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The effects of anaerobiosis on nucleotide metabolism during embryonic development of Artemia salina.

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THE EFFECTS OF ANAEROBIOSIS ON NUCLEOTIDE METABOLISM DURING EMBRYONIC DEVELOPMENT OF *ARTEMIA SALINA*

BY

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ABSTRACT

The great majority of developing embryos respond to anaerobiosis by producing one or more end-products of carbohydrate catabolism. Irreversible damage or death usually results as a consequence of these catabolic reactions. The inability of the brine shrimp, Artemia salina, to respond to anoxia by accumulating carbohydrate end-products prompted this study of nucleotide metabolism during anaerobiosis. In contrast to carbohydrate metabolism, nucleotide metabolism is not static during anaerobiosis. Interruption of development in Artemia by nitrogen has a marked effect upon all adenosine nucleotide levels, whereas the guanosine and diguanosine nucleotide levels are only slightly altered. The relationship between these changes and the unique developmental pattern of Artemia are discussed.

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INTRODUCTION

Extended periods of anaerobiosis during embryonic development usually produce morphological aberrations leading to cytolysis of the majority of developing animals. The deleterious effects of oxygen debt are usually attributable to an accumulation of end-products of anaerobic carbohydrate catabolism. The most common of the end products is lactic acid although some of the more primitive invertebrates and endoparasites accumulate other intermediates such as pyruvate and glycerol (Saz and Lescure, 1966; Von Brand, 1946). The brine shrimp, Artemia salina, offers an interesting exception to these observations and has therefore been the subject of several studies concerned with the effect of anaerobiosis on embryonic development. At room temperature, Artemia embryos can withstand 1 to 2 weeks[1] of anaerobiosis with little or no effect on either the carbohydrate stores or subsequent development (Ewing, 1966). Also, the common end-products of carbohydrate catabolism have not been detected under these conditions.

In this thesis, a study of the effect of anaerobiosis on nucleotide metabolism during early embryonic development is presented. This study indicates that unlike carbohydrate metabolism, nucleotide metabolism is not static during periods of anoxia. Marked changes in the levels of adenosine nucleotides occur whereas slight changes in the diguanosine nucleotides occur during nitrogen treatment. Although the changes in adenosine nucleotides of Artemia are similar to those

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[1] R. D. Ewing, personal communication.

reported for other embryonic systems maintained anaerobically, cytolysis does not occur in hydrated Artemia embryos even after several weeks of anaerobiosis (Ewing, 1966; Barth and Jaeger, 1947; Brachet and Ledoux, 1955). In contrast to other embryonic systems maintained anaerobically, Artemia embryos readily resume development upon aeration of the saline incubation medium. The ability of Artemia embryos to withstand anaerobiosis and other harsh conditions is of great adaptive significance and is probably related to their ability to inhabit brackish waters and saturated brines where the oxygen content is often immeasurable (Hutchinson, 1957).

MATERIALS AND METHODS

Methods of Incubation

In all experiments, dried dormant cysts of the brine shrimp, Artemia salina (Sanders Brine Shrimp Company, Ogden, Utah) were used as starting material. The cysts were sterilized by immersion in 7% antiformin solution for 15 minutes at 4°C as previously described (Nakanishi et al., 1963). Following the removal of floating cysts and debris by suction, the remaining cysts were collected and washed several times with cold distilled water on a fritted glass filter. One-gram portions of sterile cysts (about 100,000 embryos) were weighed directly into 250-ml Erlenmeyer flasks and covered with 50 ml of chilled, sterile sea water (see Appendix A), supplemented with 1000 units/ml penicillin (Squibb and Sons Ltd., Montreal) and 100 µgm/ml streptomycin sulfate (Nutritional Biochemical, Canada). The flasks containing the eggs and sterile medium were kept in an ice-bath until needed.

Development was initiated by immersion of the flasks in a shaker bath (Dubnoff), at 30°C with gentle agitation to assure an adequate oxygen supply. In those samples used for anaerobic studies, the contents of the flasks were purged continuously with highly purified nitrogen (see Appendix B) for at least one hour. After purging with N_2 the flasks were stoppered tightly and maintained at 30°C with gentle shaking until the time desired.

Extraction and Purification of Acid-Soluble Nucleotides

At varying times the contents of the flasks were collected on a fritted glass filter and washed thoroughly with distilled water. The

cysts were transferred quantitatively to a tissue grinder (Ten Broeck type) and homogenized in 25 ml of ice-cold N $HClO_4$. The homogenates were centrifuged at 12,000 g for 10 minutes and the acid-soluble fraction retained. The acid-insoluble pellet was washed with 10 ml of 0.5 N $HClO_4$ and the soluble wash collected by centrifugation and combined with the first acid-soluble fraction. The combined acid-soluble fractions were deacidified by shaking with Alamine (see Appendix C), and the neutral aqueous fraction applied to a 1 x 50 cm DEAE-cellulose column (DE-23, Whatman), prepared according to Peterson and Sober (1956), and converted to the bicarbonate form with 0.25 M NH_4HCO_3, pH 8.6. Finally the column was equilibrated with 0.002 M NH_4HCO_3, pH 8.6. The deacidified acid-soluble sample was applied directly to the column and the nucleotides eluted using a linear gradient of NH_4HCO_3, pH 8.6 (Oikawa and Smith, 1966).

When necessary, further purification was accomplished by rechromatography on columns of DOWEX-1-Cl^-, 2% (BIO-RAD), using a chloride system (Cohn, 1950). The ultraviolet absorbing fractions were identified using 280/260 ratios and on the basis of their elution sequence as described previously (Warner and Finamore, 1966), and quantitated using the proper extinction coefficients (Volkin and Cohn, 1950). Standard deviations about the mean were determined on all samples.

RESULTS

Aerobic versus Anaerobic Nucleotide Metabolism

In an earlier work, Warner and Finamore (1966) indicated that pre-naupliar development in air results in a decrease in GMP,[2] ADP, GDP and Gp_4G levels, whereas ATP and GTP show significant increases. Concomitantly, Gp_3G shows no significant change prior to hatching. When hydrated embryos of *Artemia* are incubated at 30°C in a nitrogen atmosphere and compared to control embryos, strikingly different results are obtained. These observations are shown in Table 1. Utilization of GMP, GDP and Gp_4G is suppressed by incubation in N_2, whereas ATP production is rapidly and markedly inhibited while GTP production is inhibited, but only after 5-10 hours under nitrogen. In all experiments Gp_3G levels increase slightly during anaerobiosis, whereas the controls remain unchanged.

The Effect of Nitrogen on Nucleotide Levels in *Artemia* Embryos Administered before/after Initiation of Development

The observation that *Artemia* embryos can initiate/resume development after prolonged exposure to anaerobic conditions prompted us to examine the acid-soluble nucleotide profile in hydrated embryos either pretreated with N_2 or whose development was interrupted by N_2. In one experiment, hydrated cysts were maintained under N_2 for 24 hours at 22-24°C, then aerated and permitted to develop to 2.0, 5.5, or 12.0

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[2] The following abbreviations have been used throughout this thesis: AMP, adenosine 5′-monophosphate; ADP, adenosine 5′-diphosphate; ATP, adenosine 5′-triphosphate; GMP, guanosine 5′-monophosphate; GDP, guanosine 5′-diphosphate; GTP, guanosine 5′-triphosphate; Gp_3G, P^1, P^3-diguanosine 5′-triphosphate; and Gp_4G, P^1, P^4-diguanosine 5′-tetraphosphate.

TABLE 1

Nucleotide Levels under Aerobic and Anaerobic Conditions of Incubation.[1]

Compound	0 Hour	2 Hour D[2]	2 Hour N_2	5.5 Hour D	5.5 Hour N_2	12 Hour D	12 Hour N_2
GMP	2.88 ±.35	2.18 ±.02	3.14 ±.11	2.02 ±.29	3.07 ±.03	1.79 ±.22	3.07 ±.55
ADP	0.37 ±.01	0.23 ±.01	0.29 ±.04	0.23 ±.06	0.34 ±.08	0.29 ±.13	0.28 ±.09
GDP	2.42 ±.16	1.99 ± 0	1.90 ±.14	1.92 ±.06	2.12 ±.15	1.59 ±.26	2.09 ±.01
ATP	0.07 ±.09	0.69 ±.01	0.35 ±.07	0.72 ±.30	0.28 ±.07	0.74 ±.07	0.15 ±.14
GTP	0.98 ±.16	1.37 ±.07	1.35 ±.03	1.40 ±.22	1.26 ±.18	1.38 ±.12	1.11 ±.06
Gp_3G	1.14 ±.03	1.19 ±.09	1.43 ±.17	1.11 ±.04	1.28 ±.07	1.14 ±.04	1.27 ±.13
Gp_4G	7.31 ±.29	6.84 ±.24	7.13 ±.34	6.63 ±.13	6.75 ±.28	5.67 ±.21	6.48 ±.48

[1] Concentrations are given in micromoles per 100,000 embryos ± standard deviations of the mean.

[2] Incubation in air.

hours at 30°C, at which times the acid-soluble fraction was prepared and analysed. In a second experiment, Artemia cysts were permitted to develop for 2.0, 5.5, or 12.0 hours then purged with N_2 and maintined anaerobically for 24 additional hours before analysing the nucleotides.

The results of both experiments are compared in Tables 2, 3, and 4. In general, preincubation under N_2 appears to retard the utilization of GMP and GDP compared to controls, and enhances the level of ADP in all stages examined. Pretreatment with N_2 has little effect upon ATP production during subsequent aerobic development except for the 12-hour stage. The diguanosine nucleotide picture is more complex. Although it is clear that pretreatment with N_2 enhances Gp_3G levels at all stages examined, it appears that Gp_4G is affected very little, if any, when one considers the loss of this compound to the medium by diffusion from non-viable cysts in the population examined.

When pre-naupliar development under air is interrupted by N_2 treatment (24 hrs) a somewhat different picture emerges. As development approaches hatching, N_2 treatment has a diminishing effect, if any, upon GMP and GDP levels, whereas GTP synthesis is more markedly inhibited during similar periods of development and N_2 treatment.

When the adenosine nucleotide levels are examined, it was noted that ADP and ATP levels become immeasurable when development is interrupted by N_2 (24 hrs), whereas the AMP level rises. The loss in ADP and ATP from the embryos maintained anaerobically can be accounted for entirely by the rise in AMP

Interruption of Artemia development by N_2 appears to have little effect upon the diguanosine nucleotide levels at all stages examined.

TABLE 2

The Effect of Nitrogen on Nucleotide levels in Artemia embryos before/after Development (2 hours).

Compound	0 Hour	2 Hour D[1]	24 Hour N_2 + 2 Hour D	2 Hour D + 24 Hour N_2
AMP	0.38	0.19	0.19	1.10
GMP	2.88	2.18 ±.02	2.45 ±.01	2.78 ±.27
ADP	0.37	0.23 ±.01	0.41 ±.04	0
GDP	2.42	1.99 ± 0	2.39 ±.41	1.94 ±.20
ATP	0.07	0.69 ±.01	0.61 ±.09	0
GTP	0.98	1.37 ±.07	1.48 ±.20	1.22 ±.03
Gp_3G	1.14	1.19 ±.09	1.45 ±.31	1.18 ±.16
Gp_4G	7.31	6.84 ±.24	5.90 ±.35	5.76 ±.52

[1] Development in air.

TABLE 3

The Effect of NItrogen on Nucleotide levels in *Artemia* embryos before/after Development.(5.5 hours).

Compound	0 Hour	5.5 Hour D[1]	24 Hour N_2 + 5.5 Hour D	5.5 Hour D + 24 Hour N_2
AMP	0.38	0.16		
GMP	2.88	2.02 ±.29	1.93 ±.53	1.67 ±.45
ADP	0.37	0.23 ±.06	0.62 ±.17	0
GDP	2.42	1.92 ±.06	2.07 ±.39	2.01 ±.50
ATP	0.07	0.72 ±.30	0.67 ±.29	0
GTP	0.98	1.40 ±.22	1.45 ±.15	1.17 ±.17
Gp_3G	1.14	1.11 ±.04	1.40 ±.13	1.33 ±.19
Gp_4G	7.31	6.64 ±.13	5.64 ±.32	6.02 ±.25

[1] Development in air.

TABLE 4

The Effect of Nitrogen on Nucleotide levels in Artemia embryos before/after Development (12 hours).

Compound	0 Hour	12 Hour D[1]	24 Hour N_2 + 12 Hour D	12 Hour D + 24 Hour N_2
AMP	0.38			
GMP	2.88	1.79 ±.22	2.13 ±.05	2.07 ±.37
ADP	0.37	0.29 ±.13	0.57 ±.24	0
GDP	2.42	1.59 ±.30	1.89 ±.45	1.59 ±.33
ATP	0.07	0.74 ±.07	0.34 ±.13	0
GTP	0.98	1.38 ±.12	0.97 ±.15	1.06 ±.13
Gp_3G	1.14	1.14 ±.04	1.22 ±.21	1.15 ±.10
Gp_3G	7.31	5.67 ±.21	5.35 ±.42	5.83 ±.69

[1] Development in air.

FIGURE 1

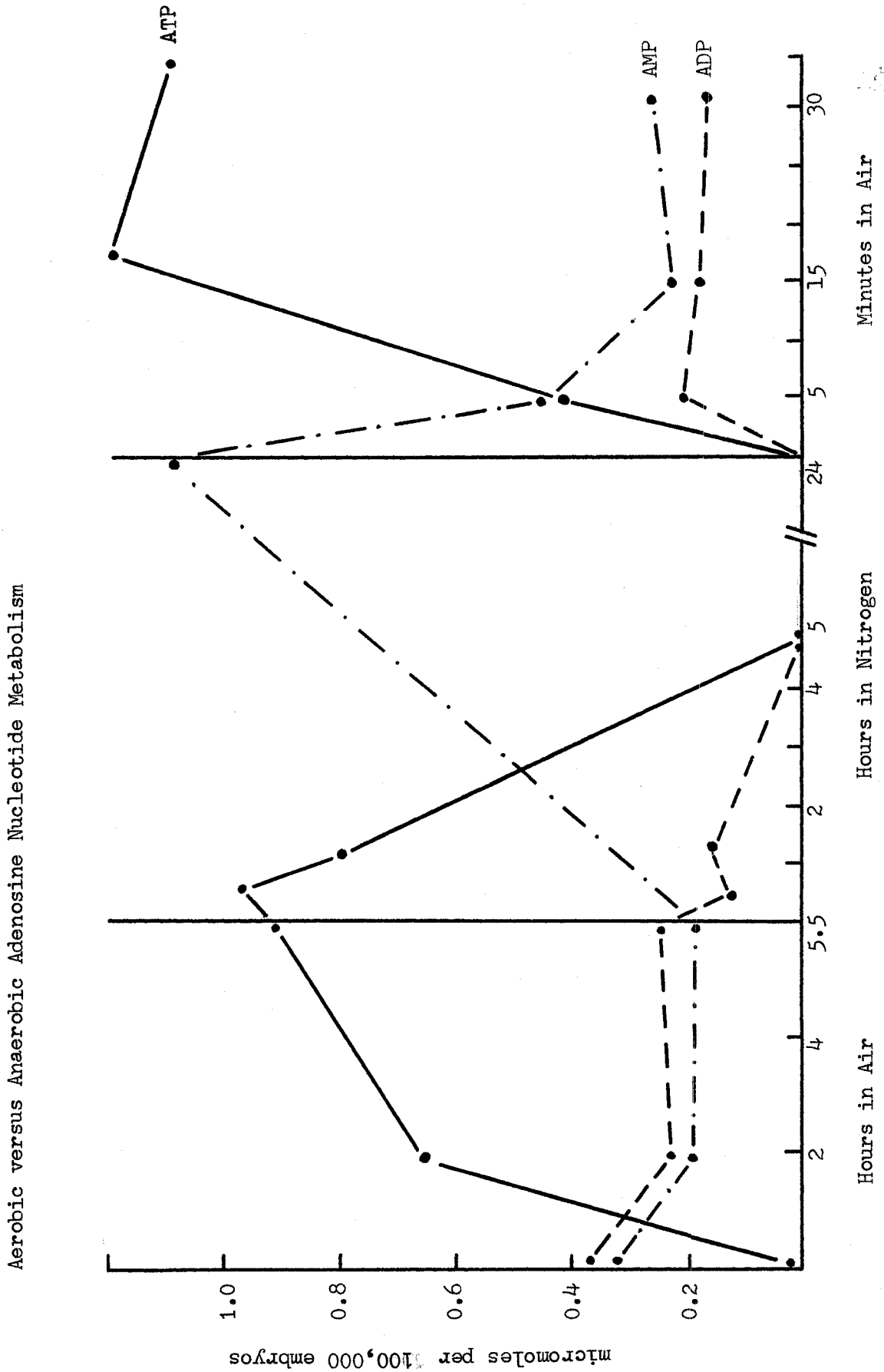
Aerobic versus Anaerobic Adenosine Nucleotide Metabolism

— · — · —, micromoles of adenosine 5′monophosphate, (AMP).

— — —, micromoles of adenosine 5′diphosphate, (ADP).

———, micromoles of adenosine 5′triphosphate, (ATP).

FIGURE 1



DISCUSSION

The impermeability of Artemia embryos to virtually all nucleotide precursors has prevented us from employing radiolabelled nucleotide precursors in this study. The only precursor that readily penetrates the hatching membrane of the pre-hatched Artemia embryos is HCO_3^- (Clegg et al., 1967; Clegg, 1967), but unfortunately Artemia cannot synthesize purine nucleotides de novo (Warner and McClean, 1968), and incorporate very little HCO_3^- into the extremely small pyrimidine nucleotide pool in brine shrimp embryos. These observations offer serious limitations to further studies of pre-naupliar development in Artemia where the use of radiolabelled precursors is mandatory

In a recent study by Ewing (1966), it was observed that although Artemia embryos do not accumulate lactic acid or other end products of carbohydrate catabolism while under N_2, Artemia nauplii produce large amounts of lactic acid when exposed to N_2 for only one hour. These studies suggest that at some point during Artemia development, a transition must occur in the ability of the embryo to synthesize lactic acid in response to anaerobiosis. Further studies from the Miami laboratory indicate that this transition period coincides with the time of hatching. In addition, experiments in our laboratory have shown that nauplii die when maintained under N_2 for 3-4 hours. In this thesis, then, all anaerobic experiments are limited to pre-naupliar stages of development when the effects of N_2 on Artemia development are reversible.

Nitrogen treatment of the newly hydrated, undeveloped cyst appears to have little effect upon the nucleotide profile compared to the untreated, dried cysts. The decrease in Gp_4G levels during prolonged

incubation of Artemia cysts in N_2 is believed due to diffusion of this nucleotide from non-viable and cracked encysted embryos in the population. Similar observations have also been reported before (Warner and Finamore, 1966). In contrast, the rise in Gp_3G during pre-development exposure to N_2 is always apparent. Since no evidence is available as to the function of this compound during development, it is premature at this time to speculate as to the reasons for the upward shift in concentration of this nucleotide anhydride.

The observation that N_2 treatment of the undeveloped cyst results in little change in nucleotide levels may explain in part, why pre-hatch development of Artemia is reversible. The ability of the brine shrimp to survive in brackish ponds and highly concentrated brines where oxygen content is often immeasurable, could be attributed to its ability to suspend nucleotide metabolism and preserve its energy stores for further development. Nucleotide metabolism resumes when the availability of oxygen increases. Additional evidence of Artemia's adaptive ability to the stress of anaerobiosis was observed when embryos undergoing pre-naupliar development were interrupted by N_2. Under these conditions Artemia embryos suspend morphogenic activity until O_2 is resupplied to the saline environment.

During the period of suspended morphogenic activity the ADP and ATP content of these embryos soon becomes immeasurable, (using our ion-exchange technique), with a concomitant increase in AMP. The nucleotide profile now remains unchanged until the embryos are aerated, at which time the ADP and ATP contents return to normal at the expense of the AMP fraction. The fact that both ADP and ATP stores are rapidly exhausted during anaerobiosis may account for the rapid and complete

cessation of carbohydrate utilization known to occur in Artemia in response to anaerobiosis (Ewing, 1966). In view of these findings, it appears that Artemia possesses a mechanism for ensuring completion of embryonic development in brackish waters by turning off anaerobic carbohydrate utilization which normally produces pyruvic and/or lactic acid whose accumulation leads to cytolysis.

Although an earlier study (Brachet, 1949) indicated that mitosis, and therefore embryonic development, is impossible in the absence of oxygen, (leading to irreversible damage and death of the organism), it has been observed that both frog and trout embryos complete cleavage under anaerobiosis then rapidly cytolyze (Brachet, 1934; Devillers, 1953). Perhaps their ADP/ATP levels are sufficient to permit completion of cleavage but that the more complex events associated with morphogenesis require additional ATP which is produced mainly aerobically. One reason for Artemia's insensitivity during morphogenesis may reside in its ability to maintain a constant number of cells throughout the period of morphogenesis (Nakanishi et al., 1962). Perhaps the deleterious effects of anoxia are not manifest in Artemia embryos due to the lack of mitosis during this period of development.

The reasons for cessation of development are probably more complex than this discussion indicates, nevertheless, Artemia may provide a system to further examine the effects of N_2 on cellular processes.

SUMMARY

1. Incubation of hydrated encysted embryos of Artemia salina under a N_2 atmosphere prevents morphological development and inhibits the utilization of GMP, GDP, ADP and Gp_4G and surpresses the synthesis of ATP and GTP.

2. When normal development of hydrated, encysted embryos is preceded by 24 hours under N_2, the utilization rate of GMP and GDP is decreased, whereas ADP and Gp_3G levels rise. Pretreatment with N_2 has little, if any, effect on ATP production and Gp_4G utilization during subsequent development.

3. When pre-naupliar development is interrupted with N_2, the ADP and ATP content rapidly falls to zero with a concommitant rise in AMP. Both diguanosine nucleotides, GMP and GDP are unaffected by N_2 interruption of development, whereas GTP production is markedly inhibited.

4. The ability of the adenosine nucleotides to control/regulate carbohydrate metabolism in Artemia in response to anoxia is discussed. Perhaps Artemia is able to withstand anoxia, by turning off nucleotide and carbohydrate metabolism, thereby preserving its energy stores for the resumption of development when the proper O_2 level is restored to all environment.

APPENDIX A

The sea water used contained the following salts (micromoles/liter): NaCl, 422; KCl, 9.4; $MgSO_4$, 25.4; $MgCl_2$, 22.7; $CaCl_2$, 1.4; $NaHCO_3$, 0.5. Sea water of this composition closely resembles that of the Great Salt Lakes region (Hutchinson, 1957).

APPENDIX B

Nitrogen, (Prepurified Grade, Matheson of Canada, Whitby, Ontario) was further purified by bubbling through a series of three flasks containing: 1) half concentrated NH_4OH saturated with NH_4Cl and copper filings, 2) $\underline{N}$ H_2SO_4 containing 4 drops phenopthalein, and 3) distilled water (Thibert).

APPENDIX C

Deacidification of acid-soluble extracts of developing brine shrimp embryos was performed by a method similar to that previously described by Warner and Finamore (1966). To the perchloric acid extract was added an equal volume of N tricaprylyl amine in chloroform (Alamine 336 s, General Mills Inc., Kankakee, Illinois) and the mixture inverted slowly several times until the aqueous phase tested neutral or slightly basic with universal indicator paper. The phases were separated by mild centrifugation and the aqueous layer retained. The organic layer was washed once with H_2O and the wash was added to the deacidified aqueous layer. The combined aqueous fractions were stored at -20°C until nucleotide fractionation on DEAE-cellulose could be carried out. Under these conditions the tertiary amine forms an acid-salt with perchloric acid and remains in the organic phase, whereas the nucleotides are inextractable from the aqueous phase.

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