The rôle of calcium in the hatching of *Globodera rostochiensis*

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SUMMARY

Experiments indicating that free Ca^{2+} is not essential for the hatching of *Globodera rostochiensis* Woll. eggs, are described. *G. rostochiensis* eggs were stimulated to hatch by solutions of decationised potato-root exudate to which a range of 0.5 to 10mM of Na^+, K^+, Ca^{2+} or Mg^{2+} chlorides were added, but only the solution containing 0.5mM $CaCl_2$ caused the emergence of significantly more juveniles from cysts than the exudate alone. When cysts were extracted with the chelating agent EGTA to remove Ca^{2-} and other polyvalent cations the number of juveniles which emerged in dilute exudate solutions containing up to 2.0mM $CaCl_2$ reached a maximum at 0.1mM $CaCl_2$, probably because some of the hatching factor ionically bound to inert materials such as the cyst walls was displaced by Cl^-. Ca^{2+} is extracted from *G. rostochiensis* cysts by decationised potato-root exudate. Concentrations of 1 to 12 mM EGTA did not significantly inhibit the hatching induced by root exudate. We found that the calcium ionophore A23187 inhibited hatching and that Ca^{2+} did not activate juveniles immobilised by 0.4M trehalose. It is suggested that the initiation of hatching may be the result of changes in eggshell permeability brought about by the effect of the hatching factor on bound Ca^{2+}.

RÉSUMÉ

Rôle du calcium dans l'éclosion de Globodera rostochiensis

Les auteurs décrivent des expériences indiquant que le Ca^{2+} libre n'est pas essentiel pour l'éclosion des œufs de *Globodera rostochiensis* Woll. L'éclosion de ces œufs a été stimulée par des solutions d'exsudats de racines de pomme de terre auxquelles des chlorures de Na^+, K^+, Ca^{2+} et Mg^{2+} étaient ajoutés en quantités variant de 0.5 à 10mM, mais seule la solution contenant 0.5mM $CaCl_2$ a provoqué la sortie d'un nombre de juvéniles significativement supérieur à celui obtenu avec l'exsudat seul. Quand les kystes étaient extraits avec de l'EGTA, agent de chélation, pour enlever Ca^{2+} et autres cations polyvalents, le nombre de juvéniles émergeant dans des solutions d'exsudats contenant jusqu'à 2mM $CaCl_2$ était maximum pour 0,1mM $CaCl_2$, probablement parce qu'un facteur stimulant l'éclosion et lié à un matériel inerte, tel que la paroi du kyste, était déplacé par Cl^-. Ca^{2+} est extrait des kystes de *G. rostochiensis* par l'exsudat décationisé. Des concentrations de 1 à 12mM d'EGTA n'ont pas inhibé significativement l'éclosion induite par l'exsudat de racine. Les auteurs ont observé que l'ionophore A23187 du calcium inhibait l'éclosion et que Ca^{2+} n'activait pas les juvéniles immobilisés par 0,4 mM de tréhalose. Ils émettent l'hypothèse que l'initiation de l'éclosion peut être le résultat de changements dans la perméabilité du tégument de l'œuf dus à l'action du facteur stimulant de l'éclosion sur le Ca^{2+} lié.

Robinson and Neal (1956, 1959), Ellenby and Gilbert (1957, 1958) and Dropkin, Martin and Johnson (1958) have suggested that metal ions may be involved in the hatching mechanism of the potato cyst-nematode, *Globodera rostochiensis* Woll. More recently Atkinson and Ballantyne (1979) revived the hypothesis that Ca^{2+} in particular has an active role in the initiation of hatching. The experiments of Ellenby and Gilbert (1958) are the only evidence that Ca^{2+} may contribute to the hatch-stimulating action of potato-root exudate. Mg^{2+}, however, was reported to have a similar effect. Clarke and Hennessy (1981) showed that experiments with the Ca^{2+}-inhibitors, ruthenium red and $LaCl_3$ did not prove that free Ca^{2+} was necessary for the initiation of hatching. Moreover, in contrast to the view that a single specific cation is involved, Robinson and Neal (1956, 1959) suggested that a mixture of Ca^{2+}, Mg^{2+}, Na^+ and K^+ ions is essential for maximum activity of the hatching factor.

We have made various experiments with the aim of clarifying the evidence relating to cation involve-

ment in *G. rostochiensis* hatching. We re-examined the effects of Ca^{2+}, Mg^{2+}, Na^{+} and K^{+} on the hatching properties of decationised potato-root exudate (DPRE) using both untreated cysts, and cysts extracted with chelating agents to remove divalent cations. We also determined the amounts of Ca^{2+} extracted from cysts by DPRE and chelating agents. We repeated hatching tests (Atkinson & Ballantyne, 1979) of the ionophore A23187. The compound is reported to be a hatching agent and might act by carrying Ca^{2+} through the egg-shell. Furthermore, we tested the effect of Ca^{2+} on juveniles immobilised in 0.4M trehalose (Clarke, Perry & Hennessy, 1978), *i.e.* with the juveniles exposed to the conditions thought to prevail within the egg.

Materials and methods

Cysts of *G. rostochiensis*, pathotype Ro1, were raised on potato plants in pots and extracted by the usual method (Shepherd, 1970). The hatching tests were made with batches of 100 cysts in three-fold or greater replication in solid watch glasses. The dried cysts were soaked for one week in distilled water, after which the water was removed and replaced by a test solution. Hatched juveniles were counted (Shepherd, 1970) after ten days.

Potato-root exudate (PRE) was obtained as described by Shepherd (1970). Decationised potato-root exudate (DPRE) was prepared by slow passage ($<$ 0.4 ml/min) of PRE (125 ml) through a column of cation-exchange resin (Dowex 50 W $\times$ 8 ; H^{+} form, 24 cm $\times$ 1.2 cm internal diameter). The first 25 ml of the eluate was discarded and the following 100 ml retained for use. The Ca^{2+} content of the eluate was used as a measure of decationisation. A typical solution of undiluted DPRE had a pH of 2.4 and contained about 200 µg dry weight of non-volatile solids/ml and $<$ 1 ppm of Ca^{2+}.

Hatching tests were carried out with solutions of 0.5, 1.0, 2.5, 5.0, 7.5 and 10.0mM of each of NaCl, KCl, $MgCl_2$ and $CaCl_2$ (Fig. 1) in DPRE and were similar to those of Ellenby and Gilbert (1958) in that sub-optimal concentrations of DPRE were used. However, we tested a more limited range of salt concentrations (Ellenby and Gilbert, 1958, tested concentrations of 0.2 to 100mM) and we did not use non-ionic solutes to compensate for any differences in osmotic pressure. Our hatching tests used many cysts in contrast to the single cyst technique of Ellenby and Gilbert (1958).

The mixture of metal chlorides ("Salts A") used by Robinson and Neal (1956, 1959) in their hatching tests contained 0.13mM KCl, 0.21mM NaCl, and either 0.29mM $MgCl_2$ and 4.06 mM $CaCl_2$ ("Salts AA") or 0.13mM $MgCl_2$ and 2.10M $CaCl_2$ ("Salts AB"), depending on whether the concentrations (given in µg/ml for "Salts A") refer to the anhydrous or hydrated salts of Mg^{2+} and Ca^{2+}. We therefore compared (Tab. 1) the number of juveniles which emerged from cysts in a 1:4 dilution of DPRE, in 1:4 dilutions of DPRE containing "Salts AA", and "Salts AB", and in 1:4 dilutions of DPRE containing 4.06mM or 2.10mM $CaCl_2$.

To observe changes in the pH of test solutions, batches of about 100 cysts were prepared as for hatching tests (see above). After soaking, the water was replaced by a 1:16 dilution of DPRE (1.5 ml) or a 1:16 dilution of DPRE containing 1 mM $CaCl_2$ (1.5 ml). The pH of the supernatant liquid was determined at intervals over a period of 3h.

The metal chelating agents, sodium ethylene-diamine-N,N,N¹,N¹-tetra-acetate (EDTA), and sodium 1,2-di(2-aminoethoxy) ethane-N,N,N¹,N¹-tetra-acetate (EGTA) were used to remove Ca^{2+} and other polyvalent cations from outside the eggs *i.e.* bound to the cyst walls and possibly elsewhere.

To determine the amounts of Ca^{2+} extracted and to test the effects of the removal of metal ions, cysts were given four successive extractions with 30mM EDTA or EGTA - two days per treatment - followed by three washings with distilled water (one day per treatment), before the solutions for hatching tests were added. The pH of the solutions of chelating agents was adjusted to 7.6 before use. The extracts were analysed for Ca^{2+} ; they contained few or no juveniles.

To determine the amount of DPRE-extractable Ca^{2+} (Tab. 4) the cysts were first soaked in distilled water at 25°. After six days, the water was replaced by a 1:4 or 1:16 dilution of DPRE (1.5 ml/watch glass), and the cysts were stored at 3°. The DPRE was changed daily. The solutions removed from batches of twelve watchglasses were filtered, combined and analysed for Ca^{2+}. The glassware for the experiments described by Figs 2, 3 and 4 was washed with EDTA, dilute hydrochloric acid and distilled water. The total Ca^{2+} content of solutions was determined by atomic absorption spectroscopy.

The effect of various solutes on the movement of juveniles was compared as described in Clarke, Perry and Hennessy (1978) and Clarke and Hennessy (1981). In particular, to test the effect of Ca^{2+} on juvenile movement in a medium comparable to that of the egg fluid (Clarke, Perry & Hennessy, 1978) we immobilised juveniles by storing them for seven days in 0.4M trehalose before adding a small volume of 1M $CaCl_2$ to the solution. The number of juveniles moving was counted at one and two days after the

addition of $CaCl_2$, and also at one and two days after dilution with water to obtain a trehalose concentration less than 0.1M.

The ionophore 23187 is only sparingly soluble in water and so is often used as a colloid, prepared by adding a concentrated solution in organic solvent to a vigorously agitated aqueous phase. We found that organic solvents tended to inhibit hatching and so used suspensions instead : the solvent was removed from a small volume of 0.01M ethanolic solution of A23187 by a current of air. The film of ionophore was scraped from the glass with a spatula and the particles suspended in the aqueous medium. Ultraviolet light showed any A23187 left in the beaker.

The solubility of A23187 in aqueous solutions was determined by spectroscopy. The aqueous phase (10 ml) was stirred in a stoppered test-tube with 0.1μmol of A23187 for 24 h at ambient temperature (about 20°) and filtered from undissolved solid. The A23187 recovered by washing the test-tube and filter (Whatman glass microfibre paper) with ethanol, was made up to 4.0 ml. The absorption spectra of aqueous extracts of A23187 showed no distinct bands in the range 200 to 400 nm. By contrast ethanol solutions of the recovered water-insoluble fractions showed pronounced bands at 228, 304 and 379 nm. Comparison of the absorption at 304 nm of the ethanolic solutions with that of a control showed that at least 88 % of the ionophore was not dissolved by the aqueous solvents (distilled water, tap water, 0.4mM $CaCl_2$ and PRE diluted 1:64 with 0.4mM $CaCl_2$) *i.e.* the aqueous solutions contained concentrations less than 1.2 μM A23187.

Significance of results (Figs 1, 2 and 3, Tab. 5) was estimated by analysis of variance following log transformation.

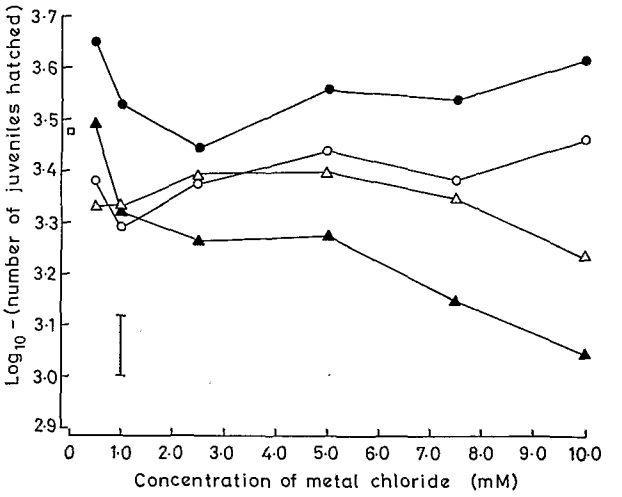

Fig. 1. Emergence of juveniles from cysts immersed in a 1 : 16 dilution of decationised potato-root exudate (white square), and in similar solutions containing 0.5 to 10mM Na^+ (white triangle), K^+ (black triangle), Mg^{2+} (white circle) or Ca^{2+} (black circle) chloride. Three-fold replication, batches of 100 cysts for each test. Mean juvenile emergence in distilled water : 92. Vertical bar ; standard error of means derived from transformed data.

Results

Figure 1 shows the number of juveniles which emerged in hatching tests with solutions containing 0.5 to 10mM K^+, Na^+, Ca^{2+} or Mg^{2+}. The results of several similar experiments showed common features, although the shape of the hatch/salt concentration curve for a particular salt varied from one experiment to another. Thus, with Na^+ at all concentrations, and with K^+ at concentrations over 2 mM, fewer juveniles emerged than with exudate alone. Occasionally, more juveniles emerged in solutions containing < 1mM K^+ than from those in exudate, although the difference was not significant ($p > 0.05$). At least as many juveniles consistently emerged in exudate solutions containing Ca^{2+} as in the unmodified exudate. Maximum emergence from cysts occured in solutions containing less than 2mM $CaCl_2$.

The number of juveniles emerging from cysts in exudate with added Ca^{2+} was never greater than twice the number from cysts treated with DPRE alone. The increase in emergence was sometimes significant ($p < 0.05$).

There was a lesser hatch in solutions of exudate containing Mg^{2+} than in those containing Ca^{2+} at the same concentrations and the hatch was usually less than in DPRE. However, occasionally in tests with 2 to 5mM $MgCl_2$, slightly more juveniles emerged from cysts than with DPRE.

The 1:4 dilution of DPRE containing "Salts AB" caused the emergence of significantly more ($p < 0.05$) juveniles than were obtained with a 1:4 dilution of DPRE alone (Tab. 1). However, as the number of juveniles which emerged from cysts treated with the DPRE solution containing "Salts AB" was similar to the number of juveniles obtained by treatment of cysts with a DPRE solution containing 2.10mM $CaCl_2$, the effects of cation mixtures were not further investigated.

Table 1

Emergence of juveniles from cysts of *G. rostochiensis* treated with (A) a 1:4 dilution of decationised potato-root exudate (DPRE) and with similar dilutions of DPRE containing "Salts AB" or 2.10mM $CaCl_2$; (B) a 1:4 dilution of DPRE, and similar dilutions of DPRE containing "Salts AA" or 4.10mM $CaCl_2$. Three-fold replication, 100 cysts/test.

Test Solution	*Mean Hatch*
(A)	
1:4 dilution of decationised potato-root exudate	2 817
1:4 dilution of decationised potato-root exudate containing "Salts AB"	4 617
1:4 dilution of decationised potato-root exudate containing 2.10mM $CaCl_2$	4 550
Glass-distilled water	150
(B)	
1:4 dilution of decationised potato-root exudate	4 630
1:4 dilution of decationised potato-root exudate containing "Salts AA"	3 465
1:4 dilution of decationised potato-root exudate containing 4.06mM $CaCl_2$	5 345
Glass-distilled water	345

Table 2

Changes in test solution pH after contact with cysts of *G. rostochiensis* (1.5 ml/100 cysts).

	pH after contact with cysts for		
Solution	*0 min*	*5 min*	*3 h*
1:16 dilution of decationised potato-root exudate	4.4	5.7	6.3
1:16 dilution of decationised potato-root exudate containing 1mM $CaCl_2$	4.6	5.2	5.7

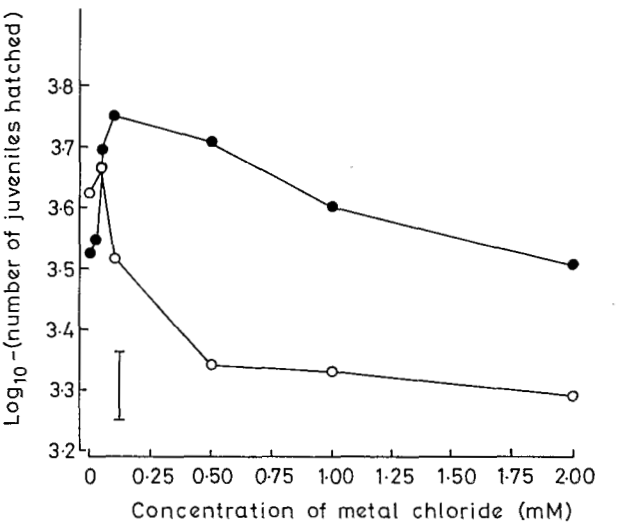

Fig. 2. Emergence of juveniles from cysts treated with 30mM EGTA or for the experiment with $MgCl_2$ 30mM EDTA, and distilled water, and then immersed in a 1:16 dilution of decationised potato-root exudate containing 0.1 to 2.0mM $CaCl_2$ (black circle), or $MgCl_2$ (white circle). Mean juvenile emergence in distilled water : 80 and 200 respectively. For both experiments five-fold replication was used with batches of 100 cysts for each test. Vertical bar ; standard error of means derived from transformed data.

Table 2 shows the changes in pH observed over a period of 3 h when cysts were immersed in a 1:16 dilution of DPRE containing 1mM $CaCl_2$.

Figure 2 shows the hatch from EGTA-extracted cysts treated with a 1:16 dilution of DPRE and a 1:16 dilution of DPRE containing 0.025 to 2.0mM $CaCl_2$. Maximum emergence was obtained with 0.10mM $CaCl_2$ and more juveniles emerged than from cysts treated with DPRE alone. At concentrations > 0.1mM of added $CaCl_2$, results resembled those shown in Fig. 1, *i.e.* juvenile emergence decreased gradually with increasing concentration of $CaCl_2$. Fig. 2 also shows the hatch from EDTA-extracted cysts treated with a 1:16 dilution of DPRE and a 1:16 dilution of DPRE containing 0.025 to 2.0mM $MgCl_2$. Emergence decreased with increase in Mg^{2+} concentration.

The amounts of Ca^{2+} removed from cysts by chelating agents at 25° as in the experiments relating to Fig. 2 are shown in Table 3. Table 4 shows that Ca^{2+} is also extracted from cysts by DPRE at 3°. Few juveniles emerged under these conditions. The cumulative values for Ca^{2+} extracted after four days by 30mM EGTA (Tab. 3), 1:4 and 1:16 dilutions

Table 3

Total calcium (ppm), given as the mean of three replicates together with the standard error, in four successive extracts at 25° of *G. rostochiensis* cysts (100 cysts/1.5 ml solution) with 30mM EGTA, and three with glass-distilled water (GDW) and then with either a 1:4 dilution of decationised potato-root exudate (DPRE) or 2mM EGTA. Mean juvenile emergence from 100 cysts, in 1:4 DPRE (8a) and 2 mM EGTA (8b), 3 700 and 150, respectively.

Extract	*Extracting solution*	*Period of treatment (days)*	*Calcium content (ppm)*
1	EGTA	2	21.53 ± 1.258
2	EGTA	2	2.30 ± 0.367
3	EGTA	2	2.15 ± 0.377
4	EGTA	2	1.53 ± 0.150
5	GDW	1	1.18 ± 0.048
6	GDW	1	0.40 ± 0.071
7	GDW	1	0.53 ± 0.184
8(a)	DPRE	10	0.65 ± 0.050
8(b)	EGTA	10	1.70 ± 0.150

Table 4

Total calcium (ppm), given as the mean of three replicates together with the standard error, in four successive 24 h extracts of *G. rostochiensis* cysts (1.5 ml/100 cysts) with 1:4 and 1:16 dilutions of decationised potato-root exudate (DPRE) and glass-distilled water (GDW) at 3°. Total Ca^{2+} (ppm) present in successive extracts of cysts.

Extract	*1:4 DPRE*	*1:16 DPRE*	*GDW*
1	7.95 ± 0.475	4.56 ± 0.179	2.33 ± 0.075
2	7.41 ± 0.190	3.80 ± 0.015	2.35 ± 0.035
3	4.67 ± 0.105	3.33 ± 0.262	1.92 ± 0.085
4	2.70 ± 0.660	2.98 ± 0.087	2.29 ± 0.690

of DPRE, and by distilled water (Tab. 4) were 23.8, 22.7, 14.7 and 8.9 ppm Ca^{2+} in the test solution (40 ppm = 1mM Ca^{2+}). The cumulative value for Ca^{2+} removed from cysts by four successive EGTA extractions over eight days was 27.5 ppm (= 0.69mM).

There was little Ca^{2+} in the 1:4 DPRE (0.7 ppm) or in the 2 mM EGTA (1.7 ppm) removed from around the cysts after the 10-day treatment (Tab. 3) at 25°.

Figure 3 shows the number of juveniles which emerged from EGTA-extracted cysts in a 1:4 dilution of DPRE containing 1 to 12mM EGTA at pH 7.2. Although hatching decreased with increase in EGTA concentration, there was no significant difference ($p > 0.05$) between the hatch in DPRE and in EGTA solutions.

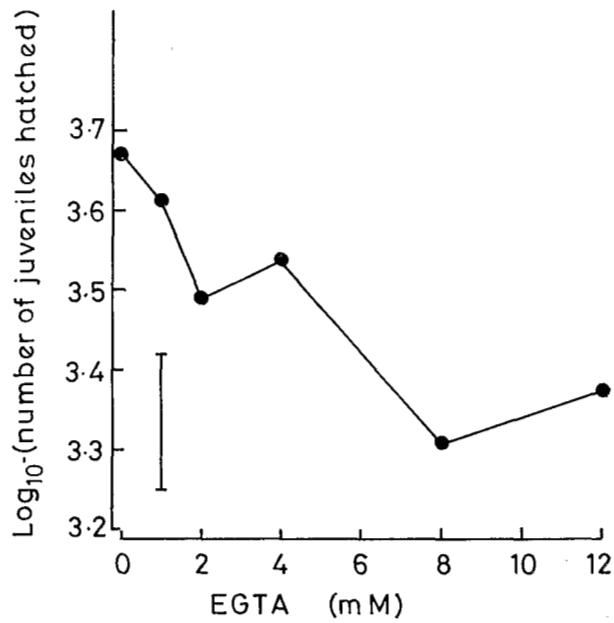

Fig. 3. Emergence of juveniles from cysts treated with 30mM EGTA and distilled water, and then immersed in 1:4 decationised potato-root exudate (DPRE) containing 1 to 12mM EGTA. Five-fold replication, with 100 cysts for each test. Mean juvenile emergence in distilled water : 217. Vertical bar ; standard error of means derived from transformed data.

Figure 4 shows that the juveniles remained inert when the 0.4M trehalose solution was made 0.2, 2.0 or 4.0mM with respect to $CaCl_2$. The juveniles became mobile however after the concentration of trehalose was reduced to less than 0.1M by water dilution.

Table 5 shows the results of hatching tests with suspensions of A23187 in various solutions. The numbers of juveniles which emerged from cysts

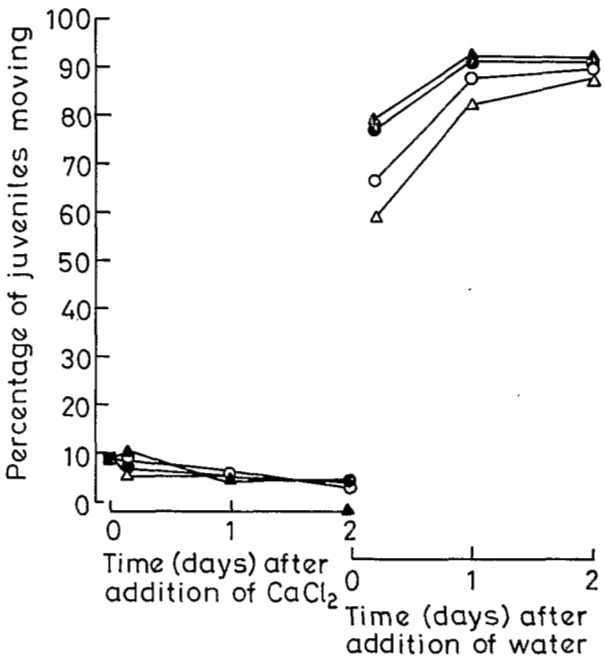

Fig. 4. Percentage of juveniles moving in 0.4 M trehalose (black square) solutions after seven days, and at 6, 24 and 48 h after adjustment of the solutions so that they contained 0 (black circle), 0.2 (white circle), 2.0 (black triangle), or 4.0 mM (white triangle) $CaCl_2$, and thereafter at 6, 24 and 48 h after addition of water.

Table 5

Emergence of juveniles from cysts immersed in potato-root exudate diluted 1:64 with distilled water or 0.4 mM $CaCl^2$, and in similar dilutions containing suspensions of the ionophore A23187 (0.10 µmol/10 ml). Five-fold replication, batches of 100 cysts for each test. The hatch obtained in a test marked * was significantly less ($p < 0.05$) than the hatch obtained in a test with the comparable solution without A23187.

Test Solution	*Mean Hatch*
Potato-root exudate diluted 1:64 with distilled water	2 205
Potato-root exudate diluted 1:64 with distilled water and containing a suspension of A23187 (0.10 µmol/10ml)	1 145*
Potato-root exudate diluted 1:64 with 0.4mM $CaCl_2$	2 580
Potato-root exudate diluted 1:64 with 0.4mM $CaCl_2$ and containing a suspension of A23187 (0.10µmol/10 ml)	930*
0.4mM $CaCl_2$	1 455
A suspension of A23187 (0.10 µmol/10 ml) in distilled water	375*
Distilled water	1 360

treated with A23187 suspended in water or with 1:64 dilutions of PRE containing A23187, were significantly less ($p < 0.05$) than the numbers which emerged from cysts in the appropriate solution without A23187.

Discussion

Hatching tests with solutions of DPRE to which metal chlorides were added (Fig. 1) gave a strikingly variable pattern of results, but they provide partial confirmation of the findings of Ellenby and Gilbert (1957, 1958). In particular, the experiments showed that the number of juveniles emerging from cysts treated with 1:16 DPRE could be significantly increased ($p < 0.05$) when the solution contained 0.5mM $CaCl_2$. Of the other chlorides tested, Na^+ tended to inhibit hatching, but low concentrations of Mg^{2+} and K^+ sometimes caused the emergence of more juveniles from cysts than were released by DPRE alone, although the increase were not significant ($p > 0.05$). Our experiments with a mixture of cations ("Salts A", Robinson & Neal, 1959) showed (Tab. 1) that similar numbers of eggs were hatched by DPRE containing "Salts A" (with concentrations of 2.10 mM $CaCl_2$ and 0.13mM $MgCl_2$), and by DPRE containing only the $CaCl_2$ component of the mixture.

Cysts treated with metal-chelating agents gave more reproducible results (Fig. 2). Hatch from cysts treated with 1:16 DPRE containing up to 2.0mM $CaCl_2$, reached a maximum at 0.1mM $CaCl_2$ and was significantly greater ($p < 0.05$) at this concentration than with DPRE alone.

Ellenby and Gilbert (1957, 1958) suggested that their experiments showed that cation transport is an essential part of the hatching mechanism and that Ca^{2+} synergised the action of the hatching factor. We believe our results (*e.g.* Fig. 2) show the effect of opposing actions by cation and anion, with Ca^{2+} inhibiting, and Cl^- enhancing hatch. We attribute the hatch-enhancing effect of Cl^- to competition

with the hatching factor anion ; when cysts are treated with solutions of DPRE, some of the hatching factor binds to basic groups present in the cyst walls, and elsewhere. If $CaCl_2$ is present, the Cl^- competes more effectively than the hatching factor anion for the basic sites and hence the concentration of the hatching factor in solution is increased. The chlorides of other metals will behave similarly (in proportion to the Cl^- concentration). The effect is however more readily detectable with $CaCl_2$ because of the less pronounced inhibitory action of Ca^{2+}. In experiments with $MgCl_2$ for example (Fig. 2) the Cl^- concentrations, at which displacement of hatching factor from basic sites occur, are also those at which there is marked inhibition by Mg^{2+}. Na^+ and K^+ also inhibit more strongly than Ca^{2+} (Fig. 1).

The removal of hatching factor (and other acids) from solution by binding to cyst wall protein might be expected to increase the pH of the dilute unbuffered medium. In keeping with our explanation, the pH of dilute DPRE left in contact with cysts for 3 h shifted nearer neutrality by almost 2 pH units. A similar experiment with dilute DPRE containing 1mM $CaCl_2$ only changed the pH by about 1 pH unit (Tab. 2).

The cyst walls (Clarke, 1968) which comprise about 34 % by weight of the cysts, contain about 72 % protein. The protein contains about 0.13 µequiv. of free amino groups (lysine and arginine) per 100 cysts. Figure 2 shows maximum hatch at 0.1mM added $CaCl_2$; at this concentration the test solution (1.5 ml/100 cysts) contained 0.30 µequiv. Cl^-, *i.e.* sufficient to displace the more weakly bound anions from the protein.

A complicating feature of the experiments such as those performed for Figure 1, is that the cysts themselves provide a source of Ca^{2+}. We have shown (Clarke, 1968, 1970) that cyst walls of both *G. rostochiensis* and *Heterodera schachtii* contain Ca^{2+} and that much of the Ca^{2+} in the cyst wall of the latter species is extractable with EDTA. Tables 3 and 4 show that both the chelating agent EGTA and DPRE removed bound Ca^{2+} from cysts of *G. rostochiensis*. The results given in Table 4 together with those of Fig. 2, show that the Ca^{2+} extracted from *G. rostochiensis* cysts by DPRE in hatching tests may be sufficient to influence the number of juveniles which emerge from cysts, and that the amount of Ca^{2+} present will depend on the concentration of extractants in the DPRE. It is noteworthy that hatching tests with DPRE containing added $CaCl_2$ show a significant enhancement of hatch relative to that obtained with DPRE solutions, only when dilute sub-optimal concentrations of DPRE are used. Also, onset of hatching may alter any existing equilibrium between free and bound ions by exposing new sites to the test solutions. Atkinson, Taylor and Ballantyne (1980) reported that eggshells removed from stimulated eggs contained more Ca^{2+} than those from non-stimulated eggs.

Our observations on the influence of cations on the hatching of *G. rostochiensis* are clearly relevant to the assay of isolates of the natural hatching factors.

According to Atkinson and Taylor (1980) the initiation of hatching may involve the uptake of Ca^{2+} and its transport through the eggshell. Calcium-chelating agents such as EDTA or EGTA inhibit cellular Ca^{2+}-transport systems. Complete inhibition can be obtained by decreasing the concentration of free Ca^{2+} to $< 10^{-8}$ M with EGTA or EDTA (see Reed & Bygrave, 1975). Fig. 3 shows a substantial hatch from EGTA-extracted cysts treated with DPRE containing 1 to 12mM EGTA. The large hatch again suggests that Ca^{2+} from outside the eggshell is not responsible either for initiating hatching or for the subsequent emergence of many juveniles from the cysts.

As evidence in support of a messenger role for Ca^{2+} in the hatching of *G. rostochiensis*, Atkinson and Ballantyne (1979) reported that the ionophores BrX537A and A23187 had some hatching activity. The authors suggested that such compounds might interfere with the normal control of hatching by carrying Ca^{2+} through the egg-shell or juvenile. We have tested one of the ionophores. In preliminary experiments we found that the solubility of A23187 in aqueous solutions ($< 1.2\mu M$) was much less than the concentrations of up to 50µM reported by Atkinson and Ballantyne (1979). Our tests (Tab. 5) with suspensions of A23187 showed that the ionophore did not stimulate the release of juveniles from cysts, nor did it synergise the hatching activity of dilute PRE ; on the contrary, it inhibited hatching. We previously reported (Clarke & Hennessy, 1978) that various crown compounds have little hatching activity and that ion transport experiments provided no evidence of the movement of Ca^{2+} through lipophilic layers by the hatching factor.

Any proposed hatching mechanism which involves a messenger role for Ca^{2+} must take into account the presence of about 0.4M trehalose in the egg fluid of *G. rostochiensis*. The ability of some sugars to complex Ca^{2+} is well known (Cook & Bugg, 1977 ; Poonia & Bajaj, 1979). Vassilev and Russev (1981) have shown that the ion activity of $CaCl_2$ decreases in the presence of sucrose. More particularly, a crystalline trehalose-calcium bromide complex has been prepared and its structure established (Cook & Bugg, 1973). Binding of Ca^{2+} by trehalose is thus likely to influence the concentration of free Ca^{2+} in the egg fluid. Fig. 4 shows that juveniles immobilised by immersion in

0.4M trehalose remained inert even when the medium contained 4mM $CaCl_2$.

Atkinson, Taylor and Ballantyne (1980) have commented on the uptake of Ca^{2+} by *G. rostochiensis* juveniles in eggs treated with PRE. We suggest the Ca^{2+} uptake may be incidental to the inferred change in permeability of the egg-shell (Clarke & Perry, 1977 ; Clarke, Perry & Hennessy, 1978) rather than indicative of the hatching mechanism. The Ca^{2+} uptake by juveniles coincides with the uptake of water by the unhatched juveniles (Ellenby & Perry, 1976) and may be part of the maintenance of the internal salt balance of the juvenile in response to water uptake.

This paper reports several experiments which suggest that the hatching mechanism of *G. rostochiensis* does not involve the transport of Ca^{2+} through the eggshell. We have shown that *G. rostochiensis* eggs hatch in test solutions in which there is a virtual absence of free Ca^{2+}. We have indicated that the addition of Ca^{2+} to DPRE probably does not increase its ability to initiate hatching, and shown that Ca^{2+} does not activate juveniles immobilised in 0.4 M trehalose.

As an alternative hypothesis for the initiation of hatching we suggest that the hatching factor may change eggshell permeability (Clarke & Perry, 1977 ; Perry & Clarke, 1981) by its effect on the bound Ca^{2+} of the eggshell.

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