On the Nature of Hatching of Heterodera schachtii. III. Principles of Hatching Activity

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Introduction

The step, in the life cycle of animals, in which the developed embryo emerges from an egg membrane or egg case to continue its growth and development is a relatively common event in the animal world, and in nematodes as in other animals this transition may be a relatively inconspicuous incident in the normal sequence of events. In plant parasitic nematodes of the *Heterodera* species, however, emergence from the cyst assumes a special significance, for the cyst stage can serve as a survival form capable of enduring adverse environmental conditions.

This aspect of nematology received considerable attention especially after the observation of Baunacke (1)[3] that leachings of host plant roots stimulated larval emergence. The emergence of larvae from cysts of *Heterodera* has been the subject of a recent extensive review (12). There is a wealth of data concerned with the stimulation of hatching, the hatching factors in root diffusates and the physical effects on the hatching assay. The available information is the product of numerous workers in various laboratories using different populations of animals, different sources of hatching stimulation, and different conditions and methods of assay. As a result numerous apparently contradictory reports have arisen.

Investigations into the hatching process have consisted for the most part of descriptions of the physical parameters, the testing of a number of diverse substances from natural or synthetic sources for hatching factor activity and attempts at the identification of a naturally occurring hatch active substance. It was clear from previous reports (11,17,18) that if hatch data were to be useful as a manifestation of the nature of hatching it would need to be obtained from reproducible assays conducted in a standard fashion with animals of as similar an environmental history as possible and with hatch factor source materials prepared in a similar fashion.

The relative unspecificity of hatch factor sources, that is

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[3] Numbers in parentheses refer to literature cited.

the large number of host and non-host plants whose root exudates or plant organ extracts are capable of stimulating the emergence of larvae from cysts of *Heterodera schachtii* Schmidt (11,18), suggests that the active substance or substances in many cases could well be relatively common products of plant metabolism. Since plant extracts and exudates are known to contain constituents and by-products of metabolism, for example, amino acids, sugars, proteins, vitamins, alkaloids, etc, it was of importance to test the hatch stimulatory activity of a number of representatives of the various classes of biologically active substances with cysts of *H. schachtii*.

Materials and Methods

The sugar beet nematode, *H. schachtii*, was reared on sugar beets (*Beta vulgaris*) grown in sand culture in the greenhouse as previously reported (14). At harvest time, approximately 3 to 4 months after the initial infestation, a crude cyst concentrate was separated from the sand by wet screening. Subsequently cysts in the concentrate were separated from the associated debris (14). The cysts, essentially pure, were then damp dried and placed on the screen in a controlled humidity box (RH 98% at 5° C) in which the gas phase was mechanically circulated. After 3 days the cysts were transferred to a static humidity box (RH 90% at 5° C) and stored until used. Since the hatch tests were conducted over a period of several years, cysts from a number of rearing lots so processed were used.

Standard sugar beet leachate dry solids consisted of lyophilized leachings obtained by misting purified sugar beet germ in the misting apparatus previously described (18). The sugar beet germ was purified by the screening and winnowing of by-products from the commercial seed processors. The leachings obtained during the first and second day of germination were discarded, for according to preliminary tests they contained no active material; the leachings of the third and fourth days were saved and lyophilized. By the fifth day decomposition began and the seed germ was discarded. The lyophilized material from a number of batches was thoroughly mixed, then stored at −15° C until used. The chemical compounds used in these assays were of the highest purity commercially available.

The hatching bioassay was conducted after the method of Viglierchio (16). The cysts were suspended at the surface of the test solution by a stainless steel screen in plastic wells containing 0.5 ml of solution sample. The cysts and solution (8 replicates per test solution of a concentration series) were incubated in a humidity chamber at 25° C. At 4-day intervals the

emerged larvae together with old test solution were withdrawn for counting and the wells refilled with freshly prepared test solution. Bioassays were discontinued after three collections. The concentration of test materials, grouped according to class of compounds, ranged from 0.1 mg per ml downwards. Concentration is given in terms of weight/volume to facilitate comparison with natural hatch factor dry solids.

In figures where more than one curve is presented for a compound the closed circles indicate the cyst response at maximum sensitivity and the open circles, minimum sensitivity. The ratio indicates the number of tests of high sensitivity to the number of low sensitivity. Whenever no substantial difference in sensitivity was observed only a representative curve is shown. For practical considerations the curves of any one class of compounds were compiled from a number of experiments. It was usually impossible to test all the members of a class at all the indicated concentrations at one time.

Robinson and Neal (8) recommended the use of a potassium, sodium, magnesium and calcium chloride solution with purified preparations of leach material for increased sensitivity in *H. rostochiensis* hatch tests. The suggested salt concentration was maintained constant though test materials were diluted.

Results

Cyst Sensitivity Cysts of *H. schachtii* reared, purified and stored as described in the previous section responded to standard hatch factor materials in a continually changing fashion throughout their useful life. At the time of harvest there was usually no hatch stimulation i.e., the hatch response to water was essentially the same as that to standard leachate. This continued

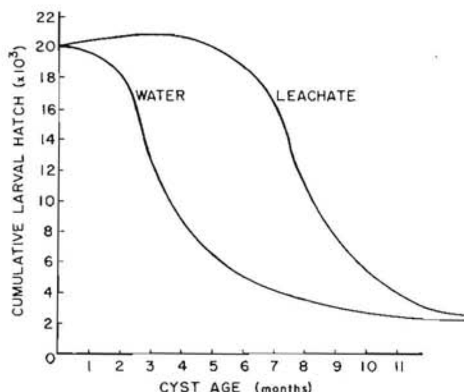


Figure 1.—The cumulative larval hatch response of *H. schachtii* to standard solutions of the dried hatch factor materials and water as a function of cyst age.

for 1 to 3 months (generalized curve Figure 1). Thereafter there was a slight maximum in the leachate response before it decreased slowly as indicated. The water response, however, decreased more rapidly so as to effect a differential response. Leachate usually provided a three- to four-fold increase in hatching over water controls; however, under certain conditions a ten-fold increase was possible. Some 9 months after the time of harvest the sensitivity as well as the differential response decreased to a value too low to be useful for emergence assay purposes. It was evident, therefore, that absolute values of hatch-

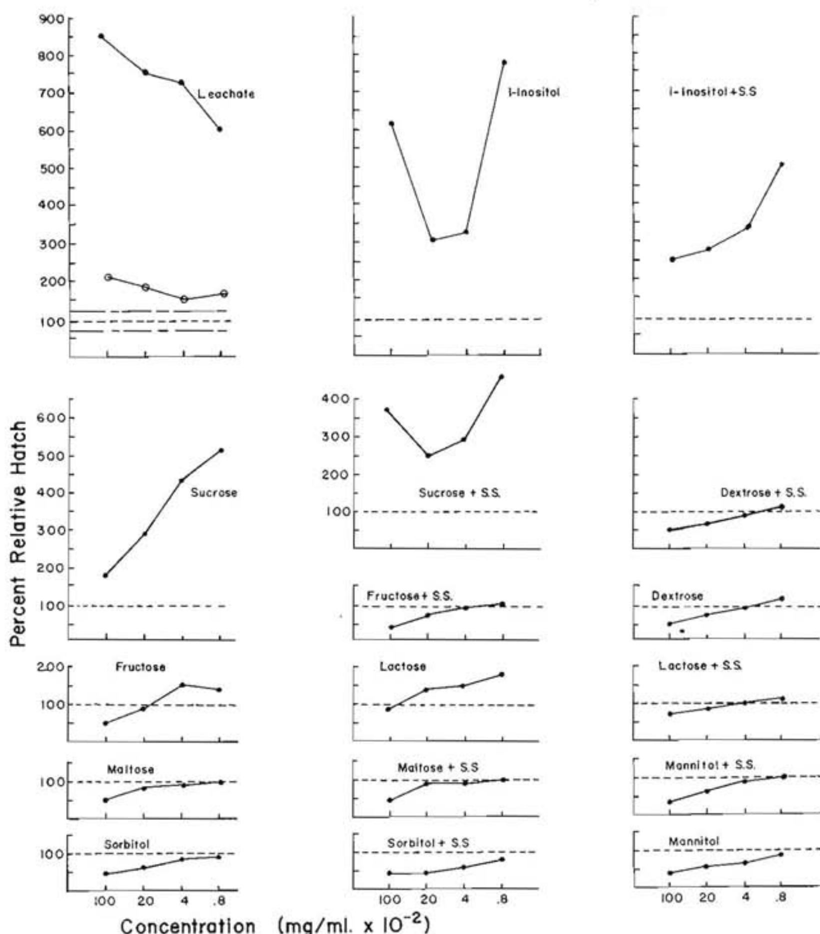


Figure 2.—The percent larval hatch response, with respect to water, of *H. schachtii* to solutions of sugars and related compounds. The horizontal dotted lines indicate the larval emergence in distilled water.

ing could be misleading. A relative measure would be more useful, consequently the hatch response curves have been given as percent relative hatch with respect to water.

Sugars and Related Compounds Of the compounds of this class tested, i-inositol, sucrose and possibly fructose and lactose possessed stimulatory activity (Figure 2). Dextrose, maltose, sorbitol, mannitol and possibly lactose with salts tended to be inhibitory. The concentration of salt solutions beneficial for hatching in *H. rostochiensis* appeared to be inhibitory in the hatching of *H. schachtii.* Except at the high concentration of i-inositol and sucrose the effect of salt solution when manifest was to reduce larval emergence. In this class of compounds, i-inositol and sucrose approached the stimulatory activity of sugar beet leachate.

Organic Acids From the observed results (Figure 3) citric, maleic, succinic, glutaric, malonic, fumaric and oxalic acids could be as active as leachate. Only 2-oxoglutaric acid and tartaric acid were found inactive. Additional tests with succinic, glutaric, malonic, fumaric and oxalic acids showed that activity was not consistent.

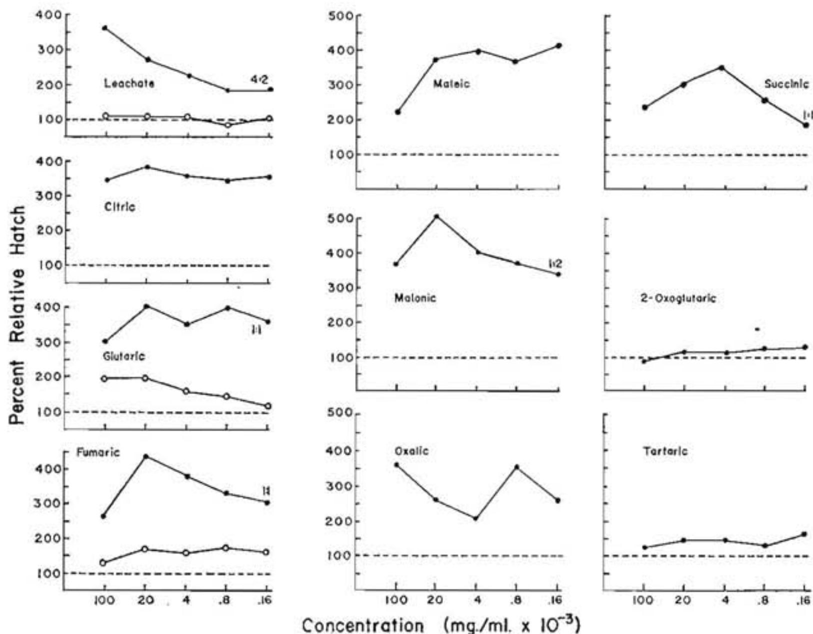


Figure 3.—The percent larval hatch response, with respect to water, of *H. schachtii* to solutions of organic acids. The horizontal dotted lines indicate the larval emergence in distilled water.

Vitamins and Related Compounds Biotin, d-calcium pantothenate, folic acid, ascorbic acid, and thiamin could possess stimulatory activity comparable to leachate (Figure 4). Riboflavin and taurine appeared to be less active whereas glutathione, pyridoxine and nicotinic acid were completely inactive in these experiments. Repeated tests with biotin, d-calcium pantothenate and folic acid confirmed that whereas biotin could be inactive, d-calcium pantothenate and folic acid could be very inhibitory. The hatch curves indicated that encysted larvae could be sufficiently sensitive to detect concentrations of vitamins on the order of 0.1 ppm.

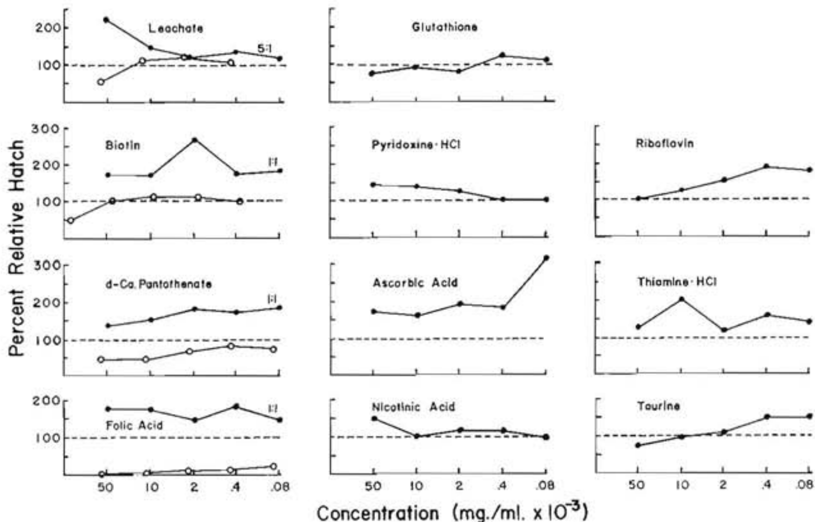
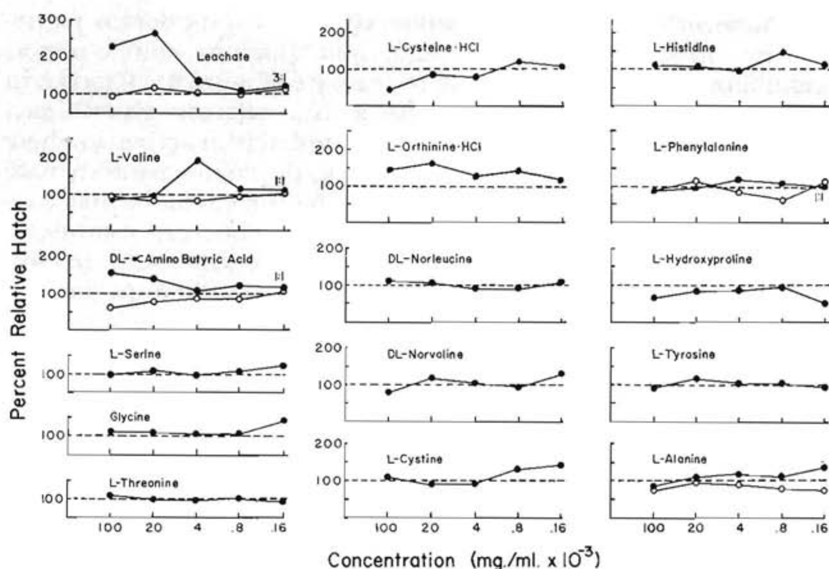


Figure 4.—The percent larval hatch response, with respect to water, of *H. schachtii* to solutions of vitamins and related compounds. The horizontal dotted lines indicate the larval emergence in distilled water.

Amino Acids The L-amino acids as a group appeared relatively inactive in hatch stimulation tests (Figure 5 and 6). Of some 25 amino acids tested only L-lysine, L-tryptophan, L-aspartic acid and perhaps L-glutamic acid possessed some ability to stimulate larval emergence from cysts. Some of the more active acids appeared to possess stimulatory power at concentrations on the order of 0.01 ppm. L-lysine was the only amino acid consistently active.



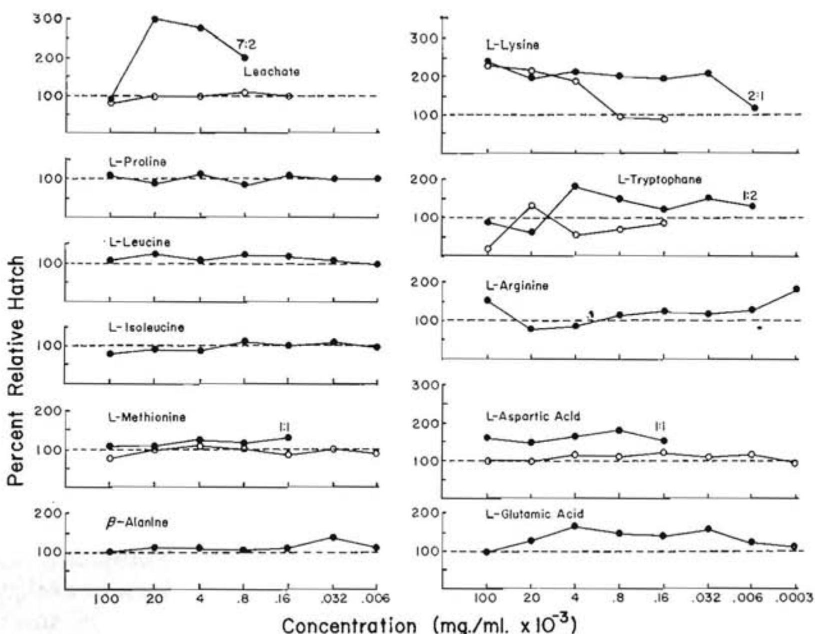


Figure 5-6.—The percent larval hatch response, with respect to water, of *H. schachtii* to solutions of amino acids. The horizontal lines indicate the larval emergence in distilled water.

Nucleosides and derivatives The hatch curves (Figure 7) showed that though cytosine, adenine, guanine, adenosine, guanosine and inosine were capable of stimulating larval emergence as much or more than standard leachate, they could also be completely inert. There is some indication, e.g. adenosine and inosine, that the coupling of a sugar to the nitrogen base increased stimulatory power; however, when the adenosine was phosphorylated to give the coenzymes of higher phosphate content, activity appeared to be lost. Coenzyme A and diphosphopyridine nucleotide were not active.

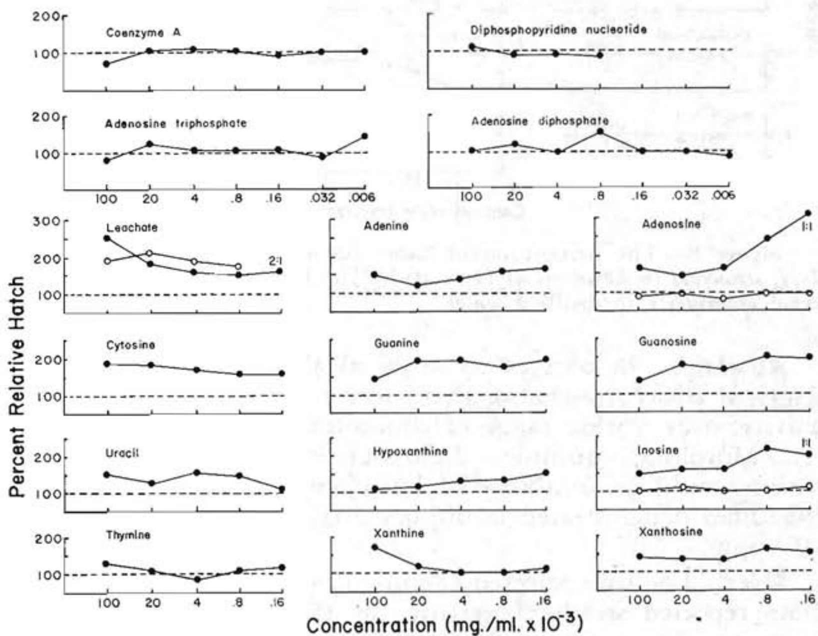


Figure 7.—The percent larval hatch response, with respect to water, of *H. schachtii* to solutions of nucleosides and derivatives. The horizontal lines indicate the larval emergence in distilled water.

Fatty Acids The higher fatty acids C_{12} and above, whether saturated or unsaturated, were inactive in hatch test (Figure 8). The inhibitory activity at the higher concentration could be attributed to the high alkalinity necessary to keep the fatty acid in solution. The lower members of the fatty acid series, acetic, propionic, and butyric acids could be inert and/or inhibitory to larval emergence. Caproic, caprylic and capric acids were able to stimulate a larval emergence comparable to leachate.

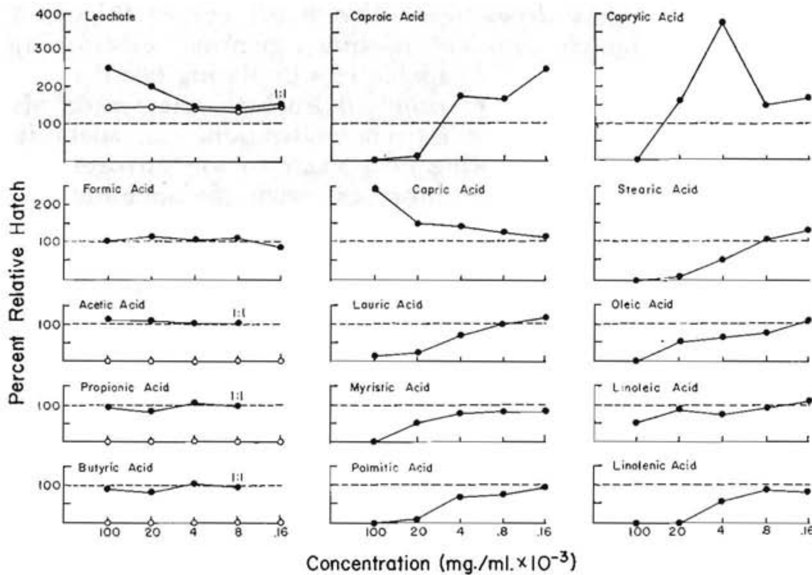


Figure 8.—The percent larval hatch response, with respect to water, of *H. schachtii* to solutions of fatty acids. The horizontal lines indicate the larval emergence in distilled water.

Alkaloids In tests of some 30 alkaloids, representatives of principal structure groups, there was a general tendency to inactivity, over a wide range of concentrations (Figure 9, 10 and 11). Morphine, quinine, digitoxigenin, strychnine, codeine, coniine could be inhibtory of larval emergence. Digitoxigenin sometimes demonstrated inhibitory activity at concentrations of 0.01 ppm.

Dyes The dyes selected (Figure 12) were the most active of those reported in the literature for the stimulation of larval emergence from cysts of *Heterodera schachtii* (12). Methyl red was comparable in activity to leachate; picric acid was more active.

Miscellaneous Biologically Active Componnds Camphor, benzoic acid, indole-3-acetic acid, salicylic acid, and 2-naphthoxyacetic acid were comparable to leachate in ability to stimulate larval emergence from cysts (Figure 13). At high concentrations β-mercaptoethylamine and salicylic acid were more active than leachate, whereas, camphor appeared to be more active at greater dilutions. Of the plant growth regulators, naturally occurring indole-3-acetic acid and synthetic 2-naphthoxyacetic acid were very active but gibberellic acid, 2,4-dichlorophenoxyacetic acid and 1-naphthaleneacetic acid were only slightly active. Thiol

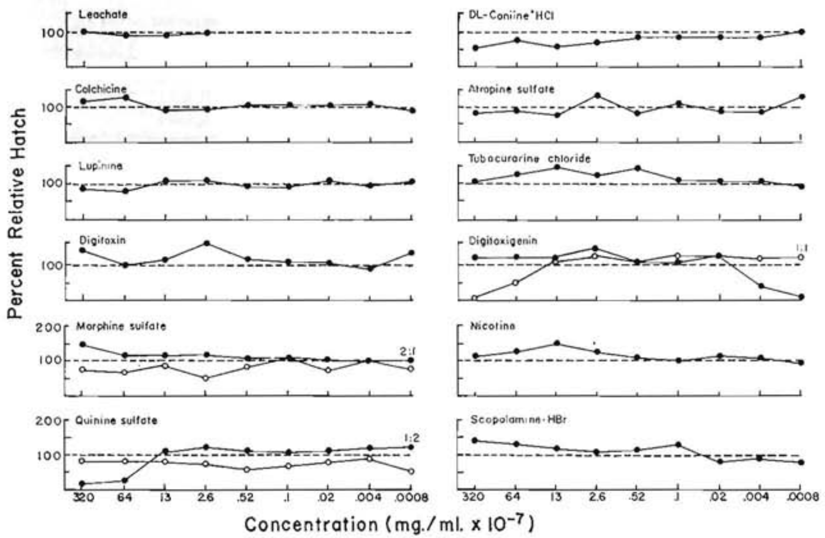


Figure 9.

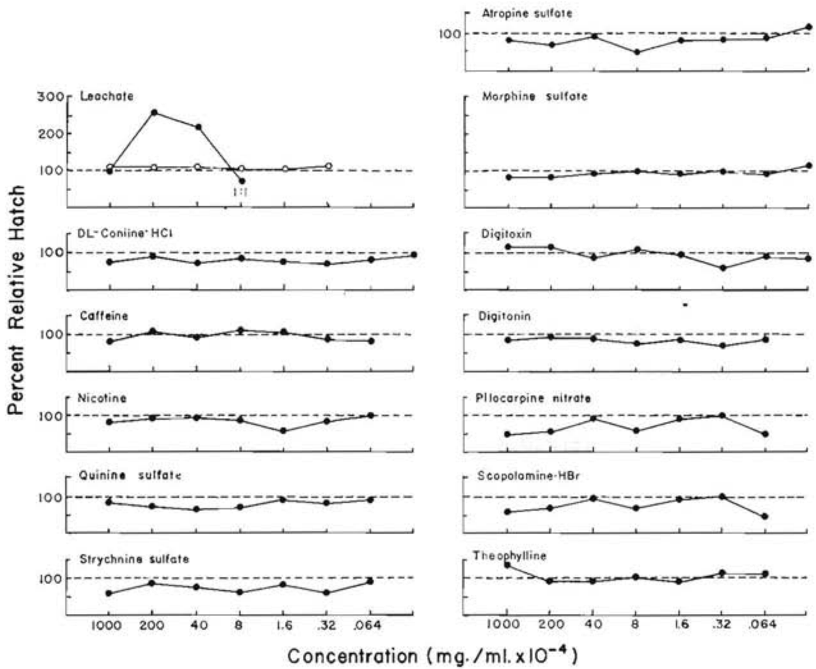


Figure 10.

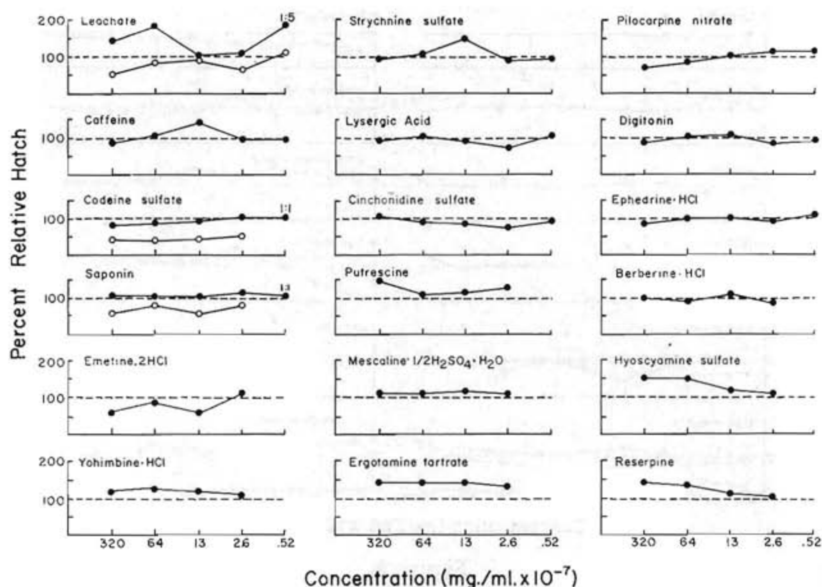


Figure 9-11.—The percent larval hatch response, with respect to water, of *H. schachtii* to solutions of alkaloids. The horizontal lines indicate the larval emergence in distilled water.

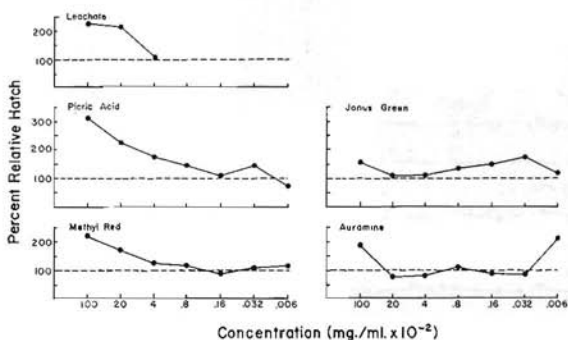


Figure 12.—The percent larval hatch response, with respect to water, of *H. schachtii* to solutions of synthetic dyes. The horizontal lines indicate the larval emergence in distilled water.

compounds could be very active in high concentrations as β-mercaptoethylamine, mildly active as thiodiglycolic acid and mercaptoacetic acid at low concentrations or inhibitory at higher concentrations as mercaptoacetic acid. Cholic acid, inert at 100 ppm, increased in activity with dilution approaching the maximum of leachate at 0.1 ppm.

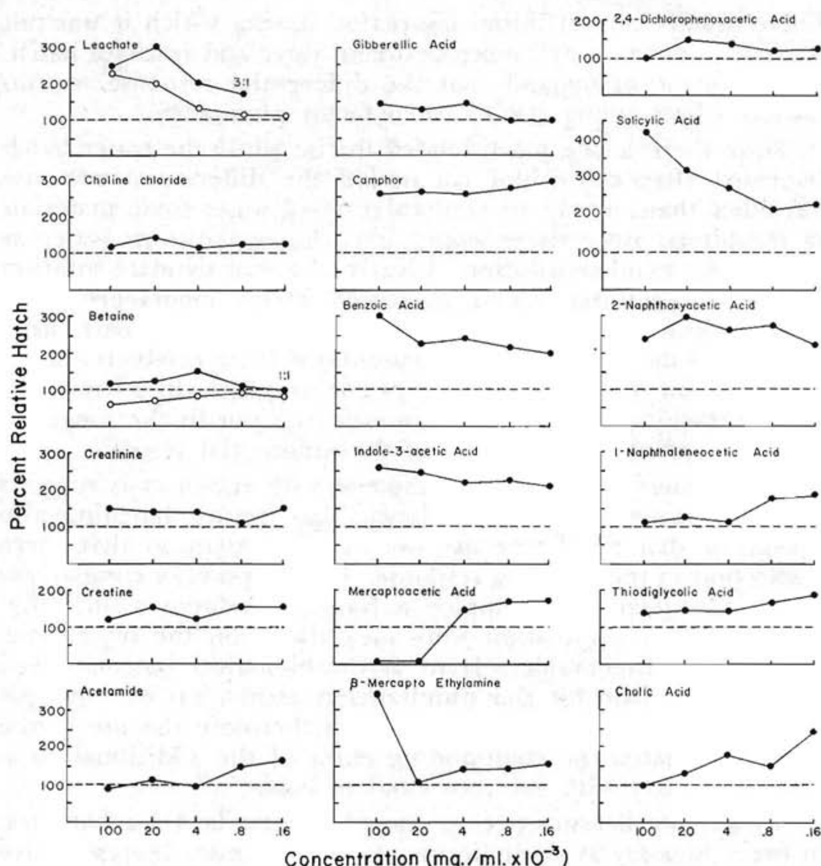


Figure 13.—The percent larval hatch response, with respect to water, of *H. schachtii* to solutions of miscellaneous compounds. The horizontal lines indicate the larval emergence in distilled water.

Discussion

The cumulative larval hatch curves indicating emergence due to water and leachate with respect to cyst age (Figure 1) are a function of rearing conditions, method of purification, technique of drying and conditions of storage. The ordinate intercept can be varied greatly by the technique of drying; if the conditions are drastic and the desiccation takes place quickly, cumulative larval hatch can be reduced to an insignificant value. Gradual drying over a period of 3 to 5 days maintains a high initial cumulative hatch. The drying is conducted at 5° C to prevent the high emergence normally occurring at higher temperatures.

There usually was an initial lag period during which it was not possible to detect a difference between water and leachate hatch. This observation suggests that the differential response was an induced effect giving rise to hatch factor physiology.

Since the leachate hatch tended to rise while the water hatch decreased after the initial lag period the differential response was other than merely an accumulation of waste toxic materials or inhibitors, since these would have leached out in water as well as root exudate solution. Clearly, the root exudate solution provided something which promoted larval emergence. The gradual decrease in hatching can be visualized in part, as a resultant of the toxic effects of accumulated waste products and/or the depletion of metabolic reserves not supplied either by water or root leachings. The primary interest was not in the longevity of hatching but in the nature of the differential response.

The change in differential response with age of cysts suggests that the requirements of the larvae that permit hatching also change or that the larvae are not all equivalent so that there is selection in the hatching response. For purposes of comparison it was advantageous to employ a frame of reference such that inhibition and stimulation were measurable on the same scale. Water, an indispensable solvent of the biological system, served as a suitable base for the simultaneous estimation of solvolysis effects and comparison of addenda. Furthermore the use of the water eliminated the confounding effect of the additional parameter necessary with the root exudate base.

It was normal practice to include a standard leachate test in every bioassay as an indicator of cyst response; representative compilations were presented in the figures of data for each class of compounds. It was evident that at one concentration the activity could range from a maximum, to intermediate, to inertness, to inhibition; since the hatch factor source, the same throughout, had not been observed to deteriorate appreciably it could reasonably be assumed that there were other larval requirements for emergence not supplied by the leachate.

The data presented in this report have shown that many biologically active compounds at the appropriate concentration and under the proper conditions were as capable of stimulating larval emergence as the crude leachate preparations. Sucrose and i-inositol of the polyalcoholic compounds could be good hatch stimulants. It was of interest to observe that sucrose was a good hatch stimulant whereas dextrose or fructose were not. This suggested that sucrose activity was not due to its normal glycolytic role, i.e. its breakdown to fructose and glucose followed by subsequent phosphorylation etc. but perhaps to its structural

entity as a α-D-glucopyranoside-β-D-fructofuranoside. The salt solutions which were important cofactors in the hatch test of *H. rostochiensis* had an inhibitory effect when used in association with sugars in *H. schachtii* hatching tests. For practical considerations their use in association with other synthetic compounds was omitted.

The high hatching activity of a number of the organic acids suggested the importance of the tricarboxylic acid cycle. Citric, succinic and fumaric acids are members of the cycle and their activity may be explained as a supplement to a deficiency of substrates. The inactivity of α-keto-glutaric acid, a constituent of the cycle, could be explained by the presence of sufficient substrate and the nontoxicity of a surplus. Maleic acid was active, perhaps because it isomerized to the trans active fumaric acid. Malonic acid activity may have been the result of competitive inhibition in the succinate to fumarate step in the tricarboxyclic acid cycle.

Many of the vitamins tested were able to stimulate larval emergence. Water soluble, nonstorable vitamins present at low levels could be consumed in a relatively short time. Their addition to correct a deficiency would permit enzymatic reactions, in which they were cofactors, to proceed thereby eliminating a block in the sequence of events leading to larval emergence.

In general the amino acids did not appear to be especially active in hatch stimulation. L-tryptophan and L-aspartic acid could be mildly stimulatory; only L-lysine appeared to be consistently active. A consideration of the processes involved in hatching suggests no need for active protein synthesis such as could be expected in the molting process. Normally tissue proteins would be expected to be catabolized in preference to enzymatic proteins, and inasmuch as the enzymatic complement was present initially for larval emergence it could be expected to remain. The active amino acids, especially L-lysine, could be involved in reactions other than protein synthesis, e.g. lipid metabolism and membrane physiology.

The hatch stimulation effected by purine and pyrimidine bases and their pentose derivatives would be explicable in terms of nucleic acid synthesis or coenzyme activity. Since hatching was unlikely to involve a burst of new cell formation or other vigorous growth activity, low nucleic acid synthesis would be expected. On the other hand it was difficult to reconcile the lack of hatch activity of polyphosphorylated nucleosides with this notion (Figure 7).

In view of the habitat and the environmental conditions in which the plant parasitic nematode lives, it is not surprising that the depot lipids are in the liquid phase. It is of considerable interest, however, that the liquid short chain saturated fatty acids C_6, C_8 and C_{10} not only were more active in the stimulation of larval emergence than the liquid unsaturated long chain fatty acids but that with the short chain saturated acids caproic increases in activity with dilution, caprylic reaches a maximum and decreases and capric decreases with dilution. It is striking to find that acetic acid, propionic acid and butyric acid could be inert or completely inhibitory to hatching.

The mode of action of alkaloids is not understood but no survey of biologically active compounds would be complete without them. They appeared to be relatively inactive except perhaps at very high concentrations. There were anomalous reactions, for example, digitoxigenin and morphine inert at higher concentrations were inhibitory at extremely low ones. There seems to be little explanation for this other than a difference between cyst lots in ability to detoxify a particular alkaloid.

The hatch stimulating ability of certain dye compounds would appear to be a result of competitive reactions. Picric acid and methyl red, for example, are not naturally occurring compounds but synthetic preparations; their structure needs correlation with that of a naturally-occurring stimulatory material for confident speculation on mode of action. This action is to be differentiated, however, from that of acids, enzymes, etc. of non-nematode origin which induce emergence by chemical rupture of the egg membrane, a mode of emergence irrelevant to this report.

Organic bases differing in structure and strength as illustrated by choline chloride, betaine, creatinine and creatine, appear to have little stimulatory power, for example, betaine could stimulate slightly or inhibit slightly. Naturally occurring indole-3-acetic acid and the synthetic analog 2-naphthoxyacetic acid were as active or perhaps more active than leachate. Other synthetic auxins, however, 2,4-dichlorophenoxyacetic acid and 1-naphthaleneacetic acid were essentially inactive. Naturally occurring gibberellic acid was also inactive.

Thiol compounds differed markedly in activity. Mercaptoacetic acid for example was inhibitory at high concentrations and mildly active at the more dilute concentrations. Thiodiglycolic acid was mildly active at all concentrations. On the other hand β-mercaptoethylamine was very active at high concentration but mildly so at dilute ones. Cystine and cysteine are

essentially inactive. There seemed to be no particular pattern in hatch stimulation activity of these sulphur compounds. Cholic acid appeared to increase in activity with dilution. In view of the interesting results with the fatty acids additional tests with sterol compounds would have been especially useful; unfortunately no others were included. Benzoic acid, camphor and especially salicylic acid were very active compounds. The nature of the hatch stimulatory powers of these compounds is unknown.

In view of current understanding, hatch stimulatory activity appears to take three forms with respect to general modes of action: metabolic—consisting of substances serving as substrates, cofactors, or coenzymes of conventional physiological systems, including anabolism, catabolism and endocrinology; pseudometabolic—consisting of substances reacting competitively in physiological reaction systems; and ametabolic—consisting of substances with non-physiological modes of action. The diversity of compounds manifesting apparent ametabolic hatching stimulation illustrates the complexity of the hatching phenomenon.

It is tempting to believe that the naturally occurring stimulation is of the metabolic type since this would be more compatible with evolutionary or survival value. Inorganic salts, carbohydrates, organic acids, vitamins, amino acids, as well as other unidentified substances, are present in the root leachings of plants (6,7,10,13). There is evidence (17) that: stimulatory substances in natural leachings can be consumed or utilized as substrate during the process of hatching, inhibitors can be present in cysts (17) and in natural leachings together with activators from host and non-host plants (18). There is great number and diversity of: plants whose leachings can stimulate larval emergence (11,18), synthetic compounds capable of inducing hatching (2,3,12,19,20,21), physical and environmental parameters important in the hatching process (11,15,17,18), and in cyst lots in readiness and capability to hatch. It would appear that the sum of these observations would be consistent with the visualization of eclosion in *H. schachtii* as being effected by the successful completion of a complex series of physiological reaction any of which could be limiting. The limiting steps would depend on the cumulative effect of the environmental influences in the rearing and storage of cysts up to the moment of hatching.

The stimulation of larval emergence of a particular cyst lot effected by root exudate would be a reflection in part of its ability to supply the metabolic requirements—substrate, organic and inorganic cofactors, inhibitors, etc. as suggested by Dropkin (4). Though crude leachate can be expected to contain inert

substances in varying proportions, the results of this investigation show that in some cases activity of the leachate could essentially be accounted for by known synthetic biologically active compounds.

It would be unlikely however that hatch stimulation of active root exudates could always be explained in terms of the better known metabolic reactions; one might expect that the likelihood would decrease with increasing specificity of parasitism (5). If hatching occurs after the fashion suggested by Rogers (9) then the search for the more specific blocks in *Heterodera* ought necessarily to take cognizance of the simultaneous occurrence of confounding blocks of more general metabolic reactions. It would be desirable to be certain that the same reaction was being measured throughout a hatch test series, and whether the larvae obtained by diverse synthetic stimulants were equivalent in physiological potential i.e. penetration, development, reproduction etc. It is apparent that for a clearer insight into the nature of hatching, experimentation needs to be conducted with more finesse than in the past.

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