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## SYNTHETIC HATCHING AGENTS FOR *HETERODERA SCHACHTII* SCHM. AND THEIR MODE OF ACTION

BY

A. J. CLARKE AND AUDREY M. SHEPHERD

Rothamsted Experimental Station, Harpenden, Herts., England

Two hundred and eighty-three compounds were tested for their hatching activity for *Heterodera schachtii* Schmidt, 31 of these, at the given concentrations, gave hatches equal to or greater than that obtained with beet root diffusate. No correlation was found between hatching activity and redox potential for the 25 redox compounds examined. The structure of the artificial hatching agents and the mechanism of hatching are discussed.

Since Baunacke (1922) found that root diffusates of sugar beet stimulated larvae to hatch from eggs of *Heterodera schachtii* Schm. many compounds have been tested for activity. Rensch (1924) found that a component of plant roots and a product of the breakdown of organic matter in soil were both more active than beet root diffusate, but they were ineffective in field experiments (Nebel, 1926). Rademacher (1930), Rademacher & Schmidt (1933) and their colleagues tested over 300 compounds, many of indefinite composition. Only a few of the compounds they found active have been studied further e.g. calcium hypochlorite (Molz, 1930, 1932; Smedley, 1936; Skarbilovich, 1953) and rivanol (3,9-diamino-7-ethoxyacridine lactate) (Winner, 1957). Winner also tested other acridine derivatives of which several were active.

Wallace (1956, 1957) tested amino acids, carbohydrates, caffeine, urea, ascorbic acid and several inorganic chlorides. He found glutamic acid and galactinol (1-O- $\alpha$ -D-galactopyranosyl-D-myo-inositol) inactive although reported active by Bauserman & Olsen (1957). Emanuelsson (1954) tried amino acids, plant growth hormones and vitamins but found only ascorbic acid appreciably active. Other active compounds are mercuric chloride (Wallace, 1956), anhydrotetrone acid (Winslow, 1959) and sodium ethylene-bis-dithiocarbamate (Steele, 1961). Streptomycin sulphate and mercuric chloride added to test solutions increased hatch, perhaps by suppressing micro-organisms (Shepherd, 1959). Shepherd (1962a) also showed that some dyes stimulate hatch.

In much of the early work, tests were qualitative and inadequate; assay methods for natural and artificial agents were improved by Fenwick & Widdowson (1958) and using these, many compounds were tested or retested (see Table I).

TABLE I  
*Classes of compound tested for hatching activity with H. schachtii*

Class of compound	No. tested	No. with hatch ratings >90	No. with hatch ratings between 10 and 90
Redox compounds	30	13	17
Acids	72	5	25
Alcohols	10	0	3
Aldehydes	10	1	3
Amines	20	0	9
Amino acids	8	0	1
Esters and lactones	14	0	4
Heterocyclic compounds (excluding pyridine derivatives)	21	1	9
Ketones	14	1	5
Phenols	30	4	14
Pyridine derivatives	9	1	3
Quinones	16	3	7
Miscellaneous organic compounds	20	2	10
Inorganic compounds	19	0	12

#### MATERIALS AND METHODS

Chemicals were mostly of analar quality, otherwise of reagent grade, and redox compounds were of known  $E_0'$  at pH 7 as supplied by British Drug Houses Ltd., except safranin O and toluidine blue which were from G. T. Gurr Ltd.

All the redox compounds were tested over a range of dilutions, 1/1, 1/4, 1/16, 1/64, 1/256 and 1/1024, starting from 10mM when the substance was sufficiently soluble in distilled water. From the results, hatch-dilution curves were drawn and the maximum hatch used to calculate the hatch rating of the compound. Hatching activity of most of the redox compounds was also tested at a single concentration of 0.05% by weight (approx. 2mM) in distilled water and in 4mM phosphate buffer at pH 7.

Other compounds were usually tested as a 3mM solution when sufficiently soluble in distilled water. Many were also tested over a range of dilutions; these are marked with an asterisk in the tables, and the concentration, hatch rating and hatch as a percentage of cyst content are for the maximum hatch obtained.

Cysts of *H. schachtii* were raised in the glasshouse and stored in the soil where they were produced, and from which they were elutriated as needed. Batches of cysts (approx. 100) were pipetted into solid watch glasses (Jones & Gander, 1962), the water removed and test substances added. Three replicates were incubated at 25°C and the hatched larvae were counted every week for 3 weeks. As controls for each batch of substances tested, the hatches in 'standard' beet root diffusate and in distilled water were also estimated. The mean percentage hatch from 44 separate tests was 50% in root diffusate and 13% in water.

Because the slope of the hatch-dilution curve differs from compound to compound, log activity (L.A.) values (Fenwick & Widdowson 1958) cannot be used

to assay hatch. Nor does the large and variable water hatch (1 to 30% of encysted eggs) provide a sufficiently stable value to compare test solutions. Similarly, the hatch from a stock of cysts in 'standard' root diffusate differs greatly in different tests (20 to 80% of encysted eggs). Both water hatch and diffusate hatch are, therefore, unsatisfactory yardsticks. Hatch expressed as percentage cyst contents is also unsatisfactory because it does not take into account variations in the responsiveness of eggs to a hatching stimulus, although it is a useful indication of the effect produced. For these reasons a hatch rating was calculated using the formula: —

$$\text{Hatch rating} = \frac{H_s - H_w}{H_D - H_w} \times 100$$

where  $H_s$  is the hatch in the test substance and  $H_D$  and  $H_w$  that in root diffusate and water respectively, all tested simultaneously. The larvae which would have hatched in water are thus discounted and the stimulus by the test substance expressed as a percentage of that by a standard root diffusate. On this scale, therefore, the diffusate hatch is represented by 100 and the water hatch by 0. For hatches smaller than in water the degree of inhibition is expressed as follows: —

$$\text{Inhibition \%} = 100 - \frac{100H_s}{H_w}$$

and is given a negative sign. Compounds with hatch ratings of 90 or more are, at the concentration specified, as active as root diffusate whereas those with a rating of 10 or less are inactive or inhibitory.

Although preferable to other measures, the main disadvantage of the hatch rating is that, because the response in test solutions, root diffusate and distilled water tends to vary independently, there are often large differences in hatch rating for any one compound tested at different times. For example, in two experiments with Bindschedler's green, the hatch was 57 and 54% of the cyst contents. The diffusate and water hatches were 28 and 19% in the first experiment and 79 and 6% in the second, giving hatch ratings of 334 and 87 respectively. Similarly, in two experiments with tolylene blue the hatch was 89 and 74%, the diffusate and water hatches were 33 and 22% in the first and 34 and 14% in the second, giving hatch ratings of 592 and 294 respectively.

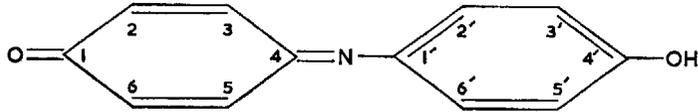
#### RESULTS

The maximum activity of the redox compounds tested, their optimum concentration and redox potential ( $E_0'$ ) are in Tables II to VI. The numbering of the ring systems in the compounds follows that used by Chemical Abstracts.

The addition of buffer to a test solution usually lessened the hatch by an amount which differed from compound to compound; this may be associated

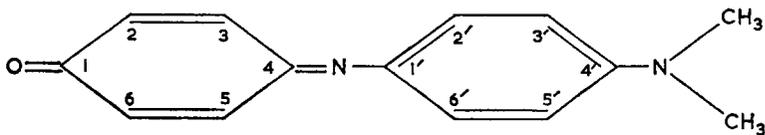
TABLE II  
Hatching tests with redox compounds

## INDOPHENOLS

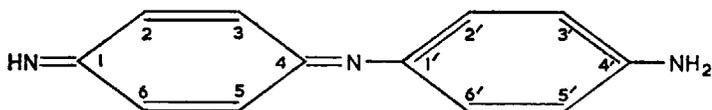


Compound	Eo'	mM Conc.	Hatch as % cyst content	Hatch rating
<i>o</i> -Bromophenol-indophenol	+ .243	3	62	† 298
<i>o</i> -Chlorophenol-indo-2, 6-dichlorophenol	+ .210	2	37	273
<i>m</i> -Chlorophenol-indophenol	+ .235	1	74	243
Guaiacol-indo-2, 6-dibromophenol	+ .163	4	42	198
Phenol-indo-2, 6-dibromophenol	+ .218	10	66	129
Phenol-indophenol	+ .235	10	83	106
Phenol-indo-2, 6-dichlorophenol	+ .214	10	82	104
<i>m</i> -Carboxyphenol-indo-2, 6-dibromophenol	+ .196	10	21	94
<i>o</i> -Chlorophenol-indophenol	+ .202	4	48	92
<i>m</i> -Tolylendiamine-indophenol	+ .125	4	44	91
<i>m</i> -Cresol-indophenol	+ .216	2	44	77
I-Naphthol-2-sodium sulphonate-indophenol	+ .128	6	22	60
<i>o</i> -Cresol-indophenol	+ .183	1	20	42
<i>o</i> -Bromophenol-indo-2, 6-dibromophenol	+ .239	2	13	27
<i>o</i> -Cresol-indo-2, 6-dichlorophenol	+ .188	10	9	18
Thymol-indophenol	+ .174	2	16	15

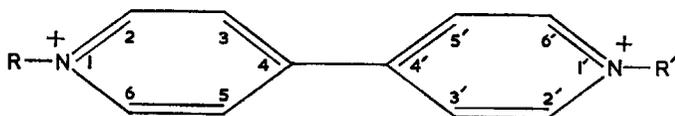
TABLE III  
INDOANILINES



Compound	Systematic name	Eo'	mM Conc.	Hatch as % cyst content	Hatch rating
Phenol blue	N,N-dimethyl-indoaniline	+ .216	0.4	42	51
Indophenol from naphthol	N-( <i>p</i> -dimethyl-aminophenyl)-1,4-naphthoquinone-imine	—	0.3	16	10

TABLE IV  
INDAMINES

Compound	Systematic name	Eo'	mM Conc.	Hatch as % cyst content	Hatch rating
Tolylene blue	N,N'-dimethyl-N-(4,6-diamino- <i>m</i> -tolyl)-benzoquinone-diimine	+ .104	3	77	273
Bindschedler's green	N,N'-dimethyl-N-( <i>p</i> -dimethyl-aminophenyl)-benzoquinone-diimine	+ .224	2	57	141
Phenylene blue	N-( <i>p</i> -aminophenyl)-benzoquinone-diimine	—	10	71	113

TABLE V  
VIOLOGENS  
(4,4'-Bipyridinium compounds)

Compound	Systematic name	Eo'	mM Conc.	Hatch as % cyst content	Hatch rating
Methyl viologen	1,1'-dimethyl-4,4'-bipyridinium dichloride	— .432	3	28	77
Benzyl viologen	1,1'-dibenzyl-4,4'-bipyridinium dichloride	— .320	3	25	65

TABLE VI  
PHENAZINE, PHENTHIAZINE and TETRAZOLIUM COMPOUNDS

Compound	Systematic name	Eo'	mM Conc.	Hatch as % cyst content	Hatch rating
Phenosafranin	3,7-diamino-5-phenylphenazinium chloride	— .252	10	52	115
Lauth's violet	3,7-diaminophenothiazinium chloride	+ .070	10	33	74
Tetrazolium salt	2,3,5-triphenyltetrazolium bromide	+ .080	1	41	66
Thionin blue	7-amino-3-ethylmethyl-amino-phenothiazine	—	3	14	25
Toluidine blue	7-dimethylamino-3-amino-phenothiazine	—	10	10	13
Methylene blue	3,7-bis(dimethylamino)-phenothiazine	+ .011	1	6	6
Safranin O	2,8-dimethyl-3,7-diamino-5-phenylphenazinium chloride	—	1	5	3

with the different behaviour of the various ionisable groups. The initial pH values of the unbuffered 0.05% solutions ranged from 3.6 to 7.0. Small changes of less than 0.5 pH units occurred and some of the compounds in Table II changed colour slightly during the tests, but these changes were shown by very active and weakly active compounds. Hatched larvae in tolylene blue and Bindschedler's green moved more actively than in root diffusate, water or the other test solutions. The histograms in Figs. 1 and 2 compare hatching activity over a range of redox potentials.

The other compounds tested are arranged in classes according to their functional groups (Table VII). Compounds with more than one functional group are arbitrarily allotted to a particular class. Table I shows the classes, number of compounds tested and their activities.

*Acids.* Many acidic compounds were examined because the natural hatching factor or factors for *H. schachtii* seem to be acids (Carroll, 1958; Clarke, 1960). Thirty of the 72 acids tested were more active than water, but most of these were less active than root diffusate.

The simple unsubstituted aliphatic mono- and di-carboxylic acids tested were inactive. The largest group of active acids were aromatic acids with the carboxyl group directly attached to the aromatic ring, i.e. substituted benzoic acids. The five most active benzoic acids have *ortho* substituents.

TABLE VII

Compound	ACIDS		
	mM Conc.	Hatch as % cyst content	Hatch rating
Nicotinic acid	*10	90	<b>318</b>
<i>o</i> -Aminobenzoic acid	*10	81	<b>250</b>
<i>o</i> -Bromobenzoic acid	*2	63	<b>143</b>
<i>o</i> -Chlorobenzoic acid	3	48	<b>108</b>
Acetylenedicarboxylic acid	*3	49	<b>103</b>
Acetonedicarboxylic acid	*30	47	75
3,5-Dinitrosalicylic acid	2	33	<b>68</b>
<i>o</i> -Nitrobenzoic acid	3	29	<b>60</b>
Salicylic acid	4	41	60
3,5-Dinitrobenzoic acid	2	15	51
<i>p</i> -Chlorobenzoic acid	0.3	24	<b>45</b>
2,4-Dichlorophenoxyacetic acid	*1	27	<b>43</b>
Ethylenediaminetetraacetic acid	0.9	23	<b>43</b>
<i>iso</i> -Nicotinic acid	4	17	42
Lactic acid <sup>1</sup>	0.6	12	<b>41</b>
Benzoic acid	2	12	40
Anisic acid	1	39	37
Diphenic acid	2	26	<b>35</b>
Dipicolinic acid	2	19	<b>35</b>

TABLE VII (Contd.)

Compound	mM Conc.	Hatch as % cyst content	Hatch rating
Citric acid <sup>1</sup>	*3	29	33
Phthalic acid	3	14	32
Phenylarsonic acid <sup>1</sup>	3	13	30
Dihydroxyfumaric acid	3	22	29
<i>p</i> -Aminobenzoic acid	4	16	26
<i>m</i> -Hydroxybenzoic acid	4	36	22
Citraconic acid	4	13	19
Homophthalic acid	3	10	14
L-Malic acid	4	8	13
<i>p</i> -Toluenesulphonic acid	3	5	13
<i>m</i> -Aminobenzoic acid	4	12	11
Cinchomeric acid	3	15	10
<i>o</i> -Nitrocinnamic acid	3	5	5
Phenylpropionic acid	3	15	5
Dihydroxytartaric acid	3	21	4
Succinic acid	4	7	3
Glycollic acid	7	6	2
Sebacic acid	3	5	2
D-Galacturonic acid	3	30	—1
<i>p</i> -Hydroxybenzoic acid	4	4	—4
Picolinic acid	4	17	—5
Gallic acid	3	32	—6
<i>p</i> -Coumaric acid	3	6	—11
Cinnamic acid <sup>1</sup>	3	11	—12
Aconitic acid	3	15	—14
Pyruvic acid <sup>2</sup>	6	16	—15
Glyoxylic acid monohydrate	5	24	—18
Oleic acid (suspension)	2	11	—19
Pyrole-2-carboxylic acid	5	24	—19
Adipic acid	3	15	—20
Indole-3-acetic acid <sup>2</sup>	1	10	—20
DL-Malic acid	4	26	—25
Quinic acid	3	13	—25
Phenylacetic acid <sup>1</sup>	4	12	—29
3-Hydroxybutyric acid	4	2	—40
Myristic acid	0.6	18	—40
Maleic acid	4	2	—43
$\alpha$ -Ketoglutaric acid	3	8	—45
DL-Tartaric acid	3	8	—49
Oxaloacetic acid	3	8	—50
Acetic acid <sup>1</sup>	8	3	—51
Ketomalonic acid sodium salt	4	7	—54
Pimelic acid	3	6	—69
Allylmalonic acid	4	5	—70
Fumaric acid	4	4	—73
trans- $\Delta^2$ -Dihydromuconic acid	3	3	—77
Suberic acid	3	4	—77
DL-Mandelic acid	3	3	—82
Mesaconic acid	2	2	—85
Itaconic acid	4	13	—90
2-Bromopropionic acid	3	0	—98
Propionic acid	7	0	—98
Oxalic acid <sup>1</sup>	6	0	—100

TABLE VII (Contd.)

ALCOHOLS			
Compound	mM Conc.	Hatch as % cyst content	Hatch rating
1,1,1-Trichloro-2-methyl-2-propanol	3	41	41
<i>iso</i> -Propanol <sup>1</sup>	9	17	16
Methanol <sup>1</sup>	*90	13	13
Ethylene glycol	8	5	4
Tetrahydrofurfuryl alcohol	5	4	0
<i>t</i> -Butanol	7	6	—30
<i>n</i> -Butanol <sup>1</sup>	7	4	—50
Ethanol	10	3	—68
Hex-3-yn-1-ol	5	1	—86
Benzyl alcohol	5	0	—98
ALDEHYDES			
Compound	mM Conc.	Hatch as % cyst content	Hatch rating
<i>o</i> -Nitrobenzaldehyde	*0.3	49	<b>102</b>
<i>p</i> -Hydroxybenzaldehyde	4	20	35
<i>m</i> -Nitrobenzaldehyde	3	12	23
<i>p</i> -Nitrobenzaldehyde	1	8	12
<i>p</i> -Dimethylaminobenzaldehyde	2	9	—30
2,4-Dihydroxybenzaldehyde	3	2	—74
Salicylaldehyde <sup>1</sup>	4	2	—74
Veratraldehyde	3	3	—84
<i>o</i> -Aminobenzaldehyde	4	3	—85
Benzaldehyde <sup>1</sup>	5	0	—100
AMINES			
Compound	mM Conc.	Hatch as % cyst content	Hatch rating
<i>p</i> -Hydroxydiphenylamine	0.8	60	82
<i>m</i> -Aminophenol hydrochloride	3	29	70
<i>o</i> -Phenylenediamine dihydrochloride	3	29	69
<i>p</i> -Phenylenediamine dihydrochloride	3	28	66
<i>m</i> -Tolylenediamine	1	49	49
Aniline hydrochloride	4	9	29
<i>m</i> -Phenylenediamine dihydrochloride	3	16	25
<i>p</i> -Nitroaniline	4	25	22
Diphenylamine <sup>1</sup>	2	12	11
<i>p,p</i> -Tetramethyldiaminodiphenylmethane	2	3	8
Choline chloride	4	14	7
<i>n</i> -Octadecylamine	0.4	14	4
<i>m</i> -Nitroaniline	3	4	—33
Aniline <sup>1</sup>	6	3	—67
Benzidine acetate	1	2	—84
N,N-Dimethylethanolamine	6	1	—95
Ethylamine <sup>1</sup>	10	0	—96
4-Aminopyridine	5	0	—99
N-Ethylaniline	3	0	—99
N,N-Dimethylaniline <sup>1</sup>	2	0	—100

TABLE VII (Contd.)

AMINO ACIDS			
Compound	mM Conc.	Hatch as % cyst content	Hatch rating
L-Cystine hydrochloride	0.7	29	23
Methionine	3	14	5
Tyrosine <sup>1</sup>	3	7	3
Glycine <sup>1, 2, 3</sup>	7	10	—39
L-Cysteine hydrochloride	4	6	—71
L-Proline	4	5	—72
L-Histidine	3	5	—75
$\beta$ -(3,4-Dihydroxyphenyl)- $\alpha$ -alanine	2	3	—80

## ESTERS AND LACTONES

Compound	mM Conc.	Hatch as % cyst content	Hatch rating
Anhydrotetronic acid <sup>4</sup>	3	28	68
Ascorbic acid <sup>2, 3</sup>	3	37	44
4-Hydroxycoumarin	0.6	28	22
$\alpha$ -( <i>p</i> -Hydroxybenzylidene)- $\gamma$ -methyl $\Delta$ $\beta$ -butenolide	0.4	14	13
Ethyl acetonedicarboxylate	2	5	4
Ethyl aminoacetate hydrochloride	4	14	—15
Amyl acetate <sup>1</sup>	4	9	—25
$\beta$ -Methyl umbelliferone	1	8	—57
Coumarin	3	10	—68
Umbelliferone	1	2	—68
Glycylglycine ethyl ester	3	2	—86
Ethyl acetoacetate	4	0	—90
Phthalide	4	1	—95
$\beta$ -Angelica lactone	5	0	—99

## HETEROCYCLES (other than pyridine derivatives)

Compound	mM Conc.	Hatch as % cyst content	Hatch rating
Picolonic acid	*0.3	45	130
Furan	3	50	57
Kojic acid	4	25	56
Thiamin <sup>2</sup>	1	36	46
Isatin	3	21	30
Pyrrrole	7	16	25
Riboflavine <sup>2</sup>	0.2	23	25
Dioxan	*30	15	17
Hexamine	4	9	12
Uric acid	0.3	18	12
2,3-Dihydropyran	6	6	5
Allantoïn	3	27	—10
Adenine	4	25	—18
Rutin	0.3	11	—18
Tetrahydrofuran	7	3	—24
Riboflavine-5-phosphate	1	17	—37
Sodium diethylbarbiturate	2	19	—45

TABLE VII (Contd.)

1,2,3,4-Tetrahydroisoquinoline	4	17	—47
Morin	0.9	4	—66
Phthalimide	3	6	—75
Caffeine <sup>3</sup>	3	7	—76

## KETONES

Compound	mM Conc.	Hatch as % cyst content	Hatch rating
Acetone <sup>1</sup>	*170	66	<b>138</b>
Ethyl methyl ketone <sup>1</sup>	*9	22	74
<i>iso</i> -Butyl methyl ketone	*40	41	33
Hexan-2,5-dione	*4	39	27
Cyclopentanone	*1	15	21
Cyclohexane-1,3-dione	5	10	19
Benzophenone	1	3	7
Mesityl oxide	5	31	—1
3-Hydroxybutan-2-one	6	11	—15
Michler's ketone	1	13	—16
5,5-Dimethylcyclohexane-1,3-dione	3	9	—32
Ketotetrahydrophenanthrene	0.6	2	—79
<i>o</i> -Hydroxyacetophenone	4	0	—94
Acetylacetone	*20	0	—100

## PHENOLS

Compound	mM Conc.	Hatch as % cyst content	Hatch rating
Picric acid <sup>1</sup>	*2	71	<b>238</b>
<i>p</i> -nitrosophenol	*10	42	<b>143</b>
Ammonium picrate	2	60	<b>108</b>
2,4-Dinitro-1-naphthol-7-sulphonic acid	1	41	<b>99</b>
2,4-Dinitrophenol	1	43	<b>88</b>
4-Chlororesorcinol	3	31	74
2,4,6-Tribromophenol	0.6	57	74
2,5-Dinitrophenol	1	32	65
<i>o</i> -Aminophenol	5	28	63
2,4,6-Trichlorophenol	2	52	58
Thymol <sup>1</sup>	3	26	48
<i>p</i> -Aminophenol	4	20	38
<i>m</i> -Nitrophenol	3	15	34
$\alpha$ -Naphthol	0.2	8	24
Pyrogallol	2	30	23
Phenol <sup>1</sup>	5	7	20
Salicylanilide	0.9	39	17
2,6-Dinitrophenol	1	12	16
Pentachlorophenol <sup>1</sup>	0.2	14	10
Salicylaldoxime	4	35	10
4,4'-Dihydroxydiphenyl	1	5	2
Catechol	5	8	1
<i>m</i> -Cresol	5	9	—8
3,5-Dinitro- <i>p</i> -cresol	3	9	—28
Resorcinol <sup>1, 2</sup>	5	5	—61

TABLE VII (Contd.)

3,4-Xylenol	4	6	—63
<i>p</i> -Nitrophenol	4	2	—86
<i>o</i> -Bromophenol	3	1	—96
<i>o</i> -Nitrophenol	4	0	—99
<i>p</i> -Cresol	5	0	—100

## PYRIDINE DERIVATIVES

Compound	mM Conc.	Hatch as % cyst content	Hatch rating
Pyridoxine hydrochloride <sup>2</sup>	*10	63	<b>153</b>
Pyridoxal hydrochloride	2	26	50
Pyridine <sup>1</sup>	6	22	35
$\alpha$ -Trippiperideine	2	28	35
Nicotinamide <sup>2</sup>	4	13	—29
$\beta$ -Hydroxypyridine	5	9	—47
Quinoline <sup>2</sup>	4	4	—52
Piperidine <sup>1</sup>	6	1	—92
$\gamma$ -Picoline	5	0	—99

## QUINONES

Compound	mM Conc.	Hatch as % cyst content	Hatch rating
2,6-Dichloro- <i>p</i> -benzoquinone-4-chlorimine	*0.1	42	<b>198</b>
2,3-Dichloro-5,6-dicyano- <i>p</i> -benzoquinone	0.3	60	<b>189</b>
2,6-Dichloro- <i>p</i> -benzoquinone	*0.4	78	<b>165</b>
Juglone	1	33	80
Purpurin	0.7	41	78
Carminic acid	1	26	77
1,2-Naphthoquinone	1	30	74
Alizarin <sup>1</sup>	2	24	43
2-Hydroxy-1,4-naphthoquinone	2	18	30
1-Hydroxyanthraquinone	1	13	15
2,5-Dihydroxy-1,4-benzoquinone	3	9	1
2,3,5,6-Tetrachloro- <i>p</i> -benzoquinone	*0.1	15	—1
9,10-Anthraquinone	1	12	—22
1,4-Naphthoquinone	2	6	—45
1,2-Naphthoquinone-4-sulphonic acid	2	7	—56
<i>p</i> -Benzoquinone <sup>1</sup>	0.3	1	—96

## MISCELLANEOUS ORGANIC COMPOUNDS

Compound	mM Conc.	Hatch as % cyst content	Hatch rating
Allylthiourea <sup>1</sup>	*4	42	<b>117</b>
Aureomycin	0.6	44	<b>100</b>
Phenylthiourea	*1	41	81
Thiourea	7	23	70
Diguanide hydrosulphate	1	47	67
Nitrobenzene <sup>1</sup>	4	29	59
Streptomycin sulphate	0.4	36	46
Dimethyl sulphoxide	6	29	43
Nitromethane	8	26	38

TABLE VII (Contd.)

$\alpha$ -Benzoin oxime	2	18	19
Ether <sup>1</sup>	140	10	19
Toluene	5	4	11
Methylene-aniline	2	12	10
Urea <sup>1, 2</sup>	8	8	5
Tannic acid	0.3	5	3
Benzanil	2	7	—23
Calcium D-pantothenate <sup>2</sup>	1	22	—26
Benzoyl peroxide	2	9	—40
1,3,5-Trinitrobenzene	0.5	7	—45
Guanidine carbonate <sup>1</sup>	3	8	—72

## INORGANIC COMPOUNDS

Compound	mM Conc.	Hatch as % cyst content	Hatch rating
Potassium permanganate <sup>1</sup>	*10	37	86
Hydrogen peroxide <sup>1</sup>	*100	31	76
Hydrochloric acid <sup>1</sup>	*3	22	62
Ferrous sulphate <sup>1</sup>	2	23	59
Potassium iodate	2	22	47
Calcium permanganate	10	35	40
Sodium iodate	3	15	36
Ferric chloride <sup>1</sup>	2	19	34
Ferrous ammonium sulphate	1	16	26
Potassium persulphate	3	11	25
Potassium ferrocyanide	1	12	13
Sulphuric acid	*2	14	11
Ammonium nitrate <sup>1</sup>	8	10	—14
Ammonium sulphate	4	10	—15
Sodium dithionite	1	6	—24
Potassium ferricyanide	2	1	—63
Sodium chlorate	5	1	—76
Sodium nitrate	6	1	—88
Potassium dichromate <sup>1</sup>	2	0	—97

<sup>1</sup> Tested by Rademacher & Schmidt (1933).

<sup>2</sup> Tested by Emanuelsson (1954).

<sup>3</sup> Tested by Wallace (1956).

<sup>4</sup> Tested by Winslow (1959).

\* The given concentration is that which gave the maximum hatch when a range of dilutions of the compound were tested. The hatch rating and hatch as a percentage of the cyst content are for this concentration.

† Bold type indicates activity comparable with root diffusate.

Of the three pyridine-monocarboxylic acids only nicotinic acid (3-carboxy-) gave a greater hatch than beet root diffusate; *iso*-nicotinic acid (4-carboxy-) was moderately active and picolinic acid (2-carboxy-) inactive.

The few active non-aromatic acids were acetonedicarboxylic, acetylenedicarboxylic, dihydroxyfumaric, lactic, citric, and L-malic acids.

*Alcohols.* The most active member of this class, 1,1,1-trichloro-2-methyl-2-propanol, was only moderately active. The other alcohols were weakly active, inactive or inhibitory.

*Aldehydes.* The aldehydes tested were mostly inhibitory. *o*-Aminobenzaldehyde and salicylaldehyde differed from their corresponding acids in being inactive. *o*-Nitrobenzaldehyde, however, was definitely active.

*Amines.* Nine of the twenty amines tested had moderate or good activity, and these were all aromatic amines.

*Amino acids.* Several types of amino acids not previously tried were tested. Cystine was slightly active; the rest were inactive or inhibitory.

*Esters and lactones.* None of the esters tested was active, but all three lactones with  $\beta$ -keto-enol groups, i.e. ascorbic acid, anhydrotetrone and 4-hydroxycoumarin, were moderately active. Ascorbic acid gave rather variable results, possibly because micro-organisms metabolised the ascorbic acid in the sterile test solutions.

*Heterocyclic compounds (other than pyridine derivatives).* Representatives of many heterocyclic ring systems were tested. The active compounds contained several quite different ring systems. Picrolonic acid, the most active compound in the group, has, in common with the active lactones, a keto-enolic group.

*Ketones.* Several simple ketones were active, especially acetone and ethyl methyl ketone. The cyclic ketones and those acyclic ketones with substituent groups other than methyl were less active.

*Phenols.* Eighteen of the thirty phenols tested had some activity. Both substituted and unsubstituted phenols were active and the activity depended greatly on the nature and position of the substituent groups. The most active phenol was picric acid, but other phenols without nitro group substituents were also very active. *Ortho*- and *para*-nitrophenol were inhibitory, but *meta*-nitrophenol was slightly active. The 2,4- and 2,5-dinitro phenols were active but 2,6-dinitrophenol only weakly so. The 2,4,6-trichloro- and tribromophenols were active, whereas the completely halogenated compound, pentachlorophenol, was inactive.

*Pyridine derivatives.* Pyridoxine hydrochloride was very active and three other compounds moderately so.

*Quinones.* Of sixteen quinones tested, ten were active. Three of the six inactive quinones were unsubstituted *p*-quinones. 1,2-Naphthoquinone was active but not 1,4-naphthoquinone. With 2,6-dichloro-*p*-benzoquinone-4-chlorimine and 2,6-dichloro-*p*-benzoquinone, the initial saturated aqueous solutions changed from a colourless or very pale yellow to pink on standing. The nature of this reaction is unknown.

*Miscellaneous organic compounds.* This group contains the organic compounds which could not be placed in the previous classes. The activity of allylthiourea (Rademacher & Schmidt, 1933) was confirmed. The parent compound, thiourea, and another derivative, phenylthiourea, are also active. Urea was inactive, although Wallace (1956) found it slightly active. Nitrobenzene, dimethyl sulphoxide and nitromethane were all moderately active. Aureomycin was very active, streptomycin sulphate moderately so.

*Inorganic compounds.* Few inorganic compounds were tested. Ferrous sulphate, ferrous ammonium sulphate and ferric chloride were all moderately active. Al-

though the hatch ratings based on the third week cumulative count of larvae are similar for these three compounds, there are, however, considerable differences in the hatches at one week (Table VIII).

TABLE VIII

*Weekly hatch of larvae from 100 cysts of H. schachtii in various iron salts, root diffusate and distilled water, with the total hatch after 3 weeks.*

Compound	1st week hatch	2nd week hatch	3rd week hatch	Total hatch after 3 weeks
Ferric chloride	1,969	2,438	1,538	5,919
Ferrous sulphate	994	4,063	1,619	6,675
Ammonium ferrous sulphate	931	3,625	613	5,169
Sugar-beet root diffusate	8,056	3,056	1,113	12,225
Distilled water	988	1,669	75	2,731

Rademacher & Schmidt (1933) found Chile saltpetre, the principle constituent of which is sodium nitrate, was moderately active. In our tests, sodium nitrate was inactive but sodium iodate, a minor constituent of Chile saltpetre, was active (Table VII).

Although most active inorganic compounds were oxidising agents, solutions of hydrochloric acid were also moderately active but ammonium chloride was inactive and other inorganic chlorides (Wallace, 1956) were only very weakly active and so the activity of hydrochloric acid may depend on traces of hypochlorite.

#### DISCUSSION

This work is part of a study of synthetic hatching agents for the cyst-forming nematodes (genus *Heterodera*), to provide information on the mechanism of hatching and also help in revealing the structure of the natural hatching agents.

As Tables II to VII show, many compounds have some activity. There are inorganic and weakly active organic compounds, which are unrelated or only remotely related to the natural hatching factors, and organic compounds that, at the concentrations specified, give hatches as great or greater than those from sugar-beet root diffusate, and may act like natural hatching factors. It should be noted that activity can also be compared in terms of the concentration needed to produce a given hatch. On this basis purified material from beet root diffusate showing optimum activity, with hatches equal to or greater than those of the root diffusate, at 0.01-0.10mM (Clarke, unpublished work) if a molecular weight of about 200 is assumed, is distinctly more active than any artificial compound yet tested.

Hatching compounds also influence the metabolism of larvae. Winner (1957) found that rivanol (2-ethoxy-6,9-diaminoacridine lactate) increased larval activity; we have confirmed this and shown that other very active hatching agents also have this effect. By influencing larval movement, a gradient of such substances

might cause larvae to travel towards a greater concentration and so act as an attractant.

*Redox potential and hatching activity.*

The active redox compounds have some features in common with the active dyes previously tested (Shepherd, 1962a), including a conjugated unsaturated system able to undergo oxidation and reduction. For this reason a correlation was sought between their redox potential and their hatching activity, but Figs. 1 and 2 show that neither in distilled water nor in buffer solution was there any obvious relationship between them. This is also true when the comparison is restricted solely to the indophenol derivatives.

Winner (1957) pointed out that a strong negative redox potential was not a criterion of hatching activity, because 9-aminoacridine ( $-0.916\text{v}$ ) was active but 3,6-diaminoacridine ( $-0.731\text{v}$ ) was not.

*Structure of redox compounds and hatching activity.*

*Indophenols.* All sixteen indophenols tested had some activity in unbuffered solution. Ten had hatch ratings greater than 90 and six between 10 and 90.

The only indophenol previously tested was an approximately 4mM solution of dichlorophenol-indophenol and that was weakly active (Emanuelsson, 1954). This was presumably phenol-indo-2,6-dichlorophenol which as a 10mM solution gave a hatch as great as beet root diffusate (Table II).

*Indoanilines.* In the indoanilines, where one of the oxygen atoms of the indophenol molecule is replaced by nitrogen as a dimethylamino group (Table II), the activity was much less than that of indophenol. Phenol blue was moderately active, while N-(*p*-dimethyl-aminophenyl)-1,4-naphthoquinone-imine was only very weakly so.

*Indamines.* In the indamine molecule, both oxygen atoms of the indophenol molecule are replaced by nitrogen. All three indamines tested were very active.

*Viologens.* Methyl and benzyl viologen had about the same moderate activity, so that replacement of methyl groups by benzyl groups had no great effect on the active centre of the molecule.

*Phenazine, phenothiazine and tetrazolium compounds.* Although safranin O is inactive (Shepherd, 1962a), phenosafranine, which differs from it only by lacking two methyl groups, is active. Methylene blue, toluidine blue and thionin blue, which are N-alkylated derivatives of the active compound Lauth's violet, are inactive or only weakly active, (Shepherd, 1962a). 2,3,5-Triphenyltetrazolium bromide was moderately active.

The active substances, then, include representatives with various types of redox behaviour. They include indophenols which normally undergo a single-step, two-electron reduction, and Lauth's violet, Bindschedler's green, phenol blue and viologens, whose reduction can take place by successive, univalent steps (Schwarzenbach & Michaelis, 1938; Michaelis *et al.*, 1940; Homer *et al.*, 1960).

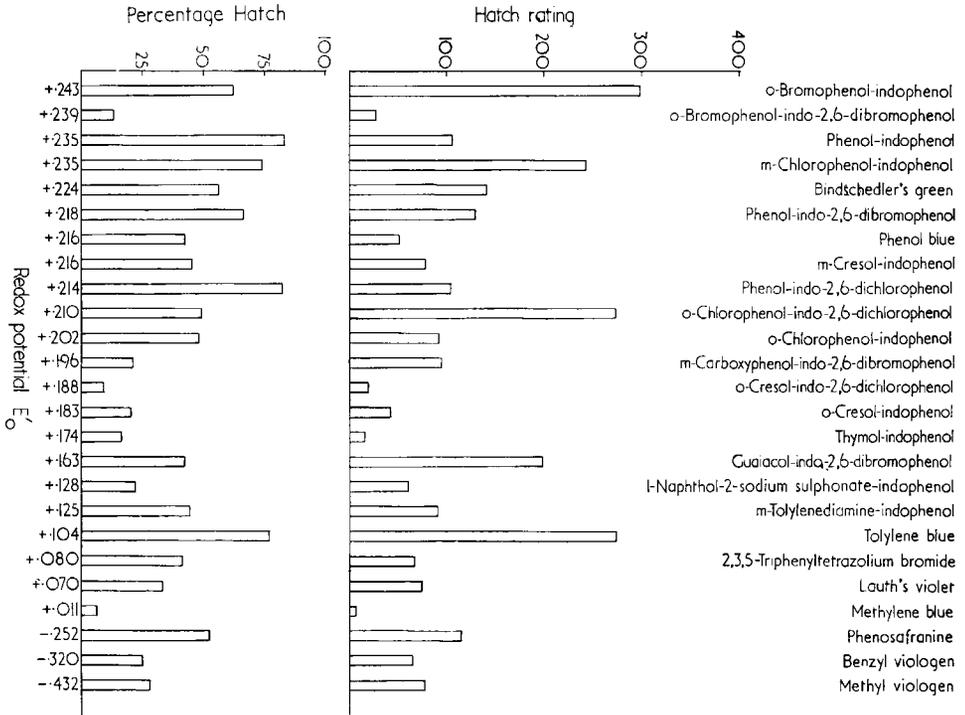


Fig. 1. Histogram of redox potential and hatching activity with *H. schachtii*. The hatching activities refer to the concentrations which gave maximum hatch when the compounds were tested over a range of dilutions in distilled water.

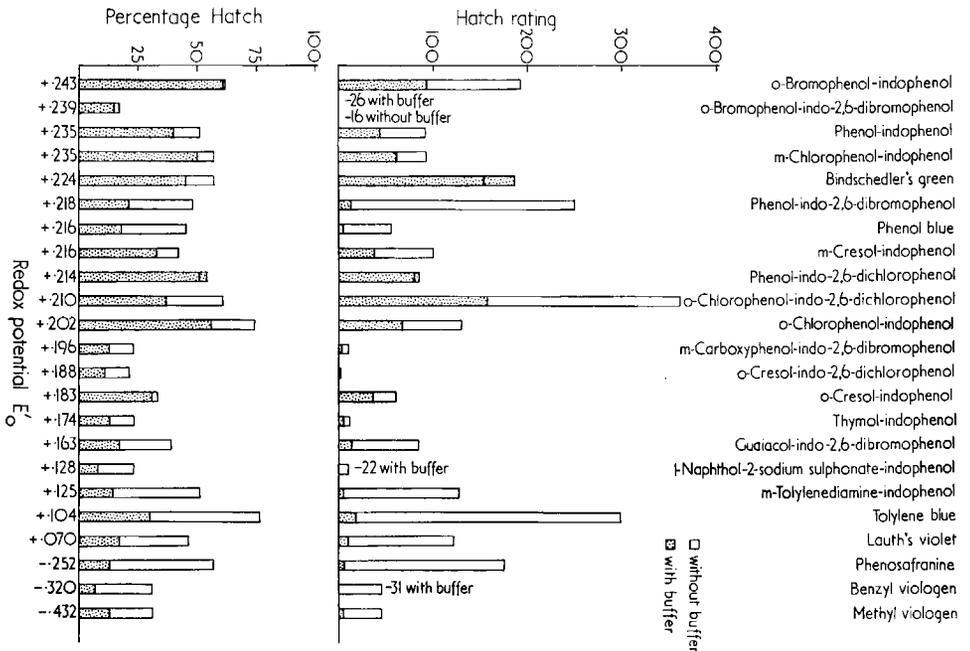


Fig. 2. Histogram of redox potential and hatching activity with *H. schachtii*. The hatching activities are for 0.05% solutions (approx. 2mM) in distilled water and in 4mM phosphate buffer at pH7.

Reduction of 2,3,5-tetrazolium bromide normally gives an insoluble red formazan (Nineham, 1955) which is usually assumed to be irreversible *in vivo* because of the insolubility of the formazan in the aqueous phase. If this is relevant, it suggests that irreversible oxidising agents can act as hatching agents.

*The relationship between oxidation-reduction and hatching activity.*

It is suggested that many hatching agents function by acting as electron acceptors (hydrogen acceptors, oxidising agents). Not all oxidising agents however are hatching agents, because of the specificity of structure required by the organism. It is also suggested that compounds which are not themselves oxidising agents, may show hatching activity because of their conversion by aerobic oxidation, either enzymic or non-enzymic, into electron acceptors of suitable structure. In support of this hypothesis are the following points.

1. In general, inorganic compounds are not very active; those which are active are oxidising agents (i.e. electron acceptors). There seems no reason to distinguish between the activity of inorganic and organic hatching agents. Although strong solutions of, for example, calcium hypochlorite and hydrogen peroxide damage cysts and eggs, and larvae that emerge under these conditions die, with dilute hypochlorite and with a wide range of concentrations of other inorganic compounds, there is no sign of damage and the larvae emerge alive, and are active.

Iron salts are anomalous, because both ferrous and ferric salts are active but it seems likely that activity in both is associated with ferric ions. The slow oxidation of ferrous compounds to ferric upon exposure to air is well-known. The results after one week's hatching in ferric chloride, ferrous sulphate and ferrous ammonium sulphate (Table VIII) support this view. Over this period, ferric chloride is the most active, ferrous sulphate less so and ferrous ammonium sulphate is inactive.

2. Many of the substances that give hatches as great or greater than root diffusate are redox compounds. They include dyestuffs (Shepherd, 1962a), indo-phenols and related compounds, quinones and simple ketones.

3. Where both oxidised and reduced forms of compounds were tested, the oxidised form was the more active (Table IX).

TABLE IX

*Hatch ratings of oxidised and reduced forms*

Oxidised form	Hatch rating	Reduced form	Hatch rating
Acetone	138	<i>iso</i> -Propanol	16
Cystine	23	Cysteine	—71
Malachite green	90	Leuco-malachite green	12
Pyridine, tripiperidine	35, 35	Piperidine	—92
Furan	57	Tetrahydrofuran	—24

4. Among the less active organic compounds, e.g. furan, pyridine, dimethyl sulphoxide, thiourea, there are many which, although not normally distinguished as electron acceptors, possess unsaturated groups able to be reduced.

5. In addition to the active compounds with readily reducible groups there are some compounds without such groups e.g. phenols and aromatic amines. These compounds may be active because of their conversion by oxidation into suitable electron acceptors. Some are sufficiently similar to the structures of compounds known to be more active, to suggest that they are converted to these active compounds. *p*-Hydroxydiphenylamine, for example, might be a precursor of indophenol, and 2,4,6-trichlorophenol might be converted into 2,6-dichlorobenzoquinone (which itself may undergo further conversion). The ability to oxidise aromatic compounds is common in a wide range of living organisms and quinonoid products are frequently formed. Little is known of suitable enzymes in *Heterodera* cysts. Ellenby (1946, 1956) found a polyphenol oxidase in young cysts of *H. schachtii* and *H. rostochiensis*.

*The structural specificity of organic hatching agents*

Purified material from beet root diffusate shows optimum activity at 0.01-0.10mM (Clarke, unpublished work), assuming a molecular weight of about 200. Some of the most active synthetic compounds show optimum hatches at 0.1-3.0mM e.g. *m*-chlorophenol-indophenol, picric acid, picrolonic acid, 2,6-dichloro-*p*-benzoquinone. The activity and speed of action of these and other organic hatching agents suggests that they may undergo little or no preceding alteration. Examination of the structures of the compounds which, at the concentrations specified, give hatches greater than beet root diffusate shows that a characteristic feature of many of them is the presence of two polarisable atoms (e.g. O, or N) connected by a conjugated unsaturated system. Juglone, for example, has two oxygen atoms, and nicotinic acid has an oxygen and a nitrogen atom at the ends of a conjugated system. The chain most common in these active hatching agents consists of five atoms, e.g. juglone HO—C=C—C=O; picric acid HO—C=C—N=O. An example of a six-membered chain is *o*-nitrobenzaldehyde O=C—C=C—N=O.

There are, in addition, compounds such as *o*-bromobenzoic acid, 2,6-dichloro-*p*-benzoquinone-4-chlorimine, 2,6-dichloro-*p*-benzoquinone and 2,3-dicyano-5, 6-dichloro-*p*-benzoquinone, which have halogen atoms at a position occupied by O or N in the above five-membered chains. The possibility exists that replacement of the halogen by hydroxyl may occur to give rise to compounds with a conjugated chain of five atoms with oxygen terminal atoms. The simple ketones and thioureas are examples of the simplest active system, two atoms joined by a polarisable bond, e.g. C=O → C<sup>+</sup>—O<sup>-</sup> and similarly for C=N etc.

The chain length, the nature of the terminal atoms, the polarisability of the chain all appear to be critical features in determining the activity of compounds. For example, *o*-nitrobenzaldehyde is active but not *m*- or *p*-nitrobenzaldehyde; *m*-nitrophenol is active but not *o*- or *p*-nitrophenol; *m*-nitrophenol is active but not *m*-nitroaniline.

The few synthetic compounds that Marrian, Russell & Todd (1949) found to hatch *H. rostochiensis* had either the structure HO—(C=C)<sub>n</sub>—CO or

$\text{RCO.NH}-(\text{C}=\text{C})_n-\text{CO}$ . They commented that these structures "are conducive to hydrogen bonding which may well play a part in the attachment of the hatching agent to some specific protein". Such structures may also determine the suitability of the compounds to function as electron acceptors.

### *The hatching mechanism*

Ideas on the cause of the hatching effect of root diffusates on the cyst-forming nematodes have differed widely (see Shepherd, 1962b, p. 11). Baunacke (1922) thought that the root diffusates might help to complete the maturation of the larvae and so cause more to hatch. Dropkin, Martin & Johnson (1958) suggested that the root diffusates altered the permeability of the egg or larval membrane to permit the passage of water and other molecules and ions. The assumption made was that lack of water is the critical factor in the inhibition of activity and hatch in the quiescent larva. Ellenby (1957) suggested a possible connection between hatching activity and ion transport. The hatching activity of hydrogen peroxide and calcium hypochlorite with respect to *H. schachtii* was attributed by Molz (1930) to the acceleration of metabolism caused by free oxygen made available to the larvae. Nolte (1956, 1958) and Kämpfe (1952) expressed similar views. Doliwa (1956) however attributed the action of these compounds, and of potassium permanganate to the oxidation of substances in the egg membrane. The possibility that some of the artificial organic hatching agents act on the egg membrane cannot be excluded. Many of the active compounds can participate in oxidation or addition reactions with  $-\text{SH}$  groups, and could thus modify proteins.

Our work on hatching agents has led us to the view that, irrespective of the immediate means by which the larvae are released from the eggs, i.e. whether by mechanical, enzymic, physical or chemical action, the mechanics of hatching are almost certainly accompanied by changes in the metabolism of the larvae. The energy demands of the hatched, free-moving larvae are presumably greater than when they are dormant within the eggs. The crux of the whole question of hatching from this viewpoint is the means by which this energy is suddenly made available and by which it sets in motion the train of events associated with hatching.

The sugars, amino acids, Krebs's cycle acids and other related compounds tested and found to lack conspicuous activity, suggest that the hatching response is not associated with the availability of a common metabolite to the unhatched larvae and energy that might be so provided. This also seems likely from the fact that the larvae remain within the egg and cyst for several years. A vitamin deficiency also seems unlikely to be connected with activity, for although some vitamins show activity, the wide range of other compounds that are also active shows the absence of the specificity that might have been expected.

Another means by which the metabolism of the larvae might be controlled at a level appropriate to a dormant state is by regulation of the oxygen supply. It has long been known that oxygen is essential for the hatching of *H. rostochiensis* (Triffitt, 1930). It is also clear that oxygen is essential both for the spontaneous

hatch of *H. schachtii* in water and for the much greater hatch obtained in beet root diffusate (Wallace, 1955a; 1955b). The experiments of Wallace (1955) showed that the spontaneous hatch of *H. schachtii* in water increased with increasing oxygen tension but the hatches obtained at atmospheric temperature and pressure under these conditions do not attain the levels reached with beet root diffusate in the presence of air. Hatching agents must thus provide some additional means for the liberation of energy, or are themselves sources of energy. Recent work (Sembdner, Osske & Schreiber, 1961) with *H. rostochiensis* shows that there is no difference in the rate of respiration of the hatched larvae and the larvae active in the egg just before hatching.

If the hypothesis is accepted that many hatching agents act either as direct electron acceptors, or give rise to electron acceptors by indirect action, then the most obvious way by which they might function is by interaction with the electron transport system and oxidative phosphorylation (i.e. the sequence of oxidation-reduction reactions associated with the production of ATP, by which the stepwise transfer of electrons (hydrogen atoms) occurs from metabolite to molecular oxygen).

One possibility is that hatching agents provide a means of by-passing a rate-limiting step in the electron transport chain. This could, for example, be achieved by providing some means for the oxidation of  $\text{NADH}_2$  and/or  $\text{NADPH}_2$ , which because of their role in dehydrogenase systems have a key position in the oxidation of many metabolites. There is evidence to suggest that hatching agents might function in this way. Non-biological hydrogen acceptors for  $\text{NADH}_2$  and  $\text{NADPH}_2$  are numerous. In particular, several of the highly active hatching agents have been reported to be particularly efficient oxidising agents for these compounds, e.g. 2,6-dichlorobenzoquinone (Wosilait & Nason, 1954) and 2,6-dichlorophenol-indophenol (Hadler & Erwin, 1963). It has also been shown that *o*-quinonoid compounds formed by the action of phenol oxidase systems can oxidise  $\text{NADH}_2$  and  $\text{NADPH}_2$ , and can in this way function as a terminal oxidase system. Various organo-nitro compounds are also known to be reduced by  $\text{NADH}_2$  in the presence of enzymes, e.g. xanthine oxidase. The ability of  $\text{NADH}_2$  and  $\text{NADPH}_2$  oxidants such as quinones to stimulate the metabolism of more organised systems i.e. in guinea-pig brain preparations (Hoskin & von Eschen, 1963), by their effect on the glucose-6-phosphate cycle has also been shown. It is of interest to note that Dahlstrom *et al.* (1964) have reported that potato root eelworm, *H. rostochiensis*, hatching factor stimulated the growth, acid production and respiration of *Aspergillus awamori*. They suggest that the hatching factor may stimulate the direct oxidation of glucose or glucose-6-phosphate in this organism.

Hatching stimulants for cyst-forming nematodes may thus provide the means of achieving the change from a form of metabolism appropriate to a dormant state to a form suitable for the active larvae. Various other parasites undergo changes in metabolism in response to changes in environment. The bloodstream and tsetse fly midgut culture forms of trypanosomes (*T. brucei* group) adapt themselves to their

environment by changes in their respiratory behaviour and their respiratory enzyme systems (Fulton & Spooner, 1959; Grant, Sargent & Ryley, 1961). Changes in respiration are also associated with the encystment of the protozoan *Colpoda cucullus* (Pigon, 1959). Some animal parasites, including nematodes, also depend on their hosts for stimuli to initiate changes leading to the development of their subsequent stages, although it is not known whether metabolic changes are involved (Rogers, 1960).

An attempt has been made to explain the hatching activity of various compounds for *H. schachtii*, advancing the idea that many hatching agents act either directly or indirectly as electron acceptors. This idea seems to cover most compounds now known to have activity.

We thank Mrs. Valerie Meaton and Miss Christine Hurley for their help with the experimental work.

#### ZUSAMMENFASSUNG

##### *Synthetische Schlüpfstoffe für Heterodera schachtii Schm. und ihre Wirkungsweise*

283 Verbindungen wurden auf ihre Schlüpfaktivität für *Heterodera schachtii* Schmidt geprüft. Das Verfahren war dem von Fenwick und Widdowson (1958) ähnlich. Die Aktivität jeder Verbindung ergibt sich aus der Schlüpfmenge, nach der Formel  $\frac{H_s - H_w}{H_D - H_w} \times 100$ , wobei  $H_s$  die Schlüpfmenge der Prüfschubstanz,  $H_D$  und  $H_w$  die Schlüpfmenge in Wurzel diffusat bzw. Wasser ist. Alle Versuche werden gleichzeitig durchgeführt. Die geprüften Stoffe sind Säuren, Aldehyde, Amine, Aminosäuren, Ester und Laktone, Phenole, Chinone und Redox-, heterocyclische und anorganische Verbindungen. 31 Substanzen gaben in bestimmten Konzentrationen Schlüpfresultate, die gleich oder grösser waren als die mit Rübenwurzel diffusat erhaltenen Werte. Zwischen Schlüpfaktivität und Redoxpotential wurden bei 25 Verbindungen keine Beziehung gefunden. Viele der aktivsten Verbindungen haben zwei polarisierbare Atome die zu einem konjugierten ungesättigten System verbunden sind, das gewöhnlich aus einer Kette von 5 Gliedern besteht, z.B. Juglon  $\text{HO}-\text{C}=\text{C}-\text{C}=\text{O}$  und Pikrinsäure  $\text{HO}-\text{C}=\text{C}-\text{N}=\text{O}$ . Es wird vermutet, dass viele Schlüpfstoffe als Elektronen-Acceptoren wirken und dass ihre Aktivität aus ihrer Wechselwirkung mit dem Elektronentransport-System entstehen kann.

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