

## A review of the cellular and developmental biology of *Artemia*<sup>1</sup>

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<sup>1</sup> We dedicate this review to Dr. Frank J. Finamore of the Oak Ridge National Laboratory in recognition of his important work on the developmental biochemistry of *Artemia*.

## Introduction

It has often been the case in experimental biology that a particular organism, by virtue of its peculiarities, provides a singularly useful means by which a specific biological problem can be examined. In this review we will describe a number of properties of this organism which render it very suitable for experimental work in developmental biology. As we extol its virtues we also point out that in some respects, it is not a very good experimental system. Indeed, some of the very same features that render it uniquely useful, also offer frustration and chagrin.

*Artemia* has attracted the attention of such notable reproductive and developmental biologists as Friedrich Brauer (1886), Franz Leydig (1851), Jacques Loeb (1901), and Joseph and Dorothy Needham (1930). During the last decade or so there has been a revival of interest in research on the developmental biology of *Artemia*, and we believe this will continue to grow in the decade ahead. We hope this attempt of ours to describe what is currently known will facilitate this growth.

Hultin and Nilsson (1980) will cover the molecular biology of *Artemia*, so we will exclude from our coverage the details of gene replication, transcription, and transition. Also, we will be dealing exclusively with the diploid, bisexual populations of *Artemia* from the Great Salt Lake and the San Francisco Bay area unless stated otherwise.

## Gametogenesis and fertilization

### SPERMATOGENESIS

Fig. 1 illustrates the adult male reproductive system (Wolfe, 1971). All structures are paired and the histology of the testis is similar throughout its length. Supporting cells and germ cells are the two cell types present (Fautrez-Firlefyn, 1949, 1951; Fautrez-Firlefyn and Fautrez, 1954), the former constituting an epithelial sheath toward the tubule periphery. The spermatogonia are found just beneath the supporting cells, usually in clusters. As spermatogenesis occurs, the stages within a cluster tend to be the same, and may be connected intercellularly according to Wolfe (1971). Mature sperms, located toward the central lumen, are spherical, non-motile and non-flagellated, but exhibit slender "cytoplasmic arms" (Brown, 1966 and 1970). There is disagreement concerning the presence (Fautrez-Firlefyn and Fautrez, 1955) or absence (Brown, 1966) of an acrosome.

Because the earlier stages (spermatogonia and spermatocytes) are located in close proximity to the hemolymph, Wolfe (1971) has suggested they are nourished in this fashion, whereas the supporting cells presumably perform this function for spermatids and sperm. The mature sperm are moved along the testes and into the vas deferens apparently by non-testicular activity, perhaps chiefly by general body movement (Wolfe, 1971). The vasa deferentia secrete seminal fluid and store sperm. Their structure is the same throughout, consisting of a secretory epithelium surrounded by longitudinal and circular muscles. The sperm are presumably nourished by the seminal fluid which contains a substantial amount of neutral mucopolysaccharide (or mucoprotein) according to Wolfe (1971). The vasa deferentia empty near the tips of the two eversible penes, in close proximity to the openings of the two accessory glands (Fig. 1). The duct of this gland does not join the sperm duct but rather

empties near the tip of the eversible penis. The function of the accessory gland secretions is not known. Several roles have been suggested (Wolfe, 1971) including lubrication of the female genital opening and ovisac, an activation substance for the sperm, and a copulatory plug. These glands are apparently homologous with the shell glands of the female.

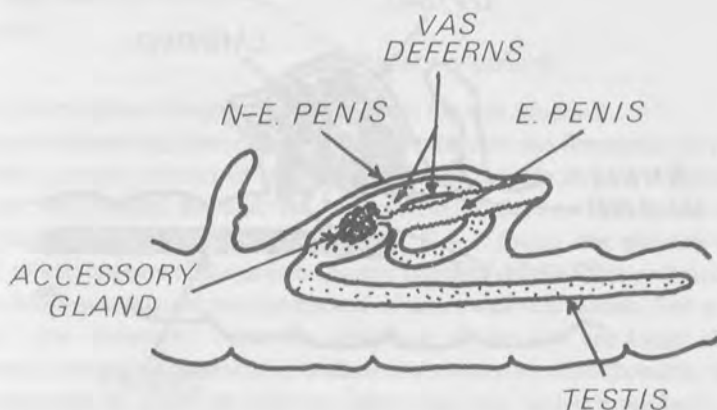


Fig. 1. Diagram of the adult male reproductive system of *Artemia* (after Wolfe, 1971).

The preceding account has been based chiefly upon the works of Wolfe (1971), who appears to have done the definitive study, and Brown (1966 and 1970). Fautrez-Firlefyn (1949, 1951) and Fautrez-Firlefyn and Fautrez (1954, 1955) have described various cytochemical details of spermatogenesis. These papers adequately cover earlier work which extends back to Leydig's (1851) study.

#### OOGENESIS, VITELLOGENESIS AND FERTILIZATION

The female reproductive system is shown in Fig. 2 based on the description by Lochhead (1941 and 1950). All structures are paired with the exception of the single ovisac, also called a uterus or brood pouch. A great deal of work has been carried out on this subject by Fautrez-Firlefyn, Fautrez, and other colleagues at the University of Ghent in Belgium and it will not prove feasible to review all of it here in any detail. Criel (1980ab) considers the morphology of the genital apparatus, including ultrastructure.

Bands of oogonia are found throughout the ovary length, being most abundant toward the ventral side. Some of these oogonia enlarge and undergo vitellogenesis, during which numerous nurse (nutritive) cells participate (Anteunis *et al.*, 1966ab). As vitellogenesis proceeds the cells enter prophase of the first meiotic division (primary oocyte) and remain at that stage as they approach and enter the opening to the enlarged lateral sacs (oviducts) where they are halted at metaphase of the first meiotic division (see Fautrez-Firlefyn, 1951, 1957; Goldschmidt, 1952). Lochhead (1941) stated that it is only while the oocytes are in the lateral sacs that copulation is effective, but Bowen (1962) was the first, to our knowledge, to provide good evidence for this, and she also showed that sperm are not stored within the females from

one mating to the next. Subsequently, cleavage of the zygote begins and embryonic development proceeds along two general lines as will be discussed later. At present we will consider some events taking place in the ovary and lateral sac.

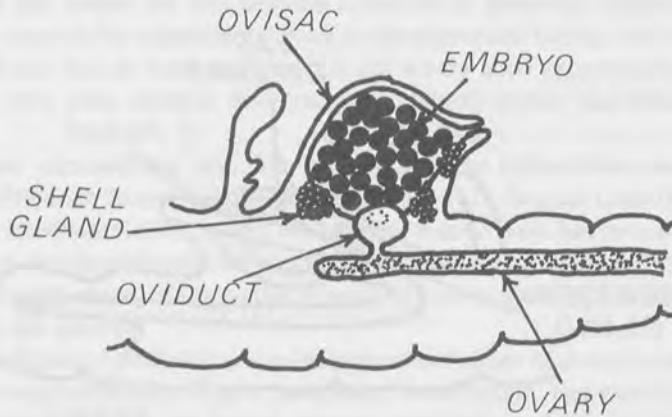
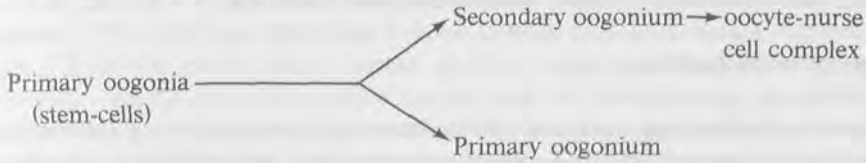


FIG. 2. Diagram of the adult female reproductive system of *Artemia* (after Lochhead, 1941, 1950).

In a brief abstract Lochhead and Lochhead (1967) describe the ovary as a tubular structure in which oogonia occupy in strip along the full length of one side, while "special somatic cells" are similarly disposed along the other side. In between these occur long twisting chains of cells which apparently can be observed only in living teased preparations. Each chain is attached at one end to the oogonial strip where the chain arises by mitosis. Within each chain the cell nearest the "special somatic cell" strip increases markedly in size relative to the others and will eventually give rise to a primary oocyte. The remaining chain cells become nutritive cells which the authors describe as polyploid, multi-nucleolate cells containing relatively small amounts of protein, but very large amounts of cytoplasmic RNA. There are roughly 70 nutritive cells per oocyte, but only two are attached at a given time. These two nutritive cells appear to supply the oocyte with cytoplasm while their nucleus degenerates (see Anteunis *et al.*, 1966b). Presumably, the process repeats when two more nutritive cells are brought close to the growing oocyte. To our knowledge this interesting sequence of events has not been commented on by subsequent workers, presumably because they were not aware of the abstract.

Iwasaki (1970 and 1973a) has described the structure of the ovary as consisting of four cell types: germinal cells (oogonia, we presume), nutritive cells, oocytes, and follicle cells. It is not clear to us whether or not the "follicle cells" represent the "special somatic cells" of Lochhead and Lochhead (1967). Iwasaki (1970) proposes that the germinal cells divide and give rise to both the nutritive cells and the oocytes. Interestingly, at least 12 nutritive cells were observed to provide for the growth of a single oocyte, and the nutritive cells are arranged in "rows", each cell closely pressed to the others. Thus, at least some of Iwasaki's observations appear to be compatible with Lochhead and Lochhead (1967).

Squire (1970) proposes that the oogonia represent a non-exhausting stem-cell population as shown in the following diagram :



Iwasaki's (1973a) irradiation study provides support for this proposal.

One of the more interesting features of oogenesis concerns the formation of yolk platelets which constitute a major portion of the mature oocyte cytoplasm and which are vital to nauplius growth. As we shall see later the yolk platelets should be considered as extremely important developmental organelles. Anteunis *et al.* (1964) found that platelets arise from a "yolk nucleus" which appears in the previtellogenic primary oocyte. This yolk nucleus (YN) is composed of a dense cytoplasmic area composed of microvesicular bodies, free microvesicles, "dense bodies", and ribosomes. Near the periphery of the YN are Golgi elements (the suggested producer of the microvesicular bodies) and numerous mitochondria. Yolk platelet formation is proposed to occur as follows: microvesicular bodies transform into "dense bodies" which leave the YN and take up residence elsewhere in the cytoplasm. Here they become much larger, perhaps by accretion, giving rise to the fully formed yolk platelets. What is remarkable is that the YN persists well beyond vitellogenesis; indeed, it is maintained through cleavage and eventually ends up in one of the "germinal initiales" (one of the two first cells to invaginate during gastrulation). This observation was made by Fautrez and Fautrez-Firlefyn (1964) who also proposed that the YN could play the role of a "genital determinant".

Another feature of importance in the oocyte is the formation of a tubular-mitochondrial complex composed of elongated mitochondria displayed in a hexagonal array with rough endoplasmic reticulum tubules arranged in between to form an elaborate coiled architecture (Anteunis *et al.*, 1966c). These authors suggested that the complex is involved with the synthesis of proteins destined to be incorporated into the yolk platelets because it appears in very young previtellogenic oocytes and precedes the appearance of the YN.

Detailed analyses of "mature" yolk platelets (*i.e.*, those in encysted gastrula) have been carried out by Warner *et al.*, (1972). We shall cover these organelles later in detail, but mention now in passing that there is good reason to believe the yolk platelets perform a far more important role in development than simply to provide nourishment for the developing embryo and larva.

Although fertilization occurs in the ovisac (Bowen, 1962) it has, to our knowledge, never actually been observed (Brown, 1970; Wolfe, 1971). Subsequent events, involving behavior of pro-nuclei, spindle formation and so forth can be found in the massive study of Benesch (1969), and particularly in papers by Fautrez-Firlefyn (1951), Anteunis *et al.* (1967) and the review by Fautrez-Firlefyn and Fautrez (1967). A recent study by De Maeyer-Criel *et al.* (1977) warrants brief description. These workers showed that the mature oocyte is not covered with any structure other than the cell membrane, and no special subcortical organelles exist. However, upon sperm penetration a fertilization membrane is progressively secreted during the first 1.5 hr after fertilization. The precursor of this "membrane" first appears as large granules in the smooth endoplasmic reticulum. These are eventually extruded

in vesicles liberated from the Golgi apparatus, onto the cell exterior where the secretion spreads over the entire egg surface, generating a perivitelline space as well as the fertilization membrane. We shall return to these observations in the following section.

### Development to the gastrula

Although the fertilized egg is extremely yolky, cleavage is complete, having apparently been described first by Artom (1907) and subsequently by several others (Gross, 1936; Fautrez-Firlefyn, 1951; Dutrieu, 1960; Benesch, 1969). Fautrez-Firlefyn (1951) describes the cleavage as intermediate between complete and partial, and transitional between spiral and equal non-spiral, but this does not appear to be in complete accord with the description by Benesch (1969). In any event, it is unusual that the blastocoel begins to form during the first cleavage division (Anteunis *et al.*, 1961). Certainly, the clearest and most complete description of cleavage is that given by Benesch (1969) who provides detailed drawings as well as a time-sequence for all cleavage stages. That author's work indicates the nuclei of the blastomeres are always contained within cell membranes, whereas Fautrez-Firlefyn (1951) and Dutrieu (1960) describe the formation of a "periblastula" in which the cell membranes of the blastomeres break down, and the yolk platelets come to lie in what was the blastocoel. Perhaps a detailed ultrastructural study would clarify this point. Gastrulation occurs by invagination initially of the two "germinal initiales", resulting in formation of the primary germ layers, which subsequently will give rise to the larval tissues (Benesch, 1969).

Once more, the group at Ghent has contributed significantly to our knowledge about this phase of *Artemia* development. Fortunately, much of this work has been reviewed by them (Fautrez-Firlefyn and Fautrez, 1967) and we will not repeat that effort here. However, a brief account of some of the work done since then will be given.

Roels (1970, 1971) presented evidence that peroxisomes, first formed in previtellogenic oocytes, release their contents into the perivitelline space during early cleavage, apparently ceasing at the 8-cell stage when the peroxisomes have essentially all but disappeared from the blastomeres. He implicates this secretion in the hardening of the "cell envelope", perhaps through cross-linking of proteins with concomitant decrease in permeability. This process continues through the blastula, at least. It is tempting to suggest that this secretion-induced hardening might play some role in the formation of the embryonic cuticle which de Chaffoy *et al.* (1978a) have described as becoming impermeable at the "second gastrula" stage (Benesch, 1969). However, de Chaffoy *et al.* (1978a) point out that this permeability change occurs only if the embryos are allowed to remain within the ovisac during development to the gastrula. Thus, if peroxisome release is involved, it is not a sufficient condition for the development of impermeability at the second gastrula stage. We will return to this point later.

A number of studies have been carried out on the effects of inhibitors and various other agents on early development (mostly cleavage). Studies prior to 1968 have already been reviewed (Fautrez-Firlefyn and Fautrez, 1967). Since then, the effects of a variety of compounds have been examined: hydroxyurea (Fautrez-Firlefyn and Fautrez, 1968b, 1969, 1970; Fautrez and Fautrez-Firlefyn, 1970ab); actinomycin-D (Fautrez and Fautrez-Firlefyn, 1973); puromycin (Fautrez and Fautrez-Firlefyn, 1976); several simple amines (Fautrez-Firlefyn and Fautrez, 1968a); cycloheximide (Fautrez and Fautrez-Firlefyn, 1974).

### The two alternative developmental paths

It has been known since the earliest published works (reviewed by Mathias, 1937; Barigozzi, 1939; Dutrieu, 1960) that *Artemia* females can either release encysted embryos ("cysts") from the ovisac (the oviparous path) or retain the embryos and give birth to swimming nauplius larvae (the ovoviviparous path). In the latter case the gastrula simply continues its development to the nauplius. By far, the most complete morphological description of this development has been given by Benesch (1969), a work we consider to be the definitive study on cell differentiation and morphogenesis in *Artemia*. It is the former "oviparous" condition we will concentrate upon, and will refer to the released encysted embryo as a "cyst". According to Benesch (1969) it is at a stage referred to as gastrula-II, an advanced gastrula. This confirms the prior observations of Fautrez-Firlefyn (1951) and Dutrieu (1960).

Some authors (Lochhead, 1940 and 1941; Mathias, 1937; Dutrieu, 1960) have observed two types of encysted embryos: one type produces nauplii shortly after the cysts are released; the other type is apparently dormant (in diapause) and must be "activated". While several factors might be involved in terminating dormancy, one of the most effective treatments is dehydration, either by osmotic withdrawal of water from the cysts or through their exposure to air of low relative humidity. Thus, when such dehydrated cysts are placed into an aqueous environment of suitable osmotic pressure, containing sufficient oxygen, the embryos will resume their development. We believe it's fair to say that the precise conditions required to terminate dormancy in freshly released cysts (and the mechanisms involved) are not known. Several interesting suggestions have been advanced (Barigozzi, 1939; Mathias, 1937; Dutrieu, 1960; Morris and Afzelius, 1967).

The other side of the coin has to do with the factors that lead either to the production of dormant cysts or nauplii within the female. We should point out that although a given female can alternate between these conditions, all of the embryos of a given brood (or clutch) follow the same path. There seems to be uniform agreement that the shell gland (Fig. 2) secretion and/or the shell itself play an important role in determining the path taken (Von Siebold, 1873; Lochhead and Lochhead, 1940; Dutrieu, 1960; Stefani, 1961; Ballard and Metalli, 1963; Benesch, 1969; Bowen *et al.*, 1966; Finamore and Clegg, 1969; Anderson *et al.*, 1970), but other possibilities exist (Morris and Afzelius, 1967, and de Chaffoy *et al.*, 1978a). Having said this, we point out that little is known concerning the details of this relationship.

The ultrastructure of the shell glands has been described in detail (Anderson *et al.*, 1970; De Maeyer-Criel, 1973), and Fautrez and Fautrez-Firlefyn (1971) have performed a number of cytochemical analyses. The origin of the various shell components has been documented by the excellent work of Anderson *et al.* (1970). The ultrastructure of the shell of the encysted dormant gastrula has been the object of several studies (Morris and Afzelius, 1967; Humphreys *et al.*, 1967; Khalaf *et al.*, 1978, 1980; Mazzini, 1978). The comparative study of anostracan egg shells by Gilchrist (1978) should also be consulted.

Fig. 3 is a diagrammatic sketch of the shell of a dormant cyst at the point of being released from the ovisac (after Anderson *et al.*, 1970; Morris and Afzelius, 1967). The region from (and including?) the outer cuticular membrane inward is apparently produced by the embryo whereas the remainder of the shell appears to originate from the maternal shell gland secretion and perhaps other components of the fluid in the ovisac. Secretion by the shell

glands into the ovisac begins as the oocytes pass into the ovisac. After fertilization and the first cleavage division, the shells begin to form at the surface, consisting at this point of a tertiary envelope of irregular thickness. The tertiary envelope is, in general, completed by 30-36 hr after fertilization, at which point the embryo is a late blastula (Anderson *et al.*, 1970). Further alterations occur in the tertiary envelope as gastrulation occurs, until the final structure (Fig. 3) is formed.

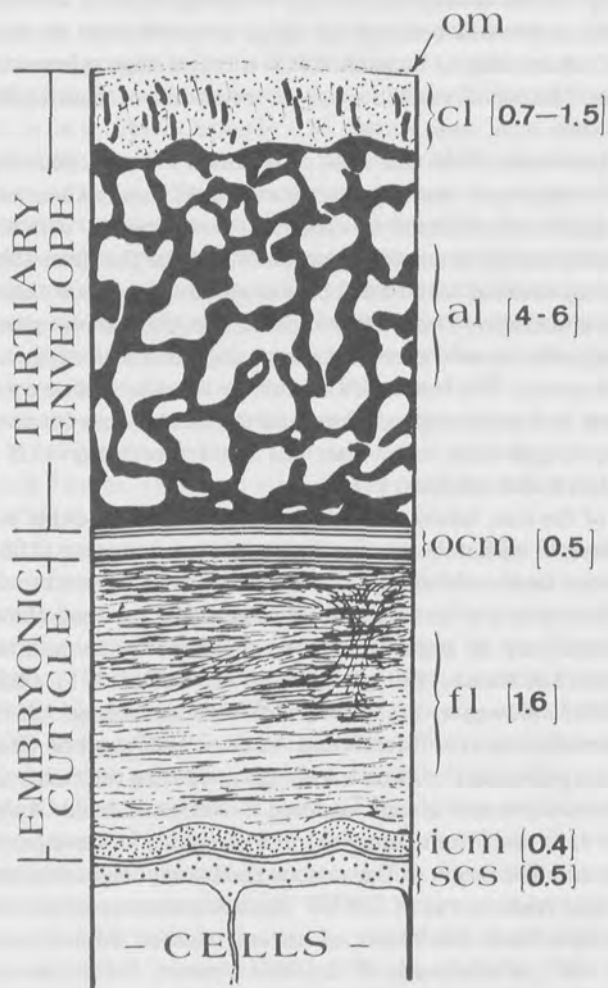


FIG. 3. Diagram of the ultrastructure of the shell of an encysted gastrula (after Morris and Afzelius, 1967; Anderson *et al.*, 1970). Abbreviations: (om) outer membrane; (cl) cortical layer; (al) alveolar layer; (ocm) outer cuticular membrane; (fl) fibrous layer; (icm) inner cuticular membrane; (scs) sub-cuticular space. The numbers given in the brackets represent the approximate thickness in  $\mu\text{m}$  given by Khalaf *et al.* (1978).

Of some importance is the observation by Anderson *et al.* (1970) that the embryonic cuticle is not synthesized by the developing embryo until after the tertiary envelope has been formed. The outer cuticular membrane appears to be the permeability barrier characteristic of the encysted gastrula after it has been dried and rehydrated (Morris and Afzelius, 1967). Thus, one would expect the pre-gastrula stages to be generally permeable to a variety of solutes but to lose this characteristic as gastrulation occurs. That is precisely what de Chaffoy *et al.* (1978) have convincingly shown by experiments on developing embryos both *in vitro* and in the ovisac. They also demonstrated specific ultrastructural changes in the outer cuticular membrane (and fibrous layer) that accompany the permeability changes. de Chaffoy, *et al.* (1978) suggest that the outer cuticular membrane might not form correctly unless the embryo is supplied with material from the maternal female. While the origin of the outer cuticular membrane remains an open question, its developmental importance is established, and it should be the object of further study.

The approach used by de Chaffoy *et al.* (1978a) is clearly a very useful one for the investigation of *Artemia* embryogenesis. It is somewhat surprising that so few studies have been performed using early embryonic stages in culture, other than those by Fautrez, Fautrez-Firlefyn, and colleagues, who were first to do so (Fautrez-Firlefyn and Van Dyck, 1961). However, the observations of de Chaffoy *et al.* (1978a) do not indicate that the particular developmental route taken will be determined by the permeability and membrane changes they observed since both kinds of embryos appear to exhibit similar changes during development. In this context it might be significant that shell glands that lack the brownish secretion ("white shell glands") exhibit significant differences in both the structure of the gland cells as well as in the secretory granules as shown by De Maeyer-Criel (1973) and Criel (1980ab). That the secretion of the "white shell glands" might promote the ovoviviparous route seems one reasonable possibility, among several others (De Maeyer-Criel, 1973).

### Properties of the dehydrated encysted gastrula (cyst)

The ultrastructure of the dried encysted gastrula has been described by Morris (1968) (also see Humphreys *et al.*, 1967; Morris and Afzelius, 1967). There can be no doubt concerning the extreme resistance of the dried cyst to various environmental insults, and several papers presented at the present symposium will add to what we will summarize here (Gaubin *et al.*, 1980; Iwasaki *et al.*, 1980; MacDonald, 1980; Metalli, 1980; Planel *et al.*, 1980). However, one fact that must be kept in mind when evaluating tolerance is the difference between the ability of the embryo to emerge from the shell, and even to hatch as a nauplius, compared with the ability of the resulting larvae to give rise to a reproductively competent adult. Since the encysted gastrula gives rise to the newly hatched nauplius without cell division (Nakanishi *et al.*, 1962) this tends to render the emergence process (and often hatching as well) much less sensitive to damage than subsequent growth, molting and development of the nauplius.

Nevertheless, the performance of the dried cysts is impressive. There seems to be no lower temperature limit since the cysts have been placed at  $-271.7^{\circ}\text{C}$  for 2 hours (Iwasaki, 1973b),  $-190^{\circ}\text{C}$  for 24 hr (Whitaker, 1940), and  $-270.8^{\circ}\text{C}$  at reduced pressure for 6 days (Skoultchi and Morowitz, 1964) with negligible or modest decreases in viability (Dutrieu, 1960). With regard to the upper temperature limit, Hinton (1954, 1968) observed negligible change in % hatch when cysts were pre-incubated at  $103 \pm 0.5^{\circ}\text{C}$  for up to 1.5 hr but longer exposures

rapidly reduced viability as judged by hatching assays. Iwasaki (1973b) obtained similar results, but carried out a much more detailed kinetic study. She found no change in viability when cysts were incubated for 9 hr at 60 °C. However, at 70 °C a decrease of about 15% hatchability occurred between 5 and 9 hr. Over this time span, the "critical" temperature would appear to be between 60-70 °C. These observations agree generally with those of Dutrieu (1960) who found the critical temperature to be above 66 °C. Thus, the dried cells of these cysts seem to be capable of exposure to a total temperature range of 330 °C for appreciable times, clearly an impressive ability. Equally impressive is their resistance to low pressure ("vacuum"). Iwasaki (1973b) detected no change in % hatch of cyst populations incubated for 48 hr at  $5 \times 10^{-5}$  torr (the longest time examined). Whitaker (1940) observed the same outcome after 6 months at  $10^{-6}$  torr, and Gaubin-Blanquet *et al.* (1976) exposed cysts to  $9 \times 10^{-7}$  torr for 38 hr with comparable results. In a study designed to remove all "residual" water from the cysts, the following treatment was applied using a B.E.T. volumetric apparatus: evacuation to  $10^{-5}$  torr, cool to -196 °C, bombard with pure N<sub>2</sub> vapor at 650 mm pressure for 0.5-1 hour, warm tube to room temperature ( $< 10^{-5}$  torr) and evacuate for one hour with a mercury diffusion pump (Clegg *et al.*, 1978). That treatment was applied for 12 consecutive cycles with no significant decrease in the hatching rate or percentage. This same treatment applied to tendon reduced it to powder. Clearly, the tolerance of *Artemia* cysts to desiccation is remarkable.

Smith and Siegel (1975), Tazawa and Iwanami (1974), and Iwanami *et al.* (1975) have examined the effects of soaking dry cysts in a wide variety of organic solvents. Negligible effects on viability were observed for a 30 day exposure to "absolute" acetone, n-butanol, n-propanol, isopropanol, ethyl ether and xylene. Longer periods of exposure showed temperature dependence in their effects (Tazawa and Iwanami, 1974). Interestingly, absolute ethanol exposure reduced viability to some extent, while absolute methanol rapidly killed the cysts (Smith and Siegel, 1975). The authors propose the latter effect results from the similarity of methanol to water.

A great many studies have been carried out on the resistance of dry cysts to various sorts of radiation treatments. The first detailed studies were performed during the early 1950's (see Bowen, 1963) using X-rays, but  $\gamma$ -rays, fast neutrons, proton beams, and high energy electrons have been studied since then, most extensively by Iwasaki at the Radiological Institute in Chiba, Japan (see Iwasaki, 1973ab, and her article in the present proceedings).

These and other well known attributes of dried cysts have no doubt provided the stimulus to utilize them in several space experiments (NASA's Biosatellite II, Apollo 16 and 17, the Apollo-Soyuz Test Project, and Cosmos 782 and 936). The outcome of these studies is still in progress (for details see Planel *et al.*, 1975, 1980; Blanquet, 1977; Gaubin *et al.*, 1979, 1980). In some of these experiments the cysts were housed on the outside of the space craft and were exposed directly to the conditions of outer space. Yet, relatively little overall effect on viability was observed for cysts not directly hit by cosmic heavy ions (Gaubin *et al.*, 1979).

It seems clear that these tolerances are a consequence of the ability of the cysts to reversibly lose practically all of their intracellular water. The biochemical and biophysical basis of reversible water loss has been the subject of considerable study (for reviews see Clegg, 1974a, 1978a, 1979). Of particular importance appears to be the accumulation of large amounts of trehalose (a disaccharide) and the polyhydroxy alcohol, glycerol during early embryonic development (Clegg, 1965). It has been proposed that glycerol (and perhaps trehalose) serve as

a "substitute" for water as the cells undergo dehydration, and this mechanism appears to be widespread in a variety of organisms capable of reversible dehydration (Crowe and Clegg, 1973, 1978; Crowe, 1971, Clegg, 1978ab).

The tolerance of hydrated cysts is another matter. Iwasaki (1973b) examined the tolerance of cysts pre-hydrated for 1, 2, and 3 hr at 4 °C in 2% NaCl and then exposed to -196 °C. No effect on viability was observed for the 1 hr pre-hydration preparation but a large fraction of the cysts were killed after 0.5 hr at -196 °C using the 3 hr pre-hydration group (maximally hydrated cysts). These findings contrast, however, with those of Dutrieu and Chrestia-Blanchine (1967) who observed no significant decrease in cyst viability after several hours exposure to -196 °C. It is difficult to reconcile these observations. To my knowledge, no published data are available on the exposure of hydrated cysts to higher than ordinary temperatures: in principle, hydration should decrease thermal tolerance appreciably.

As expected, addition of water also dramatically changes the response of cysts to various kinds of radiations (Engel and Fluke, 1962; Snipes and Gordy, 1963; Iwasaki, 1964, 1973b, 1980; Metalli, 1980). Hydration exerts a profound effect on the penetration of alcohols into cysts as judged by viability decreases (Smith and Siegel, 1975). At this point we should briefly consider the general permeability characteristics of hydrated encysted embryos. We believe the evidence shows clearly that the cysts are essentially impermeable, in the strict sense of that word, to all solutes of low volatility. There is good evidence that CO<sub>2</sub> can penetrate the cells (Clegg, 1966) probably as free CO<sub>2</sub> rather than HCO<sub>3</sub><sup>-</sup> (Finamore and Clegg, 1969). Studies using radioactive ions, amino acids, sugars, nucleotides and glycerol show that these substances do not penetrate the cells prior to emergence (Clegg, 1966, 1967; Finamore and Clegg, 1969; Conte *et al.*, 1977). One must be careful, of course, to consider adsorption of these substances by the shell components and their trapping within the extensive vesicular network of the porous tertiary envelope (Conte *et al.*, 1977). The electron microscopical study of Morris and Afzelius (1967) demonstrated convincingly that the shell is impermeable to lead phosphate, and they identified the outer cuticular membrane as the site of impermeability. That observation was confirmed and extended by the detailed work of de Chaffoy *et al.* (1978a) that we have already discussed. The only exception to this conclusion is the work of Susheela and Jayaraman (1976) who claimed that <sup>32</sup>P-inorganic phosphate did penetrate encysted embryos. But, the nature of this incorporation was not what one would expect from what is known about the nucleotide pool, and nucleotide metabolism (Warner and Finamore, 1967; Finamore and Clegg, 1969; Van Denbos and Finamore, 1974). It could be of importance that the cysts used by Susheela and Jayaraman (1976) were collected from solar salt ponds on the southeastern coast of India, and were not the ones used in the studies cited above. If indeed the Indian cysts are permeable to labelled phosphate they should prove of considerable value in the study of developmental biochemistry during the encysted period.

In the aggregate, the foregoing suggest that the cells of the dried encysted gastrula represent the most resistant of all animal cells to environmental hazards. These abilities reside within the cells themselves, as well as in the complex shell.

A matter of some interest concerns the metabolic status of "dry cysts", and its relationship to cyst longevity. With regard to the latter it seems evident that all storage conditions that increase longevity are those that suppress metabolism: low temperatures, maintenance of low hydration levels, and removal of oxygen (Bowen, 1963; Clegg, 1967, 1973). Thus, if a metabolism exists it is apparently not desirable in terms of longevity. The conclusion of one of

us is that the cysts are certainly ametabolic below water contents of 0.1 g H<sub>2</sub>O/g dry wt of cysts, and probably below 0.3 g/g, based on a wide variety of experiments (reviewed by Clegg, 1978b, 1979a) and theoretical considerations of macromolecular hydration (Clegg, 1973, 1978b). What can be said then of the observation by Sundnes and Valen (1969) that *Artemia* cysts, containing about 0.15 g/g water consumed oxygen at detectable rates in a temperature dependent fashion? But oxygen consumption need not reflect metabolic participation in dried biological systems, a great many inanimate objects "consume oxygen". (One need only place some "dry" iron shavings in a Warburg respirometer to observe this). Consequently, the uptake of oxygen at low rates in dry biological systems is completely inconclusive in the absence of additional evidence that the oxygen is in fact coupled to ongoing metabolic processes (*i.e.*, the electron transfer chain). We maintain that the encysted gastrula is ametabolic at hydrations less than 0.1 g/g and probably less than 0.3 g/g. As a result, the dry *Artemia* cyst is a true cryptobiotic form, exhibiting properties and characteristics that are neither those of living nor dead cells. Further accounts of these general considerations can be found in works by Keilin (1959), Clegg (1967, 1973, 1978b), Hinton (1968), Crowe (1971) and Crowe and Clegg (1973, 1978).

#### Resumption of metabolism and development of the encysted gastrula

Granted that the severely dried cyst is ametabolic due to water lack, it follows that rehydration must reinitiate metabolism, and a number of studies have "mapped" this hydration dependence of metabolism (reviewed by Clegg, 1978b, 1979a). A conventional metabolism (*i.e.*, one characteristic of fully hydrated cysts) is initiated at 0.65 g H<sub>2</sub>O/g dry wt ( $\pm 0.07$ ), roughly at one-half the hydration level of fully hydrated cysts ( $\approx 1.4$  g H<sub>2</sub>O/g dry wt) achieved by incubation for 3 hr in 0.1 M NaCl, or equivalent water activity (Clegg, 1978a). This minimal hydration reinitiates metabolism, but at a low rate. However, the cyst has biochemical properties that allow it to increase its water content once metabolism is initiated; the cells adaptively synthesize and accumulate glycerol which tends to decrease the osmotic differential between the inside and outside of the cyst, thus allowing for increased water uptake and a more rapid metabolism (Clegg, 1964). Thus, embryonic development is also resumed above the threshold hydration. The bulk of available evidence supports the claim of Dutrieu (1960) that, in addition to hydration at suitable temperature, the only other external requirement for resumption of development is molecular oxygen, at adequate partial pressure. The nature of the requirements that exist for the processes of emergence (splitting of the shell) and hatching (escape of the nauplius) are matters we will consider later in this review.

Certainly, the vast majority of what is known about the developmental biochemistry of *Artemia* comes from the study of the reactivated dried gastrula, its transition into a nauplius larva, and the first three or four larval stages. Before examining the biochemical findings, it is important to describe what is known regarding the morphology of this segment of development. Fig. 4 shows the overall structure of some characteristic landmarks of development. The encysted gastrula consists of 3 000 to 4 000 cells depending upon the geographical origin (Nakanishi *et al.*, 1962; Olson and Clegg, 1976). "Pre-emergence development" (PED) usually requires between 8-16 hr of incubation in optimal conditions - obviously, external conditions will strongly influence its duration. The newly emerged embryo is referred to as an E<sub>1</sub> (Nakanishi *et al.*, 1962) and is a partially formed nauplius larva.

After about 2 hr of incubation the embryo protrudes further from the shell as an  $E_2$  (Fig. 4) and this will hatch as a swimming nauplius larva after an additional 2 hr, depending on the conditions of incubation. Fig. 5 shows the composition of a population as a function of incubation time for cysts from the Great Salt Lake, Utah (Conte *et al.*, 1977; Peterson *et al.*, 1978ab). We point out that biochemical studies done as a function of time are often difficult to interpret because of the heterogeneity of these populations.

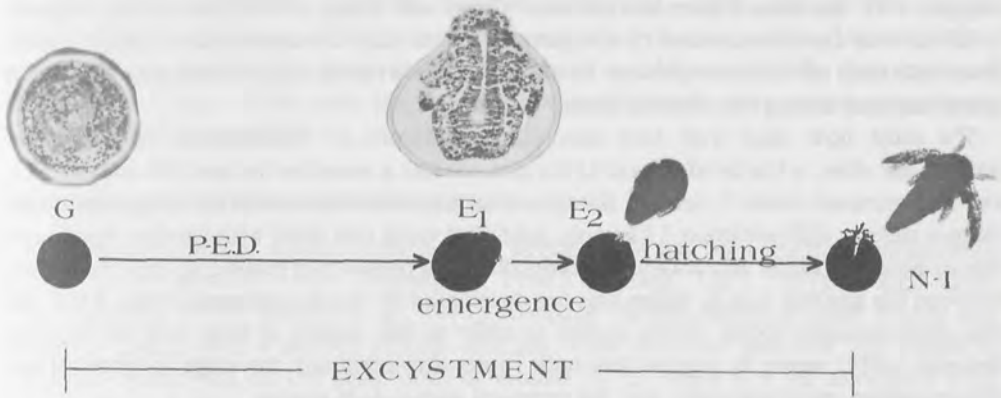


FIG. 4. Development of the encysted gastrula. The photographs given above G (gastrula) and  $E_1$  (just emerged embryo) are cross sections of these stages as seen with the light microscope. The diameter of the encysted gastrula is about 0.2 mm. N-I refers to the first stage nauplius, and P-E.D. means pre-emergence development.

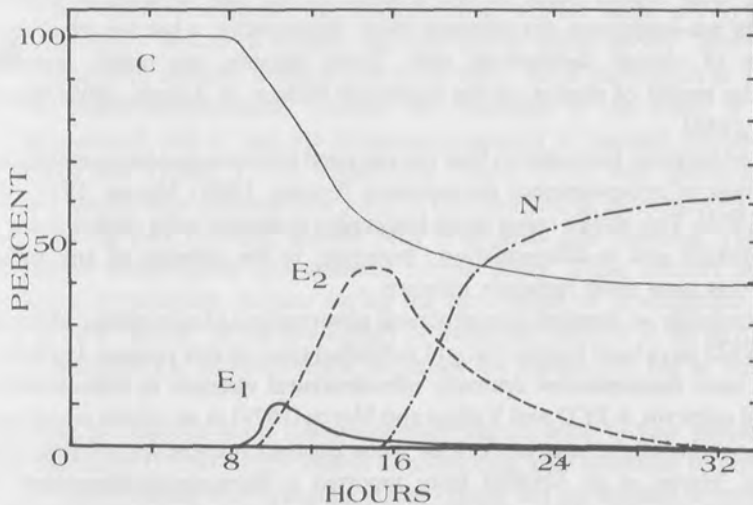


FIG. 5. Relative composition of a developing population with increasing incubation time. Abbreviations: (C) unemerged cysts; ( $E_1$ ) just emerged embryo; ( $E_2$ ) fully emerged embryo; (N) stage-I nauplius).

An observation of considerable importance is that the entire process of PED occurs without the participation of DNA synthesis (Bellini, 1960; Nakanishi *et al.*, 1963) or the occurrence of cell division (Nakanishi *et al.*, 1962). These findings have been confirmed and extended (Finamore and Clegg, 1969; McClean and Warner, 1971; and Olson and Clegg, 1978). Furthermore, it appears that most (all?) of the cell division taking place in the E<sub>1</sub>, the E<sub>2</sub>, and the first two larval stages is not mandatory to development because the E<sub>1</sub> to nauplius-II transition occurs "normally" even when DNA synthesis and cell division are essentially stopped with the drug 5-fluorodeoxyuridine (Olson and Clegg, 1978). Hence, all processes involved with the development of the gastrula into a stage II nauplius can occur with far fewer cells than normally are present. Furthermore, these results indicate that gene replication is not required during this developmental period.

We must now deal with two interrelated questions of considerable developmental importance: first, is the development of the gastrula into a nauplius the same for the dormant and non-dormant routes?; second, does pre-emergence development of the encysted embryo involve cellular differentiation? The only published work that deals with the first question is that of Benesch (1969). His drawings clearly show the presence of mitotic figures in embryos between the gastrula and E<sub>1</sub> stages that are developing by the non-dormant route. Thus, the two developmental routes would appear to differ in this respect at least. But the work of Benesch (1969) seems to suggest that both routes are essentially the same in terms of cell differentiation, morphogenesis, and the temporal sequence of events.

This second question must be asked because of Morris (1971) who hypothesized that no differentiation occurred during PED until just before the embryo (E<sub>1</sub>) emerged from the shell. Morris was apparently unaware of Benesch's (1969) study which clearly details the occurrence of differentiation of a variety of cell types, and widespread histogenesis during the entire period between gastrula and nauplius. Thus, we can conclude that cellular differentiation does indeed occur in the absence of any cell division or DNA synthesis throughout the pre-emergence development stage. Presumably, what we are observing is the differentiation of already determined cells. These features are rather important when interpreting the results of studies on the molecular biology of *Artemia* development (Hultin and Nilsson, 1980).

A number of workers have shown that the encysted embryos can be reversibly dehydrated throughout most of pre-emergence development (Ewing, 1968; Morris, 1971; de Chaffoy and Kondo, 1976). This ability raises some fascinating questions with regard to the possibility of dedifferentiation and re-differentiation; however, in the absence of any morphological observations this issue must remain a mystery.

To our knowledge no detailed ultrastructural observations of subcellular changes in intact cysts during PED have been carried out and published prior to this volume. However, Schmitt *et al.* (1973) have demonstrated dramatic ultrastructural changes in mitochondria isolated from encysted embryos in PED and Vallejo and Marco (1976) in an article which we have not been able to obtain, might have performed some electron microscopy of yolk platelets and mitochondria. Marco *et al.* (1980a) have reported a development-dependent release of mitochondria-like structures from yolk platelets during pre-emergence development, and have related these observations to various *in vitro* assays of mitochondrial enzymes. We will consider their work and that of Schmitt *et al.* (1973) in more detail later in the review.

### Biochemistry of pre-emergence development

Having documented the morphological changes that take place we will now examine the biochemistry that presumably either underlies or is a consequence of these changes. We will exclude coverage of RNA and protein metabolism since these considerations are covered elsewhere (Hultin and Nilsson, 1980). We warn the reader that we have not critically evaluated all of the techniques and methods used in the papers we cite; rather, we will generally accept the findings stated by the authors. Several reviews dealing with various aspects of the biochemistry of PED have been published (Dutrieu, 1960; Urbani, 1959; Green, 1965; a particularly good review by Huggins and Munday, 1968; Finamore and Clegg, 1969; Clegg, 1978a; and the published proceedings of a very recent symposium, Bagshaw and Warner, 1979).

#### RESPIRATION, MITOCHONDRIA, AND ANOXIA

Oxygen consumption during hydration and development has been examined extensively (Urbani, 1946; Dutrieu, 1960; Muramatsu, 1960; Emerson, 1963, 1967a; Clegg, 1964, 1976b; Morris, 1971; Schmitt *et al.*, 1973). Although the conditions of these studies and sources of cysts vary, a general pattern emerges when encysted gastrulae are placed in solutions 0.5 M NaCl (or the equivalent) at around 25 °C; there is a lag associated with hydration of about 15 to 30 min, followed by an increase in the rate of respiration for several hours, then a leveling off as the time for emergence approaches. Another increase in rate occurs at about the time of emergence and hatching. The latter increase has been ascribed by Emerson (1967a) to the increase in surface area that accompanies emergence and hatching, but there is an alternative that we will describe later. The initial increase in respiratory rate seems to be due to dramatic changes in the structure and function of free mitochondria (Schmitt *et al.*, 1973). Those workers showed that mitochondria isolated from pre-hydrated (0 °C) gastrulae are essentially devoid of cristae, and the matrix stains poorly. However, matrix staining and cristae both increase markedly over the first 7 hr of incubation at 30 °C. Nicely correlated with these morphological changes are increases in the levels of cytochrome oxidase, cytochromes b and c, and the respiratory capacity of isolated mitochondria. The findings of Marco and Vallejo (1976), Vallejo and Marco (1976), and Marco *et al.* (1980a) presented at this symposium indicate that some of this increase in mitochondria results from their release from the yolk platelets, notably after emergence and hatching. This is a rather surprising series of observations and deserves further study. These authors also ascribe a critical function to cytochrome oxidase during the resumption of development, considering this molecule to be a "sensor" of the availability of molecular oxygen in the environment: in the absence of adequate O<sub>2</sub>, developmental processes are turned off, but in fully reversible fashion.

This suggestion is related to some interesting experiments done 13 year earlier by Dutrieu and Chrestia-Blanchine (1966) which clearly showed that fully hydrated encysted gastrulae responded in a rather unique way to anoxia – they simply did not resume development upon anoxic hydration, even when incubated at room temperature for half a year. However, it was noted that upon restoration of oxygen, the gastrulae resumed development and gave rise to normal nauplii at rates, and percentages, comparable to controls. Ewing (1968) in a

completely independent study made similar observations. Dutrieu and Chrestia-Blanchine (1966) examined the total lipid and carbohydrate levels of anoxic fully-hydrated cysts, but observed no significant change in either of these after 5 months of incubation, the implication being that these metabolic pathways had been reversibly halted by anoxia. Ewing and Clegg (1969) confirmed and extended these observations, concluding that the gastrulae did not carry out a conventional carbohydrate-based anaerobic metabolism. These studies raised the question as to how the cells could maintain their integrity over many months of anoxia, if indeed they did lack an energy-generating anaerobic metabolism. This question was answered by the thorough and detailed studies of Stocco *et al.* (1972). They showed that nucleotide metabolism is not halted by anoxia, and that the stored nucleotide, diguanosine tetraphosphate (Gp<sub>4</sub>G) is slowly metabolized, presumably providing the primary source of free energy. Apparently, this free energy is somehow coupled to processes concerned with maintaining the integrity of the gastrula. Ewing and Clegg (1969) had observed that the first stage nauplius, in striking contrast to all stages of unemerged incubated embryos in PED, were killed within the first 5 hr of anoxia, and that a conventional anaerobic metabolism did occur in the nauplius, resulting in lactic acid formation. Stocco *et al.* (1972) confirmed the lethal effect of anoxia on nauplii and also found that changes in the control of guanine nucleotide metabolism take place at hatching. In view of all the foregoing, it seems that the postulated role for cytochrome oxidase advanced by Vallejo *et al.* (1980) is reasonable. Of course, it will be necessary to show precisely how cytochrome oxidase activity controls the totality of metabolic processes and development during anoxia.

Finally, we note that Stocco *et al.* (1972) did observe some changes in developmental rates and % hatch levels of cysts during long term anoxia, in contrast to Dutrieu and Chrestia-Blanchine (1966). These appear to be relatively minor differences and do not alter the general picture described above, but they do indicate something of considerable importance we will consider later: namely, the potentially different behavior and properties of different batches of cysts from the same geographical origin, as well as cysts from different origins. We will also return to the matter of nucleotide metabolism during normal development.

There is uniform agreement that the respiratory quotient of embryos in PED is very nearly unity (Dutrieu, 1960; Muramatsu, 1960; Emerson, 1963; Clegg, 1964) providing good indication that carbohydrate utilization is the substrate for respiration. Those studies also showed that the R.Q. decreases after hatching, usually to values between 0.7-0.8, indicating a switch in respiratory substrates. As we shall see in the next section, these expectations have been confirmed.

#### BULK ENERGY AND CARBON SOURCES FOR PED

We have documented the impermeability of the embryonic cuticle to non-volatile organic molecules. Hence, it is clear that all energy sources and substrates for metabolism must already be present in the gastrula as it encysts and becomes dormant within the maternal female. The bulk source of energy for PED has been established to be  $\alpha$ ,  $\alpha$ -trehalose, the non-reducing disaccharide of glucose, first demonstrated in these cysts by Dutrieu (1959, 1960). Trehalose utilization not only serves as the major, and likely the only bulk respiratory substrate, it apparently also serves as a source of carbon for glycogen and glycerol synthesis during PED (Dutrieu, 1960; Clegg, 1964) and perhaps as the carbon skeleton for a variety of

metabolites (Emerson, 1967b ; Huggins and Munday, 1968 ; Boulton and Huggins, 1977). Although a detailed study of the glycolytic pathway and Krebs' cycle in encysted embryos has apparently not been done, it seems very likely indeed that the overall pathways will be similar to those found in eucaryotic cells in general. What little evidence is available supports this expectation (Huggins and Munday, 1968 ; Huggins, 1969 ; Boulton and Huggins, 1970, 1977). Nevertheless, we must stress that this is an assumption and that a detailed examination of these pathways might reveal unique and interesting features.

At this point we digress briefly to emphasize something of great importance in evaluating published work on these cysts as well as in the design of experiments, and publication of the results. It is imperative to clearly state the geographical origin, the year the cysts were collected, and the commercial supplier, in the published work. The reason is simple : different populations (even from the same area) can exhibit different behavior and properties, and the metabolism of trehalose is an excellent example of this. In 1970, Huggins and Boulton published an abstract in which they stated findings completely at odds with earlier work (Dutrieu, 1960 ; Clegg, 1964 ; Ewing and Clegg, 1969). We successfully repeated our earlier work (Clegg, 1976a) and initiated an exchange of several letters with Dr. Huggins. Eventually, we decided to resolve the matter by exchanging samples of the cysts we each had used. Whereupon, we were able to confirm our respective observations : the seeming conflict in results was due to different behavior of the two cyst populations (ours were from the California population near San Francisco while theirs were from a location in Canada). Details of this work are given by Boulton and Huggins (1977). Other examples of this sort could be given.

In view of the importance of trehalose metabolism during PED one might postulate an important role for the enzyme(s) involved with the first step in its metabolism. Only two papers have been published on the matter. Boulton and Huggins (1977) observed no change in trehalase activity throughout PED, using 20,000 g (1 hour) supernatants as the sole source of enzyme. However, Ballario *et al.* (1978) have shown that almost all of the trehalase activity of whole homogenates of unincubated cysts, sediments at 1 000 g (10 min). Thus, the developmental change in this enzyme activity, if any, remains to be determined. Ballario *et al.* (1978) purified the enzyme 180 fold and carried out the usual characterization. It is highly specific for  $\alpha$ ,  $\alpha$ -trehalose and is, therefore, an  $\alpha$ ,  $\alpha$ -trehalose 1-D-glucohydrolase (E.C.3.21.28). Its  $K_m$  is  $4.3 \times 10^{-3}M$  (for trehalose), the pH optimum is close to 6, and its molecular weight is about 75 000. Although its regulation was not examined in detail these workers observed no effect of adding ATP or 3',5'-AMP on enzyme activity. Further study of this enzyme is clearly needed, particularly in terms of developmental changes and possible regulation. It is of some interest that the enzyme appears to be chiefly particulate since Warner *et al.* (1972) have obtained evidence that appreciable amounts of trehalose are associated with the yolk platelets. Perhaps it is worth suggesting that the initial step in trehalose utilization might not be hydrolytic and that future studies might take this into account. It seems quite possible that phosphorylytic cleavage could be involved since this would reduce the free energy requirement for subsequent utilization of the resulting phosphorylated glucose compared with free glucose.

"Balance sheet" studies have indicated that trehalose provides the substrate for glycerol and glycogen synthesis, both of which occur during PED (Dutrieu, 1960 ; Clegg, 1964). In the absence of tracer studies, this conclusion must be tentative. We have no information on the

enzymes involved with glycerol formation although a likely pathway would involve glycolysis to dihydroxyacetone phosphate, with subsequent reduction and phosphate removal. Because of the postulated role of glycerol in the emergence process (Clegg, 1964) and in the desiccation resistance of the encysted gastrula (Clegg, 1974a) a detailed study of the enzymes of glycerol metabolism should prove of interest.

Unpublished work from one of our laboratories (J. S. Clegg) has been directed toward the enzymes of glycogen metabolism and we will briefly summarize our findings (Tables I and II). These studies were performed by Dr. Z. Gunja-Smith. Glycogen synthetase activity is detectable in all stages examined, increases during PED and as the nauplius is formed, and is practically all of the "dependent" form (Table I). In contrast, glycogen phosphorylase shows no significant change during PED but increases very sharply at the E<sub>2</sub> to nauplius transition (Table II). Curiously, the gastrula and nauplius enzyme exhibit strong dependence on AMP, whereas the activity of E<sub>1</sub>/E<sub>2</sub> extracts is much less dependent on this nucleotide. These activities can be compared with the net increase in glycogen that occurs during PED (Dutrieu, 1960; Clegg, 1964).

TABLE I  
Glycogen synthetase in three developmental stages

Stage examined	Activity ( $\mu$ moles <sup>14</sup> C-glucose incorp./mg P/min) <sup>1</sup>	
	Plus G-6-P	Minus G-6-P
Gastrula	0.189	0.009
E <sub>1</sub> /E <sub>2</sub>	0.380	0.014
Nauplius-I	0.550	0.019

<sup>1</sup> 10 000 g (10 min) supernatant used for assay; Methods in enzymology. Vol. 8, p. 374. Average of two experiments.

TABLE II  
Glycogen phosphorylase in three developmental stages of *Artemia salina*

Stage examined	Activity ( $\mu$ moles Pi/mg P/min) <sup>1</sup>	
	Plus 5'-AMP	Minus 5'-AMP
Gastrula	0.51	0.067
E <sub>1</sub> /E <sub>2</sub>	0.52	0.145
Nauplius-I	1.28	0.005

<sup>1</sup> 10 000 g (10 min) supernatant used for assay; Methods in enzymology. Vol. 8, p. 526. Average of two experiments.

Except for the analysis by Dutrieu (1960) on total lipid contents we know virtually nothing about changes in lipids during PED, let alone their metabolism; a fertile area for study.

Fortunately, Schauer *et al.* (1980) have initiated such studies, and have observed differences between the total lipid content and fatty acids of encysted gastrulae compared with nauplii.

The total protein content decreases slightly during PED (Dutrieu, 1960 ; Peterson *et al.*, 1978a). However, there are significant changes with regard to the intracellular distribution of proteins, and important qualitative changes do indeed occur (Hultin and Nilsson, 1980 ; and subsequent sections of our review). Since a large fraction of the total cyst protein is in the yolk platelets (Warner *et al.*, 1972) and since these are degraded to some extent during PED (Olalla *et al.*, 1977) this is to be expected. We will return to these considerations later.

#### NUCLEOTIDE METABOLISM

Without doubt the most thorough and extensive study of metabolites in *Artemia* concerns the nucleotide pool. Virtually all of this work can be traced to the laboratory of Dr. Frank Finamore of the Oak Ridge National Laboratory. In the early 1960's, he and Dr. Alden Warner discovered an unusual guanine-containing nucleotide in the dormant gastrula (Finamore and Warner, 1963), the first in a series of studies on the nucleotides of *Artemia*. Practically all of the research on this and related subjects since then has been heavily influenced by Dr. Finamore. Indeed, his contributions to the developmental biochemistry of *Artemia*, and his influence on the field, via his students and associates, has been far reaching. It is in recognition of this that we dedicate this review to him.

The nucleotide Finamore and Warner (1963) discovered was shown by them to have the structure shown in Fig. 6. In current nomenclature it is abbreviated as G(5')pppp(5')G, or simply Gp<sub>4</sub>G. It is present in very large amounts in the dormant gastrula (about one-half of the total free nucleotide pool) and its study during the last 16 years has revealed its importance to the developmental biochemistry of *Artemia*. In addition to Gp<sub>4</sub>G, other unusual dinucleotides were discovered in the gastrula : G(5')ppp(5')G by Warner and Finamore (1965a), G(5')pp(5')G and G(5')ppp(5')A by Gilmour and Warner (1978). In terms of the total acid soluble nucleotide pool the amounts present in the gastrula are Gp<sub>4</sub>G (45 %), Gp<sub>3</sub>G (7 %), Gp<sub>2</sub>G (0.5 %) and Gp<sub>3</sub>A (0.5 %) (Gilmour and Warner, 1978). While a variety of potential origins and roles for Gp<sub>2</sub>G and Gp<sub>3</sub>A have been considered, little is known about these matters at present (Gilmour and Warner, 1978). In contrast, a great deal of information has been obtained on the metabolism and roles of Gp<sub>4</sub>G.

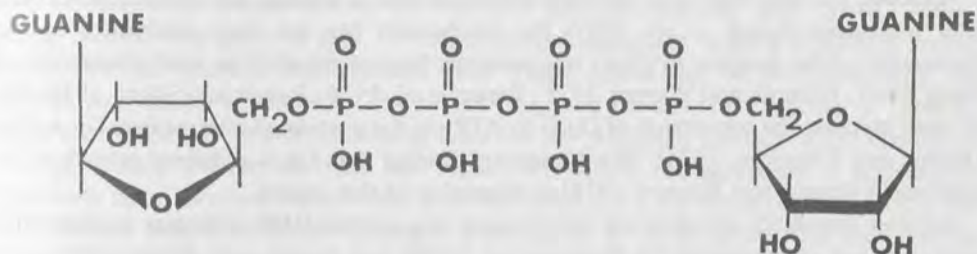


FIG. 6. The structure of guanosine 5'-tetraphospho-5'-guanosine (Gp<sub>4</sub>G) discovered in *Artemia* by Finamore and Warner (1963).

We have already described the importance of Gp<sub>4</sub>G in anoxia of hydrated gastrula (Stocco *et al.*, 1972). Now we will review the evidence that Gp<sub>4</sub>G also functions as a source of purine nucleotides for nucleic acid synthesis, particularly after emergence when a net increase in both RNA and DNA occur (Bellini, 1960; Finamore and Clegg, 1969; McClean and Warner, 1971). Interesting, *Artemia* lacks the ability to synthesize the purine ring *de novo* (Clegg, *et al.*, 1967; Warner and McClean, 1968). Consequently, it must have available an exogenous source of purines to support nucleotide and nucleic acid metabolism. Since the diet of the larvae and adults is rich in purines this poses no problem for these life cycle stages. But the encysted gastrula is impermeable to such solutes and feeding does not begin until the nauplius II stage is reached (Benesch, 1969). This hiatus in the availability of purine is bridged chiefly by Gp<sub>4</sub>G. Warner and McClean (1968) demonstrated that Gp<sub>4</sub>G is essentially absent from all stages of the life cycle except for the sexually mature female and all developmental stages from oocyte to stage II or III nauplii, in which Gp<sub>4</sub>G has essentially disappeared. They also concluded that Gp<sub>4</sub>G synthesis was limited to ovarian tissues in the female and that Gp<sub>4</sub>G was synthesized by the oocytes themselves during oogenesis. In contrast, Gp<sub>3</sub>G, Gp<sub>2</sub>G, and Gp<sub>3</sub>A are made by the developing embryo prior to the gastrula stage.

Subsequent work by Warner *et al.* (1972) showed rather conclusively that Gp<sub>4</sub>G is restricted to the yolk platelets ( $\approx 90\%$  of the total) of the encysted gastrula. That result was contrary to an earlier claim for a nuclear location (Sillero and Ochoa, 1971), which has apparently not been withdrawn (Renart *et al.*, 1976). Warner *et al.* (1972) found that tritiated GDP and GTP were incorporated into Gp<sub>4</sub>G by purified yolk platelets *in vitro* and they proposed that these cell organelles were also the biosynthetic sites during oogenesis (vitellogenesis) as well as being the intracellular storage location in later developmental stages. Further analysis of the system (Warner *et al.*, 1974; Warner and Huang, 1974) provided good support for that contention, since yolk platelets were shown to contain a Gp<sub>4</sub>G synthetase (GTP:GTP guanylyltransferase) which utilizes two GTP molecules as substrates to reversibly form Gp<sub>4</sub>G and inorganic pyrophosphate. They also demonstrated the formation of Gp<sub>3</sub>G but at a much slower rate ( $\text{GDP} + \text{Gp}_4\text{G} \rightarrow \text{Gp}_3\text{G} + \text{GTP}$ ), presumably by the same enzyme. However, competition studies showed clearly that two catalytic sites must exist if a single enzyme is involved, and these workers did not rule out the possibility that two different enzymes are involved.

In this fashion then, the encysted gastrula has a supply of purine nucleotide as well as a source of "high energy phosphate" to meet the needs of anoxia (Stocco *et al.*, 1972).

Although it is clear that Gp<sub>4</sub>G provides adenine as well as guanine for nucleic acid synthesis after emergence (Clegg *et al.*, 1967) the mechanism has not been established for the conversion of the guanine in Gp<sub>4</sub>G into adenine. Several possibilities exist (Finamore and Clegg, 1969; Gilmour and Warner, 1978; Renart *et al.*, 1976; Renart and Sillero, 1974). One of these involves the conversion of Gp<sub>4</sub>G to ATP *via* the postulated intermediate Gp<sub>4</sub>A (Van Denbos and Finamore, 1974). The subsequent finding that Gp<sub>3</sub>A is indeed present in the gastrulae (Gilmour and Warner, 1978) is interesting in that regard.

Another possibility concerns the involvement of a soluble GMP reductase isolated (from gastrulae) and characterized by Renart *et al.* (1976) and Renart and Sillero (1974). This enzyme converts GMP to IMP, requiring NADPH as coenzyme. This group has shown that IMP and XMP competitively inhibit the reductase, notably XMP whose presence produces «hyperbolic kinetics». Gp<sub>4</sub>G alters the shape of velocity *versus* (GMP) curves when XMP is

present, essentially reversing inhibition by XMP. On the basis of these and other findings they speculate that the production of adenine nucleotides from Gp<sub>4</sub>G could involve IMP as an intermediate: Gp<sub>4</sub>G → GTP + GMP; GMP → IMP; and (postulated) IMP → AMP. It is perhaps important to point out that Hernandorena (1972) has shown that IMP satisfies the purine requirement that exists for larval growth in axenic culture.

Of course, Gp<sub>4</sub>G is likely to play roles in addition to serving as an anoxic energy source and as a precursor of adenine. Warner and Huang (1974) pointed out that Gp<sub>4</sub>G synthetase could also be involved in Gp<sub>4</sub>G utilization (provided that inorganic pyrophosphate were available) supplying GTP as the product. Other enzymes from gastrulae that are likely to be involved with Gp<sub>4</sub>G utilization have also been demonstrated. Warner and Finamore (1965b) purified and characterized a diguanosine-tetraphosphate guanylohydrolase that converts Gp<sub>4</sub>G to one mole each of GMP and GTP. It is interesting that this enzyme increases in activity as the level of Gp<sub>4</sub>G falls in the developing nauplius (Beers, 1971). Vallejo *et al.* (1976) carried out further studies on what seems to be the same enzyme, showing among other things that it was present largely in the 150 000 g supernatant. They made the interesting observation that Ap<sub>4</sub> and Gp<sub>4</sub> are both extremely potent inhibitors of Gp<sub>4</sub>G-guanylohydrolase as well as another similar enzyme (dinucleosidase tetraphosphatase) that has been studied by this group (Vallejo *et al.*, 1976). Therefore, even though these nucleoside tetraphosphates have not been detected thus far in the cysts the effective concentrations required for inhibition are so low that routine screening might well miss them, and they remain potentially important. In this connection it is worth noting that Gilmour and Warner (1978) resolved over 20 nucleotide components in their thorough work on the gastrulae, and that a number of these have not yet been identified.

From the foregoing it seems quite clear that the guanine nucleotides play a major role in PED and the early naupliar stages. (For details of their participation in protein synthesis see Hultin and Nilsson, 1980). We hope that the laboratories examining these various aspects will continue their work, and that others will join them. A recent review of "unusual" nucleotides in eucaryotic cells clearly indicates their potential importance in metabolism (Silverman and Atherly, 1979), and the work on *Artemia* could prove to be of general applicability.

#### FREE AMINO ACIDS

The free amino acids have not been studied in any great detail, being limited to a few papers. Dutrieu (1960) did not quantify the free amino acids but did state the order of their apparent relative abundance based on visual inspection of chromatograms. She lists these (in order of decreasing abundance) for the dormant gastrula: aspartic acid, cysteine, serine, glycine, alanine, and glutamic acid. In nauplii the corresponding ranking is alanine, serine, glycine, proline, tyrosine, and glutamic acid. These values can be compared with the quantitative estimates of Emerson (1967b) and Clegg and Lovallo (1977) shown in Table III for dormant gastrula (A) and developing embryos (B). Although the incubation conditions were appreciably different in these two studies we believe the agreement is remarkable, particularly since Emerson's cysts were from the Great Salt Lake while ours were from the San Francisco area. Unless this agreement is fortuitous, it would appear that the embryos exercise considerable control over free amino acid levels. Obviously, the net concentrations of the free amino acids will be determined by the balance between supply from yolk platelet metabolism (and any other sources of free amino acids) and utilization of amino acids for protein synthesis, and other metabolic pathways.

TABLE III  
Free amino acids in *Artemia* encysted embryos  
( $\mu\text{g}$  of amino acid/mg dry weight)

Amino acid	Dormant gastrula		Developing embryo <sup>1</sup>		B/A	
	A. Emerson	A. Clegg and Lovallo	B. Emerson	B. Clegg and Lovallo	Emerson	Clegg and Lovallo
Cys	0.12	0.11	0.46	0.12	3.8	1.1
Asp	1.66	0.65	1.11	0.55	0.7	0.9
Thr	0.14	0.12	1.30	0.62	9.3	5.2
Ser	0.42	0.53	1.25	1.53	3.0	2.9
Glu	1.42	0.93	0.83	0.69	0.6	0.7
Pro	0.43	0.30	1.54	1.10	3.6	3.7
Gly	0.44	0.30	1.28	0.85	2.9	2.8
Ala	1.00	1.00	2.90	2.80	2.9	2.8
Ile		0.03		0.08		2.7
Leu	0.10	0.04	0.21	0.21	2.1	3.0
Tyr	0.62	0.12	0.94	0.29	1.5	2.4
His	0.13	0.05	0.32	0.09	2.5	1.8
Lys	0.19	0.11	0.33	0.21	1.7	1.9
Arg	0.45	0.19	0.69	0.22	1.5	1.2
Val	0.15	—	0.33	—	2.2	—
Phe	0.01	—	0.24	—	24.0	—
Taurine	—	1.66	—	2.31	—	1.4
Totals	7.28	6.14	13.73	9.27	1.9	1.5

<sup>1</sup> Emerson's (1967b) data are from embryos incubated for 44 hr in 1.0 M NaCl. Clegg and Lovallo's (1977) data are from cysts incubated for 48 hr in the vapor phase of 1 M NaCl.

With regard to the total free amino acid pool there is uniform agreement that this gradually increases, starting very soon after hydration is complete and PED is resumed. Reasonably similar trends and relative increases have been observed by Emerson (1967b), Bellini (1960), Dutrieu (1960), Boulton and Huggins (1977) and Clegg and Lovallo (1977). Since the total amino-nitrogen shows relatively little change during PED (Dutrieu, 1960, and references cited therein; Peterson *et al.*, 1978b) these increases in most of the free amino acids most likely reflect yolk protein breakdown. The yolk platelets are, indeed, rich sources of a variety of proteins (Warner *et al.*, 1972; de Chaffoy *et al.*, 1978b; Olalla *et al.*, 1977) and the yolk platelets are degraded during PED, at least to some extent (Olalla *et al.*, 1977). While protease activity is present in extracts of dormant gastrula and embryos in PED (Bellini, 1957; Nagainis and Warner, 1979) the level of activity has been reported to be essentially zero for four different protease activities (Osuna *et al.*, 1977). The situation is complicated by the fact that several protease inhibitors have also been shown to exist and these apparently are involved with protease regulation (Osuna *et al.*, 1977; Nagainis and Warner, 1979). It is not at all clear what the substrates for these proteases are, but the work of these two laboratories clearly indicates that such enzymes could play a far more specific role during development

than simply to hydrolyze yolk protein. Further work on *Artemia* proteases will likely provide information of considerable developmental importance.

#### ENZYME ACTIVITIES

It is beyond the scope of this review to critically examine all of the information on this subject. Much of the earlier work was carried out using very crude extracts and the enzyme activities were poorly characterized, if at all. As a result, one must view the data with some trepidation.

In previous sections we have covered, in a little detail, studies on several enzymes involved with carbohydrate and nucleotide metabolism. A number of the papers presented at this symposium deal, in considerable detail, with various enzymes: aspartate transcarbamylase (Alayse-Danet, 1980); RNA polymerases (Bagshaw, 1980; de Chaffoy *et al.*, 1980; Sastre and Sebastian, 1980; Sebastian *et al.*, 1980); poly (A) polymerase (Jeyaraj *et al.*, 1980); proteases (Marco *et al.*, 1980; Sillero *et al.*, 1980; Warner and Shridhar, 1980); cytochrome oxidase (Vallejo *et al.*, 1980); mitochondrial enzymes (Marco *et al.*, 1980a); and amylase and trypsin (Samain *et al.*, 1980). In addition, the review by Hultin and Nilsson (1980) considers enzyme activities associated with the protein synthesizing system specifically and transcriptional and translational processes in general. We did believe it would be worthwhile to compile a list of the enzyme activities that have been studied in addition to those in the present volume, and the source of this information. We have also constructed a rather arbitrary classification of these enzymes (Table IV) for the purpose of indicating their presence or absence in the dormant gastrula, and to detect general trends (if any) for changes in enzyme activity during PED and the first few larval stages. We should stress that a major problem in summarizing the data in this fashion is the difficulty of evaluating when emergence and hatching occur in the population studied, and the rates of these events. As stressed previously (Fig. 5) most studies have been carried out on mixed populations. Therefore, when we refer to an enzyme activity as increasing, for example, at "emergence" this decision must be re-examined for each specific case if one is interested in precise relationships. Also we do not imply that an increase in activity should be taken to indicate enzyme synthesis. Clearly, there is good reason to believe that enzyme activation/inhibition is of major importance in the developmental biochemistry of *Artemia*.

Inspection of Table IV reveals a few generalities. First, almost all of the enzymes that have been studied in *Artemia* exhibit some sort of fluctuation in activity during development between the encysted gastrula and the first stage nauplius. The exceptions are given in Class III. Of these, only the N-substituted aa-tRNA hydrolase has been studied in detail, the others represent rather preliminary data.

Only one of the enzymes in Class I has been shown to be synthesized *de novo*, the (Na + K)-activated ATPase (Conte *et al.*, 1977; Peterson *et al.*, 1978ab). Furthermore, it seems very likely that this enzyme is absent in the cells of embryos in pre-emergence development. We will return to its important role in the nauplius later in the review. It is difficult to say whether or not the other enzymes assigned to this class are synthesized *de novo* or are held at low activity in the gastrula due to the presence of inhibitors, or are "masked" in some other fashion. The individual papers should be consulted.

TABLE IV  
Summary of enzyme activities during development between  
the encysted gastrula and nauplius

CLASS I. — <i>Not detectable (or extremely low) in the gastrula ; appears later</i>		
<i>Enzyme</i>	<i>Comments</i>	<i>Reference</i>
(Na + K)-ATPase	De-novo synthesis at E <sub>1</sub> /E <sub>2</sub> ; peaks, falls in nauplius	Conte <i>et al.</i> , 1977 ; Peterson <i>et al.</i> , 1978ab
RNase	Sharp rise after hatching ; differs from other RNase's	Sebastian and Heredia, 1978
RNA polymerase III	Linear increase thru PED to hatching	Renard and Sebastian, 1976
RNA polymerase I	Barely detectable ; 4-fold increase thru PED (activation ?) ; declines in nauplius	D'Alessio and Bagshaw, 1977 ; Bagshaw <i>et al.</i> , 1978
DNase	Sharp rise in activity after hatching	Cervera <i>et al.</i> , 1980
Proteases (A,B,C,D)	B & C increase after hatching ; A & D do so later ; activation ?	Osuna <i>et al.</i> , 1977
aa-tRNA hydrolase	N-substituted non-specific form, increases after hatching	Miralles <i>et al.</i> , 1978
CLASS II. — <i>Detectable in the gastrula ; fluctuates during development</i>		
(A) <i>No change during PED</i>		
<i>Enzyme</i>	<i>Comments</i>	<i>Reference</i>
Dipeptidase and Protease	Reach a peak at the E <sub>1</sub> to N transition, then decreases in nauplius	Urbani <i>et al.</i> , 1952 ; Urbani and De Cesaris-Coromaldi, 1953 ; Bellini, 1957
RNase	Increase at E <sub>1</sub> , peaks, falls in nauplius	Urbani and Bellini, 1958
Alk. phosphatase	Increase at E <sub>1</sub> , continues in nauplius	Bellini, 1959
Acid phosphatase	Increase E <sub>1</sub> to E <sub>2</sub> ; levels off in nauplius	Bellini, 1959
Amylase	Increase at E <sub>1</sub> ; continues in nauplius	Urbani <i>et al.</i> , 1953 ; Bellini, 1958
Glycogen phosphorylase	Very sharp increase E <sub>2</sub> to N transition	Gunja-Smith and Clegg (unpublished data)
Lipase	Increase at E <sub>1</sub> , peak nauplius, then falls	Bellini and Lavizzari, 1958
Lactate dehydrogenase	Increase at E <sub>1</sub> , continues thru nauplius ; one isozymic form	Ewing and Clegg, 1969, 1972
Histone acetyltransferase	Increase at E <sub>1</sub> , seems to level off in nauplius ; activity on nuclear fraction basis ; activation ?	Cano and Pestaña, 1976
5'-UMPase, NADH-oxidase, G-6 Pase, Mg-ATPase (?)	Increase during E <sub>1</sub> to nauplius transition, level off or slight decline in nauplius	Peterson <i>et al.</i> , 1978b
Sucrase	Increase occurs in nauplius	Boulton and Huggins, 1977

## (B) Significant change during PED

Enzyme	Comments	Reference
RNA polymerase-I	4-fold increase PED, falls in nauplius	Renart and Sebastian, 1976 ; Bagshaw <i>et al.</i> , 1978
RNA polymerase-II	Falls, then increases PED and nauplius	Renart and Sebastian, 1976
RNA polymerase-II	Increases and levels off PED ; falls in nauplius	D'Alessio and Bagshaw, 1977 ; Bagshaw <i>et al.</i> , 1978
Cytochrome oxidase	Steady increase PED, peaks in E <sub>2</sub> and nauplius	Peterson <i>et al.</i> , 1978b
Cytochrome oxidase	Sharp increase 1st hr PED, then gradual increase	Schmitt <i>et al.</i> , 1973
Glycogen synthetase	Gradual increase PED, continues in nauplius	Gunja-Smith and Clegg (unpublished data)
Mg-activated alk. phosphatase	Seems to increase during PED, does in nauplius	Bellini, 1959
Glu-pyr transaminase	Steady increase PED, peaks E <sub>1</sub> , rises in nauplius	Emerson, 1967b
Glu-oxa transaminase	Steady increase PED, levels off in nauplius	Emerson, 1967b

## CLASS III. - No change in activity, gastrula through nauplius

Enzyme	Comments	Reference
Soluble trehalase, maltase, GPDH, PFK, LDH	20 000 g supernatants used ; all activities said not to change during development	Boulton and Huggins, 1977
N-acetylphenylalanyl-RNA hydrolase	No change during 40 hours of development	Miralles <i>et al.</i> , 178

Other enzymes examined : chitin synthetase in extracts from 3-day old nauplii (Carey, 1965) ; phosphoenolpyruvate carboxykinase and malate dehydrogenase in young nauplii (Conte, 1977) ; glutamic acid dehydrogenase in mixed populations (Emerson, 1967b) ; microsomal enzyme preparations from nauplii involved with phosphatidyl choline synthesis (Ewing and Finamore, 1970ab) ; phosphoglucomutase, phosphofructokinase, glyceraldehyde phosphate dehydrogenase, aldolase, pyruvate kinase, lactate dehydrogenase, and glucose-6-phosphate dehydrogenase in adults (Boulton and Huggins, 1970).

There seems to be little doubt that the majority of enzymes examined do exist in the dormant gastrula at appreciable levels (Class II). While the levels of activity of a number of these enzymes change little if at all during PED (Class IIa) they uniformly exhibit an increase at emergence, or shortly afterward. Whether or not this represents synthesis or "activation" remains to be seen. However, since the cells begin to divide at this point, and since increases in RNA and protein synthesis also accompany emergence and hatching (Hultin and Nilsson, 1980) it seems reasonable to us that enzyme synthesis is likely to be occurring. Even for enzymes of Class IIb which appear to exhibit fluctuations in the level of their activity during PED, abrupt changes also usually occur at the time of emergence and/or hatching. The same is true for enzymes of Class I. There seems to be little doubt that these two important

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developmental events are accompanied by significant changes in biochemistry, and that at least some of these can be directly related to morphological or physiological changes associated with differentiation. These will be considered in a later section of this review.

Finally, inspection of Table IV indicates that most of the enzymes involved with hydrolytic activity do not fluctuate during PED, whereas those involved with synthetic processes increase in activity over this period. The relationship is not exact.

#### OTHER COMPOUNDS IN THE GASTRULA, EMERGED EMBRYO AND NAUPLIUS

A considerable amount of work has been done on the pigments present in these stages. The characteristic brown coloration of the shell is due to hemoglobin-derived haematin localized in the tertiary envelope (Needham and Needham, 1930 ; Gilchrist and Green, 1960). The extent of coloration of the shell (cream to dark brown) is a result of the amount of haematin present and is not apparently due to other pigments. As discussed earlier the origin of the tertiary envelope can be traced to the secretions of the shell glands in the maternal female (Anderson *et al.*, 1970). Presumably, these gland cells process hemoglobin found in the hemolymph (which we will consider later) and release haematin during secretion into the ovisac.

It is well known that the gastrula often contains very large amounts of carotenoids (Needham and Needham, 1930 ; Dutrieu, 1960 ; Gilchrist and Green, 1960). However, there is no substantial evidence that these are synthesized by *Artemia*, of any stage ; rather, they originate from the diet. In nature this usually consists of a wide variety of algae, from which the carotenoids are obtained (Czeczuga, 1980). It is perhaps for this reason that considerable disagreement exists over the kinds of carotenoids present. However, for the gastrula and nauplius, the major carotenoids appear to be canthaxanthin and echinenone (Krinsky, 1965 ; Czygan, 1966 ; Gilchrist, 1968 ; Hsu *et al.*, 1970) ; furthermore, it appears that canthaxanthin is contained within the yolk platelets conjugated to protein (Warner *et al.*, 1972 ; Zagalsky and Gilchrist, 1976 ; de Chaffoy *et al.*, 1978b). These statements appear to hold for the San Francisco *Artemia* population. However, we should note that Czeczuga (1971, 1980) claimed the two most common carotenoids to be  $\beta$ -carotene and astaxanthin ; however, the geographical origin of the cysts was not specified.

In reading these papers we were impressed by the complexity of the situation and the reader would have to do the same to appreciate this. While considerable effort has been devoted to the identification of these various carotenoids, it is fair to say we believe, that absolutely nothing can be said regarding their function(s), at the present time, with any degree of confidence.

Mead and Finamore (1969) isolated a sulfur derivative of ascorbic acid from encysted gastrulae which had the properties of ascorbic acid sulfate. In a subsequent publication from that laboratory (Bond *et al.*, 1972) the compound was proven to be ascorbic acid-2-sulfate, the structure of which is given in Fig. 7. Mead and Finamore (1969) suggested several potential roles that this compound might play (storage of ascorbic acid during embryonic dormancy and/or as a form of active sulfate). Apparently no additional work on this compound in *Artemia* has been published.

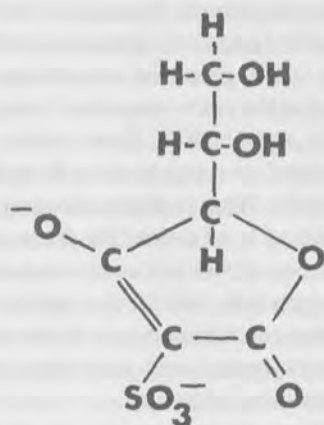


FIG. 7. The structure of ascorbic acid-2 sulfate discovered in *Artemia* by Mead and Finamore (1969). (see Bond *et al.*, 1972 for proof of structure).

### Emergence and hatching

If one observes a cyst at the time of emergence it is evident that the embryo, now a partially formed nauplius (Benesch, 1969) or "pre-nauplius", does not very gradually emerge; rather, the shell seems to "pop" as the pre-nauplius suddenly protrudes slightly (Fig. 4,  $E_1$ ). It is as if the cyst contents are under positive pressure prior to emergence. The mechanism of emergence is not established, although some information is available. Clegg (1964) observed an increase in free glycerol during PED and proposed that some of this glycerol accumulates in the extra-embryonic space (beneath the outer cuticular membrane). It was suggested that glycerol accumulation could establish an osmotic pressure gradient which, in turn, might cause (or contribute to) the rupture of the shell. To our knowledge no specialized region of the shell is involved (*i.e.*, there seems to be no morphologically-distinct area that ruptures at emergence). However, it is a fact that the anterior end of the pre-nauplius ( $E_1$ ) always is the area that protrudes at emergence. Consequently, either the embryo "rotates" to find the opening when the shell does split, or there is another device at work. One possibility is evident from the shape of the  $E_1$ , which is roughly pear shaped: the establishment of this morphology could simply result in the pre-nauplius pushing its way out, "head first" as it were. This possibility could be evaluated by calculating the stress exerted on a sphere by internal formation of such a "pear".

A series of studies by Sato (1967) implicates the participation of a "hatching enzyme" in the  $E_1$  to nauplius transition. When emergence occurs, the  $E_1$  is surrounded by two envelopes or membranes, and as the  $E_2$  is formed the outer envelope ruptures (Myint, 1956). Sato (1967) collected the filtrate of media (Herbst's artificial seawater) in which nauplii had previously hatched, and dialyzed it, presumably against artificial seawater although that is not stated. This dialyzed solution was then employed in a variety of studies, the general conclusion being that an enzyme was present which dissolved the outer envelope after the  $E_1$  emerged.

Furthermore, the outer envelope was always dissolved at the anterior part of the  $E_1$ . The enzyme was inhibited strongly by  $Fe^{+3}$  and  $Cu^{+2}$ , and activated by  $Ca^{++}$  (which, however, did not reverse  $Fe^{+3}$  inhibition). Sato (1967) proposed a mechanism of action for the process.

In other studies, it was shown that the entire process of "excystment" ( $E_1$  through hatching of nauplius) requires NaCl,  $CaCl_2$  and  $NaHCO_3$  (Sato, 1966a, 1967). Furthermore, several amino acids were found to "stimulate" conversion of the  $E_1$  to  $E_2$  stage, histidine and cysteine being the most effective (Sato, 1966b). What is most interesting is that  $\alpha$ -D-amino acids were apparently used, not the L-form which is, of course, the form required for normal amino acid metabolism and protein synthesis. Sato (1966b) does not comment on this, but does point out that his evidence indicates a non-metabolic role for the amino acids, perhaps as a chelator of heavy metal ions. The basis for that conclusion comes from studies with EDTA, glutathione and other compounds. Sato's work poses some interesting possibilities for further study, particularly with regard to the "hatching enzyme".

A comparative review of hatching mechanisms in eggs of aquatic invertebrates includes brief discussion of *Artemia* (Davis, 1968).

With regard to the escape of the nauplius from the inner envelope (hatching) it seems that no elaborate devices are employed: the nauplius becomes motile and essentially "claws its way out" (Myint, 1956).

The transition from the encysted embryo through  $E_1$  and  $E_2$  stages to the free swimming nauplius is obviously a critical one in terms of development. In addition, however, the embryo faces "ionic problems" for the first time in its existence once it leaves the protection of its ion-impermeable shell. It has, so to speak, come out into the real world. We will consider this transition in the next section.

### Nauplius development

Having reviewed the developmental changes that take place in the pre-naupliar stages we will now examine the physiology and biochemistry that is involved in nauplius development. We shall separate our presentation into three major sections, one on osmotic and ionic regulation, a second deals with the changes in respiration and respiratory pigments and the third on the primitive sensory organ, the ocellus. Obviously there are other important changes occurring in the nauplius but the lack of information precludes us from making any definitive judgements on their importance.

#### FORMATION OF THE LARVAL SALT GLAND

Osmotic and ionic regulation during naupliar growth have been examined extensively by Conte (1977), Conte *et al.* (1972a; 1977; 1979a), Hootman and Conte (1975), Hootman *et al.* (1972) and Russler and Mangos (1978). These studies have conclusively established that the hemolymph in the naupliar stage can be maintained at concentrations and composition that are considerably lower and different than the external medium. As a consequence, this mode of regulation requires the animal to possess some type of structure to permit water uptake from the medium while eliminating excess ions. The cephalothoracic structure, commonly referred to as the neck organ or neck shield, is now identified as the organ responsible for water and electrolyte equilibrium. Since its ultrastructural, physiological and biochemical

features are similar to many other types of salt extruding organs, Conte *et al.* (1972) has suggested that in the future it be referred to as the larval salt gland.

Conte *et al.* (1977) has reported on the transition of the pre-emergence embryo using the hyperosmotic trehalose-glycerol mechanism to maintain water balance, to one of hypoosmotic regulation that is entirely based upon the regulation of sodium and chloride. It is the ontogeny of sodium regulation in the nauplius that establishes control over its external environment. This system requires the presence and *de novo* synthesis of Na + K-activated ATPase membranes (Peterson *et al.*, 1978ab) and it is the timing of the increase of this enzymatic activity with the concomitant rise of the cytochrome oxidase (Peterson *et al.*, 1978b; Marco *et al.*, 1980b; Vallejo *et al.*, 1980) that suggests within the nauplius there is integration of ion transport and cellular respiration. Conte *et al.* (1980) has reported upon the specific effects of ouabain on active ion transport and glycolysis, and suggested that the pivotal point for the control of ion transport lies somewhere in the switch over from anaerobic to aerobic metabolism.

#### BIOGENESIS OF BLOOD CELLS AND ONTOGENY OF RESPIRATORY PIGMENTS

Lochhead and Lochhead (1941) reported the existence of specialized nodules that are located at the base of each trunk limb and whose function is to form the free floating blood cells. They established the nature and function of the blood cells by cytological and cytochemical analyses revealing the blood cells to be nucleated and that the nucleus in the forming cell is mitotically active. However, the cytoplasm lacked color suggesting the cells do not contain the heme-containing respiratory pigment, hemoglobin.

The cytoplasm does have soluble proteins and many inclusion bodies, some identified as mitochondria but the remainder being unidentified, yet shown to be composed of acidic and/or basic type complex proteins. Carbohydrates such as glycogen or mucopolysaccharides are absent. With regard to the function of the blood cells, physiological experiments were not performed but direct observation either *in situ* or in fixed preparations showed them to be involved in agglutination or in the nutrition of developing eggs. One function that they do not appear to be involved in is respiration.

Blood plasma (assumed to be free from blood cells) has been reviewed by Kuenen (1939) and the existence of extracellular hemoglobin by spectroscopic analyses has been reported by Lochhead and Lochhead (1941). Thus, the respiratory pigment, hemoglobin, is a soluble plasma protein and is not found packaged in blood cells. The ability to gain or lose extracellular hemoglobin was investigated by Gilchrist (1954) and shown to be dependent upon the oxygen content of the water in which the adults live. As a consequence of this finding, she also examined the effects of salinity upon hemoglobin synthesis. It was found that as salinity increases the dissolved oxygen is lower and hemoglobin synthesis is stimulated. The function of hemoglobin in adults clearly is involved in oxygen transport and is especially important to adults living in concentrated brines. The presence of hemoglobin in the nauplius was not observed by Gilchrist (1954). However, Bowen *et al.* (1969), Waring *et al.* (1970) and Heip *et al.* (1977; 1978ab) have demonstrated the existence of three distinct hemoglobins (Hb-1, Hb-2 and Hb-3) synthesized during nauplius growth. Synthesis does not occur until after hatching, with the highest rate of synthesis being observed for Hb-2 and Hb-3 in the 2-3 day old nauplius. Subsequently, on about day 20, Hb-1 synthesis dominates. Physicochemical

characterization of these hemoglobins has been reported by Moens and Kondo (1976 ; 1977) and Heip *et al.* (1980) and shown to consist of two high molecular weight globin subunits (MW  $\approx$  125 000), each containing multiple heme groups. Also, Hb-2 and Hb-3 contain one identical, or at least immunologically indistinguishable, subunit.

Respiratory rates for adults (Kuenen, 1939 ; Eliassen, 1952 ; Gilchrist, 1956 ; 1958) and nauplii (Kratovich, 1964 ; Angelovic and Engel, 1968 ; and Conte *et al.*, 1980) are quite different. Nauplius respiratory rates have also been found to vary, some investigators reporting an increase in respiratory rate with increasing salinity, others a decrease, and a few reporting that there is no significant change. In the adult and the metanauplius stages there is complete agreement that oxygen transport is facilitated by the participation of extracellular hemoglobin. How hemoglobin facilitates cellular and tissue respiration is not clear at this time.

#### SENSORY ORGANS : FORMATION OF PRIMITIVE NAUPLIUS EYE

The ocellar Anlagen appears early in the pre-nauplius stages of development. At the time of hatching these primitive photoreceptor cells, together with accessory neuronal cells, integrate into a nauplius eye. It is apparent from anatomical organization that the nauplius eye does not lend itself to be a photoreceptor organ designed for precise visualization of discrete forms as is the case for the compound eye found in the adult. Rather, the nauplius eye appears to be responsible for the phototactic responses exhibited in the locomotory behavior of the larvae. Specific anatomical and physiological details concerning the nauplius eye as found in the larva have not been investigated, but Anadon and Anadon (1980) have studied the nauplius eye as it exists in the adult. The adult "nauplius eye" is composed of two cell types : (1) large pigmented cells and (2) numerous neuronal cells referred to as retinula cells. Both types of cells are integrated into a neuronal complex which also involves interdigitation with cells comprising the cavity receptor organ. The nature of the cavity receptor organ (also known as the frontal organ) was revealed by the earlier studies of Elofsson (1966), Elofsson and Lake (1971), and Rasmussen (1971), who showed these organs to be comprised of bipolar neurons.

The physiological significance of the innervation between pigment cells, retinular cells and bipolar neurons with the optic neuropiles remains to be revealed.

#### Metanauplius and juvenile development

Very little information is available on the physiology and biochemistry that accompanies development of the metanauplius and juvenile stages. Nonetheless, we will consider two aspects that we believe to be important, one on the transition between larval and adult mechanism dealing with osmotic and ionic regulation, and a second dealing briefly with neuroendocrine control of moulting.

#### DESTRUCTION OF LARVAL SALT GLAND AND FORMATION OF THE ADULT SALT ORGANELLES

The segmentation of the post-mandibular region reveals that as each thoracic appendage develops, it becomes specialized through the addition of new structures. For example, as the trunk leg becomes specialized for adult locomotory and feeding behavior the setation of naupliar limb buds is increased along with the final development of the alimentary tract (Hootman and Conte, 1974) preparing the nauplius for gathering and digesting food obtained

from the external environment. In addition, the trunk leg segment also becomes specialized for adult respiration by forming numerous gill leaflets that increase gaseous exchange through increased surface area, allowing for increased oxygen consumption by the tissues. Finally, the trunk leg becomes modified to act as an extrarenal organ responsible for excretion of excess electrolytes. It was shown by the ultrastructural studies of Copeland (1966, 1967) that the epithelial lining of the metepipodite does contain salt organelles.

It is the development of these salt organelles that provides for the transition from a larval renal system to an adult renal system because the salt organelles along with the midgut (and possibly the antennal gland for which there is little direct evidence) become the adult renal system. Since these salt organelles are products of the segmental development of the larvae, they are not physiologically active until completion of the last instar. It is at this precise moment that the destruction of the larval salt gland begins to occur. By the time the mandibles are complete the gland has undergone complete autolysis and is reabsorbed. Apparently, the co-existence of both the adult and larval renal systems within the same individual is biologically incompatible. Alternatively, they may simply require different control mechanisms. Needless to say, this intriguing phenomenon warrants further research.

#### DEVELOPMENT OF NEUROENDOCRINE SYSTEM

The morphological features which support nauplius metamorphic development at each successive molting cycle provide sufficient evidence to question whether physiological or biochemical factors are at work in controlling timing of each molt. Since neuroendocrine factors play a major role in insect molt cycles, one would suspect that a similar circumstance might occur in *Artemia*. But, to our knowledge, there is a complete lack of information on the role of neurosecretory hormones controlling embryonic and juvenile development, a highly productive area for future research.

#### Adult development

We will briefly examine a few developmental and physiological aspects of the adult. D'Agostino (1980) reviews the system's physiology of the adult, so we will exclude the details of gastrointestinal digestion and nutrition, reproduction, cardiovascular and neuronal regulation of locomotion, and sensory physiology. We shall consider in this section primitive mechano-sensory receptors, a comparison between naupliar and adult locomotory mechanisms, the anaerobic and aerobic aspects of respiration, the renal and extrarenal system, and the development of the digestive tract.

#### SENSORY RECEPTORS : CUTICULAR RECEPTORS

The structure and function of two types of sensory receptors, (1) mechanoreceptors and (2) chemoreceptors have been reported by Tyson and Sullivan (1979 ; 1980) and Wolfe (1980). The cuticular receptors found on the first antennae were shown to be easily stained with a crystal violet solution. This simple cytochemical technique has been quite useful in identifying chemosensory cells. Additional observations by scanning electron microscopy revealed the presence of cuticular pores which supports the idea that the antennular sensilla are primarily chemosensory rather than tactile. The cuticular receptors (frontal knobs) found

on the second antennae, primarily in males, are modified tactile organs and are used in copulatory behavior. Wolfe (1980) describes the frontal knobs as spherical protuberances located along the anteromedial surface of the protopodite which bear two types of cuticular processes, cuticular spines and mechano-sensory setae. The cuticular spines are acellular and non-sensory, whereas the mechano-sensory setae are comprised of a dome-shaped supporting cell, a setal cell and a sensory neuron.

#### COMPARISON OF NAUPLIAR VERSUS ADULT LOCOMOTORY MECHANISMS

Recent studies by Barlow and Sleight (1980) and Miller *et al.* (1979) involving high speed cinematography or video-computer analysis have revealed how the propulsion of water by *Artemia* is involved in swimming and feeding behavior. In the adult, water is drawn in from the ventral side of the animal by action of the power strokes of the eleven pairs of limbs. Compression of the water between the limbs at the end of the power strokes propels it out as a jet of water behind the animal to produce the thrust. The action of drawing the water from the ventral surface enables the animal to feed by filtering the water before it is used for propulsion. Only a small fraction of the water through which the animal passes is filtered. The metachronism exhibited by the limbs improves the efficiency with which they propel water.

In the nauplii the fluid propulsion is much simpler, involving only one pair of limbs. As the limbs develop, metachronism also develops and increases the efficiency of the system and this in turn increases the maneuverability of the organism to the degree found in the adult animal.

#### AEROBIC AND ANAEROBIC ASPECTS OF RESPIRATION

The primary function of the respiratory system in animals is to transport oxygen to, and carbon dioxide from the tissues in order to maintain aerobic metabolism of the cells. As was shown in the preceding section, extracellular hemoglobin is the respiratory pigment that functions as the gas transporting molecule in *Artemia*. Oxygen consumption is a reflection of these basic requirements and past investigations on the respiratory physiology of brine shrimp have been directed toward providing measurements of basal respiratory rates of individual adult and nauplius. Unfortunately, much of this information on oxygen consumption is not in very good agreement (Conte *et al.*, 1980).

A most notable exception has been the early work of Gilchrist (1956, 1958) showing that the oxygen consumption in adult females did not change appreciably in response to increasing salinities but adult males showed a significant difference in the rate of oxygen consumption when reared in the lower salinities (35‰). Additionally, Gilchrist (1954) reported that adults of both sexes respond to low dissolved oxygen content at the higher salinities by synthesizing more hemoglobin. In a similar fashion, Declerix *et al.* (1980) and Vos *et al.* (1979) studied the effects of several environmental factors upon the respiratory physiology of adults. They found, as did Engel and Angelovic (1968), that when temperature increased, the rate of respiration also increased. If the oxygen concentration of the environmental medium diminished to levels below 1.5 ml O<sub>2</sub>/l, respiratory rates fell precipitously and the animals showed a marked reduction in locomotory movements. The level of dissolved oxygen which suppressed major functional activities (swimming, feeding, *etc.*) was shown for adult males to be around 2.0 ml O<sub>2</sub>/l at 35 ‰ salinity. Below these oxygen levels, anaerobic processes must be invoked to maintain the energetic requirements of the tissues. Declerix *et al.* (1980) have

proposed that in adults the compensatory pathway utilized by the respiratory system is primarily extracellular, namely, the stimulation of hemoglobin synthesis. They report that at oxygen levels of about 1.0 ml/l Hb-3 is the principal form that is manufactured to enrich the blood concentrations and appears to be appropriately fashioned for his purpose since it was shown to have the highest oxygen affinity with the lowest Bohr effect. In this way, the adult adjusts to hypoxic conditions by making a better oxygen trapping system and avoids or lessens the need to utilize less efficient energy producing pathways such as glycolysis.

Does this compensatory mechanism exist in the nauplius? Several facts suggest that is unlikely. First, nauplii which are less than 36 hr old cannot synthesize large amounts of Hb-3 as was shown in the ontogenetic studies of Heip *et al.* (1980). Second, nauplii utilize large quantities of glycogen at moderate levels of dissolved oxygen (about 1.5-2.0 ml/l) but produce very little lactate ( $\approx 1 \mu\text{g}$ ) from sizable quantities of glycogen ( $\approx 80 \mu\text{g}$ ). Lastly, acid secretion from the nauplius (which is not lactate) appears to be salt-dependent but independent of oxygen concentrations. What is the compensatory mechanism for the primitive respiratory system of the nauplius? Conte (1977) and Conte *et al.* (1980) propose that in the early nauplius simple passive diffusion of the respiratory gases is an adequate transport mechanism for supplying oxygen to the tissues. When dissolved oxygen concentrations fall below 1.0 ml/l, however, an intracellular facultative C-4 anaerobic pathway is stimulated in contrast to the stimulation and production of extracellular hemoglobin in the adult stage.

#### RENAL AND EXTRARENAL SYSTEM

Studies by Croghan (1958abcde), Thuet *et al.* (1968), Smith (1969ab), and Geddes (1975abc) on the adult brine shrimp have established that the hemolymph can be maintained at concentrations lower than the external medium. The compensatory mechanism that has been postulated for water and electrolyte homeostasis is that diffusive water loss incurred by the external osmotic gradient is balanced by ingesting the external brine followed by an active uptake of NaCl. The water which accompanies NaCl uptake maintains the fluid volume while excess hemolymph NaCl is removed by active extrusion against a steep electrochemical gradient via an effector organ. In the adult, the effector organs are the gill leaflets located on the middle leg segments. They have been shown to be covered with a highly permeable cuticle (Croghan, 1958b). From studies involving measurement of ionic fluxes (Smith, 1969b) and the transepithelial electrical gradient across the body wall (Smith, 1969a), the underlying gill epithelium was shown to be actively secreting ions. Thus, the alimentary tract together with the gill leaflets appear to be the organs responsible for the renal and extrarenal homeostatic mechanisms involved with water and electrolytes.

#### ALIMENTARY TRACT : WATER ABSORPTION, DIGESTION AND NUTRITION

As previously mentioned, Croghan (1958c) showed the midgut of the adult was the most probable site of water absorption. Bayly (1972) disagrees with this hypothesis and bases his argument upon the fact that the gut fluids have a low NaCl concentration despite having been derived from ingested brine. Also, this small amount of NaCl accounts for less than 30% of the total osmotically active material found in the gut fluid. Furthermore, animals ligated at the neck and anus appear capable of resisting osmotic desiccation despite the fact that they are prevented from drinking. Dall (1967) suggests that the gill leaflets are the site of water

reabsorption but there is no evidence to support this idea. It is apparent that the role of the alimentary tract in water and electrolyte balance is still unresolved.

Developmental studies by Weisz (1947), Anderson (1967), and Benesch (1969) have shown that the alimentary tract is not complete prior to the first larval ecdysis. Ultrastructural studies by Hootman and Conte (1974) showed several structural specializations suggestive of a solute (*i.e.*, NaCl) absorptive role. Chief among these is the amplification of the plasmalemma along the apical and basal cell surfaces with a concomitant rise in the level of (Na + K)-ATPase. Second instar nauplii have a cellular morphology which is consistent with the finding that the foregut, midgut and hindgut are complete, thereby permitting food and ingested fluids to be acted upon by the digestive processes.

The mechanisms of digestion and nutritional aspects of the alimentary tract will be described in the review by D'Agostino (1980).

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