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The *Orobanche* Germination Factor

3. CONCENTRATION OF THE FACTOR BY COUNTER-CURRENT DISTRIBUTION

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The chromatographic purification of crude concentrates of the *Orobanche* germination stimulant, particularly partition chromatography on cellulose, has been described in an earlier paper (Brown, Greenwood, Johnson, Long & Tyler, 1951*b*). The most active preparations thus obtained had activity values (Brown, Greenwood, Johnson & Long, 1951*a*) of $1-2 \times 10^6$, and it seemed possible that they contained a high percentage of the pure stimulant. However, as these preparations were invariably resinous and as no convenient physical property of the stimulant was known by which its purity could be gauged, other methods of purification have been investigated to ascertain whether concentrates could be obtained with even higher activity values and possibly in a crystalline form.

Much of the present paper is concerned with purification of the *Orobanche* germination factor by partitioning between immiscible solvents, the choice of solvents being limited on the basis of earlier stability studies (Brown *et al.* 1951*b*) to those boiling below 120° and neither strongly basic nor acidic.

In our earlier studies it was shown that the stimulant adsorbed on charcoal could best be eluted with chloroform-methanol and it has now been found that considerable purification of these crude eluates can be achieved by a further extraction of their aqueous solutions with ethyl acetate. This step has been included in all of our later purifications. Ethyl acetate was slightly superior to chloroform or ether for this extraction step.

The partition method of purification was extended to the use of counter-current methods, and many solvent systems were tried before a satisfactory distribution ratio of the active stimulant was achieved between the two immiscible phases. Some idea of the partition coefficient of a compound between heterogeneous solvent phases can be obtained from preliminary paper-strip chromatograms (Brown *et al.* 1951*b*). The relationship of the R_F value in partition chromatography to the partition coefficient has been discussed by Martin & Synge (1941), by Consden, Gordon & Martin (1944) and by Craig (1950).

The mixture *tert.*-butanol (4)-light petroleum (b.p. 60-80°; 5)-acetone (4)-water (2) was eventually devised and this gave an appreciable concentration of the active material in the centre tubes of the counter-current apparatus, i.e. the partition was approximately equal between the two phases. *tert.*-Butanol was selected as the alcoholic component in order to cut down any esterification of lactone or acid groupings believed to be present in the molecule of the germination factor. The use of buffered solutions in the counter-current extractions to suppress such undesirable changes seemed inadvisable in view of the instability of the factor in alkaline or strongly acid solutions and the impossibility of carrying out the biological test in the presence of buffer solutions of the usual concentrations.

The purified stimulant obtained either by chromatography (Brown *et al.* 1951*b*) or by extraction methods was only slightly soluble in water, and

for testing purposes the product was dissolved in a little methanol and a large volume of water added. The methanol was then removed under reduced pressure and the resulting emulsion used for the assay. Such emulsions could not be separated in the centrifuge so that fractional precipitation offered little promise as a means of purification.

The progress of isolation and purification of a biologically active material is determined to a large extent by the speed and accuracy of the biological testing. In this case the results of any particular

shows the results of a typical fractionation, and it will be seen that most of the active material distills at temperatures around 100° at a pressure of 10⁻⁵ mm. Although considerable loss of total activity is incurred in these distillations, the fact that the stimulant can be distilled at all is clear indication that the anhydrous concentrate is much more stable than its aqueous solutions. The ultraviolet absorption spectrum of the biologically active fraction of the distillate again showed a distinct maximum at 2450 Å. ($E_{1\text{cm}}^{1\%} = 160$).

Fig. 1. Ultraviolet absorption spectrum of the crude *Orobanche* germination stimulant (activity value, 63 000). —, ethanol solution; ---, aqueous solution.

test were not available before 7 days and consequently characteristic physical properties of the active material were sought in order that the various fractions might be assessed more rapidly.

The ultraviolet absorption spectrum (Fig. 1) of the crude concentrate before counter-current purification or even after distribution between chloroform and water did not reveal any characteristic features other than an arrest at 2300–2350 Å. and a slight inflexion at 2800 Å. However, after purification by the four-component system, *tert*-butanol–petroleum (b.p. 60–80°)–acetone–water as described above, the ultraviolet absorption spectrum of the most active fraction exhibited a distinct maximum at 2450 Å. ($E_{1\text{cm}}^{1\%} = 170$) (Fig. 2), and it seems probable that this ultraviolet absorption is a feature of the biologically active material.

Like the *Striga* germination factor (Brown, Johnson, Robinson & Tyler, 1952) the *Orobanche* factor can be distilled under high vacuum. Fig. 4

Fig. 2. Ultraviolet absorption spectrum of purified *Orobanche* germination stimulant (activity value, 100 000) in ethanol.

EXPERIMENTAL

Concentration of the germination factor by solvent extraction of the crude concentrates. The crude eluate (83.7 mg.) obtained from the charcoal (Brown *et al.* 1951*a*) was dissolved in water (20 ml.; activity of solution, 10 600) and extracted with ethyl acetate (5 × 100 ml.). The solvent was removed from the combined extracts below 40° or preferably by freeze-drying and the water-soluble fraction (6.5 mg.) of the residue (16.2 mg.) tested for activity (122 000; recovered activity, 90%). Similar experiments were carried out using CHCl₃ (85–89% activity recovery) and diethyl ether (75% activity recovery).

Counter-current extraction of concentrates of the germination factor. The material used for the counter-current extractions was obtained by eluting the charcoal with CHCl₃–methanol (Brown *et al.* 1951*a*) and then extracting an aqueous solution of the product with ethyl acetate as described above. A 25-tube counter-current apparatus (Craig & Post, 1949) was used, and after the distribution the combined phases from each tube were evaporated to dryness, the efficiency of

the separation being assessed by determinations of the weight, optical density and biological activity of each fraction. Results using the 4-component, 2-phase system, water (2)-petroleum (b.p. 60–80°; 5)-*tert.*-butanol (4)-acetone (4) are expressed in Fig. 3.

Fig. 3. Counter-current distribution of a concentrate of the *Orobanche* germination factor between *tert.*-butanol-petroleum (b.p. 60–80°)-acetone-water.

Fig. 4. Distillation of a concentrate of the *Orobanche* germination factor at 10^{-5} mm.

Distillation of the germination factor. The crude concentrate (38.4 mg.; activity 5600) was subjected to high vacuum distillation at 1×10^{-5} mm. with a very short path length,

and several fractions were taken off on a cold finger, the bath temperature being raised at the rate of 20°/hr. The weight and activity of each fraction were determined and the results are expressed in Fig. 4.

Titration of the germination factor. The concentrate (18 mg.; activity, 67 000) was dissolved in 0.1 N-tetramethylammonium hydroxide (3.0 ml.) and kept for 2 days at room temperature with exclusion of CO_2 . The solution was agitated by the passage of a stream of purified N_2 through it and titrated against 0.2 N-HCl in an electrometric apparatus with a glass electrode. From the amount of acid (0.89 ml.) required to neutralize the excess alkali, the equivalent weight was calculated to be 148.

In a further experiment the same concentrate (18 mg.) was dissolved in 0.1 N-NaOH and the solution heated at 100° for 10 min., cooled and titrated as before. This experiment gave a value of 133 for the equivalent weight. In each case the pH of the alkaline solution of the concentrate decreased on keeping.

DISCUSSION

Counter-current extraction is a useful method of purification in the case of the *Orobanche* germination stimulant, the most active material thus produced having an activity value of the same order (250 000) as that obtained by partition chromatography on columns of cellulose (Brown *et al.* 1951b), and it seems reasonable to assume that both materials contain an appreciable amount of the pure stimulant. From the experimental point of view the counter-current method is slightly easier to carry out than the chromatographic method and has been adopted in most of the recent work. The preferred method of purification of the concentrate at the present time therefore comprises (i) adsorption on charcoal, (ii) elution with chloroform-methanol, (iii) extraction of an aqueous solution of the product with ethyl acetate or, better, washing an ethyl acetate solution with water, (iv) counter-current distribution between chloroform and water, (v) counter-current distribution using the *tert.*-butanol-petroleum (b.p. 60–80°)-acetone-water mixture.

The fractional distillation of the concentrates under high vacuum with a short path length also has resulted in a marked concentration of the factor in one fraction. This is a somewhat surprising result in view of the instability to heat of solutions of the concentrates (Brown *et al.* 1951a) in polar solvents, especially water, and it therefore seems that the solvents accelerate deactivation. The rate of deactivation of slightly alkaline aqueous solutions of the factor seemed to be independent of the presence or absence of oxygen.

Only preliminary evidence is available on the chemical nature of the *Orobanche* germination factor at the present time. Moreover, in the absence of the pure factor, the necessity of working only with non-crystalline concentrates makes it very difficult to differentiate between the properties of the factor

itself and those of any impurities which might be present. However, on the basis of the comparable activity values of purified concentrates obtained by partition chromatography or by counter-current extraction, the products have been used for preliminary degradative work. Sulphur, phosphorus and halogens were absent, and the small nitrogen values (about 0.5%) obtained in several analyses are unlikely to be significant. Carbon and hydrogen determinations on various concentrates (activity, $1-2 \times 10^5$) have given values of C, 59.5–62.0; H, 7.6–8.7% ($C_{10}H_{18}O_4$ requires C, 59.4; H, 8.9%; $C_{11}H_{18}O_4$ requires C, 62.3; H, 7.6%) after drying at a pressure of 10^{-2} mm. and room temperature over phosphorus pentoxide overnight.

The concentrates are unreactive to Fehling solution, periodate, Schiff reagent and Millon reagent and do not decolorize bromine water. On the other hand, they reduce Tollens reagent and instantly decolorize neutral permanganate in the cold. They are optically inactive (in one case a solution of a sample of concentrate (56 mg.) in chloroform (3 ml.) gave $[\alpha]_D +1.1^\circ \pm 0.6^\circ$ but generally the rotation was imperceptible), give no colour with ferric chloride and only a slight yellow precipitate with 2:4-dinitrophenylhydrazine. Evidence for a lactone, ester or other potential acid group comes from a positive hydroxamate colour reaction (Feigl, 1947) and the marked fall in pH on keeping freshly prepared alkaline solutions of the factor. The infrared spectrum of a purified concentrate (activity, 1×10^5) gives evidence of a hydroxyl group (band at 2.92μ), two ester (or δ -lactone) groups (bands at 5.77 and 5.82μ) and also a methyl group (band at 7.27μ) and a double bond (band at 6.04μ).

It is evident that the *Orobanche* germination factor has some features in common with the *Striga* germination factor described in another series of papers (Brown, Johnson, Robinson & Todd, 1949;

Brown *et al.* 1952). This is apparent from the closely similar methods of extraction applicable in each case, and from the closely related physical and chemical properties of each of the concentrates. This supposition has been supported by the results of testing the effect of the *Orobanche* germination factor on *Striga* seeds and vice versa when definite germination effects were obtained in each case. It is also possible that these germination factors may bear some relation to the potato eel-worm hatching factor already examined by Todd and his colleagues (Calam, Raistrick & Todd, 1949 and succeeding papers) although active concentrates of the latter showed little effect on *Orobanche* or *Striga* seeds. The evidence for the free carboxyl group in the eel-worm hatching factor is more definite than in the plant germination factors. Nevertheless, any relationship between these compounds would be of far-reaching biological significance.

SUMMARY

1. The purification of concentrates of the *Orobanche* germination factor using extraction techniques, including counter-current methods, is described. The most active concentrates so obtained have activity values comparable to those obtained by partition chromatography.

2. The germination factor can be distilled at very low pressures.

3. Some chemical properties of the purified concentrates are described.

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