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Oestrogenic Activity of Subterranean Clover

1. THE OESTROGENIC ACTIVITY OF GENISTEIN

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Disturbances of reproduction in sheep which had been grazed on pastures dominated by an early flowering strain of subterranean clover (*Trifolium subterraneum* L. var. *Dwalganup*), so-called because it buries its seed, were described by Bennetts, Underwood & Shier (1946). The condition is characterized by infertility of the female, by uterine inertia and by prolapse of the uterus, and as a result of these disorders the lambing may fall below 10% and the loss of ewes may reach 30%. The fertility of the male is not impaired, although the male castrate exhibits extensive changes in the uro-genital tract (Bennetts, 1946, 1947), while mammary development and milk secretion are seen both in the male castrate and the virgin female. These effects, together with the constant occurrence in the infertile ewes of cystic hyperplasia of the endometrium, provided strong evidence of prolonged oestrogenic stimulation.

Oestrogenic extracts were prepared from subterranean clover by Curnow, Robinson & Underwood (1948). Robinson (1949) and Beck & Braden (1951) demonstrated the phenolic nature of the oestrogen and reported methods of preparation of purified extracts for use in the biological estimation of the amount of oestrogen in the clover. The oestrogen was later concentrated by precipitating it in the 'chloroplast' fraction of a press-juice of clover (Legg, Curnow & Simpson, 1950), and this was followed by the isolation of small amounts of genistein (5:7:4′-trihydroxy-*iso*flavone) from the chloroplast fraction (Bradbury & White, 1951). In preliminary tests genistein showed an oestrogenic activity of approximately 10^{-5} that of oestrone. Formononetin (7-hydroxy-4′-methoxy-*iso*flavone) was also isolated from the 'chloroplast' fraction but was inactive.

Since genistein is a new type of natural oestrogen it is of interest to examine its biological properties and activity in detail. The investigations to be reported below have been carried out in two centres, Sydney, N.S.W., and Perth, W.A., and consist of detailed studies of the compound by means of the Allen Doisy vaginal cornification tests as described by Emmens (1950*a*).

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EXPERIMENTAL

Materials

Genistein. Crystalline genistein, m.p. 301° (decomp.) (corr.) was used. The samples used in Sydney were synthetic and prepared by Mr R. B. Bradbury. In Perth two samples were used, one synthetic and the other a sample prepared from subterranean clover (Curnow, 1954). Mixed melting

points of the synthetic and natural compounds showed no depression and no differences were observed in their oestrogenic activities.

Formononetin. Crystalline formononetin, m.p. 258° (corr.) prepared from clover was used.

Oestradiol-3:17β. This has been used as the reference oestrogen.

Experimental animals and assay procedure

Sydney. Groups of ovariectomized mice were used in the tests described below. The general details of management and randomization of experimental groups have been described previously (Biggers, 1951), and the details of the subcutaneous and intravaginal Allen Doisy techniques of oestrogen assay have been described by Emmens (1950*a*). The oestrogens were injected twice daily (9.30 a.m. and 4.30 p.m. approx.) for 2 days. The designs, results and analyses of the experiments will be described together. Standard methods of probit analysis have been used in the analysis of results (Finney, 1952).

In all the recent work on the intravaginal action of oestrogens aqueous media have been used as solvents, e.g. 50% aqueous glycerol (Emmens, 1950*b*; Biggers, 1951; Sullman, 1952), distilled water (Biggers, 1953*a*) and aqueous protein solutions (Biggers, 1953*b*). Unfortunately, genistein in the dose required is insoluble in all these solvents. Although genistein is soluble in nut oil, this solvent has been found unsatisfactory in intravaginal tests in mice (Emmens, 1939). However, propylene glycol may be substituted for water in the intravaginal method and does not effect the activity of oestrone (Claringbold, in preparation). Since genistein is readily soluble in propylene glycol, this solvent has been used for both the subcutaneous and intravaginal tests. The genistein was suspended in the propylene glycol and dissolved by gentle heating.

A severe limitation to the intravaginal method of assaying oestrogens of very low activity is the small volume of injection which must be used to avoid leakage from the vagina. Biggers & Claringbold (1954) have shown in the albino strain of mice used in Sydney that the maximum permissible volume of each injection is 0·01 ml. In the case of genistein this necessitates a very concentrated solution. These solutions may be prepared by heating and allowing them to cool slowly to body temperature before injection. The solution remains supersaturated for several hours and may be injected easily in this state.

Perth. The methods used were essentially similar to those described above, following the methods described by Emmens (1950*a*). The oestrogens were injected subcutaneously or administered orally by stomach tube, oestradiol-3:17β as a solution, and genistein and formononetin as suspensions in peanut oil. The volume of each injection was 0·05 ml.

In both Sydney and Perth a smear consisting only of nucleated or cornified cells was read as positive and a smear containing leucocytes as negative.

RESULTS

Sydney

The dose/response line (subcutaneous administration). Six dose/response lines were obtained by the subcutaneous injection of genistein in propylene glycol using four injections in 2 days. The weighted mean slope of the lines is 6·85, S.E. 0·80, and the χ^2 test shows the six estimates to be homogeneous ($\chi^2_{[5]} = 8{\cdot}88$, $0{\cdot}2 > P > 0{\cdot}1$). The common slope was used to calculate the six individual median effective doses (M.E.D.). The six estimates of the M.E.D. are also homogeneous ($\chi^2_{[5]} = 5{\cdot}66$, $0{\cdot}5 > P > 0{\cdot}3$) and the weighted mean M.E.D. found to be 2·53 mg. (fiducial limits of error ($P = 0{\cdot}05$), 2·35 to 2·73). Thus the data do not provide evidence of secular changes in the sensitivity of the mice to the genistein such as have been found with highly active oestrogens (Emmens, 1939).

The dose/response line (intravaginal administration). Three dose/response lines were obtained by the intravaginal injection of genistein in propylene glycol using four injections in 2 days. The weighted mean slope is 0·91, S.E. 0·32, and the χ^2 test shows the three estimates to be a homogeneous group ($\chi^2_{[2]} = 1{\cdot}789$, $0{\cdot}5 > P > 0{\cdot}3$). The common slope was used to compute the individual M.E.D.'s but in this case they form a heterogeneous group ($\chi^2_{[2]} = 9{\cdot}38$, $0{\cdot}01 > P > 0{\cdot}001$). The shifts in sensitivity are similar to those reported for the intravaginal dose/response line to oestrone (Biggers, 1953*a*).

It can be seen that the slope of the intravaginal dose/response line is considerably less than the slope of the subcutaneous dose/response line.

The relative activity of genistein. Three tests were carried out to determine first the relative potency of genistein in terms of oestradiol-3:17β, and secondly the ratio of the M.E.D. for subcutaneous administration to the M.E.D. for intravaginal administration (S/L ratio, see Emmens, 1941). The dose/response lines within each test were determined simultaneously to overcome the possibility of secular shifts in sensitivity. The results are shown in Table 1.

(i) *Relative potency.* Data in Tests 1 and 2 permit the estimation of the relative potency of genistein in terms of oestradiol-3:17β when both are given in propylene glycol. Table 2 shows the partitioning of χ^2. The component of χ^2 for heterogeneity is just significant at the 5% level. However, in a situation where there are small expectations (as in this case) the usual method of calculating the heterogeneity χ^2 may lead to unduly large contributions to χ^2 (Finney, 1952). However, on computing the χ^2 by the 'longhand' method (Emmens, 1948) the χ^2 for heterogeneity is not significant ($\chi^2_{[3]} = 5{\cdot}35$, $0{\cdot}2 > P > 0{\cdot}1$). In view of this finding the dose response lines are assumed to be homogeneous. Table 2 shows that all four lines are parallel, and therefore a common slope and its variance were computed for the estimation of relative potency. The value of $b_c/(s_b)_c$ is 6·74 and the limits of error are computed by the approximate method. These limits should be only slightly narrower than the fiducial

Table 1. *Dose/response data for tests of the subcutaneous and intravaginal action of genistein and oestradiol-3:17β given by four injections in 2 days in propylene glycol*

The dose/response lines in each test were determined simultaneously. The response is shown as the number of animals positive to the number of animals in the group.

	Subcutaneous administration				Intravaginal administration			
	Genistein		Oestradiol-3:17β		Genistein		Oestradiol-3:17β	
Test no.	Dose (mg.)	Response	Dose (mμg.)	Response	Dose (mg.)	Response	Dose (mμg.)	Response
1	0·25	1:11	14	0:12	—	—	—	—
	0·5	0:12	19	1:12	—	—	—	—
	1·0	0:12	25	0:12	—	—	—	—
	2·0	2:12	34	2:11	—	—	—	—
	4·0	11:12	46	8:12	—	—	—	—
2	1·44	0:11	32	6:12	0·53	5:12	0·05	1:12
	1·80	0:11	40	6:12	0·80	3:12	0·1	3:12
	2·25	5:10	50	11:12	1·20	5:12	0·2	7:12
	2·81	7:11	62·5	9:11	1·80	6:12	0·4	9:12
	3·51	8:12	78·1	11:12	2·70	7:12	0·8	11:12
3	1·44	0:12	—	—	0·67	11:36	—	—
	1·80	2:12	—	—	1·00	12:36	—	—
	2·25	5:12	—	—	1·50	22:36	—	—
	2·81	6:12	—	—	2·25	18:36	—	—
	3·51	12:12	—	—	3·38	18:36	—	—

Table 2. *Partitioning of χ^2 for the two dose/response lines obtained with genistein and the two dose/response lines obtained with oestradiol-3:17β by using subcutaneous administration in Tests 1 and 2*

Source of variation	Degrees of freedom	χ^2	P
Parallelism	3	2·94	0·5–0·3
Heterogeneity	5	12·90	0·05–0·02

Table 3. *Partitioning of χ^2 for the dose/response lines obtained with genistein by both subcutaneous and intravaginal administration in Tests 2 and 3*

Source of variation	Degrees of freedom	χ^2	P
Parallelism:			
Between mean slopes	1	21·48	0·001
Within mean slopes	2	0·47	0·8–0·7
Heterogeneity	8	15·70	0·05–0·02

limits of error (Irwin, 1943). The relative potency of genistein in terms of oestradiol-3:17β is found to be $1{\cdot}25 \times 10^{-5}$, limits of error ($P = 0{\cdot}05$) $1{\cdot}03 \times 10^{-5}$ to $1{\cdot}51 \times 10^{-5}$ or 83–121 %.

(ii) *S/L ratio.* The data of tests 2 and 3 permit the estimation of the S/L ratio for genistein. Also the data of test 2 allow a similar estimation to be made for oestradiol-3:17β for comparison. Table 3 shows the partitioning of χ^2 for the two lines obtained by subcutaneous administration and the two lines obtained by intravaginal administration. Again the heterogeneity χ^2 is just significant, but as the χ^2 obtained by the 'longhand' method is not significant ($\chi^2_{[8]} = 9{\cdot}85$, $0{\cdot}3 > P > 0{\cdot}2$), homogeneity of the lines is assumed. Table 3 shows that while the within mean slope differences are not significant, there is a highly significant difference between the slopes obtained by the subcutaneous and intravaginal methods. Mean slopes were therefore computed from the data obtained by each route of administration and these used to estimate the appropriate M.E.D.'s.

The S/L ratio was computed from the weighted mean M.E.D.'s for each method of administration. The S/L ratio is 1·22, fiducial limits of error ($P = 0{\cdot}05$), 0·73 to 2·05.

Similar computations with the data for oestradiol-3:17β give a value of 175, fiducial limits of error, 108 to 284. Thus genistein is characterized by a very low S/L ratio and falls into the class of pro-oestrogens as defined by Emmens (1941).

Perth

Two tests were made, the first being a comparison of the activity of genistein and oestradiol-3:17β administered subcutaneously, and the second being a determination of the dose/response line obtained with genistein given orally, the two tests being done at different times. The results are given collectively in Table 4.

The relative potency of genistein in terms of oestradiol-3:17β may be calculated from the data. The χ^2 for heterogeneity of the two lines is not significant ($\chi^2_{[1]} = 0{\cdot}65$, $0{\cdot}8 > P > 0{\cdot}7$) nor is the χ^2 for parallelism ($\chi^2_{[2]} = 3{\cdot}45$, $0{\cdot}1 > P > 0{\cdot}05$). Consequently a mean slope was computed for the estimation of relative potency and its exact fiducial limits of error. This was found to be $4{\cdot}53 \times 10^{-5}$, fiducial limits of error ($P = 0{\cdot}05$) $3{\cdot}30 \times 10^{-5}$ to $5{\cdot}99 \times 10^{-5}$, or 73–132 %.

Table 4. *Dose/response data for tests of the subcutaneous and oral administration of genistein and the subcutaneous administration of oestradiol-3:17β given by four injections in 2 days*

The data obtained by subcutaneous administration were obtained simultaneously, while the data on the oral administration were obtained separately. The response is shown as the number of animals positive to the number of animals in the group.

Genistein				Oestradiol-3:17β	
Subcutaneous		Oral		Subcutaneous	
Dose (mg.)	Response	Dose (mg.)	Response	Dose (mμg.)	Response
0·25	2:20	2·5	0:20	20	3:20
0·50	7:20	5·0	3:20	30	13:20
1·00	16:20	10·0	12:20	45	18:20
		15·0	19:20		
		20·0	20:20		

This estimate of the relative potency is significantly different ($P > 0{\cdot}001$) from the estimate obtained in Sydney, being about 3·5 times as great. This is probably due to differences in technique, such as the solvent employed as the vehicle of administration. It is well known that such factors can have a profound influence on the results (Emmens, 1939; Pedersen-Bjergaard, 1939). However, the results from the two centres clearly indicate that genistein has very low oestrogenic activity.

The slope of the dose/response line obtained by the oral administration of genistein is 5·65, S.E. 0·95. The M.E.D. was computed and found to be 8·20 mg.; fiducial limits of error ($P = 0{\cdot}05$) 6·74–9·97, or 82–122 %.

Formononetin, injected at a level of 10 mg. or given orally at 50 mg., elicited no vaginal response.

DISCUSSION

Although observations of oestrogenic activity in plant material have been made frequently since the original work of Loewe (1926), who induced oestrus in ovariectomized mice with ether extracts of willow catkins and of ovaries of the water lily (*Nuphar luteum*), very few oestrogenic compounds have been crystallized from plant material. Butenandt (1932) and Butenandt & Jacobi (1933) were the first workers to crystallize and identify an oestrogen of plant origin. Their starting material was the residue from the pressure extraction of oil obtained from palm kernels. This yielded a crystalline product identical with oestrone. Skarzynski (1933*a*, *b*) reported the isolation of oestriol from female willow flowers. A highly active oestrogen was isolated from the tubers of *Butea superba*, a leguminous vine common in China and Thailand, by Schoeller, Dohrn & Hohlweg (1938, 1940). The activity was about twice that of oestrone when injected subcutaneously in rats. Butenandt (1940) described further work on this substance and gave it a formula of $C_{19}H_{22}O_6$, although the structure of the oestrogen has not yet been elucidated.

Emmens (1941) compared the median effective doses of an oestrogen when administered by the subcutaneous and intravaginal routes (S/L ratio). Several compounds were examined and two groups of substances were distinguished, one where the S/L ratio is near unity and the other where the ratio is of the order of hundreds. The substances in the first group are called pro-oestrogens and those in the second true oestrogens. All the natural oestrogens found in animals belong to the group of true oestrogens. Later, Emmens (1942) examined these substances in mice in which the vagina had been surgically divided to give separate vaginal pouches. He found that a dose of a true oestrogen which was just sufficient to cornify the epithelium of one pouch when placed on it failed to produce cornification in the second pouch. However an effective dose of a pro-oestrogen for one pouch caused cornification in both pouches. It is believed that true oestrogens act directly on the cells of the responding epithelium, whereas the pro-oestrogens are absorbed into the body and give rise to true oestrogenic substances during their metabolism.

The results presented above show that genistein is a pro-oestrogen, and presumably gives rise to oestrogenic products during its metabolism in the body. The estimates of relative potency show that genistein is of very low activity, and this suggests that its oestrogenic properties are caused by only a minor metabolite.

When an oestrogen of such low activity as genistein is isolated from natural sources, there is the danger that the oestrogenic activity is due to a contaminant and not due to the compound under examination. The amount of active substance required for such a situation to arise is of the order of the tolerance limits for impurities which are normally accepted in analytical reagents. There are two reasons, however, for accepting that genistein has oestrogenic properties: (1) the synthetic and natural materials were found to be equally active, and it is highly improbable that an active contaminant could be present in equivalent amounts,

(2) the substance has been shown to be a prooestrogen, for if the activity were due to a highly active contaminant, the S/L ratio would be expected to be high, the reverse of that actually found.

The results show that the slopes of the regression lines for each route of administration are significantly different. The slope for intravaginal administration is low and indicates that the response is very variable. A possible contributory cause of this variation may be the fact that the highly concentrated propylene glycol solutions used to precipitate in the vagina on contact with aqueous tissue fluids. To what extent this influences the vaginal response awaits further investigation.

SUMMARY

1. The relative potency of genistein in terms of oestradiol-3:17β using four injections given subcutaneously in 2 days, and propylene glycol as solvent, is $1{\cdot}25 \times 10^{-5}$, fiducial limits of error ($P = 0{\cdot}05$) $1{\cdot}03 \times 10^{-5}$ to $1{\cdot}51 \times 10^{-5}$ (Sydney experiments).

2. The relative potency of genistein in terms of oestradiol-3:17β using the same technique, and peanut oil as solvent, is $4{\cdot}53 \times 10^{-5}$, fiducial limits of error ($P = 0{\cdot}05$) $3{\cdot}30 \times 10^{-5}$ to $5{\cdot}99 \times 10^{-5}$ (Perth experiments).

3. Although these two estimates are different, they show that genistein has a very low oestrogenic activity when compared to oestradiol-3:17β.

4. The median effective doses for genistein given orally in four doses in 2 days is 8·20 mg., fiducial limits of error ($P = 0{\cdot}05$) 6·74 to 9·97.

5. Since the S/L ratio of genistein is 1·22, fiducial limits of error ($P = 0{\cdot}05$) 0·73 to 2·05, it is a prooestrogen.

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