Influence of pH on toxicity of the non-electrolytes coumarin and ethyl N-phenylcarbamate to *Spirodela polyrhiza*

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Summary

pH had a decided influence on the toxicity of coumarin when assessed in terms of the ability of *Spirodela polyrhiza* to recover from certain treatments. Ethyl N-phenylcarbamate was used subsequently, with a similar effect. The uptake of coumarin was found to be influenced by pH. However, pH had less influence when toxicity was assessed in terms of concentrations to give zero recovery or to halve growth rate.

The congruence of growth-rate depression and percentage recovery in assessing the influence of pH on the toxicity of non-electrolytes is queried.

Introduction

The influence of pH on physiological response has previously received considerable attention. Simon and Blackman (1949) contributed to the subject and reviewed earlier work, pointing out that, while pH can have a major influence on the response to weak acids and bases, it has little or no effect on the response to non-electrolytes. They concluded that if the compounds studied do not dissociate in solution then it is immaterial at what pH tests are made. This conclusion has been widely accepted.

Various criteria were used in the studies on which the above conclusion was based, e.g. inhibition of germination, growth, or respiration, induction of anaesthesia, and occasionally death of the test organism.

The studies cited often used as the criterion of effectiveness, the concentrations of the test substances required to halve biological response, this procedure being chosen on account of the greater accuracy with which such determinations are made (Blackman 1952). However, few, if any, investigations dealing with non-electrolytes used the ability (or conversely failure) of an organism to recover from a treatment and propagate. Failure to recover can be an important criterion of a treatment, e.g. in research with herbicides on water weeds, where the aim may be to destroy a population and leave no source for reinfestation.

The following investigation deals with the influence of pH on the response of the Australian duckweed *Spirodela polyrhiza** to treatment with two toxic non-electrolytes. This aquatic species was selected as the test organism because of its availability, convenience of culture in the laboratory over a wide range of pH values, and because its European counterpart *Lemna minor* was used extensively in growth studies relating to pH and toxicity (e.g. Blackman and Robertson-Cuninghame 1953). The cultural methods and treatment period described below were based on those used for *Lemna*. Coumarin was used as the main poison, being selected because some of the responses it induces in other species appear to be influenced by pH (Audus 1948), and because of its availability in radioactive form. According to Delaveau (1967), its action on plants includes an increase in DNA content, a decrease in ATP content, an increase in respiration, and an uncoupling effect. Ethyl N-phenylcarbamate (hereafter called EPC), the second poison, was used to check on the response to coumarin; it was chosen because its inhibition of respiration in yeast supports the claim that pH has little or no influence on its action (Simon and Beevers 1952).

Ability to recover was the main test used to assess the response of the duckweed at different pH levels. However, as the claims about the lack of influence of pH on the toxicity of non-electrolytes were based on a number of different tests, some attention was also given to respiration and depression of growth.

*The late Dr N. T. Burbidge, of the Herbarium Australiense, advised that this identification most closely fitted the duckweed used, but that the nomenclature of the Australian duckweeds needed critical revision.

Methods

Plant culture

Several clones of duckweed were obtained from Lake Burley Griffin (Canberra). The first, and subsequently the second clone, had to be replaced because after a year or more of laboratory culture they failed to grow adequately. All clones grew well at pH 5.15 to 7.2, and persisted for months at pH 4.05; in addition clone 1 grew for 5 months or more at pH 3.6 without producing any dead fronds (other clones were not tested at this value). In all clones reproduction was solely vegetative, mother-fronds budding off daughter-fronds which later became mother-fronds.

The duckweed was cultured at 25°C in 200-ml beakers containing 100 ml of solution, under 40-watt white Mazda fluorescent lamps giving continuous intensity of 50 W m^{-2} at frond level. The phosphate-rich culture medium used successfully by Blackman and Robertson-Cuninghame (1953) for *Lemna minor* was unsatisfactory for *S. polyrhiza*, causing the fronds to shed their roots every 7 to 10 days.

Ultimately a slight modification of Hutner's (1953) solution at one-third concentration proved satisfactory; according to Hillman (1961) most Lemnaceae grow as well or better at this dilution as in the undiluted solution. The solution used contained, in mg per litre: K_2HPO_4 133, $Ca(NO_3)_2.4H_2O$ 86, $MgSO_4.7H_2O$ 167, NH_4NO_3 13.2, KNO_3 66.5, $FeSO_4.7H_2O$ 8.3, $ZnSO_4.7H_2O$ 21.7, $MnSO_4.4H_2O$ 7.0, H_3BO_3 5.0, $Na_2MoO_4.2H_2O$ 8.3, $CuSO_4.5H_2O$ 1.3, $CoSO_4.7H_2O$ 0.33, disodium EDTA (with $2H_2O$) 167. For adjusting pH, NaOH or HCl were used. Reasonable buffering was provided by the disodium ethylenediaminetetra-acetate (EDTA), which has a number of dissociation constants; solutions adjusted to pH 3.6, 4.05, 5.15, 6.3 or 7.2 seldom changed by 0.2 unit (considered unimportant) over 2 days.

Stock plants cultured in the above medium were kept healthy for experimental use by transfer to fresh solution at least three times per week; prior to transfer they were washed with a stream of distilled water. Transfer to fresh culture solution occurred the day before an experiment. Stock plants were normally cultured at pH 5.15, though in some instances, as mentioned later, they were preconditioned at other pH levels as well. In the absence of a poison, at pH 5.15 or higher the plants doubled frond numbers in *c.* 2 days, but reproduced more slowly with decreasing pH.

Two days before an experiment stock plants were teased to give clumps with smaller numbers of fronds. In any one experiment the same number of 3-frond clumps, 4-frond clumps, and so on, were added to each beaker. As adopted by Prasad and Blackman (1965), only mother-fronds and daughter-fronds exceeding 2 mm diam. were counted, being regarded as 'plants'. Within any one experiment the fronds in the different treatments were matched for size as closely as possible, so that comparisons between treatments were more consistent than comparisons between experiments. Usually 30 plants were used per replicate (one beaker), with six replicates per treatment. The toxic solutions were changed daily, i.e. three fresh solutions were used over a 3-day period.

The growth rate decreased during a toxic treatment and the clumps soon broke up into smaller ones, usually yielding plants of single mother-fronds with daughter-fronds attached. If the treatment was not too severe, at the end of several days a few small daughter-fronds would have budded off and enlarged beyond the 2 mm limit; such fronds were kept separate from the rest and, if they recovered, were regarded as evidence that the parent plants had reproduced. Following severe treatments, fronds became partly or wholly bleached. A frond fully bleached in the region producing daughter-fronds and its adjacent tip died without reproducing (sinking within 17 days of treatment). On the other hand, fronds and daughter-fronds that recovered were apparently normal, and subsequently reproduced. The rate of recovery was largely unaffected between pH 7.2 and 5.15, but was slower at pH values below 5.15.

A plant was considered dead when it failed to produce one or more viable daughter-fronds. Recovery was measured in terms of recovered plants as a percentage of the number originally treated. This procedure eliminated errors resulting from different growth rates at different pH levels.

For dry weight values in growth inhibition studies, plants were oven-dried at 80°. Further details are given later.

Poisons: uptake of coumarin

The two poisons were twice purified by crystallization, the EPC from warm petroleum ether (B.P. 40°), the coumarin from hot water. Tritiated coumarin (Amersham, general label), used only in uptake studies, was diluted with inert coumarin, dissolved in toluene and kept at −20° to minimize self-decomposition. The toluene was evaporated from aliquots at 0°. All solutions were used at 400 μCi/litre (1.5×10^4 Bq/ml).

In the uptake of coumarin and recovery test, there were five replicates for the uptake series and six for the concurrent recovery series, with 40 plants per replicate. In the uptake series, 10 plants were harvested on days 1, 2 and 3; on day 7 only living (unbleached) plants were harvested, the assay figure being adjusted to the proportionate figure for 10 plants. At harvest the plants were rinsed four times in distilled water, patted dry with filter paper, wrapped in half a cigarette paper, then dried for 3 days at room temperature in sealed containers with phosphorus pentoxide and inert coumarin (to minimize volatilization).

The paper container, with treated plants of the one replicate, was ignited with a spark in a flask of oxygen sealed with a serum-bottle stopper. The flask was then stood on crushed ice for 30 min before being injected with 6.0 ml anhydrous methanol plus 0.5 ml Triton X-100. After a further 90 min with the flask on ice, 10 ml of a toluene solution containing 50 mg PPO and 1 mg dimethyl POPOP was added, swilled around the flask, then an aliquot was taken for counting.

Results

(a) Percentage recovery tests

Table 1 gives recovery figures for plants preconditioned at the test pH levels (exps 1–4) and treated with coumarin. It is seen that pH had a decided influence on recovery. It was not surprising that recovery figures at pH 3.6 and 4.05 were much lower in exp. 2 than in exp. 1; in the former experiment, where the plants were preconditioned for 2 months, the fronds were not growing as rapidly at the lower pH levels, and were smaller than the rest. In exp. 1 visibly different populations were used.

In exp. 3 there was a marked optimal value for recovery at pH 5.15, the recovery figure being less at pH 6.3 ($P < 0.001$), and still less at 7.2 ($P < 0.01$). It might be claimed that maximal recovery occurred at pH 5.15 because the stock plants had been cultured at that level. However, in exp. 4 with higher concentration of coumarin there was zero recovery at pH 5.15, with a significantly greater recovery at pH 6.3 or at pH 7.2 ($P < 0.001$). The influence of pH on the uptake of coumarin is considered later.

Frequently in trials at different pH levels, plants are not cultured before and afterwards at the test level. Table 1 also gives results for such a procedure (exps 5–7). Before and after the coumarin treatment plants were cultured at pH 5.15, with the exception of those which were treated at pH 5.15 – which were cultured before and afterwards at pH 7.2. It is seen from exp. 5–7 that again the pH level at which coumarin treatment was applied had a marked influence on recovery. The results, moreover, suggested the possibility of finding a coumarin concentration giving zero recovery at low pH and more than 50% recovery at a higher value. This possibility was later confirmed with clone 3 (exp. 8).

The overall impression given by the experiments listed in Table 1 (and also by preliminary ones in the course of developing the technique) was that more consistent results were obtained

Table 1 Recovery of *Spirodela polyrhiza* from coumarin treatments lasting 3 days, at different pH levels

Clone 1 used for exps 1 to 7, clone 3 for exp. 8. In exps 1 to 4 plants were subsequently cultured at test pH level. In exps 5 to 8 plants were initially cultured at pH 5.15, except those treated at pH 5.15 (cultured at pH 7.2); treated plants subsequently maintained at pre-test level. Where recovery was 0%, plants were dead at end of treatment, except for pH 4.05 exp. 7.

Exp. No.	Preconditioning period at test pH	Coumarin conc. mM	Percentage recovery from treatment at pH: 3.6	4.05	5.15	6.3	7.2
1	2 months	5.5	0	8	59	46	70
2	1 week	5.5	19	33	67	57	53
3	1 week	6.15	0	0	61	41	32
4	1 week	6.85	0	0	0	21	21
5	0	4.05	18	100	96	–	100
6	0	5.15	3	–	–	–	100
7	0	6.15	0	0	23	–	32
8	0	6.15	–	0	–	–	75
1–8	As above	0.0	100	100	100	100	100

when plants were preconditioned for 1 week before treatment and subsequently maintained at test pH. This procedure was used for the following experiments, because, under conditions of practical application, plants are seldom subjected to a sudden change, lasting for days, in the pH of the surrounding medium.

The response of clone 3 to 2.7 mM EPC was also influenced by pH: recovery was zero at pH 4.05 and 74% at pH 7.2. Results of an experiment on this clone to ascertain the lowest concentrations of coumarin causing zero recovery are given at the end of subsection *c*.

Table 2 Recovery of *S. polyrhiza* (clone 2) from 6.15 mM coumarin treatments lasting 3 days, also concurrent radioactive content

Plants preconditioned for 7 days at test pH, and maintained at test pH for recovery. Plants treated on day 0. All solutions changed on days 1 and 2; on day 3 remaining plants transferred, after a rinsing in culture medium, to further culture medium.

pH	Percentage recovery	Radioactivity as mean counts of 10 plants per 20 min Day 1		Day 2		Day 3		Day 7
4.05	0	30 500	***	42 900	***	55 800		(dead)
	***	***		***		***		
5.15	77	22 000	**	26 100	n.s.	25 400	***	4240
	***	**		*		***		***
7.2	57	17 800	n.s.	20 800	n.s.	18 800	***	2500

n.s., not significant; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, in relation to the values between which the symbols are placed.

(b) Uptake of coumarin and recovery

Clone 2 was used in this experiment to provide data for both uptake and recovery, with coumarin at 6.15 mM – the same concentration as in exp. 3 (Table 1). From Table 2 it is seen that the recovery pattern was similar to that of exp. 3, where there was zero recovery at pH 4.05, maximum recovery at pH 5.15, and significantly less at pH 7.2

Table 2 also shows, for each of days 1, 2, and 3, that counts for radioactivity lessened significantly with increasing pH. Since leaching increases with injury (Stiles and Stirk 1931), and the pH 4.05 treatment provided both the greatest injury (zero recovery) and the highest counts on days 1, 2 and 3, any loss of coumarin during the washing process at harvest is considered unimportant. The fall in counts for the pH 5.15 and 7.2 series from day 3 to day 7 can be attributed to loss of coumarin or its end products, following transfer of the plants to clean solution.

On the first 3 days the counts given by the pH 7.2 series were significantly less than those by the pH 5.15 series, though the higher pH level caused more injury. Leaching having been eliminated, the difference in counts must be attributed to a difference in metabolism at the two levels; moreover, at pH 7.2 the plants were less able to tolerate coumarin, or detoxify it, than at pH 5.15.

For both the pH 5.15 and 7.2 series there were fewer counts at day 3 than at day 2. Although differences were not statistically significant, a similar decrease was observed (though usually between day 1 and day 2) in three subsequent experiments. The reproducibility of this effect in four experiments, together with the significant and consistent increase in radioactivity of the more injured pH 4.05 series from day 1 to day 3 (Table 2), are regarded as evidence that at appropriate pH levels there is an active extrusion of coumarin. Such extrusion has been demonstrated for *Lemna minor* in relation to certain growth regulators (Blackman and Sargent 1959).

Counts were based on the whole plants without consideration of relative amounts in frond and root tissues. Nevertheless, the data are presented as showing that the reaction of the organism to an undissociating poison can be affected by external pH which influences metabolic processes such as uptake and response.

(c) Further tests for response

Claims concerning the influence of pH on biological response were based largely on its influence on respiration or growth of heterotropic organisms (Simon and Blackman 1949; Simon and Beevers 1952). *S. polyrhiza* as a phototropic organism did not readily lend itself to appropriate respiration studies. If darkness was used to eliminate complications arising from photosynthesis, it took some 9 h for the control plants to equilibrate, and, moreover, the plants became more susceptible to coumarin. Accordingly, respiration studies were abandoned, and attention was given to the influence of the two poisons on growth over an interval of 3 days. Clone 3 was used.

Increases in dry weight from days 0 to 3 of the control series (without poison) were used to provide the basic growth data. Growth changes over the same interval were determined for plants grown in coumarin at 0.17, 0.34, 0.68, 1.37 mM, and in EPC at 0.125, 0.25, 0.50 and 1.00 mM. In these ranges the relationship between growth and concentration was linear, and the ranges included the concentration to be estimated. Coumarin at 0.17 mM at pH 4.05 caused a slight, but not significant, increase in dry weight; EPC at 1.00 mM caused a significant loss in dry weight in relation to that at day 0, $P < 0.05$ at pH 4.05 and $P < 0.001$ at pH 7.2.

Concentrations required to halve growth increase were estimated by fitting straight lines to the data. The values obtained, with the 95% inverse fiducial limits (Williams 1959) given in brackets, are:

coumarin
pH 4.05 0.86 mM (0.74, 1.03)
pH 7.2 0.60 mM (0.51, 0.74)
EPC
pH 4.05 0.42 mM (0.35, 0.51)
pH 7.2 0.28 mM (0.26, 0.31)

It is seen that for both poisons the concentration required to halve growth rate is significantly more at the lower pH level, and the concentration at the lower level is 1.5 times or less than that at the upper level.

Finally, to provide a basis for comparison with the above growth responses, an experiment was done with clone 3 using higher concentrations of coumarin, increasing by 0.33 mM. The lowest concentration for zero recovery was 6.5 mM at pH 4.05 and 9.5 mM at pH 7.2.

Discussion and conclusions

The results are discussed in relation to three criteria of toxicity, viz. concentrations of the substances required to cause zero recovery, recovery from a particular concentration, and concentrations required to halve growth rate.

On the basis of concentrations to cause zero recovery, pH had comparatively little influence on the toxicity of coumarin: the concentration at pH 7.2 (9.5 mM) was 1.5 times that at pH 4.05 (6.5 mM). However, the concentration of either poison causing zero recovery at pH 4.05 allowed more than 70% of the population to recover at pH 7.2 (exp. 8, and subsequent text).

As regards growth depression, nor-

mally one would expect increased depression to result from pH conditions favouring low recovery. However, the reverse is indicated by the calculated values to halve growth rate; for both poisons the concentration required at pH 4.05 was higher than that at pH 7.2. Two inter-related explanations are offered for the apparent anomaly. (1) There is an interaction between concentration of poison and pH; thus exps 3 and 4 (Table 1) show how the pH for maximal recovery rose as coumarin concentration was increased. (2) It is likely that a poison does not have the same mode of action at low and at high concentration; in particular, coumarin concentrations required above to cause zero recovery were some 8 to 16 times those required to halve growth rate. In support of the latter explanation it is pointed out that at low concentration many poisons are capable of stimulating growth.

Earlier investigations with non-electrolytes, e.g. those of Labes (1922), who used narcotics on tadpoles, and those of Clowes and Keltch (1931), who used anaesthetics on arenicola larvae, show little or no variation with changed pH level. Possibly *S. polyrhiza* is a more sensitive test organism or, more likely, the earlier tests cited were only semi-quantitative. Obviously, *S. polyrhiza* is not as affected in response to coumarin with change in pH as *Lemna minor* is to a dissociating poison such as 2,4-D (Blackman and Robertson-Cuninghame 1953). Nevertheless, pH can have a critical influence on the recovery of *S. polyrhiza* from treatments with coumarin and EPC.

Although *S. polyrhiza* is seldom a nuisance, and the poisons tested are not conventional herbicides, the above findings have some relevance to research on the chemical control of aquatic weeds.

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