THE EFFECT OF WEAK ELECTROLYTE SOLUTIONS ON THE HATCHING RATE OF THE EGGS OF *TRICHOSTRONGYLUS RETORTAEFORMIS* (ZEDER) AND ITS INTERPRETATION IN TERMS OF A PROPOSED HATCHING MECHANISM OF STRONGYLOID EGGS

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INTRODUCTION

During experiments to determine the optimum pH for the hatching of the eggs of *Trichostrongylus retortaeformis* (Wilson, 1957) it became evident that the concentration of the buffer solutions was an important factor determining the degree of hatch after a standard time. It seemed at first sight that this was a straightforward osmotic effect on the eggs, although the buffers used were not particularly strong solutions (range = 0·05–0·2 molar). This paper describes experiments which show that weak ionic solutions exert a specific effect on the hatching eggs which is not the consequence of colligative properties. Further investigations are described which attempt to use the ionic effect as a tool to elucidate the hatching mechanism.

The interpretation of the results is based on the structure of the strongyloid egg suggested by Monne & Hönig (1954). Their investigations indicate that the outer egg membrane is a quinone-tanned protein, the inner being a wax-like lipoid. They consider that the two membranes are not combined in any way, though they think that the lipoid of the inner layer may be supported by a tenuous protein skeleton.

There appears to have been no previous experimental attempt to discover the hatching mechanism of strongyloid eggs, though it seems generally to have been assumed that the first-stage larva simply forced its way out of the egg by its movements (e.g. Veglia, 1915, on *Haemonchus contortus*). Looss (1911), however, discounted the role of larval movement in *Ancylostoma duodenale* and considered hatching to be brought about by an increase in hydrostatic pressure in the egg fluid as a result of a change in the 'vitelline' (='lipoid') layer which rendered it semipermeable at the time of hatching. He based this conclusion on the fact that eggs could be more easily collapsed in strong salt solution when hatching was due to commence. Collapse of the contents of *Trichostrongylus retortaeformis* eggs in saturated sodium chloride shows, however, that the egg membranes are not semipermeable in the way Looss suggests—i.e. preferentially permeable to water—since only the larva is distorted under such conditions.

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MATERIALS AND METHODS

For this work it was important that as little development as possible should occur in the eggs between deposition in the hosts' faeces and subjection to the experimental conditions. Faeces from rabbits infected with *T. retortaeformis* were therefore collected every 2–3 hr. and stored in water at low temperature (2–4° C.) in containers with an efficient seal to prevent evaporation. Under these conditions development is effectively stopped and the eggs retain a viability of 70–80% for as long as 6 weeks (Wilson, 1957). In most experiments, however, the eggs used were obtained from faeces no more than a fortnight old.

Eggs were extracted from faeces by centrifugal-salt-flotation (Lane, 1928; McCoy, 1929), the flotation medium being a saturated solution of agricultural salt. Two centrifugations in the salt were usually necessary to remove sufficient of the faecal debris for the purpose of experiment, the eggs being thoroughly washed in distilled water before use. Eggs extracted in this way from fresh faeces showed a viability of 90–96%.

Hatching experiments were carried out at 30° C. in moist chambers to which air was accessible, or in sealed containers. The former were covered Petri dishes with the bottoms lined with moistened filter-paper, the eggs being placed in 0·2 ml. of the experimental solution in a watch-glass inside each Petri dish. In order to minimize exchange of water between watch-glass and filter-paper the latter was moistened with the same solution as contained the eggs, 2 ml. being used in every case. In this type of culture a certain amount of evaporation was inevitable, so that results obtained with it are qualitative only. According to availability and the requirements of the experiment in hand the sealed containers were various, the following being used at one time or another.

(*a*) Conical flasks of 250 ml. capacity with the eggs in 6 ml. or less of the experimental solution.

(*b*) 50 ml. conical flasks with the eggs in 1 ml. of solution (referred to later as 'flask cultures').

(*c*) Specimen tubes 3 × $\frac{1}{2}$ in. containing the eggs in 0·1 ml. of solution ('specimen tube cultures').

(*d*) Two-drachm, screw-top, rubber-sealed bottles with the eggs in 0·2 ml. of solution ('bottle cultures').

The flasks were sealed with rubber bungs and the specimen tubes with waxed corks. The adequacy of the oxygen supply in the sealed vessels was assessed by comparison with the Petri dish moist chambers. Samples of 1300 eggs in distilled water showed no difference in hatch after 36 hr. at 30° C. in the different types of container. Nevertheless, precautions were often taken to minimize the risk of possible effects due to low oxygen tension in electrolyte solutions. Such precautions are indicated in the description of the experiments concerned.

Before introduction to the hatching containers the eggs were given one centrifugal wash in the experimental solution, being then distributed by graduated pipette in fresh solution to the cultures for incubation. For most experiments ten duplicate

samples were set up for each treatment, approximately equal numbers of eggs in the samples of each treatment being ensured by a standardization of technique in any one experiment.

Estimates of the degree of hatch were made on cultures fixed in Gilson's fluid, the number hatched from a random sample of 50 or 100 being counted for each culture. These counts were made on micro-slides without a cover-slip, the stages being randomized by agitation with a bulb pipette washed in a very weak solution of 'Teepol'. Such a method was found to be necessary because the distribution of eggs and larvae is not random in a drop under a cover-slip (Wilson, 1957). The trace of 'Teepol' prevented the drifting of larvae that otherwise occurred.

EXPERIMENTS

(1) *Hatching of eggs in solutions of* NaCl *and sucrose*

Eggs were incubated in Petri dish moist chambers at 30° C. in concentrations of sucrose from 0–0·5 M and sodium chloride from 0·025–0·1 N. The stages were fixed

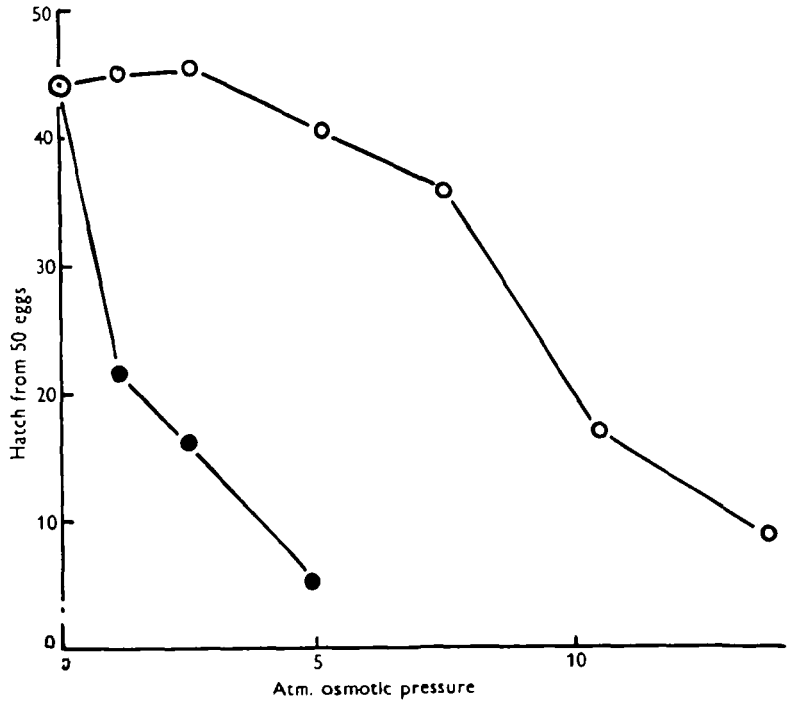

Fig. 1. Hatching of eggs of *T. retortaeformis* incubated in solutions of sucrose and NaCl for 36 hr. at 30° C. ○, mean hatch in ten sucrose cultures; ●, mean hatch in ten NaCl cultures.

in Gilson's fluid after 36 hr., estimates of the degree of hatch being made in the manner described. The mean hatch from the ten cultures for each concentration is plotted in Fig. 1 against the osmotic pressure of the solutions used.

The results show that both salt and sucrose in sufficient concentration reduce the number of eggs that hatch after 36 hr. at this temperature, but that the depression in salt solutions is manifest at concentrations where sucrose figures are little different from those in distilled water. Even if the influence of sucrose is not a straightforward osmotic effect, though it would seem likely that it is, the curves in Fig. 1 show that the effect of sodium chloride is not the consequence of colligative phenomena.

Eggs which had not hatched in either type of solution, nevertheless, contained fully developed and living first-stage larvae.

(2) *The extent of the depression of hatch by* NaCl

(*a*) *Ultimate hatch in* 0·05 N-NaCl *solution*

In order to determine whether sodium chloride permanently impaired the ability of some of the eggs to hatch, cultures were incubated in moist chambers in 0·05 N-NaCl and distilled water until maximum hatch had occurred, single cultures from the two series being fixed at intervals to determine the approximate rate of hatch. The results are plotted in Fig. 2*a*.

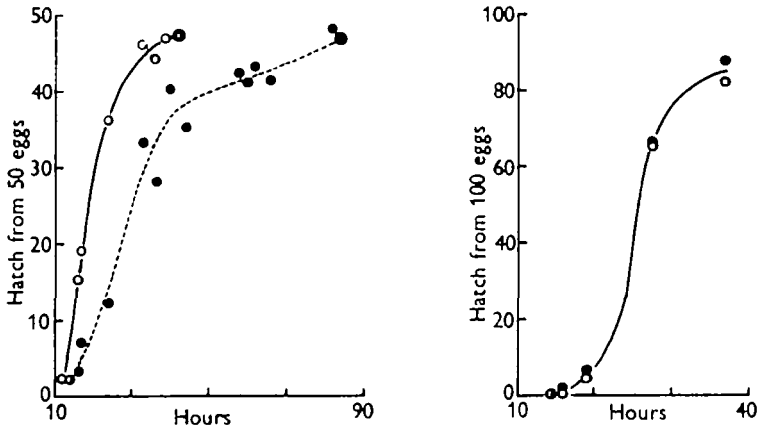

Fig. 2 (*a*) Fig, 2 (*b*)

Fig. 2. (*a*) Approximate rate of hatch of *T. retortaeformis* eggs in distilled water (———) and 0·05 N-NaCl (- - - - -) at 30° C. ⊙, mean of twenty cultures; ⊙, mean of sixteen cultures. (*b*) Rate of hatch of *T. retortaeformis* eggs in distilled water after 12 hr. in 0·05 N-NaCl (●) or distilled water (○). Each point is the mean of ten cultures.

The final mean hatch from fifty eggs in the two media was 47·3 (distilled water —twenty cultures after 42 hr.) and 46·7 (NaCl—sixteen cultures after 83 hr.), thus demonstrating that sodium chloride reduces the rate of hatch without affecting the final 'hatchability' of the eggs. The results also suggested that processes of development in the egg before hatching are not slowed down since hatching apparently started at the same time in both salt solution and distilled water.

(*b*) *Influence of* 0·05 N-NaCl *on development before hatching*

The preceding results showed that hatching normally commenced after about 12 hr. incubation at 30° C. Eggs were therefore incubated for this time in distilled water and 0·05 N-NaCl, being then thoroughly washed and distributed in Petri dish moist chambers in distilled water to discover the effect of prior incubation in NaCl. Ten cultures from each series were fixed simultaneously at intervals and the mean hatch from 100 eggs estimated. The results (Fig. 2*b*) show that the rate of hatch of eggs incubated initially in NaCl was not slower than the controls. Thus the depressing influence of sodium chloride on hatching is one concerned with the hatching mechanism itself, processes of development up to this stage not being slowed down.

(3) *The hatching of eggs in various electrolytes*

The results of experiments with buffer solutions referred to earlier suggested that an effect similar to that of sodium chloride on hatching rate would also be exerted by other ionic solutions. To confirm this, eggs were incubated in 0·05 N solutions of the following: CH_3COONa, Na_2SO_4, NaCl, K_2SO_4, KNO_3, KI, Li_2SO_4, $CaCl_2$ and $MgCl_2$.

Ten cultures for each of these solutions were incubated simultaneously in specimen tubes (see earlier) after an initial 10 hr. in distilled water. The preliminary incubation was carried out to shorten the time during which free oxygen was inaccessible to the eggs. After a total of 36 hr. (10 in distilled water and 26 in the ionic solutions) the contents of the tubes were fixed and counted. Controls were run simultaneously in 0·1 M. sucrose in order to obtain a measure of any purely osmotic effect.

The mean hatch from fifty eggs in each electrolyte is plotted in Fig. 3 against the equivalent conductance of the pure solutions at 25° C. (Harned & Owen, 1950). The highest mean hatch (25·0/50 in sodium acetate) was very much below that for the controls in sucrose (44·0/50), so that, clearly, all the ionic solutions reduced the rate of hatch to a significant degree. In addition it can be seen that a correlation exists between the depression in hatch and the conductance of the solutions, ($r = -0{\cdot}895$ for 7 D.F., $P < 0{\cdot}01$). Thus the depression in hatch is greater in solutions of ions with higher mobilities.

Since in general the permeability of a membrane to ions is proportional to the ionic mobilities the results suggest that the depression in hatch is related to the concentration of electrolyte that is able to penetrate the egg membrane in a given time. The question arises as to whether the cation or the anion is the more important constituent on the assumption that such penetration takes place. The results plotted in Fig. 3 may be analysed in terms of the mobility of the ions separately. If this is done a significant relationship ($P = 0{\cdot}02$) is found to exist between hatch and the mobility of the cation (see Table 1).

Although the value of r for the anion-hatch relationship is not significant ($P = 0{\cdot}08$) it does indicate that 39% of the variation can be attributed to the effect

of the anion. Since, on the whole, the cation was slower than the anion in each of the electrolytes used (CH_3COONa and KNO_3 were the exceptions), the correlations in Table 1 suggest that the slower ion in any one salt is the factor determining its influence. Calculations of r for the relationships slow-ion/hatch and fast-ion/hatch show clearly that this is the case, the value of the former being significant ($P < 0{\cdot}01$), that for the latter being very small and indicating an influence on the variation of

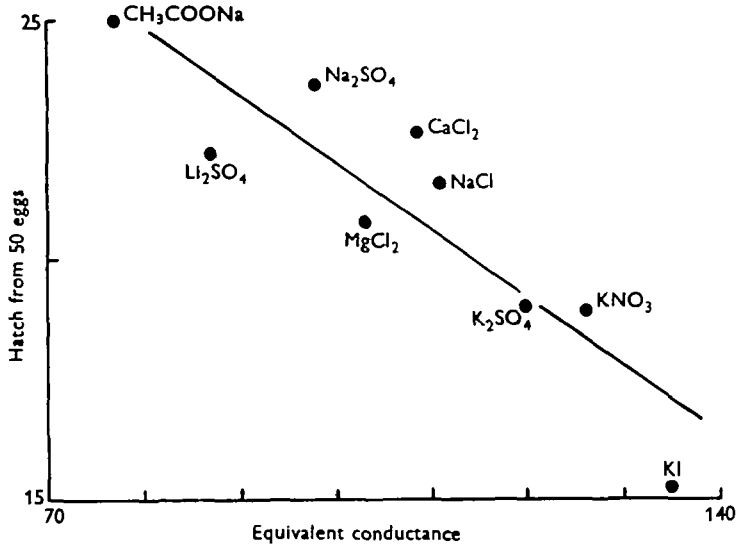

Fig. 3. Degree of hatch of *T. retortaeformis* eggs incubated in 0·05 N solutions of different electrolytes at 30° C. over the normal hatching period. Each circle is the mean of ten cultures. Control hatch in 0·1 M sucrose = 44·0.

Table 1. *Relationship between hatch* (z) *and the mobility of the cation* (x) *and anion* (y) *in each salt*

Relationship	r	Significance	Approximate % variation accounted for
x and z	−0·768	0·02 P	59
y and z	−0·623	0·08 P	39
x and y	+0·235	—	6

Table 2. *Relationship between hatch* (z) *and the mobilities of the faster ion* (x) *and the slower ion* (y) *in each salt*

Relationship	r	Significance	Approximate % variation accounted for
x and z	−0·079	—	0·6
y and z	−0·846	0·01 P	72
x and y	+0·510	—	26

less than 1 % (see Table 2). The remaining variation can be attributed to a tendency for the fast ions to be associated together, though r for this relationship (+0·510) is not significant.

Thus, while the depression in hatching rate is greater in solutions of ions with higher mobilities (Fig. 3), the effect of any one electrolyte is controlled by the speed of its slower ion irrespective of whether it is the cation or the anion. It follows from this that the effect is not an 'exchange' phenomenon in the first instance, but is the consequence of the penetration of the egg membrane(s) by both components of a salt. A further corollary is that other specific effects of different ions on the hatching mechanism are relatively unimportant, otherwise the correlation with mobility would be destroyed.

(4) *The effect of* 0·05 N-NaCl *on the water permeability of hatching eggs*

(*a*) *Water permeability of normally hatching eggs*

It has been stated by Looss (1911) that the contents of the eggs of *Ancylostoma duodenale* are more easily collapsed in strong salt solution at the time they are due to hatch. If it were found that the eggs of *Trichostrongylus retortaeformis* behave similarly this would suggest that ions exert their effects when shrinkage—i.e. water permeability—is at a maximum. The normal behaviour of eggs in this respect was therefore examined by transfer of samples of eggs incubated in distilled water to strongly hypertonic salt and sucrose solutions. (The salt solution and sucrose solution were isotonic and were compared so that any differences in their effects might be revealed.)

Eggs were incubated in a sealed 250 ml. conical flask in distilled water, samples being removed by pipette at intervals to determine the rate of shrinkage. This was done in the following way. The eggs from the pipetted sample were spun down to the bottom of a conical centrifuge tube, the supernatant was removed, and the tube was part-filled with hypertonic solution, either 2 molal NaCl, or 3·05 molal sucrose, each having an osmotic pressure of 96·2 atm. (the strength of these solutions was calculated from data supplied by Robinson & Stokes, 1949). The eggs were thoroughly mixed with the hypertonic solutions, samples were removed at intervals over a period of an hour or so and the number collapsed from a random group of fifty was recorded for each sample.

The results from the two solutions agreed in that no shrinkage occurred until just before hatching was due to commence, even if the eggs were left in the solutions for as long as 12 hr. Just before hatching commenced a proportion of the eggs became permeable to water and this proportion increased as hatching progressed.

The detailed behaviour of the eggs in the two solutions was different, however, after 13 hr. incubation. This difference was best illustrated by the 14 hr. sample, the curves for which are plotted in Fig. 4. In general, the number of eggs collapsed in sucrose solutions showed a straight line increase with time. In the salt solutions, on the other hand, there were three clearly defined components making up the total curve, namely: (*a*) an initial rapid increase in shrinkage followed by (*b*) a constant

phase over which no change was apparent (within the limits of the sampling error), and a final phase (*e*) in which further increase occurred.

Despite these differences the results show that the hatching eggs of *T. retortaeformis* conform roughly to the pattern referred to briefly by Looss for *Ancylostoma duodenale*. In the present experiments, however, a much more marked change was noted than that indicated by Looss, since it was impossible to damage healthy eggs during the prehatching developmental period—indicating that they are completely impermeable to water at this stage.

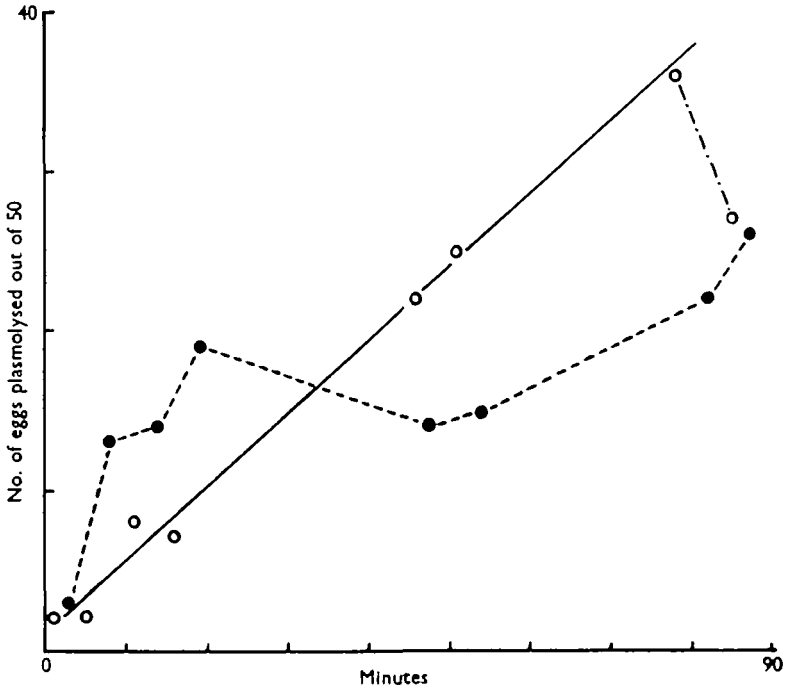

Fig. 4. Collapse of eggs of *T. retortaeformis* in isotonic sucrose (O) and NaCl (●) solutions after 14 hr. incubation at 30° C. in distilled water.

In comparing the effects of the two solutions certain errors must be taken into account which are inherent in the use of sucrose. First, it is a simple matter to identify a water-permeable egg in NaCl since the contained larva is almost immediately affected, the egg membranes retaining their shape in most cases. In sucrose, however, the initial stages of collapse are difficult to detect because the egg membranes contract slowly against the contours of the still apparently normal larva, so that the orientation of the egg plays a part in whether the effect can be seen. Unless the egg is positioned so that the head or the tail of the larva can be seen at the side the distortion of the shell cannot be detected. In addition, the final effects of hypertonic sucrose are so destructive that it is difficult to identify the remnants as eggs at all. For these reasons the number of collapsed eggs may be underestimated

and it may even appear that there has been a decrease in water permeability (e.g. the last point on the sucrose curve in Fig. 4). The main differences between the two curves cannot, however, be attributed to these errors since, even with the under-estimation mentioned, the figures for sucrose eventually exceed those for salt. The probable significance of these differences is referred to in the discussion.

(*b*) *Water permeability of eggs incubated in* 0·05 N-NaCl

With the normal picture in mind experiments were carried out to discover departures from the normal shown by eggs developing in a weak NaCl solution. Eggs were incubated in bottle cultures (see earlier) in 0·05 N-NaCl and in distilled water, bottles from the two series being removed at intervals and the degree of shrinkage estimated after 10 min. in a hypertonic solution on a slide.

After a sample had been removed from each bottle for the water permeability estimation the contents remaining were fixed in Gilson's fluid for later estimation of the degree of hatch at that time to be made. Two separate experiments were conducted, one using a strong sucrose solution (approximately 1·5 M), the other a near-saturated solution of NaCl. The time of immersion in the hypertonic media was 10 min.; the results therefore relate to the horizontal part of the NaCl curve of Fig. 4.

The results of these experiments are plotted in Fig. 5*a–d*, the estimates of hatch being the means of five counts from each bottle culture. Despite the detailed differences between the curves for salt and sucrose the results agree in showing a lower level of shrinkage in eggs incubated in 0·05 N-NaCl. This is the opposite of what might be expected on the basis of the idea earlier expressed—i.e. that the ions exert their effect at the time when the egg becomes permeable to water. If this idea had been correct shrinkage would have been greater in eggs from the weak NaCl cultures.

Two further possibilities remain depending partly upon whether water permeability is a necessary prerequisite for hatching, and partly on whether ions in solution specifically affect the attainment of water permeability. These possibilities are (*a*) that the hatching mechanism as a whole is slowed down, or (*b*) that the low shrinkage level indicates a specific effect of ions on water permeability. If water permeability was essential for hatching—and not, as it may well have been, a mere consequence—and if it was inhibited by ionic solutions, then such inhibition could perhaps have accounted for the delayed hatching rate.

The results plotted in Fig. 5*a–d* can be used to determine whether 0·05 N-NaCl has any specific effect on water permeability by calculating the 'residual' and 'total' water permeabilities for each point on the distilled water and 0·05 N-NaCl curves. In order to do this the hatching and shrinkage figures must be related in some way. According to the original method these properties were estimated on separate samples, there being no direct connexion between the figure for hatching (number hatched out of 50) and that for shrinkage (number collapsed out of 50 *unhatched* eggs). It can safely be assumed that those eggs that had hatched became permeable

to water before doing so. There remains a number of unhatched eggs (i.e. $50-h$, where h is the number hatched from 50) of which $(50-h)p/50$ are permeable to water (where p is the number collapsed out of 50 in Fig. 5 *a–d*). The value of $(50-h)p/50$ is termed the 'residual permeability' and represented by p'. The 'total permeability' for each point then becomes $(p'+h)$, since in this connexion the hatched eggs are regarded as permeable to water.

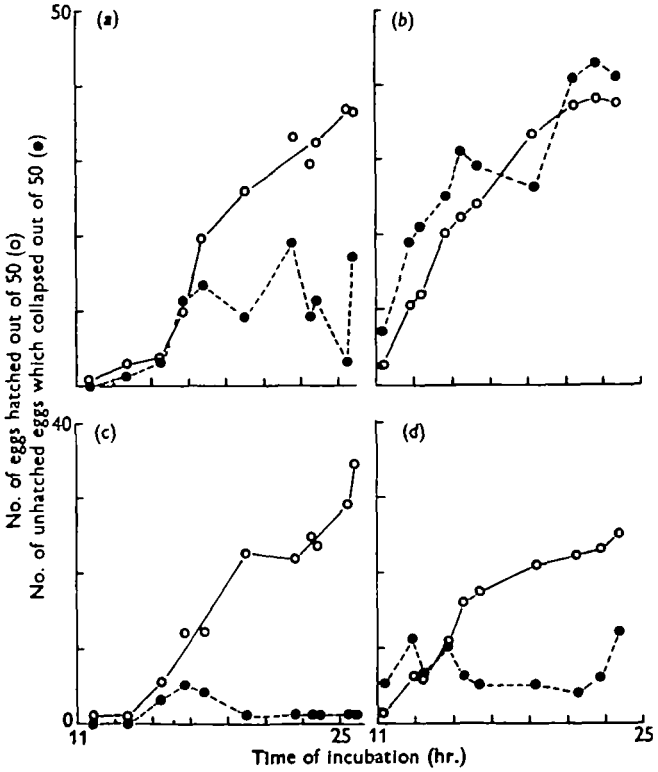

Fig. 5. Water permeability of *T. retortaeformis* eggs hatching in distilled water $(a+b)$ or 0·05 N-NaCl $(c+d)$ at 30° C. Permeability to water measured by collapse in 1·5 M sucrose $(a+c)$ and saturated NaCl $(b+d)$.

Now, if the effect of ions on hatching rate is a general slowing down of all processes, of which change in water permeability is a consequential or necessary part, then the ratio $p'/(p'+h)$ should be the same for eggs hatching in either 0·05 N-NaCl or distilled water. If, on the other hand, ions exert a specific depressing effect on water permeability, then one would expect the value of $p'/(p'+h)$ to be significantly lower in eggs from 0·05 N-NaCl.

When these calculations are made the figures for collapse both in salt and in sucrose provide the same answer, though only those for salt are represented in Fig. 6. Except for one pair of points they show clearly that $p'/(p'+h)$ is significantly lower in eggs from the 0·05 N-NaCl cultures. Thus it would appear that the change

in permeability to water is specifically inhibited by ions in solution. The fact that over the period where maximum hatching rate is attained in distilled water the number of eggs permeable to water in 0·05 N-NaCl stays at a constant low level (Fig. 5*c*, *d*) supports the view that this is in fact the means whereby hatching rate is slowed down in ionic solutions. It would follow from this that the hatching rate of eggs in distilled water is controlled by a 'second process' which normally lags behind the change in water permeability and which is not so markedly affected by ions in solution. The possible nature of this process is dealt with in the discussion.

Since it is highly probable that water permeability is controlled by the lipoid layer of the egg it is possible that the effect of ions is concerned with the stabilization of this membrane beyond a point when it would normally be broken down.

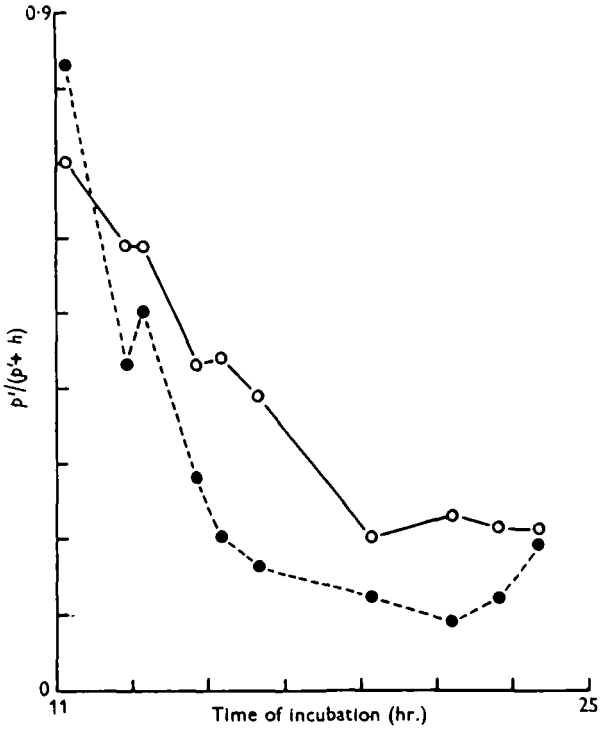

Fig. 6. 'Permeability ratio' of *T. retortaeformis* eggs incubated in distilled water (○) and 0·05 N-NaCl (●) at 30° C. Calculated from the data in Figs. 5*b*+*d* (see text).

(5) *Compounds which antagonize the effect of ions on hatch*

(*a*) *'Teepol'*

This possibility suggested the investigation of the effect of electrolytes on hatching in the presence of small concentrations of the strong emulsifying agent, 'Teepol'.

Eggs were incubated in Petri dish cultures of 0·05 N-NaCl with concentrations of $1/10^6$, $1/10^5$ and $1/10^4$ by volume of 'Teepol', the mean hatch from the ten

cultures for each treatment being estimated on material fixed after 36 hr. incubation. Before fixation, however, the eggs and hatched larvae were examined to see if 'Teepol' was toxic. Although no numerical estimate was made, there was an obvious poisoning effect of the 'Teepol' at its highest concentration, since more than the usual number of eggs had died before the larvae were fully developed. Nevertheless, all the cultures containing 'Teepol' showed a higher hatch than the culture in 0·05 N-NaCl alone, the means for the two higher 'Teepol' concentrations being significantly greater at $P=0{\cdot}05$ (Table 3).

Table 3. *Effect of 'Teepol' on hatching in* NaCl *solution*

Mean hatch from fifty eggs in				
0·1 M sucrose	0·05 N-NaCl	0·05 N-NaCl + 'Teepol' at the following volume concentrations		
		10^{-4}	10^{-3}	10^{-2}
45·5	15·3	20·8	26·7	23·0

(*b*) *Non-emulsifying compounds*

A simple theory may be deduced from the above experiment which relates the effect of 'Teepol' to its ability to emulsify the lipoid membrane in opposition to an hypothetical stabilization by ions in solution. However, the position is complicated by the fact that a number of other substances having little or no emulsifying power show a similar, or greater, antagonism to the effect of ions on hatching. Thus, the addition of the first three alkyl acetates at a concentration of 0·01 M to the 0·05 N-NaCl solution produces a hatching curve very similar to that obtained by simply increasing the 'Teepol' concentration (Table 4).

Table 4. *Effect of alkyl acetates on hatching in* NaCl *solution*

Mean hatch from fifty eggs in				
0·1 M sucrose	0·05 N-NaCl	0·05 N-NaCl + 0·01 M-CH_3COOR, $R=$		
		CH_3	C_2H_5	C_3H_7
39·8	7·3	10·1	13·8	12·4

Table 5. *Hatching of eggs in mixtures of glycine,* NaCl *and* NaOH

Estimate	Hatch from fifty eggs (mean of ten cultures) in				
	Distilled water	Glycine buffer at pH			
		8·40	9·20	9·58	10·30
1	46·8	39·2	—	33·1	37·6
2	—	32·0	33·5	—	38·5

Again, the high hatch obtained in glycine buffer solutions (Table 5) is remarkable when one considers that they contain NaCl up to a concentration of 0·1 M. Efforts to identify the salt-antagonizing agent in these solutions showed that the effect was one concerned with the properties of the mixture, and was not due to the individual influence of glycine, NaOH or pH.

Evidence also suggests that sucrose can act as an antagonist to the effect of ions on the hatching eggs.

(6) *Examination of the role of water permeability in the hatching egg*

(*a*) *Size of the egg and unhatched larva before and after the change in water permeability occurs*

Since the egg in its natural environment is almost certainly surrounded by a medium hypotonic to its contents it seems highly probable that osmotic uptake of water will take place as soon as it becomes permeable. The increase in hydrostatic pressure that results may be discernible in an increase in size, either of the egg itself, or of the contained larva, or of both.

It has been shown that delayed hatching in ionic solutions is probably the consequence of the inhibition of the change in water permeability, so that osmotic uptake of water may possibly be part of the hatching mechanism even though it cannot occur in the way Looss suggested (i.e. via the *egg* membranes, see introduction).

To examine this possibility the length and breadth of the egg and the width of the unhatched larva were measured in 0·1 M sucrose before and after change in permeability had occurred. Controls were incubated and measured in 0·05 N-NaC in which water permeability stayed at a minimum throughout. The mean sizes of thirty eggs or unhatched larvae from the different media are to be found in Tables 6*a* and *b*.

Analysis of variance shows that no significant change in size was apparent either in NaCl or in sucrose. This does not necessarily mean that no increase in hydrostatic pressure has occurred since, in the first place, the protein egg shell may be sufficiently rigid to show no distortion until breaking point is reached, and in the second place the larva is already closely confined in the egg, so that no significant increase in size need be manifest even though uptake of water raises the hydrostatic pressure. On a matter of technique, the error-variance for the measurements of the width of unhatched larvae was considerable and may easily have masked any effect that may possibly have occurred. In contrast, the egg dimensions could be more accurately determined, so that it is unlikely that any change in size did in fact occur in this case.

(*b*) *Movement of the unhatched larva just before hatch*

If the idea that hydrostatic pressure within the larva increased after the egg became permeable to water is true, then it seems likely that movements of the larva will become more restricted when hatching is imminent.

Table 6*a*. *Influence of water permeability on the size of the eggs*

Incubation (hr.)	No. of eggs that collapsed out of fifty in sat. NaCl		Mean size of thirty eggs in			
			0·05 N-NaCl		0·1 M sucrose	
	NaCl	Sucrose	Length	Breadth	Length	Breadth
11	Nil	Nil	63·3	35·8	64·0	35·4
24½	1	46	63·8	35·9	61·9	34·6

Units are mm. apparent size of the camera lucida drawing (absolute size 85–91 × 46–56 μ).

Table 6*b*. *Influence of water permeability on the width of unhatched larvae*

Incubation (hr.)	No. of eggs that collapsed out of fifty in sat. NaCl		Mean width of thirty larvae in	
	NaCl	Sucrose	0·05 N-NaCl	0·1 M sucrose
11	Nil	2	13·1	13·2
24½	3	48	13·3	13·5

Units are mm. apparent size of the camera lucida drawing.

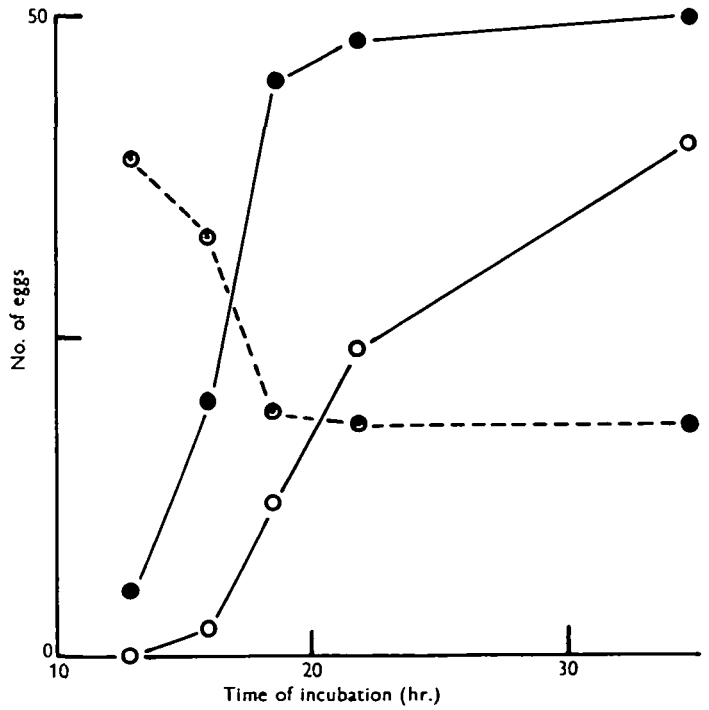

Fig. 7. Water permeability of the eggs (●), hatch (○) and mobility of unhatched larvae (⊙) of *T. retortaeformis* incubated in distilled water at 30° C. Estimates of water permeability made by plasmolysis in sat. NaCl.

Eggs were incubated in distilled water and the following properties were estimated on samples removed by pipette during the hatching period:

(*a*) degree of hatch from fifty eggs,

(*b*) the number of unhatched larvae out of fifty that would collapse in saturated salt solution, and

(*c*) the number of eggs out of fifty that contained mobile larvae when first observed.

Conditions for (*c*) were standardized as far as possible, though temperature control on the microscope stage could not be attained. Oxygen concentration was adequate throughout since the hatched larvae showed high activity.

The results (Fig. 7) show that movement decreased as hatching progressed, but, more important, mobility was at a maximum when permeability was at a minimum, and vice versa, the mobility curve being a mirror-image of the permeability curve. The results suggest that the larva is unable to move so freely just before hatching, and that this is due to the osmotic uptake of water.

INTERPRETATION AND DISCUSSION

Despite differences of shrinkage in saturated salt solution and strong sucrose solution, such experiments indicate that water permeability is essential for hatching in *Trichostrongylus retortaeformis* eggs, the specific inhibition of the change in water permeability by weak electrolyte solutions being sufficient to slow down the rate of hatch. Thus the breakdown of the lipoid layer as a water-resisting barrier appears to be an obligatory part of the hatching mechanism, though, under normal conditions, it proceeds at a rate faster than the final processes and therefore does not control the rate of hatch.

The conclusion that the rate of lipoid breakdown can be made to control hatching rate in ionic solution is supported by the partial antagonizing influence of an emulsifying agent such as 'Teepol', though the effect of various other materials in this respect cannot be explained.

In deducing the means whereby permeability to water is achieved it would seem probable that the movements of the larva are important. From the 'tadpole' stage onwards these movements become increasingly obvious, until, just before the egg becomes permeable to water, the larva can be seen gliding round and round inside its confining membrane. Such agitation of the egg fluid by the larva would assist emulsification of the inner egg layer; though the presence of some emulsifying agents in the fluid must be presupposed.

It seems likely that once the lipoid is broken down as a water-resisting barrier the difference in osmotic pressure between the external medium and the larval fluids hypertonic to it causes the larva to take up water from the surroundings and thereby to exert further pressure on the protein shell. The view that this occurs is supported by the fact that larval movements become more restricted after the egg becomes permeable to water, though no measurable swelling occurs at the same time.

It has already been explained that the figures for hatching and permeability in distilled water indicate that at least one other process is involved in hatching besides that dependent on water permeability. This process apparently controls hatch under normal conditions and is not slowed down to the same extent in electrolyte solutions. Qualitative observations of hatching eggs suggested that this second process is some sort of chemical weakening of the outer protein coat, though the more obvious experiments to test this hypothesis have proved negative. It has been found that hatching rate of eggs in samples with average numbers per sample ranging between 250 and 6750 is the same— i.e. there is no evidence for the autocatalysis of hatching eggs. In addition the supernatant from eggs that have reached maximum hatching rate does not appear to accelerate the hatching of other eggs immersed in it. If, therefore, a 'hatching factor' is secreted it must be a compound which is easily broken down once released from the egg. There is slight evidence which is being further studied that such an unstable substance is produced, but it is too tenuous to be discussed in detail here.

The evidence reported here suggests, however, that at least two distinct processes are involved in the hatching of the eggs of *T. retortaeformis*, one depending upon the breakdown of the inner lipoid membrane of the egg and increase in hydrostatic pressure inside the larva, and another subsequent process which normally controls the rate of hatch in the absence of ions in solutions. It is suggested that the first process is brought about by the agitation of the egg fluid by the larva, with the assistance of an emulsifying agent, and that its function is to enable the larva to exert pressure on the rigid protein shell. The second process is thought to be some sort of chemical weakening of the protein shell, which, in combination with the pressure resulting from the first process, enables the larva to escape.

With the possible exception of the Metastrongylidae it would seem likely that members of the Strongyloidea in general would share the same hatching mechanism as is found in the Trichostrongylidae, in view of the similarities in this part of the life-cycle of most species of the order.

The problem of exactly how the breakdown of the lipoid membrane is affected by ions in solution remains to be investigated. Speculation about the possible penetration of the shell layers poses the question as to whether control of the rate of penetration is exerted by the wax-like layer or the protein one.

At first sight it would seem likely that the effect of the ions would be to alter the egg fluid surrounding the larva—i.e. after the penetration of both shell layers. This, however, requires the wax membrane to be permeable to ions just before hatching when it would still be impermeable to water (since the main effect of salts is to delay the change in water permeability). Such a system is not impossible but appears to be rarely, if ever, encountered in biological studies. The sort of membrane required would have to be non-porous and to contain carrier molecules with an affinity for ions of the same order as the affinity of water for ions. The main objection to this theory is the fact that during development of the embryo the membrane is impermeable to ions as well as to water, otherwise plasmolysis would occur in saturated salt simply as a result of the penetration of ions. As has been indicated, developing

eggs can be left in hypertonic salt solutions for as long as 12 hr. without apparent harm. Thus, one is faced with the problem of how the carriers become modified at the time when the breakdown of the lipoid commences, since before this they appear to be incapable of ion transfer.

The difficulties of interpretation are less if one assumes that the effect is exerted between the protein and lipoid layers, either directly on the outer surface of the latter, or indirectly by an effect on an interlayer material instrumental in lipoid breakdown. The effect of ion mobility would then be an indication of the relative ion penetration through the protein shell.

The exact influence on the inner egg layer is still problematical, of course, though any explanation must be based on the apparently uniform effect of different ions and the fact that the charge on the ion plays an important part—for instance $\frac{1}{2}SO_4^{2-}$ appears to have an effect comparable with that of Cl^- (see Fig. 3).

The general idea that breakdown of the wax membrane is delayed by ions is supported by the differences in behaviour of eggs in hypertonic salt and sucrose solutions (see Fig. 4), the 'constant phase' of the salt curve being produced by this effect. These results suggest also that sucrose accelerates breakdown of the inner layer, thus supporting direct evidence of sucrose antagonism to the ion effect.

The possible adaptive significance of the effect of ions on hatch can be considered in the light of the well-known fact that the water content of the faeces of many host animals is insufficient to promote full development of strongyloid stages. Under field conditions the faeces may be subject to desiccation for some time after they are deposited, further lowering the chances of survival of the delicate first-stage larvae.

If the larva were retained in the egg under these conditions, and the lipoid coat remained intact as a water-resisting barrier, then its chances of survival would proportionately increase.

A circumstance that would follow from loss of water from the faeces would be an effective increase in the concentration of substances in solution in the faecal fluid, including any electrolytes that may be present. Thus the larva would be retained in the egg for a period inversely proportional to the water content of the faeces if the electrolyte concentration of the medium reached a significant level. Moistening of the faeces by rain or dew would dilute the faecal solutions and hasten the escape of the larva.

This must be set against the fact that various materials are able to antagonize the action of electrolytes, and some of these may be present in the faecal fluid. Only in the case of glycine buffer solutions was there any approach to normal hatching rate, however. Thus it is possible that this salt-antagonizing effect is not so important a factor under field conditions as is the concentration of electrolytes.

SUMMARY

1. The influence of solutions of NaCl on the hatching of eggs of *Trichostrongylus retortaeformis* is studied. It is shown that the effects are not the consequence of colligative properties, but are related to ionic phenomena. 0·05 N-NaCl slows down

the rate of hatch without impairing the ultimate 'hatchability' of the eggs. Processes of development up to hatching are not slowed down.

2. The effect demonstrated in the case of NaCl is shown to be shared by eight other electrolytes, the depression in the rate of hatch being proportional to the mobility of the ions in solution. On the assumption that the effect of the ions is due to a penetration of the egg membrane(s) the rate of entry is shown to be controlled by the speed of the slower ion in any one salt.

3. The influence of NaCl on the permeability of hatching eggs to water is studied. It is shown that the rate of increase in permeability is slowed down sufficiently in NaCl to control the rate of hatch. The inference that water permeability is a necessary prerequisite for hatching is made, a further hypothetical process being invoked to account for the rate of hatch in the absence of NaCl, since it is not then controlled by changes in water permeability.

4. The probability that the net effect of ionic solutions on the eggs is one concerned with the rate of breakdown of the inner wax-like layer of the egg is strengthened by experiments demonstrating that the depressing influence of NaCl is antagonized by 'Teepol', though the comparable influence of other, non-emulsifying, compounds cannot be explained.

5. The role of water permeability in the hatching mechanism is investigated.

6. A hatching mechanism of strongyloid eggs is proposed which involves two processes, the first dependent upon the osmotic relationships of the unhatched larva to its environment, the second being some sort of chemical weakening of the outer shell.

7. It is suggested that the effect of ions on hatching rate assists the 'embryonated egg' to survive under natural conditions when the hatched first-stage larva might otherwise be destroyed by desiccation.

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