The Hatching Factor of the Potato-root Eelworm

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1. The hatching factor of the potato-root eelworm was concentrated from potato-root leachings by adsorption on charcoal. The crude material, extracted from charcoal with acetone, was purified by partition between ethyl acetate and *m*-potassium dihydrogen phosphate, and ethyl acetate and a solution of potassium metabisulphite (disulphite). Neutral material was removed by extraction from phosphate buffer at pH 7.8. The yield of purified material, after chromatography on silica, DEAE-cellulose and Perlon columns, was 18 μ g./l. of potato-root leachings, and 0.04% by weight of the acetone-soluble material extracted from charcoal; 20% of the activity of the original potato-root leachings was recovered. 2. The purified material was a colourless gum. On paper chromatograms it gave a single weakly fluorescing spot, which reacted with 2,4-dinitrophenylhydrazine and aniline-xylose reagents. It was confirmed that the hatching factor is an acid, and it is inactivated at values above pH 9. Other properties were a ready oxidation by permanganate and reaction with certain carbonyl reagents. Colour tests for α - and β -unsaturated γ -lactones were negative. The hatching factor showed no light-absorption maxima at wavelengths above 210 $m\mu$; absorption bands in the infrared region (670–4000 cm^{-1}) are given. Our purified material, although a gum, gave a similar hatch to a 'crystalline' product at 640 times its concentration.

The potato-root eelworm (*Heterodera rostochiensis* Wollenweber) is stimulated to hatch from its cysts by diffusates from the roots of potato (*Solanum tuberosum* L.), tomato (*Lycopersicon esculentum* Mill.) and many other solanaceous plants. Williams (1958) found evidence of activity with plants of the genera *Atropa*, *Browallia*, *Datura*, *Nicotiana* and *Celsia*, but only the genus *Solanum* consistently gave diffusates of high activity. Extracts from all parts of potato plants, except the tubers (Janzen & van der Tuin, 1956; Carroll, Heyes, Johnson & Todd, 1958), also stimulate hatching.

In previous attempts to isolate the active substance the hatching factor in root leachings was concentrated by adsorption on charcoal or on ion-exchange columns, or by evaporation *in vacuo*. Although various methods have been tried for the purification of crude concentrates no satisfactory procedure has yet been reported.

Properties of various preparations of the hatching factor are described by Marrian, Russell, Todd & Waring (1949), Widdowson & Wiltshire (1958) and Hartwell, Dahlstrom & Neal (1960).

MATERIALS AND METHODS

Cyst stocks. *Heterodera rostochiensis* cysts from various sources were used. They were collected in the autumn and

were stored dry for up to a year, in the dark and at room temperature.

Assay procedure. The cyst-hatching technique used for the bioassay was that of Fenwick & Widdowson (1958). Tests were usually continued for 3 weeks, but the larvae were removed weekly and counted after 1 and 3 weeks. Each set of samples for assay included the standard root-diffusate solution at six dilutions (1/1, 1/4, 1/16, 1/64, 1/256, 1/1024) and the test samples at a minimum of three dilutions (usually 1/4, 1/16, 1/64). Batches of 100 cysts in fivefold replication were used for each dilution, unless otherwise stated. The cumulative larval hatch from each set of replicates was plotted against a logarithmic dilution scale. A straight line was fitted by eye to the linear part of the curve and projected to meet the line for hatch in water. The point of intersection of these lines gave, on the log scale, a relative measure of the hatching factor present, i.e. log activity (L.A. value) of the solution (Fenwick, 1952).

Widdowson & Wiltshire (1958) related concentrations of active material in samples in terms of specific activities (sp. activity = antilog L.A./mg. dry wt./l.).

As far as possible, the concentration of the test samples was arranged to give levels of hatching within the linear part of the standard curve (larval emergence/dilution).

When few larvae emerged, the hatching curve was shallow and sloped, which made the interpretation of the tests more difficult. Improved hatching curves were obtained in these circumstances by increasing the duration of the tests and counting the larvae after immersion in the test solutions for 2 and 4 weeks.

A simplified assay, in which samples were used at a single dilution and the tests lasted for 1 week only, was

used when testing many samples, e.g. fractions from chromatographic columns.

Assay standards. (i) Potato-root leachings, collected in spring, were stored at 3° in the dark in a 50l. glass carboy, standardized (Fenwick, 1952) against the appropriate cyst population and then used as the reference standard for all hatching tests during the succeeding 12 months.

(ii) A solution of the factor in acetone was prepared with material obtained by treating potato-root leachings (40l.) with charcoal, and purifying the extracts by the procedure given. This concentrate, dissolved in acetone (40ml.) and stored at 0–3° in the dark, was used as an additional standard in assay tests (diluted 1/500 in water), for 6 consecutive weeks and at 14 weeks (seven tests in all). The diffusate before treatment had L.A. 2.5 and the acetone solution (40ml.) assayed at 0.05 ml./50 ml. of water, had L.A. 2.1, a recovery of approximately 40% of the original activity.

Silica columns. Silica columns were prepared with silica (A.R. grade, Mallinckrodt Chemical Works, New York, N.Y., U.S.A.) triturated with the appropriate buffer (0.3 ml./0.5 g. of silica). The treated silica was made into a slurry with the initial eluting solvent, poured into a chromatography tube (1.2 cm. diam.) and the excess of solvent run off before use.

Ion-exchange materials. (a) A column (54 cm. $\times$ 4.0 cm.) of De-Acidite FF (–16 $\times$ 50 mesh) was used when treating large volumes of potato-root leachings. Before use the column was washed with 3% NaOH (800 ml.), then water (1.5 l.), followed by 2N-acetic acid (800 ml.) and water (2.0 l.). (b) DEAE-cellulose powder (240 g.) was washed with 0.2N-NaOH (5 l.) and then with water until the eluate was neutral. (c) Small-scale columns. A column of DEAE-cellulose (8 g.; 24 cm. $\times$ 2 cm.) in the formate form was prepared by washing the powder first with 0.1N-NaOH (200 ml.), then with water until the eluate was neutral. A 4N-formic acid solution (500 ml.) in acetone–water (1:1, v/v) was passed through the column, followed by water until the eluate was neutral. Finally, the column was washed with acetone–water (1:1, v/v). Other DEAE-cellulose columns in the formate form were prepared similarly.

Perlon columns. Columns of Perlon powder (20 g.; 18.0 cm. $\times$ 2.2 cm.) were prepared for use by washing with 0.1N-NaOH (200 ml.), then water until a neutral eluate was obtained, followed by 0.1N-HCl (200 ml.) and water until free from acid. The Perlon (polycaprolactam) powder (Farbwerke Hoechst, Werk Bobingen, Germany) was a gift from Dr H. G. C. King, Pedology Department, Rothamsted.

Potato-root leachings. Potatoes (*Solanum tuberosum* L., variety Arran Banner) were grown in sandy loam in 6½ in. (top diam.) flower pots, some in glasshouses and some in the open. The pots were kept on sloping asbestos-topped benches, and leached by first saturating the soil with tap water and then adding more (50–100 ml.). Leaching was started within 7 days of the shoots emerging and was repeated on alternate days for 6 weeks. Leachings were also obtained from potato plants (about 40 per bed, 2.5 m. $\times$ 1.1 m. $\times$ 26 cm.) grown in soil over a layer of shingle on asbestos-topped benches. The beds were leached on alternate days by spraying with water until 10l. of leachings was collected. With both methods the leachings drained into channels on the benches and were collected in pails and then transferred to 50 l. glass carboys.

Preparations of crude concentrations of the factor. Of the

three methods mainly used as the first treatment of the potato-root leachings, adsorption of the active material on charcoal was better than concentration by evaporation or adsorption on ion-exchange materials.

(a) Adsorption on charcoal. To the yellow–brown leachings (40 l.) were added conc. HCl (30 ml.) and decolorizing charcoal (120 g.). The liquid was stirred thoroughly and left to stand for a day, when the clear colourless supernatant liquid was poured away. The charcoal was resuspended in the 2–3 l. of remaining liquid, and the suspension filtered on a Buchner funnel. The charcoal was transferred to a beaker where it was washed with successive portions (4 $\times$ 300 ml.) of acidified acetone (0.4 ml. of conc. HCl/100 ml. of acetone). At each washing the charcoal was stirred with the solvent to get a uniform suspension, which was then filtered. The combined washings were evaporated to dryness at 10–20 mm. pressure in a rotating evaporator with an external bath temperature of 34–40°, to leave a mixture of organic and inorganic material (0.6–2.0 g.).

(b) Concentration by evaporation. Potato-root leachings (20–26 l.) were acidified with acetic acid (1 ml./l.) and concentrated to 50/75 ml. at 30–40° and 10–20 mm. pressure. The pH was kept at about 5 by adding further acetic acid. The sludge obtained was brought to pH 2 with conc. HCl, and extracted with ethyl acetate (40 ml., 12 $\times$ 20 ml.). Evaporation of the extracts *in vacuo* left a gum (0.3–0.8 g.).

(c) Adsorption on De-Acidite FF. Potato-root leachings (20 l.) were run through a column of De-Acidite FF (54 cm. $\times$ 4 cm.) in the acetate form. The column was then washed with water (2 bed vol.), allowed to drain and eluted with a mixture of acetone, water and conc. HCl (700 ml.:300 ml.:35 ml.), which gave a golden-yellow eluate. Elution was continued with acetone and water containing conc. HCl (700 ml.:300 ml.:9 ml.) until the eluate was colourless. The combined eluates yielded a gum (0.5–2.0 g.) when evaporated *in vacuo*.

(d) Adsorption on DEAE-cellulose. DEAE-cellulose (240 g.) in the free base form was added to potato-root leachings (40 l.) containing acetic acid (100 ml.). The mixture was stirred for 30 min. and the DEAE-cellulose recovered by filtration. The DEAE-cellulose was shaken with a mixture of acetone (700 ml.), water (300 ml.) and conc. HCl (40 ml.). The resulting slurry was poured into a chromatography tube, and the solvent mixture run off. The column was further eluted with acetone–water (7:3, v/v; 1 l.). The combined eluates yielded a gum (0.5–1.5 g.) on evaporation *in vacuo*.

Purification of the crude concentrates. The residues obtained by the above procedures were submitted to the purification procedure outlined in Scheme 1.

All extractions were made in 100 ml. centrifuge bottles so that any emulsions that formed could be broken by centrifugation. As the hatching factor is readily destroyed by alkali, solutions were kept below pH 8. Solvents were removed by evaporation *in vacuo* in a rotary evaporator with an external bath temperature of 30°. Active material was stored in acetone solution at –10°.

The crude residue (4–14 g.) was shaken with ethyl acetate (20 ml.) and m-KH₂PO₄ (15 ml.). The upper, ethyl acetate, phase was removed, and extraction continued with fresh portions of ethyl acetate (12 $\times$ 20 ml.). After removal of solvent from the combined extracts, the residue (A) was taken up in ethyl acetate (20 ml.) and the solution shaken with potassium metabisulphite (4 ml. of saturated

solution, 4 ml. of water). The ethyl acetate layer was removed and the aqueous phase extracted with more ethyl acetate (12 × 20 ml.). Removal of ethyl acetate left a resin (B) which was treated with 0.57 M-K₂HPO₄ (20 ml.). The pH of the solution was adjusted to pH 7.8–8.0 by adding 5 M-K₂HPO₄ to the stirred liquid. The solution was extracted with ether (5 × 20 ml.), then ether–ethyl acetate (1:1, v/v; 8 × 20 ml.). The residual aqueous phase was brought to pH 2 with conc. HCl and the active material extracted with ethyl acetate (12 × 20 ml.). Evaporation of the extracts left a yellow gum (C) which was further purified by chromatography.

A solution of the resin (C) in acetone (100 ml.) was run through a DEAE-cellulose (formate form) column (16 g.; 17 cm. × 4.0 cm.). Elution with 1 mM-formic acid in acetone was continued until the yellow eluate gave way to colourless fractions (total volume of eluate 1800 ml.). Yellow–brown impurity was left adsorbed on the column, whereas most of the hatching factor was contained in the acetone eluate. Further elution of the column with 0.5 N-formic acid in acetone brought off a small amount of a more impure but active material. Overloading of the column showed either by adsorbed yellow–brown material occurring throughout the column, or, with too much inorganic acid, by the upper part of the column failing to retain yellow–brown material; when either happened, the 1 mM-formic acid eluate was evaporated to dryness and the process repeated with a fresh column.

The acetone solution was evaporated to dryness and the residue (D) dissolved in chloroform (20 ml.). Silica (1.0 g. treated with phosphate buffer, pH 2; 0.6 ml.) was added to the chloroform solution, and the solvent removed *in vacuo*. The yellow powder of gum adsorbed on silica was thoroughly mixed and added to the top of a column (3 cm. × 1 cm.) of silica (2 g. treated with phosphate buffer, pH 2; 1.2 ml.) made up with light petroleum (b.p. 60–80°)–chloroform (2:1, v/v). The residues of the gum adsorbed on silica were treated with chloroform (20 ml.) and with more buffered silica (0.2 g.). The solvent was removed and the powder added to the top of the column.

The column was eluted successively with the following mixtures (v/v) of light petroleum (b.p. 60–80°) and chloroform: 2:1 (30 ml.), 1:1 (30 ml.), 2:3 (30 ml.), and then with chloroform (200 ml.). Each eluent brought off a yellow fraction. A yellow band remaining on the column after elution with chloroform was removed with ethyl acetate. The bulk of the hatching factor was in the chloroform eluate, but there were small amounts in the 1:1, 2:3 and ethyl acetate eluates.

Evaporation of the chloroform eluate left a clear pale-yellow gum (E), which was triturated with successive portions of diethyl ether (10 × 20 ml.). Most of the hatching factor passed into solution. The ethereal extracts were run through a short column of silica (1 g.; treated with m-KH₂PO₄, 0.6 ml.) and followed by a 20 ml. of ether. The pale-yellow eluate was active and some yellow–brown impurity was retained on the column.

The ether eluate was evaporated and the residue taken up in sodium-dried ether (100 ml.) and filtered to separate the soluble active material from small amounts of inorganic impurity (see below). After removal of ether the residue (F) was added to DEAE-cellulose (formate form) column (8 g.; 21 cm. × 2.2 cm.) in successive portions of acetone–water (1:1, v/v; 10 × 10 ml.). Twelve 10 ml. fractions of

1:1 eluate were first collected, and then 40 × 10 ml. with 1 mM-formic acid (in acetone–water, 1:1, v/v). The hatching factor was distributed through the 1 mM-formic acid fractions and further elution with more concentrated formic acid brought off only very little more.

It was important with the above column of DEAE-cellulose (a) that it initially was free from formic acid and (b) that material applied to it was free from traces of mineral acid, phosphate buffer etc. Otherwise the initial eluate of acetone–water (1:1, v/v) contained active material, as it did when the acidic impurities eluted with acetone–water were excessive. Fractions from each elution were therefore assayed. Some indication of the course of the elution of impurities, particularly of those eluted with acetone–water (1:1, v/v), was obtained by reading the light-absorption of the various fractions at 333 mμ against water. Absorption at this wavelength by the hatching factor is negligible.

The most active fractions of the 1 mM-formic acid eluate were combined and evaporated to dryness. The small amount of gum (G) left was treated seven times with 10 ml. of water and each 10 ml. portion was run on to a Perlon column (20 g.; 18.0 cm. × 2.2 cm.) made up with water. After the seven 10 ml. fractions had been collected, the column was eluted with acetone–water (1:9, v/v) and 20 × 10 ml. fractions were collected. Subsequent elution with acetone brought off inactive impurities. The active material was present in the acetone–water eluates. As with the eluates from the DEAE-cellulose column, the presence of active material in the fractions was detected by hatching tests, and elution of impurities could be followed from the absorption of the fractions at 333 mμ (read against water). As before, fractions were combined according to their hatching activity. Evaporation of these eluates gave a colourless gum (H), which was immediately dissolved in either ether or acetone. This purified material was used as a starting material for chemical investigations.

Samples were taken for assay at stages (A), (B) and (C), and diluted so that the concentration was directly related to that of the parent root diffusate. Thus where the material was derived from 40 l. of root diffusate, a 0.05 ml. sample would be taken from a 40 ml. acetone solution of (A), (B) or (C), and made up to 50 ml. with water after neutralization with a dilute solution of calcium hydroxide. The complete assay for a batch of root diffusate included the parent root diffusate, the filtrate after charcoal adsorption, and samples (A), (B) and (C). Purification of the product after stage (C) was followed in separate bioassays. All samples obtained from fractions from a chromatographic column were assayed simultaneously, together with a sample of the starting material.

Paper chromatography. A descending one-dimensional technique was used with Whatman no. 1 paper. Solvent systems used were: (a) 0.1 M-KH₂PO₄; (b) phenol–water (4:1, w/v) (as used by Massey & Neal, 1953); (c) m-NH₄Cl; (d) acetone–water (1:19, v/v) (Widdowson & Wiltshire, 1958); (e) butanan-2-one–water (3:50, v/v); (f) butan-1-ol–90% formic acid–water (10:3:10, by vol.; upper phase); (g) chloroform–2-methylbutan-2-ol–90% formic acid–water (136:24:3:80, by vol.; upper, i.e. aqueous, phase). Material purified to stage (C) from subsequent stages was made up in acetone solution (2–6 mg./ml.), and applied (11 × 0.005 ml.) to Whatman no. 1 paper. Development of the chromatograms was stopped when the solvent front had travelled

Scheme 1. Flow sheet of the purification procedure.

12 in. from the point of application of the sample. After drying at room temperature, the paper was cut into strips of equal R_F width (0.05 R_F unit), and each strip placed in a flask containing water (50 ml.). The flask was shaken occasionally during the next 30 min., and the solution then assayed in three- to five-fold replication at three dilutions. Paper chromatograms were examined for fluorescent material under ultraviolet light (Hanovia Ltd.) model 18 lamp. Other means of detection used were Brady's reagent, and for chromatograms developed with solvent systems (b), (d) and (g), the aniline-xylose reagent of Wood (1958).

RESULTS

Concentration of the hatching factor. Most of the active material in acidified potato-root leachings was removed by charcoal (3 g./l.) and 31% was recovered from the charcoal in the acetone extract. Various other methods of treating the charcoal, such as hot extraction with acetone, elution with methanol or acetone-water mixtures, did not increase the recovery. A yield of 20% (average of ten experiments) of the activity of the original potato-root leachings was obtained by concentration by direct evaporation. Passage through a column of De-Acidite FF (acetate form) removed 95% of the activity from the potato-root leachings, and 23% (average of ten experiments) of the initial activity of the leachings was recovered from the column in the acid eluates. However, to do this required repeated elution with acid because the amount of hatching activity released tailed off very gradually.

Treatment of potato-root leachings with DEAE-cellulose absorbed only about 55% of the hatching factor. The amount recovered in the acetone eluate of the cellulose was 10% (average of four experiments) of the initial; the eluate end-point was much sharper than with De-Acidite FF.

Purification of crude concentrations. Ethyl acetate extracts hatching factor from aqueous solutions provided that these are acid and contain salt. In treatment of the active brown gum obtained by evaporating the acetone washings from charcoal, as shown in Scheme 1 to reach product (C), 90% of the material in the original gum was eliminated while retaining 85% of the hatching activity. The further procedures to get from product (C) to product (H) eliminated more than 98% of the material in product (C) while retaining about 75% of its hatching activity. In one experiment done with 280 l. of potato-root leachings the residue obtained on evaporating the acetone washings of the charcoal weighed 12.7 g., and products (A), (B) and (C) 4.6, 2.2 and 1.3 g. The weights of later stages were 249 mg. (D), 84 mg. (E), 70 mg. (F), 32 mg. (G), 5 mg. (H). Hence the yield of the final product (H), which had 20% of the hatching

activity of the total initial activity, was equivalent to 18 μ g./l. of the starting material.

Properties of the hatching factor. The hatching factor dissolved readily in acetone, ethyl acetate, methanol or ethanol, less readily in ether, chloroform and water, and was insoluble in light petroleum, benzene or carbon tetrachloride. Our work confirmed that it was an acid and was rapidly inactivated at values above pH 9.

A solution of the hatching factor in ethanol (5 mg./100 ml.) gave a smooth light-absorption curve devoid of maxima at wavelengths greater than 210 m μ . Maxima did not appear when the ethanolic solution was made alkaline or acid, with enough potassium hydroxide or concentrated hydrochloric acid to give 0.1 N solutions. The infrared spectrum of the hatching factor dissolved in chloroform (8 mg./0.2 ml.) showed bands at 2920 (s), 2850 (s), 1712 (s), 1610 (w), 1468 (w), 1379 (m), 1162 (m), 1038 (w) cm.⁻¹.

Each of the following tests was made with 1 mg. of hatching factor, where applicable with procedures given by Cheronis (1954). The hatching factor reacted rapidly with 2% potassium permanganate solution, but caused little or no discoloration of bromine-water. It gave a positive iodic acid test. When mixed with a saturated solution of 2,4-dinitrophenylhydrazine in 2 N-hydrochloric acid, a solution of the hatching factor became turbid after 5 min., and slowly deposited a slight orange-yellow precipitate. Dimedone did not produce a precipitate. The factor reacted with 2,3,5-triphenyltetrazolium bromide, and Benedict's and Tollens's reagents, but not with Molisch's and Schiff's. It slightly changed the colour (from yellow to a light tan) of alcoholic ferric chloride (cf. Marrian *et al.* 1949). A chloroform solution of the hatching factor gave no coloration with tetranitromethane. It gave a positive iodoform test, and gave a red solution (light-pink on dilution) in a Zimmermann test (Corker, Norymberski & Thow, 1962). Tests for α - and β -unsaturated γ -lactones with sodium nitroprusside and 2,6-dichlorophenol-indophenol (Kuehl, Linstead & Orkin, 1950) were negative, as was the test with ceric nitrate reagent. Treatment with aqueous sodium nitrite, which gives a purple colour with α -unsubstituted tetric acids (Haynes & Plimmer, 1960), gave a pale-yellow solution.

Paper chromatography. Solvent systems were limited to acid and neutral material because the hatching factor is unstable in alkali. Polar systems were needed for the hatching factor to run as a discrete spot. The R_F values of the hatching factor were obtained by plotting the R_F values of the strips (0.05 R_F unit in width) against the number of larvae hatched by the washings.

The following results (expressed as the cumulative larval hatch for 100 cysts over a 3-week period,

for successive strips from R_f 0.65 to 0.95) were obtained with solvent system (c): 48, 50, 142, 875, 721, 49. The hatch in the other strips did not differ significantly from that in water (i.e. 25 larvae/100 cysts). The R_f values obtained with the various solvent systems were, for maximum hatching activity: solvents (a) and (c), 0.80–0.85; solvents (b), (d), (e), (f) and (g), 0.85–0.90. Hatching activity travelled as a spot with large R_f values and with activity usually detectable over a range 0.15–0.20 R_f unit. Activity was recovered almost completely from the chromatograms. The active regions of the chromatograms showed a weak light-blue fluorescence in ultraviolet light; they also showed an orange spot after treatment with Brady's reagent, and with solvent systems (b) and (d)–(g) a brown spot after spraying with aniline–xylose reagent (Smith, 1958).

Solvent system (f) was used by Carroll *et al.* (1958) for the chromatography of the hatching factor, but they failed to find any active material on such chromatograms. Widdowson & Wiltshire (1958) reported R_f about 0.70 with solvent system (d), but with our conditions, using the same solvent system, the region of maximum hatching activity was at R_f 0.85–0.90. With this system additional solvent in the lower part of the chromatography chamber and working at 15°, or colder, helped to prevent distortion of the active spot.

Upward elution chromatography of the hatching factor from roots of tomato plants in solvent system (b) (Massey & Neal, 1953) gave hatching activity centred at R_f 0.84. In our experiments, using the hatching factor from potatoes, maximum hatching activity was in the region R_f 0.85–0.90 with the solvent system (b) and upward or downward development. Paper chromatograms developed with aqueous phenol had to be thoroughly air-dried, because traces of phenol inhibited larval hatch and caused irregular results. Water-saturated phenol was also a suitable eluent for developing chromatograms.

DISCUSSION

Among the procedures previously used to obtain crude preparations of the hatching factor from dilute aqueous solutions, only those mentioned by Calam, Raistrick & Todd (1949), Carroll *et al.* (1958), Widdowson & Wiltshire (1958) and Hartwell *et al.* (1960) have applied to large volumes of raw diffusate. For most of our preparations, the hatching factor was first concentrated by adsorbing it on charcoal. Although this removed nearly all the activity from the diffusate, the average recovery by elution of the charcoal was only 31%, and the proportion differed considerably at different times. An average recovery of 60% was reported by

Widdowson & Wiltshire (1958) in 34 experiments with small volumes of diffusate. Calam *et al.* (1949), who also used charcoal adsorption, reported recoveries of 60–70% of the activity, but Carroll *et al.* (1958), using an improved bioassay technique, reported recoveries of 10% or less.

Asatoor & Dalglish (1956) suggested the use of charcoal deactivated with stearic acid for the selective adsorption of aromatic compounds from aqueous solutions. This deactivated charcoal readily adsorbed the hatching factor present in potato-root diffusate, but less activity was recovered from phenol eluates than was obtained from untreated charcoal by elution with acetone–water (Widdowson & Wiltshire, 1958). Acetone or acetone–water mixtures, which eluted the hatching factor from deactivated charcoal, also eluted the adsorbed stearic acid.

The losses are greatest during the preparation of the crude extracts, and the great increase in activity for unit weight of product obtained from our further procedures leads to only small losses of activity. Extraction of active material from small volumes of aqueous solution with ethyl acetate proved much superior to the solvents (ether, butan-1-ol and phenol) previously used to extract the hatching factor because it combines satisfactory extracting properties with volatility. After its use column chromatography gave a colourless, very active, gum. The hatching factor is a readily oxidizable acid, probably with little carbon–carbon unsaturation, and containing the group $\text{CH}_3\text{CO}-$. Other workers (Marrian *et al.* 1949; Widdowson & Wiltshire, 1958; Carroll *et al.* 1958; Hartwell *et al.* 1960) have obtained active materials by different methods, but there is too little information to assess the relative purity of these preparations. However, the product obtained by Widdowson & Wiltshire (1958) can be further purified by the methods reported here. Marrian *et al.* (1949), who used tomato-root leachings as their principal source of the hatching factor, fractionated the crude material obtained by adsorption on charcoal by unsatisfactory methods that largely or completely inactivated the material. Carroll *et al.* (1958) reported preliminary work on alternative procedures, but give no details. Some points of difference exist between our preparation from potato-root leachings and those obtained by Marrian *et al.* (1949) and Hartwell *et al.* (1960) from tomato roots. Marrian *et al.* (1949) reported that an ethanolic solution of their material showed a strong inflexion at 285 m μ , and gave a reddish-brown coloration with ferric chloride. Our preparation showed neither of these properties. Hartwell *et al.* (1960) reported the isolation of active crystalline material from tomato-root leachings. The hatching factor obtained from potato-root leachings by our pro-

cedures was too soluble in the solvent mixture (*tert.*-butyl alcohol, ethanol, ether) used by Hartwell *et al.* (1960), or in the individual components, to permit crystallization. The product after four crystallizations had little activity and at a concentration of 0.5 $\mu\text{g./ml.}$ gave a hatch of fewer than two larvae/cyst. The responsiveness of *H. rostochiensis* cysts to the hatching factor differs from time to time, but a typical result with our purified product at a concentration of 0.05 $\mu\text{g./ml.}$ was a hatch of 19 larvae/cyst during a period of 1 week; successive fourfold dilutions of this solution similarly hatched respectively 11, 7 and 4 larvae/cyst. Hence our product that failed to crystallize gave a similar hatch to that reported for the crystalline material when our product was 640 times as dilute.

Diffusate from tomato and potato roots give similar hatching curves (larval emergence/dilution) with *H. rostochiensis* cysts, and the differences reported between the hatching factors for the two plants probably indicate difference in degree of purity rather than of the structure of the active material.

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