FURTHER EVIDENCE FOR THE PRESENCE OF AN ENDOGENOUS GONADOTROPHIN-LIKE PLANT FACTOR, "PHYTOTROPHIN": ISOLATION AND MECHANISM OF ACTION OF THE ACTIVE PRINCIPLE

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Abstract

A proteinaceous factor extracted from each of five different plant species elicits both gonadotrophin-like responses in animals and morphogenetic responses in plants. Of the species tested, legumes contained highest amounts of this factor. Extraction was performed by binding foliage protein extracts to a column of polymerized gonadotrophin antibodies and subsequently eluting with a suitable buffer medium. Haemagglutination inhibition testing indicated antigenic properties similar to those of the animal sex hormone, human chorionic gonadotrophin. In the barley endosperm test both the animal gonadotrophin and the released plant factor, designated "phytotrophin", inhibit gibberellin-induced reducing sugar production.

I. INTRODUCTION

Gonadotrophins in mammals are a group of glycoproteinaceous sex hormones usually produced in the anterior pituitary gland at the base of the brain and which function as triggers of steroidogenesis in the male and female gonads. Human chorionic gonadotrophin (HCG) differs in source, being present in the chorionic fluid of the embryo sac in pregnant females. Working under the hypothesis that a certain analogy possibly exists between plant and animal hormone systems, Leshem (1967), Leshem and Lunenfeld (1968), and Leshem *et al.* (1969) have shown that HCG has several marked morphogenetic effects on plants.

Indirect evidence that an HCG-like substance exists in plants was obtained with anti-HCG serum. HCG induced and anti-HCG serum inhibited promotion of root number and growth in cuttings of *Begonia.*

Recently Leshem *et al.* (1972) have reported in brief the isolation of such a plant gonadotrophin, the name for which we suggested be phytotrophin, from φυτόζ (phytos) = plant; τρεφειν (trephein) = feeding; of like etymology to the mammalian hormone.

In this report we present a detailed description of an improved anti-HCG polymer system and elution medium enabling release of a greater amount of bound phytotrophin than before, evidence for gonadotrophic-like properties of the freed substance, and data on its growth-promoting properties in plants and mammals.

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II. Materials and Methods

(*a*) *Preparation of Protein Extract*

Four hundred grams of *Begonia semperflorens* cv. Indian Maid were blended in 200 ml cold 1M Tris–HCl buffer, pH 9·0, which contained 0·001M EDTA, 0·001M ascorbic acid, and 0·001M mercaptoethanol. The brei was filtered through triple layers of cheesecloth and centrifuged for 15 min at 5000 *g*. The supernatant was collected and centrifuged again for 30 min at 10,000 *g*. Proteins were salted out with 40% neutralized $(NH_4)_2SO_4$ and, after standing at least 2 hr at 4°C, centrifuged for 30 min at 10,000 *g*. The pellet was dissolved in 20 ml of the above buffer and dialysed against 0·1M phosphate buffer saline (PBS) (pH 7·2) until no traces of the $(NH_4)_2SO_4$ were left. Extraction from other species was essentially similar, varying only in pH and molarity of buffer. Rate of change of the buffer depended upon the moisture content and the natural pH of each plant species.

(*b*) *Preparation of Antiserum*

Antiserum to HCG was prepared in rabbits by three intramuscular injections, at weekly intervals, of 1 ml Freund's complete adjuvant containing 5000 i.u. HCG (Ikapharm Industries, Israel) of specific activity 4200 i.u./mg. Thereafter at 10-day intervals three booster injections each of 2500 i.u. were given. The immunized rabbits were bled by cardiac puncture and blood was allowed to coagulate for 1 hr at 37°C and overnight at 4°C. The immune serum which contains the HCG antibodies was separated by centrifugation at 2500 *g* for 10 min and stored at −20°C.

(*c*) *Preparation of the Anti-HCG Polymer*

Sodium sulphate was added to 20 ml of HCG antiserum to obtain an 18% solution and allowed to stand for 1 hr at 30°C. A white precipitate composed of the serum globulins (including the HCG antibodies) was formed, and this was centrifuged down for 30 min at 10,000 *g*. The pellet containing the HCG antibodies was dissolved in 20 ml 0·1M PBS (pH 7·2) containing 0·1M $NaHCO_3$ and 0·8 g sodium carboxymethylcellulose (CMC) (Fluka) in 40 ml water (solution A). A like volume of water in which 0·8 g agarose was dissolved was brought to pH 5·0 with 0·1N NaOH, its temperature elevated to 42°C, and then mixed with solution A. 1 ml of aqueous solution of 0·25 g *N*-3-dimethylaminopropyl-*N*-ethylcarbodiimide hydrochloride (DAPC) (Sigma) and 20 ml of anti-HCG serum, prepared as above, were added alternately to the mixture, drop by drop, while stirring.

At this stage the unpolymerized proteins may be washed out of the polymer, and the polymer lyophilized and stored in the cold, or directly lyophilized and washed before use.

Column preparation was achieved by imbibition of 0·5 g dried polymer in 100 ml phosphate buffer (PBS) for 2 hr and loading on a 2 by 10 cm glass column. The column was washed with PBS until no traces of protein, as shown by spectrophotometric readings were left. The column was further washed with 100 ml 0·1M $NaHCO_3$ and 50 ml 4M guanidine–HCl buffer (pH 2·5) in order to eliminate any other non-specific adsorbed protein. PBS was again applied until the optical density of the eluate registered no protein, and pH was restored to 7·2.

(*d*) *Phytotrophin Extraction*

Plant protein extract (5 ml) was added to the column contents and allowed to stand for 24–48 hr at 4°C. During this period any plant substance antigenically resembling HCG binds to the polymerized antibodies. The column was washed with PBS to eliminate all non-bound plant protein and subsequently the specifically bound protein was eluted with 50 ml 4M guanidine–HCl buffer, pH 2·5, and 100 ml PBS. Previous experimentation indicated that the protein eluted in this manner retains biological activity. We also found that saturation of the polymer with 0·1% rabbit serum albumin (RSA) and addition of 0·1% RSA to the buffer system enhanced release and increased biological longevity of gonadotrophin and phytotrophin. The eluted protein was dialysed against PBS to eliminate traces of guanidine and to restore the pH to 7·2, and subsequently tested by haemagglutination inhibition in order to ascertain its specific gonadotrophin-like immunological properties. In cases when 0·1% RSA was added, the hormone was salted out together with the RSA by 70% $(NH_4)_2SO_4$, washed twice with saturated $(NH_4)_2SO_4$, and kept frozen.

(*e*) *Haemagglutination Inhibition*

This was performed as outlined by Stavitsky (1964) and modified by Avrameas *et al.* (1969). In practice the assay was carried out by preparing a series of tubes containing a serial 1 : 2 dilution of HCG antibody serum in normal serum. The titre is terminated when a standard amount of HCG-coated erythrocytes no longer produces a haemagglutination effect. If HCG or plant extract containing HCG-like material were first added to the anti-HCG serum and incubated with it (1 hr at 37°C) before addition of the coated erythrocytes, it would react with part of the antibodies contained in the serum and cause inhibition of the haemagglutination, which is expressed by the decrease of antibody titre.

(*f*) *Biological Activity*

(i) *In Animals*

The protein extracted from *B. semperflorens* was salted out with 20% (fraction A), 40% (fraction B), and 60% (fraction C) $(NH_4)_2SO_4$. These fractions were dialysed against saline buffer and subjected to haemagglutination inhibition testing as described above. Immunoactivity was detected in fractions B and C and these were used for bioassay of gonadotrophin-like activity in subsequent experiments. The bioassay employed was the mouse uterus method (Loraine and Brown 1959) whereby protein extract, phytotrophin, or HCG was injected into immature female Swiss Albino mice; after a 4-day period the mice were dissected and increase in uterine weight, as compared to a control group, was measured.

(ii) *In Plants*

The parameter here measured was root growth and a standard mung bean test (Chandra *et al.* 1971) was employed.

When interaction with gibberellin-like activity was determined, the barley endosperm test (Coombe *et al.* 1967) was employed. Since the incubation period in the present experiment was 24 hr it was essential to stabilize the phytotrophin with 0·1% RSA, and therefore all the incubation media in all treatments contained RSA as well. Embryo-less endosperm halves were incubated in 5 μg/ml gibberellic acid (GA_3) containing, per litre, 62·5, 125·0, or 500·0 i.u. HCG or phytotrophin equivalents.

III. Results

(*a*) *Phytotrophin in Plants*

Extracts were made from fresh weight foliage samples of *Begonia semperflorens* cv. Indian Maid; *Chrysanthemum morifolium* cv. Vibrant; *Chloris guayana* (Rhodes grass), *Medicago sativa* (lucerne), and *Trifolium alexandrinum* cv. Musgavi (clover). Samples used were taken from fields in the south of Israel in the spring, summer, and autumn of 1971 and phytotrophin content assessed by haemagglutination inhibition by comparison with calibration series with HCG. Results are presented in Table 1.

From these results it can be seen that gonadotrophin-like immunological activity was detected in all investigated species, representing four different plant families.

(*b*) *Gonadotrophic Activity of Protein Fractions B and C in Animals*

Dialysed 0·5-ml aliquots of protein fractions B and C in PBS were injected three times, at daily intervals, into immature female Swiss Albino mice. The increment of uterine weight after 4 days is presented in Table 2. From the table it is

apparent that compared with the buffer controls both protein fractions have a marked enhancing effect on uterine growth, and that of fraction C exceeds that of fraction B.

TABLE 1

PHYTOTROPHIN CONTENT OF FOLIAGE OF VARIOUS PLANTS

Activity of protein extracts obtained as outlined in Section II(*a*). The extract tested was the dialysed 40% $(NH_4)_2SO_4$ precipitate. Final activity was determined by haemagglutination inhibition and interpolation with an HCG calibration series

Species	Phytotrophin content (i.u. HCG per 100 g fresh wt. equivalents)	Sampling date
Begonia semperflorens	500–600	9 March, 5 April
	150–200	3 June
	No reaction	1 July
Chrysanthemum morifolium	250–300	20 June
Chloris guayana	70–100	22 July
Trifolium alexandrinum	800–1000	7 March
Medicago sativa	3000–3200	20 March*
	No reaction	3 May
	400–500	1 July
	200–2500	8 September*

* Sample taken from a field where cattle showed reproductive aberrations.

(*c*) *Gonadotrophic Activity of Isolated Phytotrophin in Animals*

Protein fractions B and C showed gonadotrophic activity but do not necessarily pinpoint the specific factor responsible for it. After isolation of phytotrophin as outlined above, it was injected into immature female mice.

TABLE 2

GONADOTROPHIN-LIKE EFFECTS OF PROTEIN FRACTIONS B AND C IN 22-DAY-OLD SWISS ALBINO MICE

Each mouse received injections of 0·5 ml PBS with or without HCG, protein fraction B, or fraction C for 3 successive days. On the fourth day mice were dissected and fresh weight of uteri determined. Each treatment was applied to 10 mice

Treatment	Mean weight of uterus (mg)	Coefficient of variation (%)
Control (saline buffer)	58·7	27·6
20 i.u./HCG per ml	106·4	19·7
Fraction B*	98·2†	16·2
Fraction C*	119·0	12·7

* Equivalent to 20 i.u. HCG per millilitre.
† Slight inflammation noticeable.

From the results shown in Table 3 it is apparent that phytotrophin may be the responsible factor. Furthermore, it can also be seen that a higher dose of phyto-

trophin as compared to HCG (Table 2) is required to elicit the same biological response.

TABLE 3

GONADOTROPHIN-LIKE EFFECT IN SWISS ALBINO MICE OF PHYTOTROPHIN EXTRACTED FROM *BEGONIA SEMPERFLORENS* AND *MEDICAGO SATIVA*

Phytotrophin was obtained by release from columns as outlined in Section II(*d*). Three successive injections, each equivalent to 15 i.u. HCG per millilitre were applied. Experimental detail of bioassay as in Table 2 with the difference that mice were 3 days younger and hence uterine weights lower

	Mean weight of uterus (mg)	Coefficient of variation
Control	19·1	11·7
Phytotrophin from *B. semperflorens*	29·9	5·8
Phytotrophin from *M. sativa*	32·6	7·6

(*d*) *Rooting Studies*

Results of application of isolated phytotrophin to mung bean hypocotyls are presented in Table 4. The phytotrophin showed significant activity in promoting root initiation. There is a difference in response to equal doses of HCG and phytotrophin, the latter's activity exceeding that of HCG. The effect of indolebutyric acid (IBA) is greater than that of phytotrophin, but when they are applied together the effect on numbers of roots per hypocotyl is additive.

TABLE 4

ROOTING EFFECTS PRODUCED IN MUNG BEAN HYPOCOTYLS GROWING IN ROOTING MEDIUM CONTAINING HCG, IBA, OR PHYTOTROPHIN

Duration of experiment 7 days. Relative values expressed as means of four replicates of five plants each. Different lower-case letters in a given vertical column indicate statistical significance at $P < 0 \cdot 05$ as determined by analysis of variance

Treatment	Relative No. of roots per hypocotyl	Relative fresh wt. of roots per hypocotyl
Control	100^{a}	100^{a}
Phytotrophin* equiv. to 500 i.u./l	276^{b}	140^{ab}
Phytotrophin equiv. to 1000 i.u./l	387^{bc}	156^{b}
HCG, 500 i.u./l	114^{a}	132^{a}
HCG, 1000 i.u./l	135^{a}	116^{a}
IBA, 5 μg/ml	485^{bc}	219^{c}
Phytotrophin, 500 i.u.+IBA, 5 μg/ml	570^{d}	205^{bc}

* Extracted from foliage of *B. semperflorens* as in Table 3.

(*e*) *Interaction with GA_3-induced Effects*

Since it was previously shown that pharmaceutical HCG inhibits the GA_3-induced capacity of barley endosperm halves to produce amylase (Leshem and Lunenfeld 1968), it was considered of interest to ascertain whether phytotrophin

manifested similar behaviour. Results given in Figure 1 are a typical series, in which embryo-less barley endosperm halves were incubated with GA_3 in the presence of HCG or phytotrophin. It is apparent that both HCG and phytotrophin inhibit gibberellin-like activity and that this effect increases with concentration. It can also be noted that for the same concentration (in i.u.) the phytotrophin had a more pronounced inhibitory effect than HCG.

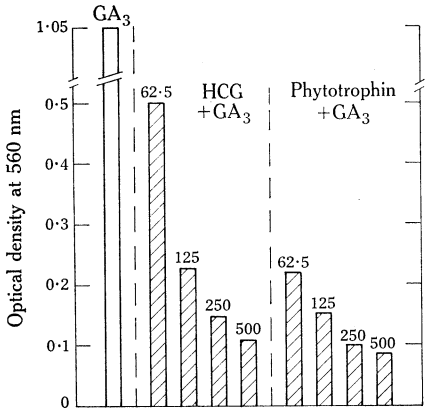

Fig. 1.—Phytotrophin and HCG inhibition of GA_3-induced reducing sugar production in embryo-less barley endosperm halves, expressed as optical density at 560 nm. Three endosperm halves of cv. Esperance were incubated for 24 hr at 26°C. Incubation medium included 0·01% rabbit serum albumin. Four replicate means. Numbers at head of columns represent concentrations in i.u./ml of HCG or phytotrophin equivalents.

IV. Discussion

The presence in plants of a principle resembling HCG in foliage extracts of all five species tested is indicated by results summarized in Table 1. The content varies considerably between the species and within the species between sampling dates. However, highest concentrations were in the leguminous species—clover in spring (7 March) and lucerne in the early autumn or spring (8 September or 20 March). These species are respectively winter and summer forage crops in Israel. The activity in general shows a rough correlation to intensity of seasonal growth.

Tables 2 and 3 show that uteri of immature Swiss Albino mice respond to protein fractions B and C, and to phytotrophin, thus further indicating specific gonadotrophin-like properties.

The presence of this factor may explain the observations that cattle and sheep may come on heat after grazing on legume fields at certain stages of plant development (Millington *et al.* 1964; Rossiter and Beck 1966), this phenomenon being caused by formation of ovarian cysts consisting of persistent Graafian follicles which do not burst. Confirmatory evidence is that the plants which indicated an unusually high content of phytotrophin (2500 and 3000 i.u. per 100 g, as seen in Table 1) were obtained from fields in which, at the time of sampling, the above physiological aberrations in the grazing animals were observed.

In plant systems the isolated phytotrophin has promotive effects on initiation and growth of roots in mung bean hypocotyls (Table 4), this being in accord with our previous results (Leshem *et al.* 1972) with tomato cuttings. Table 4 also indicates that phytotrophin has an effect exceeding that of HCG at equivalent concentrations and that it acts additively with IBA on the number of roots produced. Concerning the mechanism of action, it was shown (Leshem and Lunenfeld 1968) that applied

HCG possibly acts via regulation of endogenous gibberellin levels and also by inhibition of GA_3 already present in the system, an observation recently confirmed (Duffus *et al.* 1971) in other laboratories. In the present investigation phytotrophin, freed and detected as above, manifests similar physiological activity in the barley endosperm test (Fig. 1).

From this figure it is apparent that for equivalent amounts, as calibrated in international units and estimated from a haemagglutination inhibition series, phytotrophin has a greater inhibitory effect than HCG on action of GA_3. From the above it is proposed that phytotrophin is an endogenous plant factor of immunological and physiological resemblance to mammalian HCG. It is active as a gonadotrophin in immature female mice and stimulates morophogenesis in plants. To date its endogenous occurrence has been ascertained in five species representing four diverse plant families, both monocotyledons and dicotyledons. Its mechanism of action in plants may be by means of control of GA_3 activity, possibly akin to the gonadotrophin control of steroidogenesis in mammals, but other possibilities, including effects on the IAA–IAA peroxidase system described by Galston and Hillman (1961), cannot be excluded (Leshem *et al.* 1969).

At present work is being carried out on the production of phytotrophin antibodies which, when subjected to suitable polymerization, should afford a more efficient and specific binding medium than the anti-HCG polymer.

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