Nucleotide Metabolism during Brine Shrimp Embryogenesis*

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SUMMARY

Changes in the amounts of acid-soluble nucleotides during embryonic development of the encysted egg of the brine shrimp, Artemia salina, have been determined. Significant quantities of diguanosine tetraphosphate, the most abundant compound in the nucleotide pool, are apparently not utilized until hatching, at which time the amount falls precipitously. Diguanosine triphosphate, a homologue of the tetraphosphate, does not change appreciably in amount throughout development, but may participate in a reaction catalyzed by a nucleotide phosphokinase. Adenosine triphosphate increases over 5-fold during the first few hours of development and again after hatching, but is virtually absent from the dormant cyst. Early development is associated with the rapid appearance of nucleotides in the hatch medium. These nucleotides appear to arise by diffusion from cracked and nonviable eggs and not by an active secretory process of the normally developing embryo.

EXPERIMENTAL PROCEDURE

Incubation Method for Brine Shrimp Development—In all experiments, dried dormant cysts of the brine shrimp, Artemia salina (Saunders Brine Shrimp Company, Ogden, Utah), were used as starting material. Ten grams of dried cysts were suspended in 250 ml of ice-cold antiformin solution (6) and stirred gently for 20 min. After removal of all floating cysts and debris by suction, the remaining cysts were collected by filtration through a sintered glass filter and washed with ice-cold distilled water. The washed cysts were air-dried for 10 min and then through a sintered glass filter and washed with ice-cold distilled water. The washed cysts were air-dried for 10 min and then 1-g portions containing about 100,000 cysts were placed into each of 16 covered crystallizing dishes (90 x 50 mm). The cysts were covered with 45 ml of either Wardley's hatch mix (Wardley Company, Long Island, New York) or artificial seawater (6) supplemented with 1000 units per ml of penicillin (sodium citrate buffered, Squibb and Sons, Ltd., Montreal) and maintained at 30° with gentle shaking until the desired time. Under these conditions, emergence (i.e. egression of the embryo still enclosed in a transparent hatching membrane from the cracked shell) began at about 5.5 hours and hatching (emergence of a swimming nauplius from the transparent membranous sac) began at about 11.5 hours. Thirty-six hours after the onset of excystment, 65 to 70% of the starting cysts have hatched into swimming nauplii. Although a higher yield (80 to 85%) may be reached in an additional 12 hours of development, many nauplii begin to die during this period unless transferred to a salt solution of higher osmotic pressure. For this reason, nauplii populations older than 36 hours were not taken for analysis and discussion in this paper but have, instead, been analyzed in the accompanying paper (10).

Quantitative Extraction of Free Nucleotides—At desired time intervals (0, 2, 5.5, 9, 12, 22, 27, and 36 hours), the contents of two crystallizing dishes were pooled, filtered through a sintered glass funnel, and thoroughly washed with distilled water. Both the cysts and the nauplii were divided into equal parts and each part was homogenized in 25 ml of ice-cold 1 N HClO4 by the use of a tissue grinder of the Ten Broeck type. The homogenates were combined and then centrifuged at 15,000 x g for 15 min, and the supernatant fraction was retained. The acid-insoluble pellet was washed once with a small amount of the HClO4 solution and centrifuged, and the soluble fraction was pooled with the first supernatant fluid. The combined clear supernatant fluids were kept at 0° until deacidification.

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1 The abbreviations used are: diguanosine tetraphosphate, P1,P4-diguanosine 5'-tetraphosphate; diguanosine triphosphate, P1,P4,P6-diguanosine 5'-triphosphate.
Effective in quantitatively removing the perchloric acid from the to remove traces of acids from mixtures of inorganic compounds from certain protein hydrolysates (11) and have been employed subsequently these amines became useful in removing mineral acids ing to long chain aliphatic amines in chloroform (11). Subse- viously identified in the dormant cyst with a formate-formic acid extractants from tissue homogenates (e.g. charcoal, forma-

Deacidification of HClO₄ Extract with Amines of High Molecular Weight—Although several techniques are widely used to remove acid extractants from tissue homogenates (e.g. charcoal, formation of insoluble salts, and ether extractions), the simplest method for the quantitative recovery of the nucleotides, particularly the diguanosine type, proved to be the deacidification method de- vised by J. X. Khym. This method employs extraction of the acid with high molecular weight amines dissolved in an organic solvent. Earlier it had been reported that many mineral and organic acids could be removed from aqueous solutions by binding to long chain aliphatic amine in chloroform (11). Subse-

Deacid$cation$ with Amines of High Molecular Weight—Although several techniques are widely used to remove acid extractants from tissue homogenates (e.g. charcoal, formation of insoluble salts, and ether extractions), the simplest method for the quantitative recovery of the nucleotides, particularly the diguanosine type, proved to be the deacidification method devised by J. X. Khym. This method employs extraction of the acid with high molecular weight amines dissolved in an organic solvent. Earlier it had been reported that many mineral and organic acids could be removed from aqueous solutions by binding to long chain aliphatic amine in chloroform (11). Subsequently these amines became useful in removing mineral acids from certain protein hydrolysates (11) and have been employed to remove traces of acids from mixtures of inorganic compounds (12, 13). For our purposes the tertiary amine, tricaprylyl amine (Alamine 336s, General Mills, Inc., Kankakee, Illinois), was effective in quantitatively removing the perchloric acid from the aqueous tissue extract by forming an amine salt in the organic phase. For use, 1 N amine in chloroform was prepared and washed several times with 1 N NaOH, with water until neutral, and then with 0.01 M NaCl. To the clear perchloric acid extract was added a slight excess of the amine-chloroform solution, and the two-phase system was mixed thoroughly by inversion until the aqueous phase appeared neutral or slightly basic when tested with Universal indicator paper. Following separation of the two phases, the organic phase was washed twice with distilled water. The water washes were pooled with the first aqueous phase, which contained the bulk of the ultraviolet-absorbing material. When desired, the amine remaining in the aqueous phase can be removed by washing the aqueous phase with chloroform, and the excess chloroform can be removed by filtration through Whatman No. 1 filter paper. This method is rapid and quantitative, and it minimizes loss of nucleotides. Following deacidification, all samples were stored at -20°C.

Fractionation of Nucleotides on DEAE-cellulose—The DEAE-cellulose used (DE II, Whatman) was prepared according to Peterson and Soher (14). Prior to use, the cellulose was con-

FIG. 1. Fractionation of 2-hour acid-soluble nucleotides. The deacidified extract was made 0.002 M with respect to NH₄HCO₃, pH 8.6, and applied to DEAE-cellulose (HCO₃⁻ form) column, 1 × 50 cm. The column was washed with 0.002 M NH₄HCO₃, pH 8.6, and the nucleotides were eluted with a linear gradient of NH₄HCO₃, pH 8.6, as indicated. Recovery of ultraviolet-absorbing material was always greater than 95%.

RESULTS

Metabolism of Purine Nucleotides—The analysis of all DEAE-cellulose fractions is summarized in Table I. Although six guanine-containing compounds are found in large amounts at all developmental stages studied, no free guanine could be found. It is noteworthy that in the dormant cyst approximately 90% of all nucleotides contain guanine (50% of which is diguanosine tetraphosphate), whereas virtually no ATP is present (less than 1% of the total nucleotides). However, resumption of embryonic development by immersion in seawater is characterized by a marked synthesis of ATP.

Changes in the diguanosine nucleotides during development are more clearly represented in Fig. 2. Although the level of diguanosine triphosphate does not change appreciably throughout development, the amount of diguanosine tetraphosphate declines markedly. Preemergence development (0 to 5 hours) is characterized by a 14% drop in diguanosine tetraphosphate. During emergence (5 to 11 hours), however, there is no significant change in the diguanosine tetraphosphate level, whereas hatching (11.5 to 36 hours) is characterized by a rapid decrease in the amount of this nucleotide.

The metabolism of the other guanine-containing compounds is represented graphically in Fig. 3. Whereas guanosine and GTP levels increase appreciably prior to emergence, GMP and GDP levels fall noticeably. During emergence and hatching, however, guanosine, GMP, GDP, and GTP all decline at the same rate.

The rapid synthesis of ATP during early development is shown in Fig. 4. Prior to emergence, there is a 5-fold increase in ATP and a smaller concomitant decrease in AMP and ADP. During emergence there is little change in all adenosine nucleotides, but, during hatching, ATP production resumes once again along with a slight increase in ADP and decrease in AMP levels.

Metabolism of Pyrimidine Nucleotides—As Table I shows, Artemia cysts contain a very small pool of pyrimidine nucleotides (about 5% of the total) as compared to purine nucleotides. Although the first few hours of development show a decline in
### Table I

**Guanosine and nucleotide concentrations at various times of development**

The analyses were performed as described in the text. Concentrations are given in micromoles per 200,000 embryos.

<table>
<thead>
<tr>
<th>Compound</th>
<th>0 hr</th>
<th>2 hrs</th>
<th>5.5 hrs</th>
<th>9 hrs</th>
<th>12 hrs</th>
<th>22 hrs</th>
<th>27 hrs</th>
<th>36 hrs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Guanosine</td>
<td>4.30 ± 0.50</td>
<td>5.32 ± 0.36</td>
<td>5.78 ± 0.26</td>
<td>5.50 ± 0.43</td>
<td>5.30 ± 0.28</td>
<td>4.78 ± 0.03</td>
<td>4.72 ± 0.25</td>
<td>4.45 ± 0.45</td>
</tr>
<tr>
<td>CMP</td>
<td>0.42 ± 0.05</td>
<td>0.25 ± 0.05</td>
<td>0.25 ± 0.03</td>
<td>0.23 ± 0.03</td>
<td>0.15 ± 0.03</td>
<td>0.32 ± 0.01</td>
<td>0.34 ± 0.00</td>
<td>0.26 ± 0.03</td>
</tr>
<tr>
<td>AMP</td>
<td>0.76 ± 0.38</td>
<td>0.32 ± 0.29</td>
<td>0.29 ± 0.26</td>
<td>0.26 ± 0.23</td>
<td>0.19 ± 0.15</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UMP</td>
<td>0.51 ± 0.30</td>
<td>0.22 ± 0.27</td>
<td>0.22 ± 0.22</td>
<td>0.30 ± 0.33</td>
<td>0.56</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UDP-X</td>
<td>0.86 ± 0.84</td>
<td>0.91 ± 1.01</td>
<td>1.21 ± 1.43</td>
<td>1.52 ± 1.70</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GMP</td>
<td>4.14 ± 0.17</td>
<td>2.00 ± 0.25</td>
<td>1.79 ± 0.34</td>
<td>1.64 ± 0.26</td>
<td>1.45 ± 0.30</td>
<td>1.12 ± 0.22</td>
<td>1.15 ± 0.19</td>
<td></td>
</tr>
<tr>
<td>ADP</td>
<td>1.00 ± 0.05</td>
<td>0.42 ± 0.10</td>
<td>0.43 ± 0.10</td>
<td>0.51 ± 0.08</td>
<td>0.49 ± 0.10</td>
<td>0.60 ± 0.19</td>
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<td></td>
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<tr>
<td>GDP</td>
<td>3.50 ± 0.08</td>
<td>2.54 ± 0.18</td>
<td>2.39 ± 0.13</td>
<td>2.01 ± 0.14</td>
<td>1.54 ± 0.21</td>
<td>1.22 ± 0.23</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATP</td>
<td>0.41 ± 0.14</td>
<td>2.50 ± 0.17</td>
<td>2.21 ± 0.16</td>
<td>2.81 ± 0.06</td>
<td>2.93 ± 0.19</td>
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<tr>
<td>GTP</td>
<td>3.57 ± 0.09</td>
<td>4.22 ± 0.42</td>
<td>3.91 ± 0.32</td>
<td>3.79 ± 0.14</td>
<td>3.47 ± 0.29</td>
<td>3.23 ± 0.19</td>
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<tr>
<td>Diguanosine triphosphate</td>
<td>1.76 ± 0.06</td>
<td>1.74 ± 0.11</td>
<td>1.75 ± 0.09</td>
<td>1.69 ± 0.07</td>
<td>1.63 ± 0.23</td>
<td>1.51 ± 0.21</td>
<td>1.41 ± 0.13</td>
<td></td>
</tr>
<tr>
<td>Diguanosine tetraphosphate</td>
<td>16.95 ± 0.44</td>
<td>14.72 ± 0.43</td>
<td>13.80 ± 0.48</td>
<td>11.23 ± 0.84</td>
<td>9.88 ± 0.70</td>
<td>9.53 ± 0.40</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Totals</td>
<td>38.27</td>
<td>35.55</td>
<td>34.76</td>
<td>33.12</td>
<td>30.47</td>
<td>27.92</td>
<td>27.19</td>
<td></td>
</tr>
</tbody>
</table>

*Plus or minus standard deviation of the mean with n-1 degrees of freedom.*

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**Fig. 2.** Amounts of the diguanosine nucleotides at various stages of development. O--O, micromoles of diguanosine tetraphosphate; ●●●, micromoles of diguanosine triphosphate.

**Fig. 3.** Changes in guanosine and the guanosine nucleotides during development. O--O, micromoles of guanosine; ●●●, micromoles of GMP; □□□, micromoles of GDP; △△△, micromoles of GTP.

**Fig. 4.** Variations in adenosine nucleotides during development. O--O, micromoles of ATP; ●●●, micromoles of ADP; □□□, micromoles of AMP.

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CMP and UMP, the UDP-X fraction remains fairly constant prior to emergence. On the other hand, emergence and hatching show little change in CMP and UMP, whereas UDP-X increases significantly.

**Appearance of Ultraviolet-absorbing Material in Hatch Medium**

Shortly after immersion of washed cysts into seawater, large quantities of ultraviolet-absorbing material appear in the hatch medium (Fig. 5). Analysis of the ultraviolet-absorbing material from a 2-hour sample shows the presence of all Artenia nucleotides in amounts not too different from those in the dormant cysts. After emergence, ultraviolet-absorbing materials continue to appear in the medium, but at a reduced rate. It is presumed that the material arises by diffusion from cracked and nonviable cysts and is not actively excreted from viable cysts and developing embryos. In addition to the ultraviolet-absorbing material, P1 and P2 also appear in the hatch medium and the kinetics of their release is similar to that of the ultraviolet-absorbing compounds.

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1 A. H. Warner, unpublished observation.
A critical analysis of nucleotide metabolism during brine shrimp embryogenesis has been hampered by impermeability of the cyst to phosphate and to labeled compounds often employed in studying nucleic acid and nucleotide metabolism. Consequently, we have limited this study to variations in nucleotide concentrations during embryonic development. The presence of large quantities of diguanosine nucleotides in Artemia cysts suggests that these compounds may be the primary source of energy for chemical bond synthesis accompanying resumption of development in the encysted egg. The data in Table I show a decline of 2.2 μmoles of diguanosine tetraphosphate prior to emergence. Nucleotide analysis of the 5.5-hour hatch medium indicates that at least 1.5 μmoles of diguanosine tetraphosphate appear in the hatch medium prior to emergence. Although the decrease in diguanosine tetraphosphate in the developing embryo cannot be completely accounted for in the hatch medium, it is difficult to assess the importance of this apparent difference because of the presence of P1,P4-diguanosine 5'-tetraphosphate asymmetrical pyrophosphohydrolase (8) and phosphatases in the hatch medium. These degradative enzymes are also liberated into the hatch medium and continually attack those nucleotides appearing therein, thus making any data on nucleotide levels in the hatch medium difficult to interpret. Nevertheless, it is not unreasonable to suggest that very little, if any, diguanosine tetraphosphate is catabolized prior to hatching.

During hatching and subsequent development, however, diguanosine tetraphosphate declines rapidly. Based on one of our earlier observations (8), utilization of diguanosine tetraphosphate as a source of energy by the action of P1,P4-diguanosine 5'-tetraphosphate asymmetrical pyrophosphohydrolase should result in a concomitant increase in GMP and GDP, whereas diguanosine tetraphosphate is catabolized prior to hatching.

Prior to emergence, both GMP and GDP fall markedly, whereas guanosine and GTP increase concomitantly. The level of diguanosine triphosphate does not change appreciably, an observation not inconsistent with the above model. Such a mechanism might be useful in providing the necessary amount of GTP for early development, whereas diguanosine tetraphosphate might serve in an entirely different capacity (10).

Experiments in vitro are now in progress to test further the above model.

Another nucleotide of presumed importance to the developing brine shrimp is ATP. However, unlike most other tissues, encysted eggs of the brine shrimp contain very little ATP. Nevertheless, resumption of development by immersion of the cysts in seawater is accompanied by a rapid increase in ATP (apparently not entirely at the expense of pre-existing AMP and ADP), an increase which suggests synthesis de novo or conversion from another purine nucleotide. Although ATP production levels off during emergence, it resumes subsequent to hatching. Recent evidence suggests a conversion of guanosine nucleotides, possibly diguanosine tetraphosphate, to adenosine nucleotide (or nucleotides) during postembryonic development of Artemia (10). Such a mechanism may also function prior to hatching of Artemia. Although it has not been fully elucidated, this biochemical mechanism is under further investigation by one of us.4

Finally, if one considers the total nucleotide population at each developmental stage analyzed, along with nucleotide material liberated into the hatch medium, there appears to be a gradual increase in the nucleotide content up to hatching and then a decrease throughout the remainder of embryonic development. The decrease in acid-soluble nucleotides is probably due in part to the onset of RNA and DNA synthesis after hatching (19).

Although the experimental data presented herein suggest several possible metabolic roles for the diguanosine nucleotides during brine shrimp development, it still remains to be shown whether these compounds are associated with encystment. Experiments are now in progress with the use of thin shelled ovarian eggs (summer eggs) that give rise to living nauplii ovoviviparously, and it is possible that these studies will more...
fully elucidate the role or roles of the diguanosine nucleotides in the encystment process.

Acknowledgments—We wish to express our thanks to Mr. J. X. Khym for devising the deacidification method. Also, we extend our appreciation to Dr. Leslie R. Sabina for a critical reading of the manuscript and to Mr. Daniel K. McClean for his invaluable technical assistance.

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