The Potato Eelworm Hatching Factor

1. THE PREPARATION OF CONCENTRATES OF THE HATCHING FACTOR AND A METHOD OF BIOASSAY

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The potato eelworm, *Heterodera rostochiensis* Wollenweber (Figs. 1 and 4), is a strain or subspecies of the small nematode, *H. schachtii*, and until comparatively recently was generally described under the latter name. Eelworms parasitize a variety of economically important crops and are a serious menace to agriculture in many parts of the world. The parasite was first described by Schacht (1859); it was named *H. schachtii* by Schmidt (1871), who recognized it as a prevalent pest on sugar beet in Germany. In that country, after 1836 when sugar beet first became a farm crop, steadily increasing production without any regard to rotation led to a vast increase in eelworm infestation, with the result that between 1870 and 1880 crop failures became frequent. Only by suitable rotational cropping and intensive cultivation could the pest be brought under control. In England, Massee (1913) described the disease known as 'potato sickness', the symptoms being slow growth, spindly stems and unhealthy foliage, and noted that it seemed to be associated with the presence of eelworms in the soil. Increases in the disease and in eelworm population were rapid in the period of intensive potato cultivation during the 1914-18 war, and potato sickness has now become, in this country, one of the most serious and widespread of potato diseases, having become even more prevalent during the recent world war. It should, perhaps, be mentioned that the severity of potato sickness may also be affected by unknown factors, as it cannot always be directly correlated with the population of potato eelworm cysts (cf. Buckhurst & Fryer, 1930; Ellenby, 1942).

Although *H. schachtii* had been reported as attacking, amongst other plants, beet, potatoes, oats and peas, it was observed that each strain became specialized to a single host and lost its ability to parasitize others. With this specialization came small changes in morphology, so that a subdivision of *H. schachtii* became necessary. The potato strain, characterized by the almost spherical shape of the mature cysts (Fig. 2) as distinct from the lemon-

shaped cysts of the beet strain, was given the name *H. rostochiensis* by Wollenweber (1923).

The life history of *H. rostochiensis* is well known. The larvae hatched from cysts in the soil enter the growing potato plant after some 17 days by drilling a hole through the root wall. There the worms are true parasites until they attain maturity, when the males escape into the soil and the females remain attached to the roots by the head. The body of the fertilized female swells to a white roundish cyst full of undeveloped eggs, gradually becomes brown and then contains several hundred fully formed larvae (Figs. 5 and 6), and drops off into the surrounding soil as a mature cyst. The cysts lie dormant in the soil until stimulated to hatch by the growing of a new crop of potatoes. They can remain dormant but viable for several years, and indeed cases have been reported where cysts were still in part viable after 10 years. The disastrous effect of continued cropping on the same soil is thus readily understood, as is the value of a rotational system with several years between successive potato crops.

The hatching of larvae from cysts of *H. rostochiensis* occurs in response to a specific stimulus due to a secretion from the roots of the host (Fig. 7). This appears to have been first realized by Baunacke (1922); in the absence of the stimulus the cysts remain dormant, and hardly any larvae hatch (Fig. 3). Baunacke tried the effect of various chemical substances as hatching agents and claimed that ferric chloride, potassium permanganate and bleaching powder had some effect. O'Brien & Prentice (1930) showed that potato eelworm cysts could be made to hatch with 'potato root excretion' but not with secretions from beet, rape, mustard or oats. Triffitt (1930) was the first worker to investigate 'potato root excretion'. This author showed that it was non-volatile and reasonably heat resistant, and that it retained its activity for some time in sterile water. Later, Hurst (1935, 1937) attempted to isolate the hatching factor from leachings of soil in which potatoes were growing. By evaporation and ethanol precipitation he obtained powders which brought about hatching at a limiting dilution of

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1/500,000. On very scanty evidence he formed the opinion that the factor was an amino-acid and stated that several amino-acids, in particular tyrosine, showed some activity.

The isolation and structural elucidation of the potato eelworm hatching factor are problems of considerable scientific interest, but might also have very important practical consequences for agriculture. Much work has been devoted to the control of the pest, but no really satisfactory means of eradication has been found. Various chemicals have been used to treat the soil; of these, calcium cyanamide and chloroacetate seem to have shown some promise, but are uneconomic. More recently, dichloropropene-dichloropropane mixtures have been used to control, if not to eradicate, eelworm; such mixtures have been recommended in this country for the control of *H. marioni* in greenhouse plants (cf. Ministry of Agriculture and Fisheries, 1947). Recently, Ellenby (1945) has reported satisfactory results in experiments in which allyl isothiocyanate is applied to soil infested with eelworm; these findings, which parallel the earlier observations of Smedley (1939) with phenyl isothiocyanate, may explain the low incidence of eelworm attack on potatoes grown together with white mustard on infested soil (Morgan, 1925). The main difficulty lies, of course, in the dormancy of the cysts, for until the larvae emerge they are largely protected from attack by many chemical agents. This being so, an obvious alternative would be to apply a hatching factor to infected soil in the absence of potato or other plants capable of being parasitized by the eelworms; the emergent larvae could be allowed to die in the absence of a suitable host, or alternatively, would be readily open to attack by chemical agents. The natural hatching factor might well prove too complex for economic synthesis on the large scale needed if it were to be so used, but if its structure were known it might be hoped that some simpler related substance might have a similar action and be more easily prepared.

The investigations described in the present paper were initiated in 1939 at the London School of Hygiene and Tropical Medicine and at Winches Farm, St Albans, where the production of material was first undertaken with the help of Miss M. Oliver; they were transferred to the University of Manchester at the beginning of 1941 and continued there. At the outset it was clear that the methods employed by Hurst (1935, 1937) on potato root excretion were too cumbersome. Following a suggestion made by Prof. R. T. Leiper, whose assistance in the early stages of the work is gratefully acknowledged, it was decided to use the leachings from growing tomato plants instead of potatoes, since he had found that such leachings were highly active in hatching potato eelworms; moreover, tomatoes have the added

advantage that they are more conveniently grown and handled in bulk indoors. The method finally adopted for preparing crude solid concentrates of the hatching factor is described in detail below. Plants were grown in soil in pots or trays and watered frequently, the leachings being collected. From the leachings, active material was adsorbed on activated charcoal from which it was eluted with aqueous acetone; evaporation of the eluate under reduced pressure gave a brownish solid showing high activity when tested on potato eelworm cysts. The amounts of material obtained were not very large. For example, continuous cultivation of 4000–5000 plants yielded in the course of 1 year some 27 g. of the crude solid mentioned above; this solid had an average Relative Activity (R.A.) of 6.5 (see p. 518) and probably contained less than 2% of the factor itself.

EXPERIMENTAL

Isolation of crude material containing hatching factor

The preparation of hatching factor concentrates can be conveniently divided into four sections: (1) leaching, (2) adsorption, (3) elution and (4) evaporation.

(1) *Growing and leaching of tomato plants.* As production of fruit was not required the plants were kept quite small; constant over watering had in any case a bad effect on their development. Such experiments as were carried out showed that the most active leachings were obtained when the plants were 6–9 in. high with vigorously growing roots. It was thus necessary to keep a good supply of new plants coming along and to discard them after about 3 months, since by that time the yield of hatching factor had fallen off very markedly. Old plants could be 'reconditioned' by cutting off the tops and replanting the roots in fresh soil, but on the whole it was found better to discard them and use new seedlings.

The plants were established under the usual conditions. The seedlings were planted in 3 in. pots containing a suitable tomato-growing compost. After about 6 weeks they were well developed and leaching was begun. To facilitate collection of the leachings the pots were kept on tables with sloping tops made of asbestos sheeting or of boards covered with rubber 'roofing felt'. The tables were usually about 8 ft. long with a slope of 3 in. to one end, and some 4 ft. 6 in. wide with a slope of 3 in. towards a central gutter to carry off the leachings to an enamelled container. A table of this size held 215 plants. Watering was carried out on alternate days using water at the same temperature as the greenhouse. The space of about 1 in. between the earth and the top of each pot was usually filled with water which was allowed to soak in and the process repeated once. From 4000–5000 plants treated in this way, the yield of leachings was about 100 l./day, giving about 500 mg. of crude active solid when worked up as described below.

(2) *Adsorption.* The leachings, which were light orange in colour and had pH about 6.5, were poured without filtration into vessels containing charcoal (British Drug Houses Ltd., 'Activated Charcoal for Decolorizing Purposes' was found most suitable), 1 g. charcoal being used/l. of leachings. The charcoal gradually settled out on standing, and after 1 day the supernatant liquid was siphoned off and discarded. The charcoal adsorbate was collected on a Buchner funnel and

Fig. 1. *Heterodera rostochiensis*. The potato eelworm.
Magnification, $\times 130$.

Fig. 2. Cysts of the potato eelworm.
Magnification, $\times 30$.

Fig. 3. Cysts of the potato eelworm soaking in water;
two worms have hatched. Magnification, $\times 30$.

Fig. 4. Potato eelworms hatched from
cysts. Magnification, $\times 30$.

Fig. 5. A dissected cyst of the potato eelworm; note the immature larvae and a few mature worms. Magnification, $\times 30$.

Fig. 6. Immature larvae and part of a mature eelworm. The typical coiled form of the worm in the larva can be discerned. Magnification, $\times 130$.

Fig. 7. Cysts of the potato eelworm soaking in a solution of hatching factor. Twenty worms have hatched (cf. Fig. 3). Magnification, $\times 30$.

sucked as dry as possible. The loss of activity in this process was probably about 10%. Various other adsorbents were tried and found inferior to charcoal.

(3) *Elution*. The moist charcoal adsorbate was generally collected and eluted in 1.5 kg. batches by stirring with 8 l. of aqueous acetone (70% acetone) in four lots. The eluate was pale yellow; the charcoal recovered could not be used again and was discarded.

(4) *Evaporation*. The filtered eluate was evaporated under a pressure of 15 mm., the temperature being kept below 30° and the evaporation residue finally dried in a desiccator at room temperature over H_2SO_4 . The product was a light brown powder, readily soluble in water, and contained some 60–70% of the active material in the leachings. It formed the starting material for all the investigations here described. During the year 1940, before the concentration procedure had been standardized, a total of 76 g. crude solid of average R.A. 1 was obtained from plants grown at Winches Farm, St Albans. During 1941, 27 g. solid of average R.A. 6.5 was obtained from the same source, together with 9.5 g. of average R.A. 6.2 from the Hawthorndale Laboratories of Imperial Chemical Industries Ltd.; stages (1) and (2) in the concentration were carried out at these places and the charcoal adsorbate was then worked up during 1940 in London and subsequently at Manchester.

Preliminary studies on the crude hatching factor

The crude solid obtained as above appeared to consist largely of a mixture of salts (mainly calcium) of organic acids. As a preliminary to concentration studies, the stability of the factor at various pH values was examined. The results obtained are shown in Table 1. It is evident that the factor, although moderately stable at pH 1–7 (allowing for some latitude in the biological assay values), is extremely rapidly deactivated at pH values above 7, both at 100° and at room temperature. In practice, the active principle in the crude solid frequently appeared to be much less stable than the values in Table 1 would suggest, activity being all too often

other than very small quantity, has throughout seriously hampered chemical investigation.

As the active principle appeared to be acidic, various attempts were made to effect purification by precipitation as a metallic salt. These failed, the salts being uniformly too soluble in ethanol and their preparation being accompanied as a rule by marked loss of activity which was not restored on acidification. The factor was evidently of low molecular weight, since it diffused through a collodion membrane, and no marked concentration was obtained by electrodialysis. The crude solid gave no characteristic colour reactions, and, although it gave a reaction with carbonyl reagents, the active principle itself did not seem to be ketonic, since treatment of a solution with Brady's reagent and removal of the small precipitate of 2:4-dinitrophenylhydrazones had no marked effect on its activity.

BIOASSAY OF THE HATCHING FACTOR

Before serious chemical investigation of the potato eelworm hatching factor could be undertaken it was necessary to devise a reliable bioassay method. The literature revealed no method meeting our requirements, and comparatively little was known of the factors influencing the hatching of cysts. The usual method employed was to place a number of soaked cysts in dishes (e.g. deep solid watch glasses), add root excretion from potatoes and count the larvae produced. Hatching was usually allowed to continue for several weeks. Triffitt (1930) followed this procedure using, as a rule, single dishes at 25°. Franklin (1940) stressed the use of several dishes for each test in order to make possible a statistical examination of results, and drew attention to the unreliability of methods then available. Gemmell (1940) criticized this technique and preferred to study the hatching of single cysts. Using a method of his own, in which about 100 single cysts are treated, he examined cysts obtained from different potato-growing areas and showed them to differ in their hatchability, although they were otherwise alike. In particular, he drew attention to the important fact that, although cysts may appear perfect and be full of embryonated eggs, on test a considerable number always fail to hatch. In Gemmell's experiments hatching was allowed to continue for some months.

For chemical tests, such methods as the above were too slow and unreliable. It was, therefore, decided to carry out a series of investigations aiming at a quantitative method for testing concentrates for hatching activity, which could be carried out in a few days at any time of the year. It seemed possible that this might be achieved, despite the seasonal variation in hatchability, reported by Triffitt (1930), if attention were paid to source and pretreatment of

Table 1. *Stability of hatching factor in crude solid under varying pH conditions*

pH	Half life at 100° (min.)	Half life at 25° (hr.)
1.1	6	Very great
1.8	9	"
2.7	15	"
3.6	15	"
4.8	8	"
5.8	17	"
6.8	8	"
8.1	2.5	1.5
9.1	1.5	—
10.4	0.25	0.15
11.2	0.25	—
12.1	—	0.0

lost for no apparent reason. This instability, coupled with the difficulty of accurate bioassay and the fact that the starting material was difficult to obtain in

eelworm cysts as well as to conditions of hatching. The cysts used were obtained from two sources; sample *A* from eelworm-infested soil in Hertfordshire and sample *B* from similar soil in Chat Moss, Lancashire. The preparation of hatching factor used throughout the experiments, and described as 'Standard Solid', was a sample of crude solid material obtained in early experiments on tomato-root excretion in which 5 g. charcoal were used for each litre of leachings, and the aqueous acetone eluate was evaporated to dryness. Several grams of this product were set aside for use as a standard preparation; under suitable conditions it gave good hatches with viable cysts at dilutions from 1/20,000 to 1/40,000. Sample *A* cysts were on the average smaller and hatched more rapidly than those in sample *B*, although the latter were quite satisfactory to work with, and were otherwise very similar to those of sample *A* in behaviour. The specific objective of the experiments carried out was a simple test method, and not an exhaustive study of all the important factors in cyst hatching.

hatching. Excluding cysts which failed to hatch at all, larval counts varied from 1 to 195, while the standard deviation of individual results was considerably greater than the mean.

Sample B cysts. These cysts were isolated by the usual flotation method from soil obtained from Chat Moss, Lancashire, through the kindness of Dr R. Stewart and Dr V. E. Henderson of Manchester University. Collection was made in August 1941, from a field in which potatoes were growing (the fourth successive crop), so that the cysts were probably at least a year old. As they varied considerably in size, the cysts were graded into three groups by sieving, approximate numbers/sack of soil being: (1) held by 40 mesh sieve, 22,000; (2) held by 60, passed by 40, mesh sieve, 60,000; (3) held by 100, passed by 60, mesh sieve, 60,000. The cysts obtained were stored at about 23° in a desiccator over saturated K₂CO₃ solution giving a relative humidity of some 50%. The intermediate group (2) was selected for general use and is described as sample *B*. As the technique of sorting previously used was rather tedious, the following method was adopted. The cysts were placed in a dish of water, stirred thoroughly and left for 2 days, after which time those that floated were rejected. Those which sank were sorted under

Table 2. *Hatching of single cysts from Sample A*

	Exp. 1	Exp. 2
	45 cysts treated with 1 ml. Standard Solid, 1/40,000 in tap water, for 3 days at 24°	45 cysts treated with 1 ml. Standard Solid, 1/20,000 in tap water, for 3 days at 24°
Hatches ...	11, 8, 0, 11, 10, 9, 27, 33, 1, 10, 0, 1, 0, 16, 3, 13, 0, 34, 7, 1, 0, 0, 0, 1, 0, 21, 0, 2, 0, 1, 7, 36, 1, 34, 46, 2, 4, 0, 1, 2, 1, 51, 0, 1, 0	13, 73, 63, 15, 0, 1, 0, 0, 0, 12, 38, 1, 1, 19, 96, 4, 23, 0, 24, 41, 21, 46, 50, 24, 26, 16, 13, 1, 64, 1, 74, 25, 70, 0, 20, 195, 0, 1, 54, 0, 7, 34, 32, 0, 5
Total hatch	406	1203
s.d. among individual results	14	36
Mean hatch	9	27
s.e. of mean	±2.1	±5
Hatching no larvae (%)	29	20
Hatching only 1 larva (%)	20	13
s.e. of mean hatch on the basis of a count of 80 cysts	1.5	4
	(=17% of mean hatch)	(=15% of mean hatch)

Sample A cysts. The soil was supplied by the late Mr A. C. W. MacDonald of Winches Farm, St Albans, and was collected in the autumn so that most of the cysts were probably young. The cysts were isolated from the soil by a flotation method followed by rolling off on a Bristol board, as described by Fenwick (1940), and were stored at room temperature in a closed jar. When required for use, batches of cysts were soaked for 2-4 weeks in water at 24° and then sorted under a dissecting microscope by Miss M. Oliver. Viable cysts were selected by their appearance in conjunction with the results of dissections of small numbers of samples for this purpose, and were kept in water at 24° until used, except in experiments dealing with the effect of temperature of storage.

The hatching of single cysts was examined as described by Gemmell (1940), and the results of two experiments are given in Table 2.

From these results it is evident that not only did many apparently normal cysts fail to hatch, but there were also great variations in the rate of

a large lens in a strong light, when viable cysts could be readily distinguished by their uniform light brown colour and resistance to pressure with a blunt glass needle. The selected cysts were normally kept in water at 22° and used after about 3 weeks.

Table 3. *Hatching of single cysts from sample B*

(25 cysts treated with 1 ml. Standard Solid, 1/30,000 in tap water, for 3 days at 23°.)

Hatches ...	3, 0, 0, 15, 0, 7, 7, 7, 0, 4, 4, 4, 49, 5, 17, 0, 0, 0, 0, 0, 43, 5, 4, 0
Total hatch	174
s.d.	13
Mean hatch	7 ± 2.6
Hatching no larva (%)	44

On the basis of a count on 80 cysts, the s.e. of the mean would be 1.5, or ±21% of the mean hatch.

From Table 3 it is clear that the behaviour of sample *B* cysts is rather similar to that of sample *A*, and that the variable distribution of good and inert cysts among the dishes makes a high standard deviation inevitable.

It was noticed in the course of sorting the Chat Moss cysts that in the case of the smaller cysts (groups 2 and 3) approximately one-third appeared viable, whereas the proportion of good cysts in group 1 was much less and nearly 90 % were empty. It is of interest to note that a quantity of soil obtained from the same field 8 months later (after the potato crop had been harvested) was found to contain some 70 % more cysts/sack and there were virtually no large cysts present; the proportion of viable cysts was about the same as in sample *B*.

Earlier workers (e.g. Triffitt, 1930) have reported marked seasonal variation in ease of hatching. To check this, a portion of sample *B* cysts was set aside over saturated potassium carbonate under the roof tiles during the autumn and winter of 1941-2, when they were exposed to considerable variations in temperature, frost and snow being frequent. At intervals of about 1 month, samples were removed, sorted as above described and the viable ones soaked for 3 weeks at 22° and tested for hatchability by incubating in groups of 15 for 3 days at 23° with 1 ml. of Standard Solid solution (1/40,000). Simultaneous tests were made on samples from a second batch stored continuously at 23°. In both batches it was observed that cysts hatched fairly well (about 150 larvae from 15 cysts) in August, but this gradually fell until, by the end of November, very low hatches (10-20 from 15 cysts) were obtained. Hatching remained very difficult throughout December and January, and began to improve again thereafter. Since it was shown by both batches of cysts, the variation in hatchability is evidently seasonal and not due simply to temperature of storage. In later work on the eelworm hatching factor we have always observed this effect and, indeed, great difficulty has been encountered in doing any reliable testing of concentrates during the winter months.

In the above experiments, cysts were usually soaked in water for at least 3 weeks before a hatching test was made. This practice was adopted as a result of experiments on the effect of soaking on hatching. A batch of viable sample *B* cysts was placed in water, and samples were removed and tested with Standard

Solid solution (1/30,000) from time to time. The results are given in Table 4. The actual hatches are low as the experiment was made in January when cysts are rather difficult to hatch, but the effect of soaking is evident.

Table 4. *Effect of soaking cysts*

Period of soaking (days)	Mean hatch/dish of 20 cysts (3 days at 23°)
2	0
8	1
15	12
18	12
21	71
23	53

The effect of temperature on hatching and on soaking was examined in other experiments. In one of these, sample *A* cysts were kept at 24° for some weeks and then incubated at various temperatures with Standard Solid (1/30,000). The results are shown in Table 5. In the experiment leading to the results in Table 6, sample *A* cysts were sorted after 3 days soaking, and batches were then kept soaking for 11 days at various temperatures before hatching in the usual manner. Although these experiments are only very rough (their extension, although desirable, was rather beyond the scope of our present researches), the importance of temperature emerges quite clearly.

Table 5. *Effect of temperature on hatching*

Temperature during hatching	Mean hatch/dish (15 cysts; 3 days)
6°	2
Room temp. (about 15°)	16
24°	608
37°	7

Table 6. *Effect of temperature of soaking*

Temperature of soaking	Mean hatch/dish (15 cysts; 3 days at 24°)
6°	4
Room temp. (about 15°)	270
24°	680

For setting up a rapid testing method experiments were carried out to determine the time required to give a suitable hatch and maximum reproducibility. The results of a typical experiment are summarized in Table 7.

Table 7. *Mean hatch per dish*

Dish no. ...	Mean hatch per dish																Standard deviation	Mean hatch
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16		
Day 1	111	48	29	18	125	61	51	87	33	6	85	80	142	75	31	135	42	70±10.5
2	239	230	160	70	316	236	251	341	250	75	255	275	325	315	145	350	85	233±21
3	274	325	210	104	420	285	346	540	305	125	356	316	431	403	207	463	120	319±30
4	340	263	206	119	415	287	300	552	333	152	385	377	425	365	227	570	127	332±32

Table 8. *Calculation of standard error of means using different numbers of dishes*

Day	Using 6 dishes of 15 cysts	Using 4 dishes of 15 cysts
1	17.1 or $\pm 24\%$ of hatch	21 or $\pm 30\%$ of hatch
2	34.7 or $\pm 15\%$ " "	42.5 or $\pm 18\%$ " "
3	49 or $\pm 15\%$ " "	60 or $\pm 19\%$ " "
4	52 or $\pm 16\%$ " "	63.5 or $\pm 19\%$ " "

In this experiment 16 dishes were prepared each containing 15 cysts (sample A) to which 1 ml. Standard Solid solution was added. The dishes were incubated at 23° and counts made after 1, 2, 3 and 4 days. Table 7 shows the hatches obtained in each dish together with the standard deviations of the means.

In Table 8 the results have been further subdivided to give standard deviations calculated for groups of six or four dishes. It will be seen that hatching had practically ceased by the third day and that the standard deviation had fallen to a steady proportion, about 10% for 16 dishes, by the second day. With six or four dishes the standard deviations were 15% or 18–19%. Although it was convenient to use conditions where the number of larvae were from 180 to 200/dish, Table 3 shows that the standard errors of means were similar when the counts were much lower. From the results given it will be seen that with three to four dishes containing a total of 45–100 cysts standard deviations of about 20% are to be expected. General experience confirms this. For the purpose of a reproducible test it is the number of cysts used that is important, and these should be distributed among three to four dishes. Experiments showed that increasing the volume of solution above 1 ml. had little effect, and 1 ml. was usually used with 15–30 cysts/dish.

Fig. 8. Effect of hatching factor concentration on larval hatch.

A number of experiments was made on the effect of concentration of hatching factor on the hatching of cysts. In each case it was found that the number of larvae hatched was proportional to the concentration of factor up to a certain concentration,

above which the hatching decreased. The result of one such experiment is shown graphically in Fig. 8. In the testing method which was finally adopted (see below) tests were always carried out on the first part of the curve, where concentrations are below those giving the maximum hatch, and it was assumed that the number of larvae which emerged was proportional to the concentration of the factor. It should, however, be noted that, on account of the high standard deviations usually obtained in proportion to the means, the hatch is a rather crude measure, even where a large number of dishes is used in each test (cf. Table 7). It has, nevertheless, served as the basis of the test method which we have used throughout the work on the eelworm hatching factor described in this and succeeding papers. The testing procedure is given in detail below.

Testing of materials for hatching activity

The practice adopted has been to compare the activity of unknown materials with a standard preparation. For this purpose a large sample of crude solid from tomato root leachings was set aside at an early stage in the investigations. This material was obtained by evaporating the eluate from a charcoal adsorbate prepared, using 5 g. charcoal/l. of leachings and is the material described as Standard Solid throughout the present paper. It gave good hatches from viable cysts at dilutions of 1/20,000 to 1/40,000 and was allotted a Relative Activity (R.A.) of 1.

Tests were carried out as follows: a suitable number of cysts (usually 20), previously sorted and kept in water for 3–4 weeks at 23°, was counted into each dish (deep solid watch glasses are convenient) using a Pasteur pipette; the water was drained off with a fine pipette and replaced by 1 ml. of the active solution. The dishes were now covered with squares of glass and incubated at 23–34° for 2–3 days, by which time 50–200 larvae had emerged in each dish. Counting was carried out directly under a low-power microscope. Never less than three dishes were used and where possible four or five were preferred both for the unknown and the standard.

It occasionally happened that single dishes showed counts markedly different from the others in the same group; it was customary to reject any count which was greater than five times, or less than one-fifth of, the other counts. Experience showed that the mean values were reasonably reliable, and when repeat estimations were carried out very similar results were usually obtained. After a test was completed, the cysts could be kept for a few days in water and used again; after several tests they tend to become mouldy and should then be discarded.

To estimate the activity of an unknown substance, it was dissolved in water at a suitable dilution and applied to one group of cysts, while to a control group a solution of Standard Solid at, say, 1/40,000 dilution

was added. After 2–3 days incubation, the emerged larvae were counted and from the result the R.A. of the unknown substance calculated.

In a typical experiment, an active concentrate was diluted to 1/2,000,000 and compared with Standard Solid at 1/40,000 with the following result:

Standard 1/40,000: hatches (2 days, 15 cysts/dish)—144, 57, 112, 150; mean 116.

Unknown 1/2,000,000: hatches (2 days, 15 cysts/dish)—165, 89, 163, 120; mean 134.

From the result the unknown substance had R.A.

$$\frac{2,000,000 \times 134}{40,000 \times 115} = 58.$$

It will be observed that a dilution was chosen (by trial and error) for the unknown substance which gave about the same hatch as the control. In these circumstances the importance of the ratio concentration/hatch was diminished, since two solutions giving the same hatch would be expected to contain the same concentration of factor.

In the past, workers have usually stated the maximum dilution at which materials show any activity. This is a very unsatisfactory method of expressing activity, partly because cysts vary in sensitivity and partly because it is difficult to decide the point at which hatching due to active material becomes significant, since even in water a few worms may hatch out. The test method we have described is easy to carry out and gives results quickly.

Experience in many trials has shown that it is fairly reliable, and occasions on which it has given misleading results have been very few. Its accuracy is not very high, but the great individual variation among eelworm cysts makes it difficult to achieve precision in assay. In the work described in this and succeeding papers we have employed mainly cysts from Chat Moss, Lancashire, and from Cambridgeshire (for which we are indebted to Messrs F. G. W. Jones and F. R. Petherbridge); we have also used successfully cysts isolated from soil in the Newcastle area obtained through the kindness of Dr C. Ellenby.

SUMMARY

1. A method is described for the preparation of crude solid concentrates of the potato eelworm hatching factor by leaching the roots of growing tomato plants with water, adsorbing the factor on charcoal and eluting with aqueous acetone.

2. The effect of various conditions on the hatching of eelworm cysts has been studied, and a reasonably reliable bioassay method for the hatching factor has been worked out.

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