The *Striga* Germination Factor

2. CHROMATOGRAPHIC PURIFICATION OF CRUDE CONCENTRATES*

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In the first paper of this series (Brown, Johnson, Robinson & Todd, 1949*a*), the purpose of which is the determination of the chemical nature of the stimulant liberated from actively growing roots and responsible for the germination of seeds of the angiospermous parasite, *Striga hermonthica*, the preparation of crude concentrates was described as well as a method for biological assay. It was found that the stimulant could be adsorbed on to activated charcoal and eluted with 70 % aqueous acetone and that the material thus obtained contained carbohydrates including free pentoses. On testing a wide variety of sugars for their effects in the germination of *Striga* seeds, a powerful stimulation was obtained with dilute solutions of D-xyloketose (I), the maximum germination being recorded at a concentration of 1×10^{-6} mg./ml., and some stimulation at 1×10^{-7} mg./ml.

D-Xyloketose resembled the natural stimulant in two other important respects. In the first place the biological activities of dilute aqueous solutions of both disappeared within 72 hr. on keeping at 15° or within 1 hr. at 100° and, secondly, it was found (Brown, Johnson & Robinson, 1949*b*) that the substance released from the roots of the host, e.g. *Sorghum vulgare*, and D-xyloketose both stimulated root extension. It therefore seemed very probable that the stimulating action of the concentrates obtained from the exudates of the host roots was either similar to, or identical with, D-xyloketose. Certain other sugars, e.g. D-fructose, D-fructose 1:6-diphosphate and L-sorbose, promoted slight germination with *Striga* seeds, but no substances other than carbohydrates gave any such stimulation

* An earlier paper by Brown *et al.* (1949*a*) is taken to be Part 1 of this series.

within the same concentration range and time as was given by the natural stimulant.

The work on the germination of *Striga* seed has been continued along two lines. The action of sugars has been examined further and this will be reported in a separate paper. The present communication deals with some of the work concerned with the isolation and concentration of the stimulant itself.

Paper-strip chromatography has been applied to the study of the compositions of the crude concentrates and also to the determination of the distribution of active substances on the chromatograms. An examination of the carbohydrates present revealed a pentose with chromatographic properties very similar to arabinose, but no xyloketose has ever been detected in any of the samples tested. Besides arabinose, four other carbohydrates were generally present. One of these was identified as D-glucose, previously isolated as the *o*-nitrophenylhydrazone (Brown *et al.* 1949*a*) and another possibly as 2-ketogluconic acid and the others which were not identified were probably di- or poly-saccharides. At the same time, five or six amino compounds were also shown to be present in traces by the ninhydrin reaction, and by direct comparison with standards, three of these were shown to have properties closely related to norleucine (or leucine), valine and tyrosine. A full investigation of the nature of these carbohydrates and amino-acids was deferred when other chromatograms were examined for the location of biologically active material by the method previously described (Brown, Greenwood, Johnson, Long & Tyler, 1951*b*). A chromatogram obtained with the crude fluid in which roots of *Sorghum* had been growing showed four separate zones of activity, two of these being in a region in which carbohydrates occur and one immediately behind the solvent front (Fig. 1). Moreover, it was evident that this last component, which was not a simple carbohydrate or amino-acid, was probably the most important constituent of the complex. Chromatograms of acetone-ether eluates of the crude stimulant showed much the same distribution, although the contribution of the material which travelled close to the solvent front was relatively greater in these cases (Fig. 2). The difference between the two chromatograms

suggested that the elution had involved a greater concentration of the component with the high R_f value.

Fig. 1. Paper-strip chromatographic analysis of crude *Striga* germination factor.

Fig. 2. Paper-strip chromatographic analysis of crude *Striga* germination factor after extraction with acetone-ether.

These observations formed the basis of the subsequent development of the investigation which has been directed towards the isolation of the active material which on the chromatograms was located immediately behind the solvent front. Although little is known at the present time of the detailed chemical structure of this substance, the investigation has revealed that in many respects it is very similar to the stimulant involved in the germination of *Orobanch* seeds (Brown, Greenwood, Johnson & Long, 1951*a*), although, whereas the *Orobanch* material is relatively stable in acid solutions (Brown *et al.* 1951*b*), solutions of the *Striga* stimulant are not. Both germination factors are very unstable in alkaline solution. The techniques employed in the purification of the *Orobanch* stimulant have also been used in the present work.

In the earlier work on the isolation of the *Striga* germination factor (Brown *et al.* 1949*a*), 70% aqueous acetone was used for the elution of the stimulant from the charcoal, but this solvent has now been replaced by 75% acetone-ether as a result of a comparative study of a range of eluting solvents (Table 1). All solvent evaporations were carried out under reduced pressure at temperatures $< 35^\circ$ in an atmosphere of nitrogen, and the pH of the solutions was maintained at 5.5–6.5 by the addition of small

quantities of sodium bicarbonate solution, where necessary. By this means, crude concentrates were obtained which varied in appearance between pale-yellow amorphous solids and colourless glasses depending on the inorganic content, and which had activity values (Brown *et al.* 1949*a*) ranging from 1000 to 50,000 on the arbitrary scale.

The second stage in the purification has been an extraction with an organic solvent, and of those investigated (Table 3), diethyl ether is the most satisfactory and this step has been included in all the later preparations. It was frequently observed during these extractions that the percentage recovery of activity exceeded 100 and a similar effect was also encountered in the *Orobanch* series. It is attributed partly to the retention of substances in the aqueous phase which inhibit the germination of the *Striga* seeds, and partly to inaccuracies in the method of biological assay.

For the further purification of the germination factor various methods have been investigated, including fractional distillation at very low pressures, chromatography and counter-current extraction, the last largely forming the subject of a later paper. Although the distillation method caused considerable deactivation and decomposition, it was possible, nevertheless, to obtain an intermediate fraction with a considerably enhanced activity (1×10^6), and this result is of significance in view of the relative instability of the compound in aqueous solution. The active fraction from the distillation was subjected to a bath temperature of 60–115° for 4–5.5 hr., whereas an aqueous solution of the stimulant loses its biological activity completely after 1 hr. when heated at 100°.

In view of the success of the paper-strip chromatograms in separating the active material from sugars and amino-acids, chromatography on cellulose appeared to be an attractive possibility for the purification of the concentrates on a scale large enough to provide material for chemical studies. Alumina was not used as this had proved unsatisfactory in the case of the *Orobanch* stimulant. Water-saturated *n*-butanol was the preferred solvent for use with the cellulose columns as less biological deactivation was experienced in the absence of acetic acid. The stimulant was thus eluted in a sharply defined zone and, after removal of the solvent, material was obtained with activity values in the order 1×10^6 to 1×10^7 .

EXPERIMENTAL

Adsorption of biologically active material on charcoal

The technique adopted was essentially the same as that described previously (Brown *et al.* 1949*a*). Seedlings of the host plant, *Sorghum vulgare*, were grown in tanks (12 in. diameter $\times$ 6 in. depth; approx. 1000 seedlings/tank) con-

Table 1. *Elution of crude concentrates from charcoal with various solvents*

Solvent	Weight of charcoal (g.)	Volume of solvent (ml.)	Weight of eluate (mg.)	Activity	Percentage recovery
Acetone	100	3 × 100	83	500	3.0
Ether (peroxide-free)-methanol	140	(a) ether (100) + methanol (100); (b) ether (100) + methanol (30); (c) ether (3 × 100)	13.3	800	0.7
Ether (peroxide-free)	250	Continuously extracted for 20 hr.	30	750	1.0
Ethyl acetate-methanol	120	(a) ethyl acetate (100) + methanol (30); (b) ethyl acetate (100) + methanol (20); (c) ethyl acetate (3 × 120)	183	25	8.5
Pyridine	100	5 × 120	712	500	27.4
Chloroform-methanol	70	(a) CHCl ₃ (75) + methanol (30); (b) CHCl ₃ (75) + methanol (20); (c) CHCl ₃ (3 × 100)	79.6	500	4.4
n-Butanol-methanol	100	(a) n-butanol (90) + methanol (40); (b) n-butanol (110) + methanol (20); (c) n-butanol (3 × 100)	150	7	0.2
Acetone-ether (3:1)	440	5 × 500 (mixture stirred during 20–30 min. with each extraction)	178	10,000	100
Acetone-ether (1:1)	100	5 × 120 as in previous extraction	74.9	7500	41

taining distilled water (1.5 l./tank) and carbon (18 g./tank; Sutcliffe and Speakman 'Genster' brand, previously thoroughly washed, first with water and then with a 3:1 acetone-ether mixture). Floats consisting of muslin stretched across closed glass rings carried the seedlings on the surface of the water. It was established that this proportion of water to charcoal gave the maximum yield of crude concentrates and that a reduction in the relative amount of charcoal gave a diminished yield of active material from each tank. 100–120 tanks were set up each week and placed for 5 days in a near-dark room at 25°, and with this technique, after eluting the product from charcoal (see below), some 5–10 mg. of crude concentrates were obtained from each dish and the total weekly production was about 400 mg. of material, probably containing about 2% of the pure germination factor.

Elution of biologically active material from charcoal

The eluting powers of a variety of solvent systems on the wet charcoal were investigated and the results are tabulated below. All evaporations were carried out under reduced pressure (water pump) in an atmosphere of N₂, the temperature of the solution being kept below 35°. The pH of the solutions was maintained at 5.5–6.5 during the evaporations by the cautious addition of NaHCO₃ solution and allowance for this has been made in Table 1.

Stability of crude concentrates

A solution of the crude concentrate (activity, 1×10^5) was divided into four fractions and a range of pH values obtained by the addition of 0.01 N-H₂SO₄ or 0.01 N-NaOH respectively (pH values, 2.4, 4.7, 7.0 and 8.0). Each sample was boiled for 10 min. and after cooling, the pH was readjusted to 7.0 and the activity tested. In each case this was found to be <1.0.

The experiment was repeated with four samples of a solution (activity 5×10^4) adjusted to pH values 1.95, 3.85, 7.0 and 7.95 respectively. These were stored at 4° and the

activity values of neutralized portions of the solutions determined after various periods. The results are shown in Table 2.

Table 2. *Variations of activity with time on keeping concentrate solutions of the Striga germination factor at different pH's*

Initial pH	Activity values		
	18 hr.	42 hr.	66 hr.
7.95	1	1	1
7.0	250	350	3
3.85	350	8	1
1.95	75	40	1

Preliminary purification of the crude concentrates by solvent extraction

Aqueous solutions of the crude concentrates were extracted with a variety of organic solvents, and the activity of aqueous solutions of the material extracted into the organic phase determined in each case (Table 3).

Examination of the crude concentrates by paper-strip chromatography

The method used was similar to that already described in the case of the *Orobanch* germination stimulant (Brown *et al.* 1951*b*). The chromatograms were run on strips of filter paper (Whatman no. 1; 49 × 17 cm.), using as solvents either the upper layer of a n-butanol-acetic acid-water mixture (4:1:5) or n-butanol saturated with water. An aqueous solution of the crude material was applied all along the starting line of the chromatogram, the paper was dried at room temperature and then developed with the solvent system. The completed chromatogram after drying was cut crosswise into narrow strips (1 × 17 cm.) which were extracted separately with water (1 ml. per strip) and each extract tested biologically. Typical results are represented graphically in Figs. 1 and 2.

Table 3. Further purification of crude concentrates of the *Striga* germination factor by solvent extraction

Volume of original aqueous solution (ml.)	Concentration (mg./ml.)	Activity	Solvent for extraction	Weight of material extracted into organic solvent (mg.)	Activity	Percentage recovery
4.8	6.5	10 ⁴	Ether (6 × 10 ml.)*	3.6	10 ⁶	> 100
5	6.5	10 ⁴	Ethyl acetate (6 × 10 ml.)	5.0	0.75 × 10 ⁶	> 100
5	6.5	10 ⁴	CHCl ₃ (6 × 10 ml.)	5.8	10 ⁶	> 100

* As the aqueous layer still gave a positive biological test, nine ether extractions were used in all subsequent work.

Partial purification of the stimulant by chromatography on cellulose

The columns were prepared by introducing a slurry of cellulose (H. Reeve Angel and Co., London) in water-saturated *n*-butanol into the chromatogram tube (23 × 1.9 cm.) and thoroughly washing the cellulose with more solvent before use. The material was brought on to the column, the chromatogram developed and successive fractions of the eluate collected. Each of these was evaporated to dryness below 35° in an atmosphere of N₂ and the residues tested for biological activity. Examples of typical chromatograms are as follows:

(a) An aqueous solution (4 ml.; 31.9 mg./ml.; activity 10,000) of an acetone-ether (3:1) eluted crude concentrate was introduced on to a cellulose (23 g.) column, and developed with water-saturated *n*-butanol. After the fractions (total volume approx. 120 ml.) had been collected the column was extruded and extracted with water (150 ml.) and the filtered solution treated in a manner similar to the chromatogram fractions. The activity of each fraction was determined in the usual way and the results are shown in Table 4.

Table 4. Chromatography of an aqueous solution of a concentrate of the *Striga* germination factor on a column of cellulose, (a)

Fraction no.	Volume of fraction (ml.)	Weight of residue after removal of solvent (mg.)	Activity
1	40	1.7	0.5
2	20	9.2	1 × 10 ⁶
3	22	3.6	100
4	40	4.1	0
Eluate from extruded column	—	111	0

(b) An acetone-ether (3:1) eluted crude concentrate (421 mg.; activity 10,000) after ether extraction yielded a concentrate (23.5 mg.; activity 0.75 × 10⁶) which was dissolved in water (4 ml.) and chromatographed on cellulose (23 g.) as in (a) (Table 5).

(c) An acetone-ether (3:1) eluted crude concentrate (384 mg.; activity 25,000) after ether extraction yielded a concentrate (13.3 mg.; activity 1 × 10⁶), a portion of which (8.1 mg. in 3 ml.) was chromatographed as above (Table 6).

Table 5. Chromatography of an aqueous solution of a concentrate of the *Striga* germination factor on a column of cellulose, (b)

Fraction no.	Volume of fraction (ml.)	Weight of residue after removal of solvent (mg.)	Activity (× 10 ⁶)
1	11	4.9	0
2	4	4.1	2.5
3	9	14.7	2.5
4	4	1.5	0.25

Table 6. Chromatography of an aqueous solution of a concentrate of the *Striga* germination factor on a column of cellulose (c)

Fraction no.	Volume of fraction (ml.)	Weight of residue after removal of solvent (mg.)	Activity
1	40	2.2	0
2	10	4.6	1 × 10 ⁷
3	12	2.8	2500
4	15	0.6	10

High vacuum distillation of concentrates of the stimulant

(a) A concentrate of the *Striga* germination stimulant (26.4 mg.; activity 75,000) was subjected to high vacuum fractional distillation in a micro apparatus. The activities of the various fractions were determined and the results are shown in Table 7.

(b) As (a). Concentrate (16.4 mg.; activity 1 × 10⁶). (Table 8).

Carbohydrate content of crude concentrates

Solutions of the concentrate (40–60 mg./ml.) were examined for carbohydrates by paper-strip chromatography with the upper layer of a *n*-butanol-acetic acid-water mixture (4:1:5) and using for development the aniline oxalate reagent of Partridge (1950), which distinguishes pentose (pink spots) from hexose (brown spots). As a preliminary to the chromatographic separations, the inorganic material was removed from the solution either by ion-exchange resins or, more simply, by methanol extraction of the solid when the bulk of the salts did not dissolve. The *R_f* values

Table 7. *High vacuum fractional distillation of a concentrate of the Striga germination factor*

Fraction no.	Bath temperature (°)	Pressure ($\times 10^{-4}$ mm.)	Weight of fraction (mg.)	Activity
1	<60	5.5	14.5	0
2	60-115	8.0	5.6	1×10^6
3	115-150	8.0	2.1	Trace
4	150-200	1.4	0.9	0
Residue (methanol-soluble fraction)	—	—	0.5	0

Table 8. *High vacuum fractional distillation of a concentrate of the Striga germination factor*

Fraction no.	Bath temperature (°)	Pressure ($\times 10^{-4}$ mm.)	Weight of fraction (mg.)	Activity
1	<60	5	5.4	Trace
2	60-115	7	5.2	1×10^6
3	115-165	3	1.3	Trace
Residue (methanol-soluble fraction)	—	—	1.0	0

obtained in typical chromatograms of the crude concentrate are as follows (figures in parenthesis; starred spots were red, others brown), and they are compared with the values obtained with a series of standard sugars: (A) *Striga* concentrate (40 mg./ml.; 0.241*, 0.202, 0.126*, 0.087, 0.036); glucose (0.201); ribose (0.321*); arabinose (0.233*); maltose (0.088); raffinose (0.055). (B) *Striga* concentrate (40 mg./ml.; 0.255*, 0.210, 0.144*, 0.105); glucose (0.215); arabinose (0.267*); rhamnose (0.421); xylose (0.298*). (C) *Striga* concentrate (60 mg./ml.; 0.207*, 0.162, 0.102*, 0.068, 0.034); maltose (0.071); glucose (0.160); fructose (0.209); arabinose (0.211*); sucrose (0.100); xylose (0.247*); 2-keto-gluconic acid (0.090*). (D) *Striga* concentrate (57 mg./ml.; 0.236*, 0.186; 0.123*, 0.100, 0.066); maltose (0.106); galactose (0.164); raffinose (0.059); xyloketose (0.324*).

Amino-acid content of crude concentrates

Solutions of the concentrate (40-60 mg./ml.), after removal of the bulk of the inorganic material as before, were examined for amino-acids by paper-strip chromatography using the *n*-butanol-acetic acid-water mixture as the solvent and ninhydrin as the developing reagent. The details of typical chromatograms are as follows (R_F values in parenthesis as before) and data are provided for standard amino-acids for comparative purposes: (A) *Striga* concentrate (60 mg./ml.; 0.613, 0.443, 0.360, 0.280, 0.206, 0.147); norleucine (0.613); valine (0.430); tyrosine (0.352); tryptophane (0.447); glucosamine (0.146). (B) *Striga* concentrate (48 mg./ml.; 0.646, 0.443, 0.363, 0.207, 0.146); valine (0.441); aspartic acid (0.168); glutamic acid (0.229). (C)

Striga concentrate (48 mg./ml.; 0.637, 0.459, 0.387, 0.216, 0.137); alanine (0.228); threonine (0.182); norleucine (0.624); valine (0.412); tyrosine (0.347).

SUMMARY

1. Paper chromatographic examinations have demonstrated that the stimulant properties of crude preparations of the *Striga* germination factor may be due to a complex of substances. Using a *n*-butanol-acetic acid-water mixture as solvent, the most active component of the complex is located immediately behind the solvent front and is not a simple sugar or an amino-acid.

2. The purification of concentrates of the germination factor has been partially standardized in that carbon adsorption and elution with acetone-ether mixtures followed by ethereal extraction of the product so obtained, have been adopted as the initial stages.

3. Chromatography on columns of cellulose has been applied to the purification of larger quantities of the concentrates to yield resinous products with high biological activity.

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