SALT EFFECTS ON EGGS AND NAUPLII OF ARTEMIA SALINA L.

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INTRODUCTION

Among the organisms inhabiting strong brines, the Arthropods are well represented. Crustacea (Abonyi (1), van Doune (2), Schmankewitsch (11)), Coleoptera (von Lengerken (9), Hase (4)), Diptera (Jones (6)), and Hemiptera (Entz (3)) abound in solutions which often contain more than 25 per cent solids and which should be, according to certain authors (Schmidt (12)), azoic.

While the number of protists with naked cell surfaces is also high in these solutions, and protoplasm should, therefore, be able to absorb water and nutrient from this unusual environment, the arthropods have the advantage that this unusual plasmic mechanism need be confined only to the mesodaeum, as the remainder of the body surface in contact with the *milieu ambiant* is effectively chitinized.

In the case of *Artemia salina*,* the brine shrimp, used chiefly in our investigations, this can be easily demonstrated by means of vital dyes which, in the uninjured animal, penetrate only the columnar epithelium of the mid-gut (see, for other opinions, Martin and Wilbur (10)). It has been known for a long time that *Artemia* is capable of living for days in solutions of potassium permanganate, potassium bichromate, and silver nitrate.

This fact might suggest that the organism is particularly resistant to these substances; a conclusion, however, that is not warranted by the facts. For, as Dr. Herbert Warren has shown in this laboratory, the organism possesses in the muscular mechanism of its loop-shaped proctodaeum a most efficient means of hermetic closure of the gut.

* For the extensive literature, see Abonyi (1).

Experimentally, this may be tested by placing the animals in sea water to which a fine suspension of barium carbonate has been added. The presence of this substance in the gut can be immediately ascertained, and it was found that actual ingestion of the particles was often delayed for more than 2 days after the animals were placed in the solution.

Artemia, therefore, has a means of checking effectively the passive intake of the liquid environment (it is a *Strudler*, in the sense of Lang).

If we want to test the effect of various substances in the environment upon *Artemia*, the voluntary closure of the proctodaeum might obscure the results to a marked degree. The variable results obtained by us with adult shrimps may be chiefly ascribed to this factor.

As the gut is formed in the late nauplius stage, before the second instar, it seemed advisable to work with the nauplius stage exclusively.

The eggs of *Artemia* are available, the year around, in huge quantities. In the spring when the salt crust in the salines becomes dissolved and the environment reaches a favorable salinity, the "winter eggs" of the shrimp swell and burst. The nauplius, after having passed through about fourteen instars (Heath (5)), reaches maturity and produces eggs. By this time, the pool has become more concentrated with the advanced season. The eggs float on the dense brine, the wind drifts them leeward, and by further evaporation of the pool the eggs are beached in long windrows, often about a half inch thick and 3 to 4 feet wide.

The yield in eggs of a two-acre saline near Marina, California, over the 1928–29 season, was conservatively estimated by us at 100 pounds. About twenty pounds of eggs were cleaned by screening and washing, and showed, even after one year, almost 100 per cent "germination." *Artemia* is, therefore, available as a homogeneous and easily transported material and is well worthy, because of its curious behavior, to become a physiological standard object.

Ecdysis

The eggs are spheroidal, somewhat angular objects; in a dry condition, often with a concavity. They average 200μ in diameter. The color varies from creamy white to almost blackish brown. A mass of the eggs has a sepia brown color. Spangenberg (13), who described the ecdysis of *Branchipus* (*Branchinecta*), points out that the process in *Artemia* is quite similar and that the eggs possess two membranes. The outer (*Eikapsel*) is hard and unyielding, chiefly composed of chitin; the inner (which arises from the chorion) is very thin and yielding, and is proteinaceous in nature. When *Artemia* eggs are placed in sea water, the outer capsule will burst after about 20 hours at room temperature. The nauplius will be still enclosed in the inner membrane, which is still attached to the outer capsule by the chorion, but hangs down from it, as Spangenberg says "wie die Gondel am

Ballon." After a few hours, the thin membrane (called "membrane" in this paper) ruptures and the nauplius swims around.

It was found, early in this investigation, that the ecdysis of the inner membrane could be incited by a much wider variety and range of solutions than the appearance of living nauplii. The second process, while dependent upon the first, can only be caused by solutions of very definite composition, showing that *Artemia*, instead of being indifferent to chemical environment, is indeed very sensitive to it.

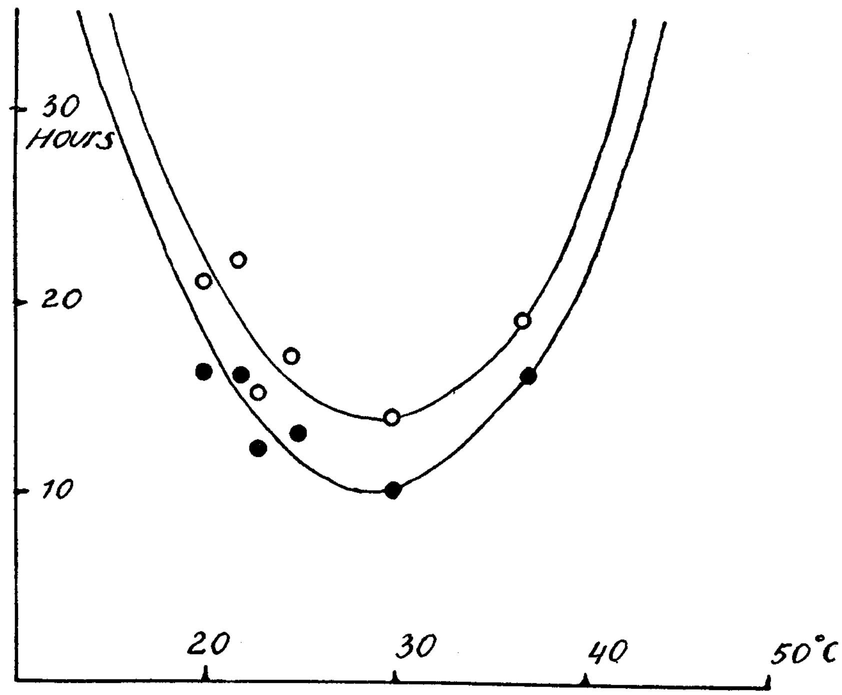

FIG. 1. Influence of temperature on the incubation time of nauplii (solid dots) and on membrane formation (open circles).

The rapidity of both membrane formation and the ecdysis of the nauplius is markedly influenced by temperature. Fig. 1 shows that optimal temperature for both processes lies around 30°, although the speed is still considerable at 22°C. Eggs were placed in 50 cc. flasks in the solution to be tested, and incubated at 22°C. The frequency and speed of both membrane formation and nauplii ecdysis were noted several times during the day. As abnormalities, we observed in certain solutions the loosening of the inner membrane prior to the appearance of the nauplius and the mass death of nauplii immediately after ecdysis.

The Influence of Hydrogen Ion Concentration

As it was found that the eggs hatched well in pure NaCl solutions and also in phosphate, mixtures were made of NaCl 1 mol, NaH_2PO_4 1/30 mol, Na_2HPO_4 1/30 mol, HCl 0.1 mol, and NaOH 0.1 mol. Membranes were formed in the entire range of pH 1.7 to pH about 13 (0.1 mol NaOH). Nauplii hatched in 0.1 mol NaOH and also in very acid solutions (pH = 2). It is hardly probable, therefore, to support the contention of Labbé (7), who claims that the distribution of the

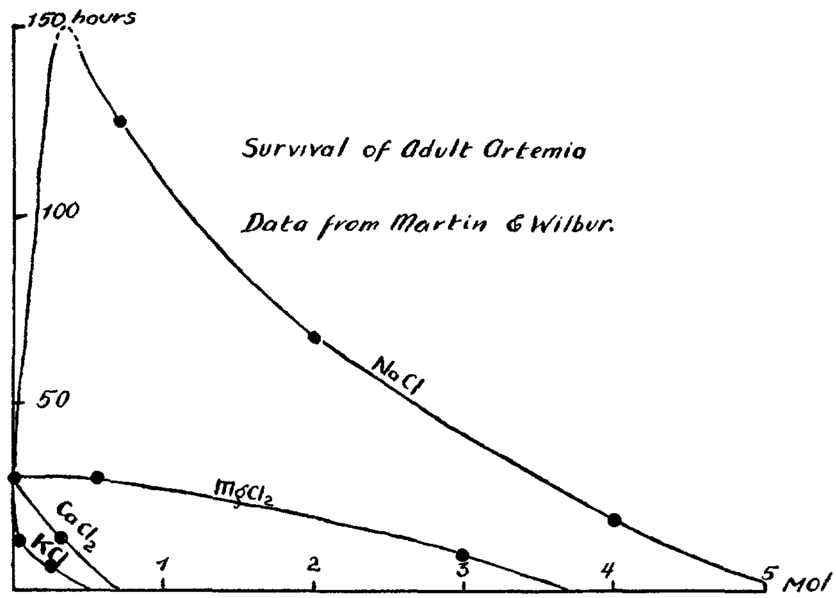

FIG. 2. Survival of adult *Artemia* as a function of salt concentration (after Martin and Wilbur).

brine organisms is dependent upon pH. Moreover, evidence is accumulating to show that the limiting influence of acids and bases depends more upon the concentration of the undissociated acids than upon the pH.

Effects of Concentration

As Martin and Wilbur (10) observed, the adults of *Artemia* survive in solutions of $MgCl_2$, $CaCl_2$, HCl, and NaCl. The astonishing fact

TABLE I

	Membrane formation	Swimming nauplii	Toxic to membrane formation	Toxic to nauplii	Toxic to both membrane formation and nauplii	Membrane separated
Sea water	+	+	—	—	—	—
Urea	0.01–1.5 M*	0.01–0.6 M	—	0.7 M	—	0.01
NaCl	0.01–3.5	0.01–3.5	2.5 M	—	4.0	0.01
NaCNS	0.01–0.78*	—	—	0.01	—	—
NaBr	0.01–2.0*	0.01–2.0*	—	—	—	0.01
NaI	0.01–0.7*	—	0.01	0.01	—	—
Na_2SO_4	0.01–2.0	0.01–2.0*	—	—	—	0.01
$NaNO_3$	0.01–0.70*	0.01–0.70*	—	0.56	—	—
KCl	0.01–0.78*	—	—	0.01	—	0.01
KI	0.01–0.70*	—	0.01	—	0.01	0.01
KBr	0.01–0.70*	—	—	0.01	—	0.01
K_2SO_4	0.01–0.50	—	—	0.10	0.50	0.10
KNO_3	0.01–0.70*	—	—	0.01	0.65	0.10
LiCl	0.01–0.78*	—	—	0.01	—	0.01
RbCl	0.28*	—	—	—	0.28*	0.28*
NH_4Cl	0.01–0.78*	—	—	0.01	—	0.01
$BaCl_2$	0.01–1.4*	—	—	0.01	—	0.15
$CaCl_2$	0.01–1.7	0.01–1.4	1.8	1.5	1.8	0.01
$MgCl_2$	0.01–1.5	0.01–0.8	1.6	0.9	1.6	0.01
$SrCl_2$	0.01–1.2	0. 3–0.6	1.3	0.65	1.5	0.55

* Highest concentration tried.

TABLE II

Salt	Membrane	Nauplii	Remarks
NaCl	0.5	0.35	Survival >6 days
NaBr	0.45	0.1	Survival >6 days
$NaNO_3$	0.5	0.1	Survival >6 days
Na_2SO_4	0.42	0.2	Survival >6 days
NaI	>0.7	....	
NaCNS	0.6–0.7	....	
KCl	0.38	....	
KBr	>0.7	....	
KI	0.4	....	
NH_4Cl	0.40	....	
$CaCl_2$	0.20	0.40	Survival 4 days
$MgCl_2$	0.36	0.45	Survival 4 days
$SrCl_2$	0.20	0.50	Survival 2 days
$BaCl_2$	0.1–0.2	....	
Urea	1.20	0.40	Survival 2 days

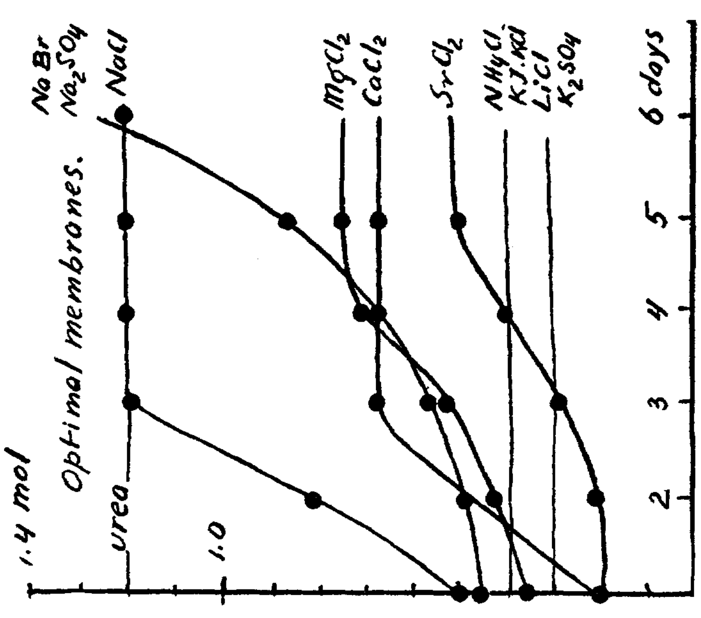

Fig. 3

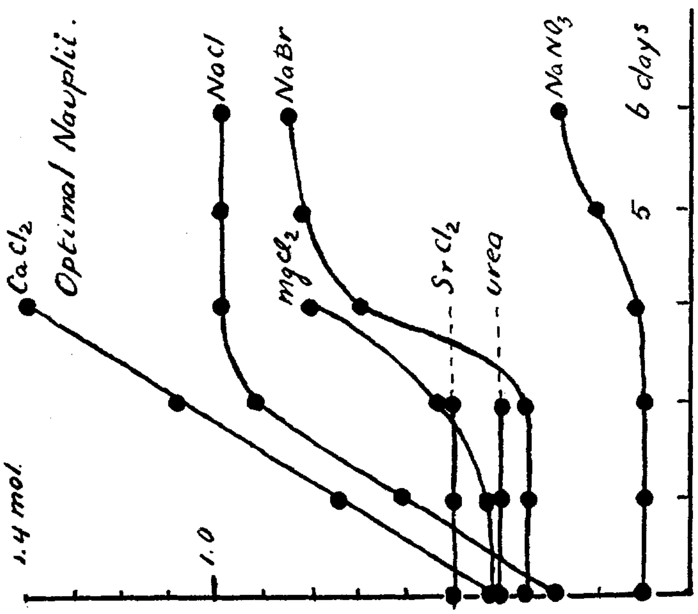

Fig. 4

Fig. 3. Optimal membrane formation in various solutions as a function of time

Fig. 4. Optimal development of nauplii in various solutions as a function of time

was brought out that KCl was very toxic (see Fig. 2). This fact was substantiated by us, for the nauplii failed to develop in solutions containing more than 0.1 mol KCl. The following table gives the results of our work with solutions of simple salts. In certain solutions, where bacterial growth was apt to occur within 2 days (Urea, $NaNO_3$), the results may be influenced by this factor.

The incubation time is, in some instances, dependent upon concentration; but the osmotic effect (if present) is entirely overshadowed by the chemical influence of the particular salts. Figs. 3 and 4 show the maximal appearance of membranes, in respect to nauplii in various concentrations of the same salt, as a function of incubation time. Table II shows the "optimal" concentrations (highest frequency of membranes and nauplii formed) for various salts.

When we take from this table only those solutions that support life, we find:

Average for	Optimal for membrane formation	Optimal for Nauplii
	mol	*mol*
Na salts	0.48	0.24
Alkaline earths	0.23	0.45
Urea	1.20	0.40

With the exception of Na_2SO_4, which showed high values (0.42 mol, 0.2 mol) membrane formation might be caused, at least partly, by osmotic processes, as the values increase with diminished dissociation. No such osmotic factor is suggested for the appearance of nauplii, however. The range of NaCl concentrations suitable for life in *Artemia* (0–3.5 mol) is so great, moreover, that it would be difficult to account for this phenomenon by osmotic theory.

As remarked before, *Artemia*, far from being indifferent to the chemical environment, is intensely specialized. Sodium salts alone appear to be the most favorable, and the anions also play an important rôle. There actually remain only NaCl and NaBr as solutions capable of causing normal ecdysis; for even the chlorides of Ca and Mg, while able to permit life for a few days, are definitely toxic.

Antagonisms

Early in our work, it was found that filtered sea water, to which traces of KNO_3 and K_2HPO_4 were added (in order to induce the development of algae) was a convenient solution for bringing *Artemia* to maturity *ab ovo*. Sea water contains K, Mg, and Ca and, moreover, potassium salts were added.

The natural environment of *Artemia* is often very rich in magnesium, so the fact suggested itself that a certain antagonism should be present.

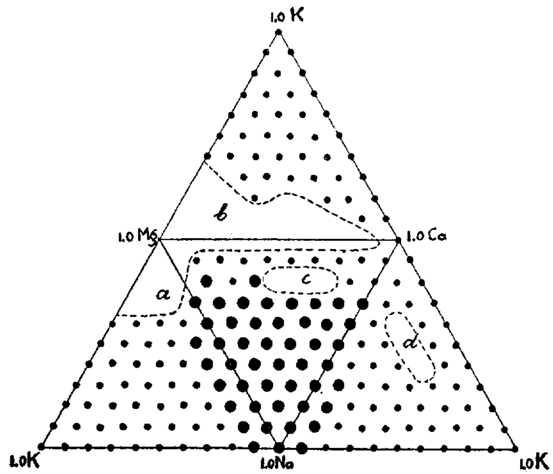

FIG. 5. Diagram illustrating salt antagonism in *Artemia*. The small dots represent membrane formation, the large dots represent nauplii. In the areas *a*, *b*, *c*, and *d* no development took place.

In our work, we have tried only three and four salt combinations, namely, the chlorides of Na, K, Mg, and Ca, always to a total molarity of 1 M.

The objection may be raised that this gives only a one-sided picture, inasmuch as the nature of the antagonism may vary with the concentration (as one of us has demonstrated for *Dunaliella*). Also, it gives no picture of the influence of anions, which is apparently secondary but not negligible. However, we had to stay within the normal limits of experimental possibilities, as the present study already involved several thousand cultures.

Fig. 5 gives the result of the work with three salt combinations.

The small dots represent salt combinations in which membrane formation alone was observed; the heavy dots represent combinations in which nauplii appeared and remained alive for several days.

It may be seen directly that sodium is able to antagonize K in proportion of 0.9 to 0.1 molar. This antagonism persists after addition of Mg up to 0.5 mol $MgCl_2$ and, upon addition of $CaCl_2$ up to 0.3 molar. In the areas *a*, *b*, *c*, and *d*, no development of any kind took place. In order to obtain the complete four-salt picture, the three outer triangles in Fig. 5 may be rotated on their central sides, making the three points (marked K) coincide. In that way, a tetrahedron is

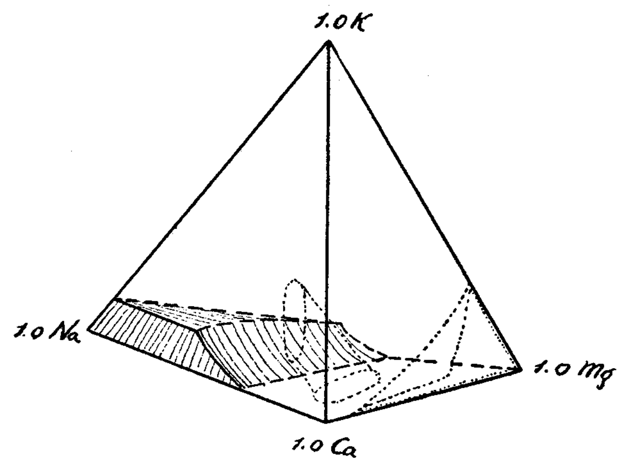

FIG. 6. Three dimensional diagram showing range of development of *Artemia nauplii*.

formed and it remains only to determine the physiological possibilities of the combinations represented by points inside that solid. Instead of tabulating the results obtained, we shall represent them diagrammatically in Fig. 6. Here, the dotted lines delimit the "forbidden areas," while the shaded area represents the salt combinations in which nauplii appear and remain alive for several days. The remainder of the tetrahedron represents salt combinations in which membrane formation, only, was observed.

Artemia represents, therefore, an organism that is very sensitive to potassium salts. The toxic effect of these salts may be antagonized to a small degree by sodium and, to a lesser extent, by magnesium and calcium. Martin and Wilbur (10), who did the pioneer work on salt antagonism in *Artemia*, report that "in two experiments, one with 0.1 mol NaCl + 0.2 mol $MgCl_2$; the other with 1.38 mol NaCl +0.162 mol $MgCl_2$ +0.03 mol $CaCl_2$, mobility persisted more than 300 hours on the average. No other mixtures gave better results than pure NaCl solutions."

The solutions described by these authors are all situated close to the point in Fig. 4 marked 1.0 Na. It is, therefore, possible that the adult has slightly more restricted salt requirements than the nauplius. It must be pointed out, however, that survival time of adults is not a good quantitative measure, as explained earlier in this paper.

Artemia and Its Natural Environment

During the evaporation of sea water in the solar salines, *Artemia* appears at an early stage. The reason why it does not occur in the open ocean is probably a question of food, combined with its total defenselessness.* *Artemia* continues to increase until most of the $CaSO_4$ is precipitated from the sea water. (When the "brine worms" die, the brine is transferred from the pickle pond to the saltern.) At this point, the molarity in NaCl is about 2.7 and, therefore, not high enough to cause death. The potassium increases up to 0.1 molar only in very concentrated brines, so this factor may hardly account for the death of the shrimp. Inasmuch as *Artemia* disappears together with *Dunaliella*, its principal food, this might account for its disappearance, although the red bacteria, which cause the red coloration of the bitterns, may be used to a certain extent. It is most probable, however, that the increasing Mg content of the brine is more important. This factor, combined with the sudden depletion of the food supply, might well account for the disappearance of the "brine worm."

The distribution of *Artemia* in the inland salines checks well with the facts observed in the laboratory. In desert lakes, rich in potassium, *Artemia* is signally absent; while *Ephydra*, the brine fly, occurs in great masses. In Great Salt Lake, which is chemically like a sea

* Students of Professor G. E. MacGinitie report *Artemia salina* in a haul from Elkhorn Slough, California, which is directly connected with the open ocean.

water concentrate, *Artemia* is present, also in the small "playa"-like pools near Marina, California, which contain a solution of NaCl and $NaHCO_3$ with very little potassium.

SUMMARY

Eggs of *Artemia salina* L., the brine shrimp, are easily obtainable in large quantities. Ecdysis takes place in two stages: (a) extrusion of the inner membrane, and (b) ecdysis of the nauplius from that membrane. The conditions which allow for the former are much more varied than those for the latter.

Nauplii form in only solutions of a few sodium salts; and, in Mg, Ca, and Sr salts, potassium is very toxic.

The possible environment for the nauplii (1 M total molarity) has been ascertained for chlorides of Na, K, Mg, and Ca.

The facts observed account for the peculiar distribution of the organism.

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