in the extracts were determined by spectrophotometry. Genistein, $E_{1\text{cm}}^{1\%}$, 262.5 m μ . (max.) = 1400. Formononetin, $E_{1\text{cm}}^{1\%}$, 249 m μ . (max.) = 1000.

Substance	Aqueous phase		
	50% ethanol (v/v)	60% ethanol (v/v)	70% ethanol (v/v)
Genistein	0.975	1.39	2.37
Formononetin	0.185	0.361	0.645

Fig. 1. Absorption curves of genistein (---) and formononetin (—) in ethanol solution.

Spectrophotometry. The absorption spectrum, over the range 220–300 $m\mu$., was observed for each fraction in solution in ethanol. A typical series of curves for the fractions is shown in Fig. 2.

Fig. 2. Ultraviolet absorption of a series of chromatograph fractions of subterranean-clover phenolic material. Fractions 10, 14 and 16 contain genistein; fractions 1, 7 and 21 do not.

Fig. 3. Infrared absorption curves of (a) synthetic genistein and (b) chromatograph fraction from subterranean clover. Bands due to impurities in the chromatograph fraction are dotted. (Grubb-Parsons S3A Spectrometer equipped with sodium chloride prism. Solid samples ground with paraffin oil.)

Fractions 9–16 had maxima at 262.5 $m\mu$. and were all similar to the absorption curve of genistein (Fig. 1). On injection in ovariectomized mice, only the fractions 9–16 showed oestrogenic activity, whereas fractions 1–8 and 17–21 were inactive. Recrystallization of fractions 9–16 yielded genistein, identified by its m.p. and u.v. absorption. As a final proof of identity, fraction 10 showed absorption in the infrared region, 860 cm^{-1} to 1200 cm^{-1} , very similar to that of synthetic genistein, as shown in Fig. 3, with impurities showing absorption at 1030 and 910 cm^{-1} .

In the crude fractions, however, the absorption at the minimum (231 $m\mu$.) was greater than that for pure genistein, whereas the maximum absorption (262.5 $m\mu$.) was low. To compensate for the impurities, an arbitrary line was calculated giving assumed values for the impurities of $E_{1\%}^{1\text{cm.}}$ (231 $m\mu$.) = 450 and $E_{1\%}^{1\text{cm.}}$ (262.5 $m\mu$.) = 320. If in Fig. 4 the observed values for the fraction are $e = E_{1\%}^{1\text{cm.}}$ (231 $m\mu$.) and $f = E_{1\%}^{1\text{cm.}}$ (262.5 $m\mu$.), then

$$500x + 450n = e \quad \text{and} \quad 1400x + 320n = f,$$

where x is the proportion of genistein present and n the 'proportion' of impurities. From these equations:

$$x = (0.96f - 0.68e) \times 10^{-3},$$

and

$$n = (2.98e - 1.06f) \times 10^{-3}.$$

To test the validity of the assumed values for the absorption by impurities, x and n were calculated for a number of fractions and the theoretical curve was drawn for the calculated mixture. There was close agreement between the observed and calculated absorption.

The similarity between the absorption by impurities in the genistein fractions and the absorption by the non-genistein fractions suggested that the same formula may be used to estimate genistein from the curves obtained from the

Fig. 4. Arbitrary straight-line absorption assumed due to impurities in chromatograph fractions. From the values shown, the proportion of genistein present in the fraction is calculated as: $x = (0.96f - 0.68e) \times 10^{-3}$.

whole phenol fraction without chromatography. A typical application of this procedure is demonstrated in Fig. 5, which shows close agreement between the observed and calculated curves.

Thus, by reading the values for $E_{1\text{ cm.}}^{1\%}$ (231 m μ .) and $E_{1\text{ cm.}}^{1\%}$ (262.5 m μ .), the amount of genistein present in the phenols may be calculated. A slide rule was constructed for the purpose of making the calculation. This has been applied to a considerable number of extracts and provides a reasonably accurate method of estimation of genistein in clovers. Similar correction methods have been applied by Morton & Stubbs (1946) in vitamin A determinations and by Sveinsson, Rimington & Barnes (1949) in porphyrin determinations. Genistein added to clover was recovered to the extent of 96–109% in six different estimations.

Micro-method of estimation of genistein in clover

Extraction and purification. Two leaves, of similar age, were taken from each clover plant. Both were weighed, one dried at 100° for the determination of dry matter content, and the other placed in a $3 \times \frac{1}{2}$ in. test tube and refluxed with a cold thimble for 10 min. with 1 ml. ethanol. The ethanol solution was decanted into a second tube graduated at 0.6 and 1.0 ml. The leaf residue was refluxed

Fig. 5. Observed absorption curve (—) of phenols from subterranean clover (no. P₄) and calculated curve (---). For explanation see text.

with a further 0.5 ml. ethanol for 5 min. and the extract decanted into the graduated tube. The combined extracts were evaporated by using a stream of air or nitrogen directed into the tube, keeping the tube warmed to about 70° in a glycerol bath, until the volume was reduced to 0.6 ml. Water was added to bring the volume to 1.0 ml. and 0.5 ml. benzene was added. The tube was rotated at an angle of 45° from vertical in a mechanical rotator at about 140 rev. min.⁻¹ for 10 min. This procedure avoided the formation of emulsions and gave an efficient extraction, the benzene layer being dark green in colour whereas the aqueous alcohol layer was almost colourless.

With the use of a pipette inserted to the bottom of the tube, 0.5 ml. of the aqueous phase, free of the benzene phase, was drawn up and a volume equivalent to 2.5 mg. of fresh clover placed in a small tube and evaporated to dryness by the technique described above.

Paper chromatography. Following the method of Bate-Smith & Westall (1950), the extract was dissolved in a minimum of butanol containing 1% HCl (w/v) and transferred with a micro-pipette to a paper strip (Whatman no. 4) 5 mm. wide, care being taken to keep the length of the applied spot within a 5 mm. limit. Control strips were spotted similarly with 2.5 and 5.0 μ g. genistein.

The strips were placed in an atmosphere saturated with the vapour of the aqueous phase of a butanol-acetic acid-water mixture (40:10:50, v/v) for 24 hr. The solvent mixture, butanol-acetic acid-water (40:10:20, v/v), was then introduced for ascending chromatography. After the solvent front had moved approx. 30 cm. (18 hr.) the strips were dried in a current of air in the fume hood and sprayed with aqueous 0.1% (w/v) FeCl₃ solution, freshly prepared from a 10% stock solution. The pink genistein spot (R_F = 0.9) was compared with the control strips.

Throughout a series of several hundred separations it was found impossible to estimate genistein content by measuring spot length. Also, the separation of genistein from the pigments present was not always complete, so that only a visual approximation of genistein content, by comparing the extract chromatograms with the controls, was possible.

RESULTS

The results of a series of determinations on different clovers by using silica gel chromatography and ultraviolet absorption measurements are given in Table 2.

Table 2. *The genistein content of several clovers*

30 g. fresh clover were used and the results are calculated on the basis of 100 g. dry matter.

No.	Description	Ethanol extract				Residue (g.)	Genistein (g.)
		Benzene phase (g.)	60 % ethanol phase				
			Neutrals (g.)	Acids (g.)	Phenols (g.)		
1	<i>T. subterraneum</i> var. <i>Dwalganup</i>	10.3	0.5	0.4	1.6	76.4	0.740
2	<i>T. subterraneum</i> var. <i>Burnerang</i>	11.3	0.5	0.4	1.4	86.6	0.400
3	<i>T. subterraneum</i> var. <i>Red Leaf</i>	8.2	0.5	0.2	0.9	76.4	0.550
4	<i>T. subterraneum</i> var. <i>Tallarook</i>	7.7	0.4	0.5	1.2	85.7	0.565
5	<i>T. repens</i>	7.9	0.2	0.1	0.2	75.8	Nil
6	<i>T. fragiferum</i>	9.4	0.6	0.5	0.2	73.4	Nil
7	<i>T. pratense</i>	15.5	0.5	0.1	0.6	76.6	Nil
8	<i>Melilotus alba</i>	9.3	0.4	0.3	0.2	71.2	Nil

The four subterranean clovers contained large amounts of genistein, whereas the others contained no trace of genistein. With the micro-method these studies have been extended to a large number of clovers and the results will be given in a further communication. The genistein content of many samples of the *Dwalganup* strain has now been determined and these results will be available for publication shortly. The figure for *Dwalganup* in Table 2 is a good representative one.

DISCUSSION

The preparation of the 'chloroplast' fraction of clover, although of use in providing a concentrate in readily stored form, is effective in retaining only about 3% of the active compound. Quantitative estimations of the intake of genistein by sheep, based on the amount present in this fraction, have been most misleading.

The isolation of substantial amounts of genistein from fresh subterranean clover, however, makes it likely that this isoflavone is responsible for the breeding problem in sheep maintained on pastures dominated by this plant species. A sheep eating 1 kg. of dry matter per day would have an intake of about 7 g. genistein daily. Genistein, on oral administration in mice has a median effective dose (M.E.D.) of 8.2 mg. (Biggers & Curnow, 1954) so the sheep receives about 850 mouse M.E.D.'s of genistein daily. With injected stilboestrol complete infertility is produced in ewes receiving 0.1 mg. or approx. 4000 mouse M.E.D.'s per day for 6 months (Underwood, Shier & Peterson, 1953). It is not unlikely that a degree of infertility equivalent to that often induced by clover grazing (20–60%) would result from the injection of 0.02 mg. (approx. 800 mouse M.E.D.'s) of stilboestrol daily, especially if the treatment were carried on for more than one season, as occurs with clover ingestion. Although it is dangerous to make assumptions regarding the activity of genistein in the sheep from the behaviour in mice, it is reasonable to suppose that a somewhat similar response to that with stilboestrol will be given by the daily 850 mouse M.E.D.'s of genistein.

It is possible also that genistein is transformed in the rumen of the sheep to a more active oestrogen. There is evidence (Beck & Curnow, unpublished data) that treatment of clover 'chloroplast' with alkalis yields a small amount of a second oestrogen with at least ten times the activity of genistein.

SUMMARY

1. From the 'chloroplast' fraction of subterranean clover (*Dwalganup* strain) 12 mg. genistein per 100 g. dry matter of clover was isolated.

2. From fresh clover 369 mg. genistein per 100 g. dry matter was isolated.

3. A method of estimation of genistein in plant material and a micro-method of detection of genistein in small amounts of plant material have been developed.

4. Four subterranean clovers studied contain genistein, whereas four other species contained no genistein.

5. The *Dwalganup* strain of subterranean clover contained 740 mg./100 g. dry matter, sufficient to account for the oestrogenic activity of the plant and therefore probably responsible for the infertility in sheep grazing on subterranean clover pastures.

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