The Potato Eelworm Hatching Factor

2. PURIFICATION OF THE FACTOR BY ALKALOID SALT FRACTIONATION. ANHYDROTETRONIC ACID AS AN ARTIFICIAL HATCHING AGENT

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Methods for the preparation of crude solid concentrates of the potato eelworm hatching factor from leachings of tomato roots and for the bioassay of the factor being available as a result of work described in the preceding paper (Calam, Raistrick & Todd, 1949), experiments directed to the further concentration and eventual isolation of the active principle were undertaken. It was clear from preliminary experiments that, if only on account of a tendency to inactivation, attempted purification through metallic salts was unlikely to be successful, at least on the crude solid. Substantial purification could, however, be effected by extraction with organic solvents; thus from ether extracts of acidified aqueous solutions of crude solid a brownish glass was obtained having a Relative Activity (R.A.) about 25 and about one-quarter the weight of the starting material. (Relative Activity is defined by Calam *et al.* 1949, p. 518.) This step, which results in little loss in activity, was accordingly included in all later concentration procedures. The product was strongly acidic and gave on titration an equivalent value of about 200; addition of excess alkali and back titration indicated the presence of lactonic material. It contained small amounts of nitrogen, but was free from phosphorus, halogens or sulphur.

As the product obtained by ether extraction could not be crystallized, and was almost certainly a complex mixture, it was treated with various alkaloids and the alkaloidal salts subjected to fractional precipitation. Brucine and quinine proved the most suitable bases, although neither was very satisfactory, as there was frequently complete loss of activity. It was noticed, too, that when the R.A. of a brucine salt fraction had reached about 50 it was difficult to raise it further without heavy loss. Although most of the earlier work was done on brucine salts, it was later found that quinine salts were equally useful. Examples of fractionations with both alkaloids are given below. The products were amorphous, but had fairly sharp melting points and gave reasonably consistent analytical values. The purest brucine salts showed R.A. 60–65. It was observed on a number of occasions that inactivation of the most highly active brucine salt fractions, on standing, on precipitation with solvents or on treatment with

a trace of alkali, yielded in nearly equivalent amount an inactive brucine salt which crystallized in rosettes of tiny needles and gave analytical values very close to those of the amorphous active salt. Such inactivation, with almost quantitative conversion to a crystalline salt, has not always been observed, perhaps because the amorphous active salts from different runs varied in their degree of heterogeneity. Treatment of the purified active brucine salts with acid, followed by continuous ether extraction, gave a highly acidic resin, active as hatching factor at dilutions of 1 in 10^7 to 1 in 10^8 ; a very similar, although biologically inactive, resin was obtained by the same treatment applied to the inactive crystalline salt. The properties of these materials, so far as they were examined, are recorded in the experimental section. The evidently heterogeneous products were so intractable, and the amounts available for study at any one time were so small, that it would be unwise to draw any conclusions beyond that the hatching factor—for which we propose the name *eclepic acid*—is an acid probably containing a lactone group. It was decided to defer further investigation of the natural material until larger amounts could be made available.

Since the hatching factor is obtained by leaching the roots of growing potato and tomato plants, a number of substances which might occur in plant roots were tested for possible hatching activity. They included aneurin, riboflavin, nicotinamide, pantothenic acid, pyridoxin, liver and yeast concentrates of the other B vitamins, ascorbic acid, heteroauxin, traumatic acid and a urine concentrate which was believed to contain auxins *a* and *b*; none of them showed any activity. Contrary to the statements of Hurst (1937), who was probably misled by the biological test method he used, tyrosine, glutamic acid, lysine picrate and picric acid were found to have negligible activity. The high acidity and probable lactonic nature of *eclepic acid*, however, led us to test also a number of tetrone acid derivatives and other compounds, viz. carolic acid (Clutterbuck, Raistrick & Reuter, 1935), tetrone acid (β -hydroxy- $\Delta^{\alpha:\beta}$ -butenolide), α -methyltetrone acid, α -benzyltetrone acid, α -carboxymethyltetrone acid, α -acetyltetrone acid, α -bromotetrone acid,

methyl tetronate, methyl α -methyltetronate, methyl α -benzyltetronate, acetyl α -benzyltetronate, benzoyl α -methyltetronate, α -carbethoxytetronic acid, α -carbethoxy- γ : γ -dimethyltetronic acid, γ : γ -dimethyltetronic acid, α -bromo- α -methyltetronic acid, O-tetra-acetyl- β -glucosidyl α -methyltetronate. (Details of new compounds are given in the Experimental section.) None of these compounds showed any activity as hatching factors.

Anhydrotetronic acid, however, for which structure (I) has been established (Marrian, Russell, Todd & Waring, 1947), did show pronounced hatching factor activity at pH values below 3. The free acid at a dilution of 1/2000 was fully active and still showed distinct activity at a dilution of 1/8000. At a dilution of 1/2000, anhydrotetronic acid gives as large hatches as the best samples of natural hatching factor. This remarkable property is not shared by any of a number of close relatives of anhydrotetronic acid; isopropylidene-bis-tetronic acid (Wolff, 1901), ethylidene-bis-anhydrotetronic acid (Wolff, 1901) and bis-tetronic acid (Wolff, 1913) are all devoid of hatching activity.

The discovery that anhydrotetronic acid shows hatching properties similar to those of the natural factor, although the degree of activity on a weight basis was clearly very small compared with that of eclepic acid, led to systematic attempts to synthesize other active substances; these will be described in later communications. It also led to a preliminary study of the feasibility of artificially hatching eelworm cysts in sand. Although the experiment (described on p. 523) was on a small scale and the results only qualitative, there was a significant reduction in the larval content of cysts in sand which had been treated with anhydrotetronic acid.

EXPERIMENTAL

Concentration of eclepic acid by ether extraction. Crude solid eluate prepared from tomato leachings (Calam *et al.* 1949; 1 g., R.A. 6) was dissolved in dilute H_2SO_4 (7.5 ml. water + 1.5 ml. 2N- H_2SO_4) and extracted continuously with peroxide-free ether for 12 hr. Evaporation of the ethereal extract followed by drying off *in vacuo* at room temperature gave a yellow acidic resin (212 mg., R.A. 28). Repetition of the procedure on many occasions using crude eluate of varying activity consistently gave products 3 to 4 times as active as the starting material. The product was free from S, P and halogens, but contained small amounts of N (1–2% estimated by the Dumas method); it was strongly acidic, behaving on titration as though it had an equivalent of about 200. Addition of excess alkali, warming and back titrating

indicated the presence of an additional concealed acid group (probably a lactone group). Numerous attempts were made to prepare metallic salts from this material, but no crystalline products were obtained and extensive inactivation occurred. Brucine, cinchonine and quinine gave amorphous salts which appeared to retain a considerable proportion of the original hatching activity, and those from brucine and quinine were selected for further study.

Fractionation of brucine salts. The above ether-free concentrate (0.15 g.) was dissolved in water (8 ml.), brucine (0.5 g.) added and the mixture shaken thoroughly. After 30 min. the solution was decanted and the residue washed with water. The combined solution and washings were filtered and evaporated to dryness *in vacuo*. The resulting crude brucine salt, which usually had R.A. 5–7, was ground under ethanol, when most of the active material dissolved, and the dissolved material was then subjected to repeated fractional precipitation by adding first ether and then light petroleum (b.p. 40–60°) and repeating the process. By this means a small amount of amorphous active salt (m.p. indefinite 165–190°) was finally obtained of R.A. 55–65, but the process involved heavy loss. Attempts to purify beyond this point usually resulted in inactivation, and the activity of the best fractions did not rise. A typical fractionation is set out in Table 1.

A sample of purified brucine salt, m.p. 180–183°, of R.A. 58 was dried over P_2O_5 (0.1 mm. and analysed. (Found: C, 62.6; H, 6.5; N, 4.9%.) When the active material was kept in ethanolic or aqueous solution it lost its activity, and on several occasions a crystalline inactive salt could then be isolated in a yield of 60–90%. This inactive material separated from ethanol in rosettes of colourless needles of rather indefinite m.p. 180–200°. Analysis of a recrystallized sample, m.p. 198–204°, gave values very similar to those obtained for the active salt. (Found in material dried at 50°/0.1 mm. over P_2O_5 : C, 63.5; H, 6.4; N, 5.2; loss on drying 4.0%.) If the active salt were reasonably pure this might suggest inactivation by isomerization.

Fractionation of quinine salts. The above concentrate prepared by acid ether extraction (0.15 g.) was treated directly with quinine (0.14 g.) in ethanol (3.5 ml.) giving a solution of pH 6. Ether (0.5 ml.) and light petroleum (11 ml.) were added and the precipitate (83 mg., R.A. 22), which contained practically all the activity, was spun off. This precipitate was stirred with acetone and the insoluble portion (R.A. 15) removed. Addition of ether gave a precipitate (R.A. 15) which was removed and the solution concentrated to small bulk and precipitated with light petroleum. The product (26 mg.) had R.A. 54 and repetition of the precipitation procedure gave a salt of R.A. 70. (Found in material dried at 35°/0.1 mm. over P_2O_5 : C, 64.6; H, 7.1; N, 5.9%.) Material of this composition and of R.A. 70–90 could frequently be obtained by this procedure, but at times inactivation would occur during fractionation for no obvious reason.

Properties of eclepic acid prepared from active brucine salt. When the brucine salt was dissolved in water, acidified with H_2SO_4 and continuously extracted with ether, an extract was obtained which, on evaporation, gave eclepic acid as a yellowish gum. The preparation of the free acid was accompanied by some loss of activity, but the best specimens (R.A. about 200) showed full hatching activity (i.e. comparable to 'Standard Solid' (Calam *et al.* 1949) at 1/40,000) at a dilution of 1/10⁷. Direct titration gave an equivalent of 258, while addition of excess alkali and back titration showed an

Table 1. *Fractionation of crude active brucine salts*

additional concealed acid group, possibly lactonic in nature. A molecular weight determination by the Barger method gave a value of about 300, but excessive dilution of the solutions which had to be used made the result unreliable. (Found in material dried at 50°/0.1 mm. over P_2O_5 : C, 55.7; H, 5.7%.) The product gave no reaction for carbonyl groups, and catalytic hydrogenation gave a variable uptake of hydrogen corresponding to that required for 1 double bond in a molecular weight of 300–400. The free acid showed only very feeble optical activity in dilute aqueous solution ($[\alpha]_D$ about +1°).

Synthesis of tetric acid derivatives

Methyl tetronate. Excess ethereal diazomethane was added to tetric acid and when evolution of N_2 ceased the solution was evaporated. Recrystallization of the residue from benzene/light petroleum and finally from a small amount of benzene gave the *ester* as colourless needles, m.p. 67°. (Found: C, 52.3; H, 5.3. $C_6H_8O_5$ requires C, 52.6; H, 5.3%.)

Methyl α -methyltetronate. Methylation of α -methyltetric acid with diazomethane in the usual manner gave the *methyl ester* as a colourless liquid which distilled at 160–170°/15 mm. (Found: C, 56.3; H, 6.4. $C_6H_8O_5$ requires C, 56.2; H, 6.3%.)

Methyl α -benzyltetronate. Methylation of α -benzyltetric acid with diazomethane gave the *methyl ester* as a colourless liquid, b.p. 155–160°/5 $\times 10^{-3}$ mm. (Found: C, 70.1; H, 6.0. $C_{12}H_{12}O_5$ requires C, 70.6; H, 5.9%.)

Acetyl α -benzyltetronate. α -Benzyltetric acid was acetylated by heating on the steam bath with excess acetic anhydride for 2 hr. Acetic anhydride and acetic acid were removed under reduced pressure and the residue recrystallized from cold methanol. The *acetyl derivative* formed

colourless needles, m.p. 69–70°. (Found: C, 67.1; H, 5.4. $C_{13}H_{12}O_4$ requires C, 67.2; H, 5.2%.)

α -Carbethoxy- γ - γ -dimethyltetronic acid. Diethyl malonate (32 g.) dissolved in dry ether (100 ml.) was allowed to react with sodium wire (4.6 g.) overnight. To the cooled mixture α -bromoisobutyryl bromide (23 g.) dissolved in dry ether (100 ml.) was added; after 24 hr. dilute H_2SO_4 (100 ml. of 3N) was added and the aqueous layer separated and evaporated under reduced pressure at room temperature. The sticky solid was purified by precipitation from benzene solution with light petroleum and finally recrystallized from a minimum of water. The *acid* formed colourless needles, m.p. 83–84°. (Found: C, 53.6; H, 6.0. $C_8H_8O_5$ requires C, 54.0; H, 6.0%.)

O-Tetra-acetyl- β -glucosidyl α -methyltetronate. Dry powdered silver α -methyltetronate (2.4 g.) was added to a solution of α -acetobromoglucose (4.1 g.) in dry benzene (10 ml.), and the mixture shaken for 10 hr. AgBr was removed and the filtrate set aside. The *glucoside* slowly separated as colourless needles and, after recrystallization from benzene/light petroleum, had m.p. 160–161°. (Found: C, 51.6; H, 5.5. $C_{19}H_{24}O_{12}$ requires C, 51.3; H, 5.4%.)

The hatching of eelworm cysts by anhydrotetric acid

Anhydrotetric acid is strongly acidic and differs from eclepic acid in that it is only active at pH values below 3. The results of a typical experiment in which the behaviour of anhydrotetric acid was compared with the natural substance are summarized in Table 2.

Table 2. *Hatching of eelworms by anhydrotetroneic acid at different pH values*

(The hatches are means of three dishes, each containing thirteen cysts incubated 3 days at 22°.)

Anhydrotetroneic acid (1/2000)		Standard solid (R.A. 1) from tomato root leachings (1/30,000)	
pH	Hatch (larvae)	pH	Hatch (larvae)
2.65	83	3.22	83
4.40	10	4.82	93
5.76	3	6.67	71
6.98	2	6.98	19
8.03	10	8.04	21

The structure (I) of anhydrotetroneic acid gives no explanation for this behaviour. The absorption spectrum does not change on dissolution in alkali, and alkaline solutions become active once more on acidification. It would thus appear that the hatching mechanism can only be started by anhydrotetroneic acid over a narrower pH range than by the natural substance. From the above experiment it can be seen that anhydrotetroneic acid has an R.A. of about 0.07 compared with over 200 for eclepic acid.

When cysts are exposed to anhydrotetroneic acid, the larvae emerge in the usual way (cf. Calam *et al.* 1949) being coiled and moving freely. This contrasts with the effect produced by certain other substances, such as bleaching powder solution or picric acid, which cause larvae to emerge in an angular, lifeless condition, and so can hardly be described as true hatching agents.

The results of an experiment in which eelworm cysts were mixed with sand and treated with anhydrotetroneic acid for 35 days is summarized in Table 3.

Sand (100 ml.) washed with HCl and water was placed in glass jars and 100 cysts and 20 ml. water well mixed with it. The anhydrotetroneic acid was dissolved in water and added after 1 week. The jars were kept at 15–20° in a room where they were not exposed to sunlight, and were occasionally watered to keep the contents moist. It was noticed that with higher concentrations of anhydrotetroneic acid the upper layer of sand became tinged with purple. Soil could not be used because it was impossible with it to reduce the pH to 3 or less, and even with 600 mg. of anhydrotetroneic acid per jar the sand mixture only just maintained this level. The jars were examined after 35 days.

It will be seen that where anhydrotetroneic acid was used the cysts were no longer viable, so that the degree of hatching was perhaps less than it might otherwise have been. In another set of jars, which were kept for 92 days, even the untreated cysts were non-viable, suggesting that complicating factors may have been operative. Under normal conditions, cysts will continue to hatch for long periods without dying. Thus when 135 cysts containing 13,635 larvae were placed, for a few days, in hatching factor solution and water alternately, 6466 larvae emerged during the next 4 months and more were still slowly hatching. Dissection of a number of cysts at this stage showed that all had contributed some larvae, maximal and minimal numbers per cyst remaining being 223 and 36 at the start and 130 and 5 afterwards. It would appear that in the above experiment with sand some unknown factor was causing the death of the cysts.

Table 3. *Reduction in content of cysts by anhydrotetroneic acid*

(Readings after 35 days; initial content, 101.6 larvae.)

Anhydrotetroneic acid		pH of extract	Acid recovered		Hatchability of recovered cysts	No. of cysts recovered	Larval content of recovered cysts	Mean reduction in larval content
(mg./jar)	(cwt./acre)		Titration (mg.)	Hatching test				
0	0	4.2	0	—	+	84	97.9	
0	0	4.3	0			93	105.8	
0	0	4.3	0			52	103.0	
0	0	4.4	0			79	92.0	
Mean		4.3	0			77	99.8	1.8
50	6.7	3.75	27			84	97.6	
50	6.7	3.95	26	—	—	70	104.0	
50	6.7	3.85	30			84	90.3	
Mean		3.85	28			79.3	97.3	4.3
200	27	3.45	77			82	97.9	
200	27	3.5	98	±	—	93	93.1	
200	27	3.4	115			92	95.7	
Mean		3.45	97			89	95.6	6.0
600	80	2.85	300			105	77.3	
600	80	2.85	365	+ +	—	95	92.8	
600	80	2.75	362			107	88.5	
Mean		2.8	342			102.3	86.2	15.4

From Table 3 it will be seen that the reduction in number of larvae per cyst after 35 days was 1.8, 4.3, 6.0 and 15.4 with 0, 50, 200 and 600 mg. anhydrotetrone acid per jar. Statistical analysis shows that there is, in fact, a significant correlation between amount of anhydrotetrone acid added and the reduction in number of larvae. When a parallel series of jars were examined after 92 days a similar effect was observed.

SUMMARY

1. The potato eelworm hatching factor can be extracted with difficulty from acid aqueous solution by ether, and this process has been employed as a first stage in the purification of crude concentrates from tomato root leachings. The hatching factor is an acid, and for it the name eclepic acid is proposed.

2. The preparation of highly active brucine and quinine salts from eclepic acid concentrates is described, as also are some of the properties of the resinous free acid prepared from them. The most active preparations of eclepic acid, although still impure, show full hatching activity on eelworm cysts at a dilution of 1 in 10^7 to 1 in 10^8 .

3. No appreciable hatching activity was shown by a variety of substances likely to occur in plant leachings or by several amino-acids and tetrone acids. Anhydrotetrone acid, however, was found to possess definite hatching properties, being fully active at a dilution of 1/2000. A preliminary pot experiment showed that it could bring about artificial hatching of eelworm cysts in sand.

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The Potato Eelworm Hatching Factor

3. CONCENTRATION OF THE FACTOR BY CHROMATOGRAPHY. OBSERVATIONS ON THE NATURE OF ECLEPIC ACID

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From the preliminary investigations described in Part 2 of this series (Calam, Todd & Waring, 1949) it was clear that unless considerably larger amounts of starting material could be made available, little progress towards the isolation and structural elucidation of the potato eelworm hatching factor (eclepic acid) was likely. Although the factor evidently had a comparatively low molecular weight, the small amount of it present in tomato root leachings, coupled with its great instability, made chemical investigation extremely difficult. Fortunately, larger supplies became available through the generous help afforded by the Agricultural Research Council, which arranged for the growing and leaching of tomato plants on a large scale by essentially the method described in Part 1 (Calam, Raistrick & Todd, 1949) to be carried out at the Experimental Station, Cheshunt. There a stock

of some 150,000 tomato plants was maintained under glass during the period May 1943 to September 1944. Production in 1944 was seriously affected by war damage at the station and ceased in the autumn of that year. The total amount of crude solid eluate obtained during the period was 218 g. with an average Relative Activity (R.A.) 9 (Calam, Raistrick & Todd, 1949), batches ranging from R.A. 4-13; under this arrangement then, we could extract about 12 g. crude solid per month, which, although small, was very much more than was available to us in our earlier work. The investigations described in this paper were all done on this material, referred to throughout as 'crude solid'. Assuming that R.A. about 200, the value shown by the most highly purified eclepic acid preparations obtained, represents something approaching the activity of pure eclepic acid, then on assay the total material we