likely to give a lower R.A. figure than one giving 50. Thus the correction involves a proportionality factor, in this instance of 50/14.1. The crude R.A. figure is multiplied by this factor to give the corrected R.A., which for the data of Fig. 3 is 1300.

It is probable that the errors involved in the R.A. determination are large. When the same sample of seed has been used in duplicate determinations, the error does not exceed 100 %. It may, however, be as great as 300% when the results involve several assays prepared on different occasions with different batches of seed, such as the data involved in following the amounts of stimulant recovered at various stages in a series of operations designed to concentrate and to purify the natural stimulant. Although the percentage errors are large it may be emphasized that the R.A. scale ranges from 1 to about 10,000,000, and the range is sufficiently large to enable large differences in activity to be evaluated.

The percentage recovery provides a figure for the proportion of the natural stimulant that has been retained in any purification procedure. Thus if a solution (total volume V ml.) with corrected half-value X yields purified material (W mg.) with R.A. Y then the percentage recovery is $100 WY/VX$. The errors in the estimation of percentage recovery are likely to be larger than those of the R.A. determination since they involve the errors of at least two

independent estimations. Occasionally, the percentage recovery exceeds 100 and these excessively large values must be attributed either to the removal of inhibitory material or to the errors of estimation.

SUMMARY

1. *Orobancha minor* is an angiospermous parasite, and the stimulant that the seed requires for germination may come from a variety of species. Linseed which is being used as the source of the germination factor in this investigation is not normally parasitized by *Orobancha minor*.

2. The bulk preparation of the stimulant is described. The method involves growing the roots of seedlings in water, from which the stimulant is recovered by adsorption on to charcoal.

3. The techniques that are being used for the determination of the 'relative activity' of various preparations, and for the calculation of percentage recovery of the stimulant during purification are described.

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The Stimulant Involved in the Germination of *Orobancha minor* Sm.

2. CHROMATOGRAPHIC PURIFICATION OF CRUDE CONCENTRATES

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The procedure adopted for the adsorption of the chemical factor responsible for the germination of *Orobancha minor* from aqueous solutions by means of active charcoal has been described in the previous paper (Brown, Greenwood, Johnson & Long, 1951), where the technique adopted for the biological assay was also described. Solutions of the stimulant were unstable under certain conditions, as evidenced by

loss of biological activity, so that it was apparent that the design of any purification procedure would depend on the nature and extent of this decomposition. The stability of solutions of the stimulant under various conditions of temperature and pH have therefore been examined. The experiments have been carried out with the standard assay fluid (Brown *et al.* 1951) which, when withdrawn from the

culture jars, is at approximately pH 5. Samples of this solution stored at 4° without adjustment of pH have been found to retain their stimulating capacity with very little or no loss over a period of 4 months. On the other hand, at pH 9 there was a rapid decrease in the stimulating capacity. The rate of inactivation was greater at higher temperatures at all pH levels, and especially so under alkaline conditions (Table 1). It was also observed that on heating alkaline solutions of the stimulant there was an appreciable decrease in the pH (Table 2), but no such change was obtained in solutions with pH < 5. These experiments indicated that the solutions of the factor were most stable between pH 3 and 4, and that alkaline treatment and excessive heating would have to be avoided throughout the isolation.

Several solvents have been examined for the elution of the stimulant from the charcoal, and the results of a series of comparative experiments are shown in Table 3, from which, on the basis of the 'Relative activity' (R.A.) (Brown *et al.* 1951) and the percentage recovery figures, it will be apparent that elution with chloroform-methanol is the preferred method. This has been used in most of the subsequent work. The inactivation of the stimulant by prolonged heat treatment has already been described, and it is therefore essential that the removal of solvent from solutions should be carried out at as low a temperature as possible. Preliminary freeze-drying of the charcoal before extraction with solvents seems, however, to offer no advantage.

The material as eluted from the charcoal was undoubtedly very crude, and a variety of techniques has been investigated for the purification of concentrates prior to chemical degradative studies. The most promising results have come from separations based on partition chromatography, described in the present paper, and those based on extraction methods which will be discussed in a later paper.

Examination of the eluates from the charcoal by paper-strip chromatography (Consden, Gordon & Martin, 1944), using *n*-butanol-acetic acid-water (Partridge, 1948) as the solvent system, has shown that the greatest part of the total activity is to be found in the fractions which travel very close to the solvent front and which are quite distinct from the fractions containing carbohydrates. This was established by drying the completed chromatogram and then cutting it into horizontal strips of convenient dimensions. Each of these was extracted with water and the individual extracts were then tested for biological activity. The results of typical experiments are shown in Figs. 1 and 2, the former being the chromatogram of an acetone eluate and the latter that of a chloroform-methanol eluate. The 'tailing' effect of the biologically active material in Fig. 1, which is not so apparent in Fig. 2, is probably

caused by the larger amount (24 mg.) used in the former chromatogram as compared with that (5 mg.) used in the latter.

The zones near the solvent front containing the active fraction appeared as weakly absorbing bands when the chromatograms were used as filters between an ultraviolet lamp and photographic paper (Markham & Smith, 1949), and it was also noted that these zones reduced Tollens reagent when this was sprayed on to the paper.

Brown, Johnson, Robinson & Todd (1949) have shown that certain sugars induce germination in *Striga*, and that sugars are present in the concentrates of the germination factor prepared from the *Sorghum* host. *Orobanche* also responds to *Sorghum* preparations, although less vigorously than to those from linseed cultures, and an examination was therefore made of the nature of the sugar content of the acetone eluate from the linseed preparations. The presence of reducing sugar in the crude concentrate was indicated by a Molisch test and by the ready reduction of Fehling solution. A reducing sugar determination (Somogyi, 1945) gave a value corresponding to 5–10 % (calculated as glucose) of the crude extracts. The individual sugars were separated by means of paper chromatography, seven compounds being detected, of which two were identified as glucose and arabinose and a third possibly as 2-ketogluconic acid. The implications of the presence of 2-ketogluconic acid will be discussed in a later paper.

The chromatograms were also examined for components other than carbohydrates. Amino-acids were absent (ninhydrin reaction), but two phosphate-containing fractions (R_f 0.13 and 0.29) were detected by spraying with a reducing molybdate solution under special conditions (Hanes & Isherwood, 1949). No reactions on the paper were observed with ferric chloride or diazotized sulphamic acid.

Simple chromatography on neutral alumina proved to be of little value for the purification of larger quantities, as the stimulant was too strongly adsorbed and could not be eluted. However, in view of the separation of active material from carbohydrates achieved by paper-strip chromatography, larger scale chromatography on columns of cellulose has been used with a degree of success. Nearly all of the active material was contained in the fractions which moved very close to the solvent front, and the product obtained from these fractions after removal of the solvent gave R.A. values of $1-2 \times 10^5$. There was also little loss of total activity. Data for typical chromatograms are shown in Tables 4 and 5.

In the course of this work, a wide variety of synthetic compounds has been tested for activity towards *Orobanche* seeds. None has so far been found to be active in the concentration range $1/10^3$

to 1/10⁶, and in this respect the situation with *Orobanche* differs from that observed with *Striga* (Brown *et al.* 1949) where certain sugars were found to be active.

The following compounds have been examined: laevulinic acid, β -ketoadipic acid, catechol, *o*-benzoquinone, cinnamic acid, heteroauxin, sucrose, D-fructose, D-glucose, D-xyloketose, D-glucosone, D-glucuronic acid, D-2-ketogluconic acid and Ca salt, D-5-ketogluconic acid and Ca salt, methylglyoxal, DL-malic acid, fumaric acid, succinic acid, citric acid, pyruvic acid, adenylic acid, L-cysteine, glutathione, *p*-aminobenzoic acid, vitamin B₁, nicotinic acid, calcium pantothenate, β -alanine, vitamin C, coumarin, anhydrotetroneic acid, aconic acid, 3-hydroxycoumarin, 3-acetonylcoumarin, homoparaconic acid, patulin, α -methyltetroneic acid, 7-hydroxy-4-methylcoumarin, α : β -penteno- δ -lactone, α : β -angelicalactone, α : β -hepteno- γ -lactone, γ -(spirocyclohexyl)- α : β -buteno- γ -lactone, α -(4-hydroxy-3-methoxybenzylidene)- γ -methyl- β : γ -buteno- γ -lactone (Marrian, Russell & Todd, 1949).

EXPERIMENTAL

Stability of the stimulant. The standard fluid (10 ml.; Brown *et al.* 1951) was placed in each of six Pyrex tubes and adjusted electrometrically by the addition of either dil. NaOH or HCl to a range of pH values (0.96–8.8). The tubes were heated at 100° in a water bath and the stimulating capacities of the

samples were measured at intervals (Table 1). Observations were also made on the change in acidity with time in the several series, and these results are shown in Table 2.

Table 1. *Rate of inactivation of standard fluid at different pH levels at approximately 100°*

(Data represent 'half-values', see Brown *et al.* 1951.)

Time (hr.)	Initial pH						
	0.96	3.62	4.37	6.18	6.85	8.02	8.83
0	50	50	50	50	50	50	50
2	<2	30	17	5	1	0	0
5	0	22	14	2	0	—	0
20	—	9	6	—	0	0	0
50	—	2	1	1	—	—	—
80	—	1	1	1	—	—	—
100	—	1	0	0	—	—	—

Table 2. *Change in acidity during heating of standard fluid at 100° with different initial pH levels*

Time (hr.)	pH values						
	0.96	3.62	4.77	6.18	6.85	8.02	8.83
0	0.96	3.62	4.77	6.18	6.85	8.02	8.83
2	0.96	3.64	4.92	5.92	6.72	6.85	7.05
100	—	3.59	4.68	4.98	6.00	6.20	6.85

Elution of the stimulant from charcoal. The results of a variety of elutions with different solvents are shown in Table 3. Methanol was added to a number of the water-

Table 3. *Formation and properties of crude eluates from charcoals given with various solvents*

Solvent		Weight of charcoal (g.)	Weight of eluate (mg.)	Appearance	Weight of water-soluble fraction of eluate (mg.)	Relative activity	Recovery (%)
(a)	Acetone	5	4.9	Yellowish glass	4.9	100	1
(b)	(i) Ether (50 ml.) and methanol (20 ml.)	27	18.8	Nearly colourless glass	15.9	31.6	1.9
	(ii) Ether (50 ml.) and methanol (6 ml.)						
	(iii) Ether (3 × 50 ml.)						
(c)	Ether (cold) (3 × 50 ml.)	35	31.9	Nearly colourless glass	14.3	1,500	14.3
(d)	Ether (hot) 8 hr. constant extraction	35	42.3	Nearly colourless glass	24.3	300	5
(e)	(i) Chloroform (50 ml.) and methanol (20 ml.)	27	21.2	Nearly colourless glass	13.1	18,000	90
	(ii) Chloroform (50 ml.) and methanol (6 ml.)						
	(iii) Chloroform (3 × 50 ml.)						
(f)	Pyridine (5 × 30 ml.)	15	67.3	Dark-brown oil	67.3	1,770	46
(g)	(i) Ethyl acetate (30 ml.) and methanol (10 ml.)	15	9.9	Nearly colourless glass	5.6	18,000	70
	(ii) Ethyl acetate (30 ml.) and methanol (3 ml.)						
	(iii) Ethyl acetate (3 × 30 ml.)						
(h)	(i) <i>n</i> -Butanol (30 ml.) and methanol (10 ml.)	29	12.2	Nearly colourless glass	9.9	4,800	22
	(ii) <i>n</i> -Butanol (30 ml.) and methanol (3 ml.)						
	(iii) <i>n</i> -Butanol (3 × 30 ml.)						
(j)	Acetone (50 ml.) and ether (20 ml.) (× 5)	29	14.7	Nearly colourless glass	5.2	14,400	64

immiscible solvents in order that the efficiency of the extraction of the wet charcoal should be increased. The extracts were clarified by filtration through Supercel (Johns-Manville Co. Ltd.) and for the biological assay the crude concentrates were extracted with water (5 ml.).

Distribution of the stimulant on paper-strip chromatograms

The acetone eluate (333 mg.) was extracted with dry methanol, the insoluble inorganic salts separated and the solvent removed from the filtrate. The residue (122 mg.) was dissolved in water (2 ml.), and a portion of this solution (0.4 ml. containing 24.4 mg. concentrate) applied to a Whatman no. 1 filter-paper strip (49 × 17 cm.) and developed during 24 hr. with the *n*-butanol-acetic acid-water system (Partridge, 1948), after which the solvent front was 38 cm. in advance of the starting line.

Fig. 1. Histogram showing distribution of stimulant on a paper-strip chromatogram of an acetone eluate after development with *n*-butanol-acetic acid-water mixture.

Fig. 2. Histogram showing distribution of stimulant on a paper strip chromatogram of a CHCl_3 -methanol eluate after development with *n*-butanol-acetic acid-water solvent mixture.

A similar experiment was conducted with a portion (1 ml. containing 5 mg. of concentrate) of an aqueous extract of the material obtained from a CHCl_3 -methanol eluate. After development of the chromatogram as in the previous experiment, the solvent front was 29.5 cm. in advance of the starting line. The papers were then dried *in vacuo* at room temperature. In an initial experiment the distribution of the stimulant on the paper was explored by allowing seeds of

Orobancha minor to germinate in contact with portions of the actual paper, but it was later found that more vigorous germination was obtained in hanging drop cultures with fluids prepared by extracting portions of the chromatogram with water. This method has the additional advantage that the technique of the test is essentially the same as that used in the assay of stimulant preparations. The chromatograms were therefore divided into a number of strips parallel to the solvent front, each strip was eluted with water for several hours and the percentage germination in each of the fluids so obtained was found from the totals in five replicate drops of each fluid mounted in the usual culture chambers and incubated at 26° for 4 days. In another experiment the procedure of preparing the chromatogram was followed, but without using any stimulant material. The paper was cut up and extracted in the usual way, but no germination was recorded from any part of the paper.

In the experiment represented diagrammatically in Fig. 1, the chromatogram from the starting line to the solvent front was cut into 19 strips each 2 cm. wide. Each strip was soaked overnight in distilled water (2 ml.) at approximately 4° and the fluids tested in the manner described above. In the case of the chromatogram of the CHCl_3 -methanol eluate, narrower strips were taken in the biologically active region and the results are plotted in Fig. 2.

Distribution of sugars on a paper chromatogram

A chromatogram of the crude eluate was prepared similarly to the one described above. After development for 24 hr. with the butanol-acetic acid-water mixture, the position of the sugars was determined by spraying the paper with aniline oxalate solution (Partridge, 1950). Seven spots were obtained: (i) pink, R_f 0.49; (ii) pink, R_f 0.22, probably arabinose; (iii) brown, R_f 0.19, probably glucose; (iv) brown R_f 0.16; (v) pink, R_f 0.13, possibly 2-ketogluconic acid; (vi) brown R_f 0.10, probably a polysaccharide; (vii) brown, R_f 0.05, probably a polysaccharide. The position of the arabinose, glucose and 2-ketogluconic acid spots agreed with those of authentic specimens run on the same chromatogram. The presence of pentoses was confirmed by a positive Miltzer (1946) reaction on the crude stimulant solution. As the pentoses have greater R_f values than the hexoses, spot (i), which moved faster than any of the known pentoses, may have been a tetrose. However, erythrulose (Müller, Montigel & Reichstein, 1937), which as a ketose should be the most mobile of the three tetroses, had R_f 0.42 and gave a brown spot with aniline oxalate.

Chromatography on cellulose

A concentrate of the stimulant (77 mg.; B.A. 1000) from an ether-methanol extraction of the charcoal was dissolved in the upper layer (10 ml.) of the *n*-butanol-acetic acid-water mixture (Partridge, 1948) and a fraction (1 ml.) of the solution retained as a control. The remainder (9 ml. containing 69 mg. of crude stimulant of B.A. 1000) was brought on to a column (23 × 2 cm.) of powdered cellulose previously washed with the same solvent (150 ml.). The chromatogram was developed with more of the solvent and the eluate was collected in four 40 ml. fractions. The column was then extruded and the cellulose extracted with water (100 ml.). After removal of the solvent from the control and the various fractions at 0.1 mm. pressure and temperature <40°, the

residues were extracted separately with water (2 ml.) and the solutions tested for biological activity. The results are shown in Table 4.

Table 4. *Results of first chromatogram of the crude stimulant on cellulose columns*

Fraction	Total residue (mg.)	Water-soluble fraction (mg.)	Relative activity of water-soluble fraction
1	8.5	5.5	43,500
2	21.7	15.5	480
3	7.1	7.1	0
4	8.7	8.7	12.5
5	19.6	19.6	0
(from extruded column)			
Material applied to column	69.0	60	1,280

A similar experiment was carried out using a CHCl_3 -methanol extract of the charcoal (25.7 mg.; R.A. 7000) on powdered cellulose (10 g.) prewashed with the *n*-butanol-acetic acid mixture (200 ml.) as used in the previous experiment. The results obtained with 20 ml. fractions are shown in Table 5.

Table 5. *Results of second chromatogram of the crude stimulant on cellulose*

Fraction	Total residue	Volume of water used for extraction (ml.)	Water-soluble fraction (mg.)	Relative activity of water-soluble fraction
1	14.5	5	10.7	19
2	7.7	2	2.9	173,000
3	3.6	2	0.3	2,000
4	1.3	2	0.6	19
Material applied to column	25.7	—	—	7,000

DISCUSSION

It is clear that the stimulating properties of the linseed fluids are in a great measure and perhaps wholly due to the material which travels close to the solvent front in a *n*-butanol-acetic acid-water partition chromatogram, and which has an R_f value greater than 0.8 under the conditions specified. This material does not correspond with any of the carbohydrates detected on the chromatograms. The active

region near the solvent front is a wide one, and it is possible that more than one active substance is concerned.

Substantial purification of the stimulant was achieved by the use of partition chromatography on cellulose columns, but in view of the resinous nature of the active material it was not possible to determine its purity. Other methods of purification have therefore been examined to ascertain whether concentrates could be prepared with comparable or even greater biological activity, and the results obtained in these investigations will be described in the succeeding paper of this series.

The relative stability of the stimulant in weakly acid solutions and its instability in alkaline solutions may well indicate the presence of an acidic or potential acidic (e.g. lactone) grouping. The reducing powers are also of great interest, but the significance of this property will only be clarified by further investigations on the purification and chemical structure of the stimulant.

SUMMARY

1. The stimulant required for the germination of *Orobanche minor* Sm. is unstable in alkaline solutions but moderately stable in weakly acid media.

2. Examination of a variety of solvents for the preliminary elution of the stimulant from charcoal has shown that a chloroform-methanol mixture is the most efficacious.

3. A method has been developed for the examination of the compound by paper-strip chromatography. With the *n*-butanol-acetic acid-water mixture, the factor travels very close to the solvent front and is quite distinct from the common carbohydrates. The method has been extended to partition chromatography on columns of cellulose, and concentrates with relative activity values of $1-2 \times 10^5$ have been obtained.

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