3. Interference in the estimation of ascorbic acid by nitrous acid may be overcome by the use of amidosulphonic acid, which reacts rapidly with nitrous acid and so protects ascorbic acid from oxidation.

4. The nitratase enzyme of *Escherichia coli* may be completely inhibited by potassium cyanide or sodium azide, without affecting the enzyme systems associated with the reduction of dehydroascorbic acid.

5. The presence of potassium cyanide in a concentration of 0·001 M accelerates the conversion of dehydroascorbic acid to diketogulonic acid, but sodium azide in a similar concentration has no effect. Sodium azide may, therefore, be used to prevent the formation of nitrite by *Escherichia coli* without any disadvantageous effect on the reduction of dehydroascorbic acid to ascorbic acid.

6. The rate of reduction of the oxidized forms of other enediols has been studied. These include D-*iso*ascorbic acid, hydroxytetronic acid, reductic acid, reductone (enol of tartronaldehyde) and the reductants present in a glucoreductone solution. All these substances with the exception of reductone were reduced, but at a slower rate than dehydroascorbic acid.

We wish to thank Dr R. H. M. Robinson for his help in preparing the bacterial suspensions, Dr J. Barker for his interest and advice, and Mr D. Wardale for his technical assistance in carrying out the work.

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

1. ASSAY TECHNIQUE AND BULK PREPARATION OF THE STIMULANT

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Among the parasitic plants, the subfamily Rhinanthoideae and the closely related Orobanchaceae are unusual in that all the species of these groups are root parasites. Those of the former group have green leaves and are only partially dependent upon other plants, but the Orobanchaceae are complete parasites which lack chlorophyll and have a much reduced and modified structure. They consist of little more than an erect flowering shoot above the ground and they produce many thousands of minute seeds, each no more than about 0·3 × 0·2 mm. in external dimensions. The base of the shoot is attached below ground to the root system of the host plant.

A number of species of *Orobanche* have considerable economic importance in that they damage a variety of crops, particularly in Mediterranean climates. Even in this country, *O. minor*, the lesser broomrape, may cause partial or complete failure of clover. The seeds of many species of these parasites normally germinate only when they are in the immediate vicinity of a host root. According to Lindley (1853) this condition was first demonstrated by Vaucher with *Orobanche*. The phenomenon was subsequently investigated by Koch (1887) again with *Orobanche*, by Heinricher (1898) with *Lathraea* and by Pearson (1912) with *Striga*.

It has been shown that the influence of the growing root is due to a chemical stimulant which is released from it and which is required by the seed in germination. Saunders (1933) induced germination in *Striga lutea* seeds by irrigating them with water that had percolated through sand containing growing maize roots, and Barcinsky (1934) and Chabrolin (1934) reported similar results with *Orobanche cumana* and *O. speciosa* respectively. It was further shown by these two workers that a dry residue could be obtained from aqueous extracts of roots and other parts of host plants which, when redissolved in water, promoted the germination of the parasite seed.

The chemical nature of the stimulant for *Striga* is being investigated in these laboratories and Brown, Johnson, Robinson & Todd (1949) have reported that a crude concentrate of the natural stimulant contained sugars and that certain pure sugars promoted the germination of the seed. The present paper is the first of a series in which the results of a parallel investigation on the stimulant for *Orobanche* will be reported.

In the first place, the range of stimulating organisms was investigated in order to decide the most suitable source of the germination factor. Not all of the species of *Orobanche* are narrowly host specific; thus whereas *O. hederae* is normally restricted to ivy (it has been reported on a few other members of the Araliaceae), other species such as *O. speciosa*, *O. cumana* and *O. minor* parasitize a wide range of hosts. *O. minor* Sm. with which this investigation is concerned has a particularly wide host range, and has been reported as occurring on more than sixty different hosts.

In the total host-parasite relationship two phases must be recognized. In the first the host root stimulates the seed to germinate and induces it to produce an independent seedling; in the second the seedling becomes attached to the root of the host and thereafter exists parasitically upon it. The restriction of host species (at least with *O. minor* and *O. speciosa*) refers in many instances to the second phase; during the first, stimulation can probably be given by the roots of a much wider range of species, and it can be given by roots which are never parasitized. Chabrolin (1934, 1935) showed that *O. speciosa*, for which a stimulant is produced by *Vicia faba* and other plants which are parasitized, may also be germinated by the roots of *Cicer arietinum*, *Linum usitatissimum* and a number of other species which are not. The significance of this phenomenon in relation to angiospermous parasites as a whole has been discussed by Brown (1945).

A comparison of clover, maize, sorghum and linseed has led us to adopt linseed (var. Blue-flowering Plate) as the stimulant source in this investigation.

The bulk preparation of the stimulant has been carried out by an extension of the method used by Brown *et al.* (1949) for the preparation of the *Striga* factor. The roots of intact linseed seedlings were grown in water and the active material adsorbed from the aqueous solution by purified activated charcoal. The subsequent elution and concentration of the factor is described in the succeeding papers and most of the present communication is devoted to a description of the assay technique employed. This technique is also based on that developed by Brown *et al.* (1949) for use with *Striga*. The procedure, however, has been further elaborated and certain modifications have been introduced that are required by the peculiarities of the germination process in *Orobanche*.

The assay depends on the condition that percentage germination decreases with increasing dilution of a stimulating solution. Different samples of seed or the same sample at different times, however, vary in the magnitude of their response to the stimulant and the results obtained with any given experimental preparation must therefore be correlated with the results obtained with a standard solution. These variations in seed behaviour, which involve both the maximum percentage germination which may be attained and the concentration of stimulant required to produce a particular percentage germination, are profoundly affected by certain treatments applied before the seeds are exposed to stimulant solutions. The assay technique involves, therefore, preparation or pretreatment of the seed, the actual germination procedure, and the preparation of the standard fluids. Each of these aspects is described separately in the experimental section. The treatment of the assay technique is completed by descriptions of the general design of a single assay and by a consideration of the errors of the method.

EXPERIMENTAL

Assessment of various stimulating organisms

Conditioned seeds were sprinkled over a sheet of moist filter paper laid against a vertical sheet of glass resting in a shallow trough of water. Evaporation from the filter paper was limited by securing a second sheet of glass at a distance of 3 mm. from the first and separated from it at the vertical edges with a wax seal. Seedlings with their roots between the two sheets of glass were placed along the upper edges of the two sheets. The roots grew downwards over the surface of the filter paper carrying the *Orobanche* seeds. The effects of the roots of clover, maize, sorghum and linseed were investigated. It was found that the most vigorous germination was given by linseed and the least by clover. Maize and sorghum gave effects intermediate between those of the other two. It may be noted that seedlings of maize, sorghum and linseed which are not normally parasitized gave a stronger stimulating effect than those of clover which is a normal host.

The effect of linseed was striking and well defined. Whereas with the other species germination was restricted to a zone a few centimetres wide along the course of the stimulating root, with linseed germination after 7 days was observed over the whole surface of the filter paper which covered an area 8 × 10 cm. With other techniques the stimulating effect of roots of pelargonium, ivy, wheat, barley, oats, potato and willow was investigated. Stimulation was observed with all these, but in no case was it as great as with linseed.

Bulk preparation of the stimulant

Seeds (150–200) of linseed were germinated on pieces of muslin each laid on a sheet of filter paper moistened with a few drops of a dilute calcium hypochlorite solution in shallow dishes kept in the dark in a constant temperature room at 25°. The seeds germinated in the dishes and on the

3rd day each net carrying growing seedlings was stretched over a frame of glass tubing and supported on the surface of 1–1·5 l. of distilled water in an aluminium bowl. One hundred bowls were set up each week, and the cultures were continued at 25° in the dark for a further 5 days, at the end of which time the undiluted fluid in the bowls promoted the full germination capacity of the parasite seeds.

The solution was treated with charcoal (4·25 g./l.) which was allowed to settle, filtered off and formed into a dry cake by removal of water in a press. After the charcoal treatment the filtrate was biologically inactive and was discarded. The bulk preparation of the stimulant was carried out at Leeds, and the active material sent to Cambridge, after adsorption on to the charcoal.

Considerable difficulty has been experienced in obtaining suitable grades of charcoal for this work and even with the grade finally selected (Sutcliffe, Speakman and Co., Genster charcoal) preliminary purification was necessary. The charcoal (500 g.) was washed successively with water, methanol, ether, ethyl acetate, acetone and finally water (500 ml. of each) and then separated and dried as far as possible by suction in air when it still contained adsorbed water (65–70 g.). After removal of the solvent from the combined extracts from the charcoal, the water-soluble fraction (357 mg.) of the residue (853 mg.) contained phosphate and sulphate ions but no chloride and was strongly acidic. It showed inhibitory effects on the growth of germinating *Orobanche* seedlings. The inhibition was particularly marked on the epidermis of the root and gave an appearance similar to that described by Burström (1949) for the inhibiting effect of di-*n*-amylacetic acid on the growth of wheat roots.

ASSAY TECHNIQUE

Pretreatment of the seed

Chabrolin (1938) reported that with *Orobanche* more vigorous germination is obtained when the seeds are stored moist before the stimulant is applied to them. Brown & Edwards (1944) found with *Striga* that with a standard solution percentage germination increased as the period of incubation at 25° under moist conditions was prolonged over a period of 14 days. A similar condition has been observed by us with *Orobanche*. The change in germination capacity with time of pretreatment is shown by the data of Fig. 1 which were obtained by placing seeds on a moist sintered-glass disk in a Petri dish, incubating them at 25°, removing samples at intervals and treating them with a standard solution. It is evident that under the conditions of the experiment germination increases over the first 14 days and unlike the position with *Striga* thereafter remains more or less constant. Fig. 1 shows only results obtained up to the 32nd day. We have, however, examined a culture over a period of 350 days, and noted only a slight decrease in germination capacity from the 14th day to the end of the experimental period. The germination capacity of other cultures has, however, often decreased more rapidly and the origin of this variability is being investigated.

The change in germination capacity over the first 14 days of pretreatment is clearly an important condition for the assay and in the normal routine, pretreatment dishes were set up at intervals and seeds were taken from them only after they had been incubated for at least 21 days.

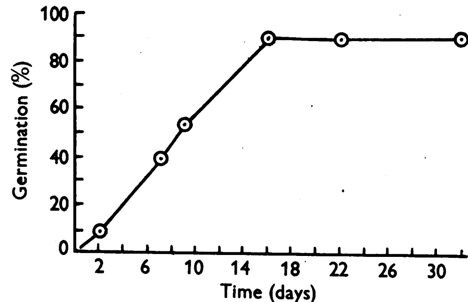

Fig. 1. Effect on percentage germination of incubating seed in moist condition at 25° before application of a standard stimulant solution. Time along abscissa refers to period of incubation.

All the seed used in this investigation belonged to the species *O. minor* Sm., most of which was obtained from a badly infested clover field near Canterbury in August 1948. We are deeply indebted to the staff at the Royal Botanic Garden, Kew, for help in the identification of the species.

Germination test

Percentage germination was determined in hanging drop cultures. This technique is that originally adopted by Brown & Edwards (1944) with *Striga*, since it is the most convenient method for incubating and observing seeds as small as those of *Striga* and *Orobanche*. But whereas Brown & Edwards used single drop cultures, we have used a multiple drop technique by which 25 drops, which represented five replicates for each of five solutions, were incubated within a single flat chamber, which was formed from two glass plates each about 8 cm. square separated by a square frame of glass rod about 3 mm. thick and closed along the edges with a mixture of equal parts of vaseline and embedding wax. The 25 equally spaced drops hung from the lower surface of the upper sheet of glass.

The cultures were set up in the following manner. A glass plate was placed over a sheet of paper of the same size divided into 25 equal squares and the exposed surface of the plate was rubbed with a cotton wool pad charged with liquid paraffin. Five drops (each 0·01 ml.) of an experimental solution were then delivered each above the centre of one of the squares on the paper in a horizontal row, the other four rows being treated similarly each with a different solution. Enough seed for a complete experiment was transferred from the pretreatment dish to a separate vessel, and from this to the drops by means of a platinum spiral which formed a short cylinder into which the seeds were pressed. The spiral

carrying the seeds was brought lightly into contact with each drop and delivered into it 25–50 seeds, approximately the same number of seeds being placed in each drop. The platinum spirals were carefully flamed before they were used for each set of transfers. When the 25 drops had been charged with seeds, the plate on which they rested was covered by a lid formed from a second sheet of glass to which a rod frame had been attached with the wax mixture. The sandwich-like unit thus formed was made into a closed chamber by a second wax seal established between the plate carrying the drops and the glass rod frame. The culture chamber was finally inverted with a swift circular motion of the hand bringing the drops to the lower surface of the upper sheet of glass.

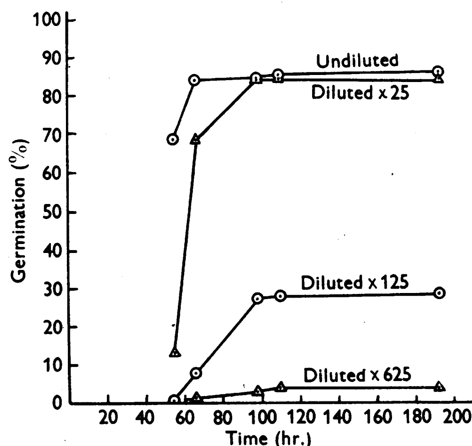

Fig. 2. Change in percentage germination with time after application of an undiluted standard solution, and of solutions diluted ×25, ×125 and ×625.

This multiple drop technique is more convenient than the single drop method since it is considerably more rapid. The trace of paraffin on the surface from which the drops hang prevents them from running together and water does not distil from the drops on the lower plate when the cultures are placed on a metal shelf in the incubator.

After incubation for 4 days at 26° the cultures were examined microscopically, and the total number and the number of seeds that had germinated in each drop were counted and the percentage germination calculated from the totals in each category in the five replicate drops for each treatment.

It has been found that 26° is the optimum temperature for germination and that 4 days are usually required for maximum germination to be attained at this temperature. The change in percentage germination after the 2nd day is shown by the data of Fig. 2. It is evident that in this series of observations the maximum was reached after about 96 hr. Certain cases have been observed in which there was a slight increase after 96 hr., but seed showing this characteristic was not used in the assay.

Preparation of the standard

In this connexion a standard similar to that used in the *Striga* investigation has been adopted. It was prepared by growing 20 seedlings of linseed on a muslin float on the surface of distilled water (50 ml.) in a stoppered jar incubated for 5 days at 26°. In certain circumstances the stimulant is highly unstable and fresh preparations were therefore made at frequent intervals.

It is extremely difficult to secure the same growth in twenty seedlings on different occasions and we have reason to believe that our standard varies slightly. Recently, in order to secure greater uniformity the standard has been prepared from the pooled fluids from four jars cultured simultaneously and further work on this aspect is proceeding.

General design of an assay

The assay involves the determination of germination capacity with a series of dilutions of a standard, with a series of dilutions of an experimental fluid, and with water, the experimental fluid normally being one in which the solute concentration is known. A series with water alone is normally included in the assay, not as with *Striga* in order to assess the percentage of independent germination, but as a check on the independence of the drops in the multiple hanging-drop chamber. If the drops creep laterally, then those with stimulant in them should affect those without it. With *Orobanche*, unlike the position with *Striga*, independent germination is rare.

The results of a typical assay are shown in Fig. 3 and Table 1.

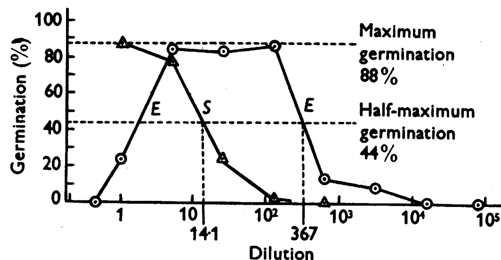

Fig. 3. Results of a single assay. *E* indicates change in percentage germination with dilution of an experimental solution, the R.A. of which is being determined. Figures along abscissa indicate dilution of an original solution. Solution *E* at 1 has a concentration of 1 mg./1 ml. *S* indicates change in percentage germination with dilution of a standard fluid.

Quantitative scales. Two quantities, relative activity (R.A.) and percentage recovery, have been used in this investigation. Relative activity is a measure of the germination-promoting capacity of any preparation, and is defined as the highest dilution of a solution having a concentration 1 mg./ml. which gives half the percentage germination promoted by the undiluted standard. Frequently, the initial concentration of the experimental solution is not 1 mg./ml. and in such cases an appropriate proportionate adjustment is applied to the 'half-value'. The term 'highest dilution' is used, but, as with the case shown in Fig. 3, the undiluted fluid may give negligible germination, and this may first increase and then decrease as the dilution is continued. The results of the assays are plotted and the

Table 1. *Seed counts involved in the assay illustrated by Fig. 3*

Fluid tested	Dilution	Seed counts in each of the five drops No. germinated/Total no. of seeds					Totals	Germination (%)
Water	—	0/25	0/32	0/35	0/29	0/38	0/159	0
Water	—	0/40	0/36	0/27	0/37	0/34	0/174	0
Standard fluid	1*	30/31	27/34	21/27	30/32	31/34	139/158	88·0
	5	21/24	22/25	28/39	32/38	28/43	131/169	77·5
	25	11/21	7/33	6/26	6/25	4/35	34/140	24·3
	125	2/18	0/25	0/36	1/30	0/26	3/135	2·2
	625	0	0	0	0	0	0	0
Extract no. 026 A. at a concentration of 1 mg./ml.	1/2·7†	0	0	0	0	0	0	0
	1*	6/28	17/33	13/35	1/29	0/29	37/154	24·0
	5	34/36	27/31	38/41	28/37	32/42	159/187	85·0
	25	26/30	21/27	17/21	16/18	27/32	107/128	83·6
	125	27/30	27/31	30/38	41/46	41/47	166/192	86·5
	625	13/28	3/38	14/53	3/38	12/35	45/192	23·4
	3,125	3/16	0/20	1/22	1/21	4/26	9/105	8·6
	15,625	0	0	0	0	0	0	0
	78,125	0	0	0	0	0	0	0
	390,625	0	0	0	0	0	0	0

* Undiluted. † Dilution 1/2·7 ≡ concentration of 2·7 mg./ml.

Maximum germination in undiluted fluid, 88 %.

The half-values (the dilution promoting 44 % germination) determined by interpolation arithmetically or graphically: standard fluid, 14·1; extract no. 026 A, 367.

Factor for seed sensitivity = 50/14·1.

'Relative activity' of extract no. 026 A = 367 × 50/14·1 = 1300.

R.A. values found by interpolation. For the data of Fig. 3 the value is 367. This, however, is a crude figure to which a further correction must be applied to correct for the variability of the seed.

Ideally, the assays should be made with a strain of known genetical constitution, and with seed in a constant metabolic state. In this instance, however, these requirements cannot be fulfilled since the seed can only be obtained in sufficient quantity from a wild population and only at one period of the year. During storage, after-ripening changes no doubt occur and further, the conditions during pretreatment have not been completely standardized. As a result of these several uncontrolled variables, the reaction of the seed used in the assay is not constant, so that the same percentage germination with different samples of seed may be given with different concentrations of the stimulant.

The correction is based on the results given by a series of dilutions of the standard fluid, and is again based on the 'half-value'. With the seed of Fig. 3 the half-value is given with a dilution of 14·1, but usually it is given at a dilution of 50, and this value is taken as an arbitrary standard from which all the corrections are calculated. Clearly seed giving a half-value with the standard of 14·1 is

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. *Orobanche 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 *Orobanche 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.

It is a pleasure to record our thanks to Prof. A. R. Todd, F.R.S., for his continued interest and advice throughout this work. We are grateful to the Agricultural Research Council for providing financial support for the development of this investigation at Leeds and our thanks are also due to Glaxo Laboratories Ltd. for a grant to one of us (A. G. L.).

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The Stimulant Involved in the Germination of *Orobanche 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 *Orobanche 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