

Purine-mediated phosphate influence on *Artemia* morphogenesis.

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Summary. Inorganic phosphate as well as organic phosphate in the form of glycerophosphate or pyrimidine nucleotides can be used by *Artemia* larvae to provide dietary purine bases and nucleosides with the phosphate required for their efficient utilization. Earlier results showed that purine nucleotides were efficiently metabolized without additional phosphate. The purine salvage pathway of *Artemia* is now established.

Basic nutritional research on *Artemia* purine requirement is especially interesting in developmental biology because larvae reared in purine-deficient cytidylic acid-rich nutritive media develop supernumerary gonopodes on the abdominal segments. The most efficient inducers of abdominal gonopodes are the guanine compounds (guanine, guanosine and guanylic acid) found to be least efficient to meet growth requirements. The relationship between growth rates and morphogenetic changes is not known. The specificity of a reduced purine:pyrimidine ratio in switching-on the expression of the genetic information coding for abdominal gonopodes is strengthened after showing that larvae reared in purine-deficient cytidylic acid-rich nutritive media did not develop abdominal gonopodes when cytidylic acid is used as a phosphate donor.

Introduction

As stressed by Gordon (1959), the biochemical peculiarities of an organism may make it ideal for research in a certain field ; experiments that would be difficult or impossible with most other organisms, may be easy with a special one. Contrary to most organisms, the Utah strain of *Artemia* (Crustacea : Anostraca) is incapable of synthesizing the purine ring de novo (Clegg *et al.*, 1967, Warner and McClean, 1968). This incapacity is expressed by an essential dietary purine requirement. Contrary to most eukaryotes, *Artemia* is equipped with a GMP-reductase (Renart and Sillero, 1974). The functioning of this enzyme could be inferred from the finding that guanylic acid, as well as adenylic acid or inosinic acid, is used by *Artemia* to satisfy its purine requirement (Hernandorena, 1972). In the same paper, I reported that the efficiency of purine bases and nucleosides was reduced as compared to that of nucleotides. It was not clear why nucleotides should be superior to bases or nucleosides since the former group of compounds

are not transported efficiently across the cell membranes and probably must be converted to nucleosides or bases prior to entering the cells. The present results resolve this paradox and the purine salvage pathway of *Artemia* is established after showing that additional phosphate is required for the efficient utilization of purine bases and nucleosides.

Artemia purine requirement is of special interest because larvae reared in purine-deficient cytidylic acid-rich nutritive media develop supernumerary gonopodes on the abdominal segments (Hernandorena, 1970, 1977, 1979). The discovery that cytidylic acid can be used by *Artemia* larvae as a phosphate donor to increase the dietary efficiency of purine bases and nucleosides serves to strengthen the idea of the specificity of a reduced purine:pyrimidine ratio in switching-on the expression of the genetic information coding for abdominal gonopodes.

Material and methods.

The Utah strain and the method developed by Provasoli and d'Agostino (1969) for its axenic culture were used. The purine and pyrimidine supplies in each experiment have been indicated in the results. The pyrimidine requirement was met by cytidylic acid (CMP), except in one experiment using uridylic acid (UMP) instead of CMP, and thymidine was always omitted. The sources and qualities of the purines and pyrimidines used are presented in detail in table 1. The molecular weights have been included in order to compare the molar ratios. The concentrations are expressed in mg per 100 ml of nutritive media (mg %).

Culture conditions and analytical procedures are identical to those previously reported (Hernandorena, 1979). Twenty-five to one hundred animals were used for each experimental condition. The growth rate was expressed as a numerical index, defined by Provasoli and d'Agostino (1969), and very nearly approximates the number of moults. The growth index (GI) is given for day 14. Morphogenesis was checked by microscopical examination of anesthetized animals.

Results

Figure 1 illustrates the difference in the ability of adenosine-3'-(2') phosphoric acid (AMP) and adenosine (AR) to satisfy the purine requirement of larvae reared in nutritive media containing a 20 mg % CMP concentration.

As seen in figure 2, growth rates increased with an AR supply increasing from 20 to 40 mg % only when the CMP concentration increased. When the CMP was replaced by UMP, the growth rates increased but to a lesser extent (fig. 3).

These results did not fit with my previous experiment on the interdependence of purine and pyrimidine requirements (Hernandorena, 1979). I suspected that the reduced efficiency of AR as compared to AMP might result from a reduced phosphate availability and that CMP or UMP were serving as phosphate donors. In order to test this hypothesis, the glycerophosphosphate ($\text{Na}_2\text{-}\beta\text{-glycerophos-}$

TABLE 1
Sources and qualities of dietary purines and pyrimidines Calbiochem products are A grade.

	Abbreviation	Source	Quality	M.W
Adenosine - 3' - (2') phosphoric acid	AMP	Calbiochem	Free acid monohydrate	365.2
Adenosine	AR	NBCo		267.2
Adenine	A	NBCo		135.1
Guanosine - 3' - (2') phosphoric acid	GMP	Sigma	Free acid	363.2
Guanosine	GR	Calbiochem	Hemihydrate	292.2
Guanine	G	U.S.B. Co		151.1
Inosine 5' - monophosphate	IMP	Sigma	Free acid	348.2
Inosine	IR	NBCo		268
Hypoxanthine	HX	NBCo		136.1
Cytidine - 3' - (2') phosphoric acid	CMP	Calbiochem	Free acid	323.2
Uridine - 3' - (2') phosphoric acid	UMP	Calbiochem	Disodium salt monohydrate	386.2

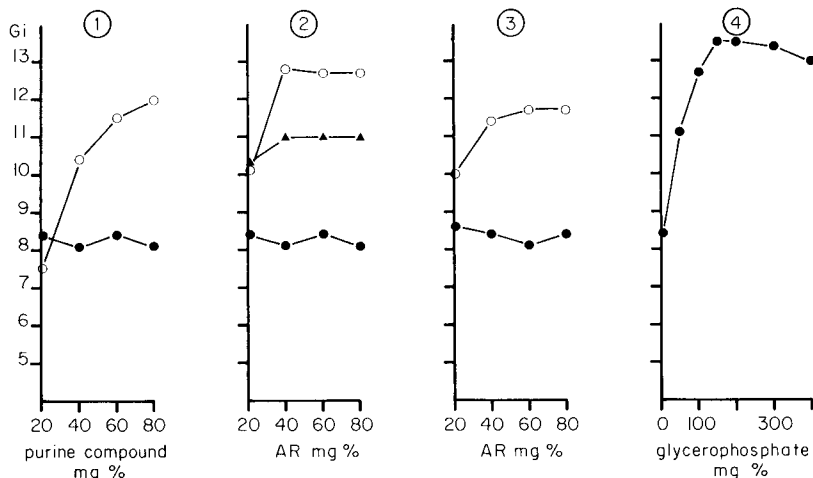


FIG. 1 (*). — Dietary efficiency of AMP ○—○ and AR ●—●. CMP = 20 mg %.

FIG. 2. — Effect of CMP on AR efficiency. CMP = 20 mg % ●—● ;
 CMP = 40 mg % ▲—▲ ; CMP = 100 mg % ○—○.

FIG. 3. — Effect of UMP on AR efficiency. UMP = 20 mg % ●—● ;
 UMP = 100 mg % ○—○.

FIG. 4. — Effect of glycerophosphate on AR efficiency. AR = 60 mg % ; CMP = 20 mg %.

phate pentahydrate) concentration of 50 mg % in the standard medium defined by Provasoli and d'Agostino (1969), was increased up to 400 mg %. As seen in figure 4, the growth rate of larvae reared in nutritive media containing 60 mg % AR and 20 mg % CMP increased when the glycerophosphate concentration rose up to 150 mg %. The ability of AR to satisfy the purine requirement thus depended on phosphate availability.

That inorganic phosphate could also be used by *Artemia* larvae to increase the efficiency of AR is shown in figures 5 and 6. Sodium phosphate mono-H and potassium phosphate di-H could serve as phosphate donors. By increasing the salt concentration above those indicated, precipitation occurred and larval growth was impaired. Consequently, glycerophosphate was retained for further experiments. A standard glycerophosphate concentration of 50 mg % was always present in the nutritive media.

The efficiency of adenine (A) (fig. 7) was significantly increased by glycerophosphate, by that of guanine (G) was not (fig. 8).

The dietary efficiencies of the purine bases (fig. 9), nucleosides (fig. 10) and nucleotides (fig. 11) were compared in larvae grown under identical conditions *i.e.* with CMP constant at 20 mg % and with a constant additional glycerophosphate

(*) In all figures, the ordinate is the growth index (GI) for the 14th day of development. The abscissa is the concentration of the tested compounds expressed in mg for 100 ml of nutritive media.

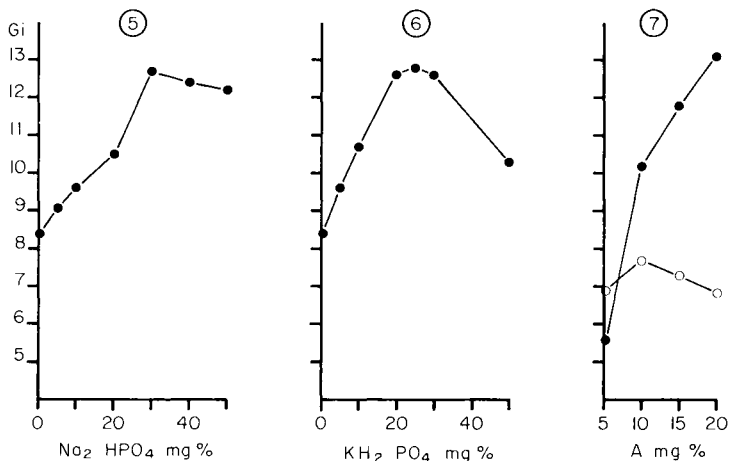


FIG. 5. — Effect of sodium phosphate on AR efficiency. AR = 60 mg % ; CMP = 20 mg %.

FIG. 6. — Effect of potassium phosphate on AR efficiency. AR = 60 mg % ; CMP = 20 mg %.

FIG. 7. — Effect of glycerophosphate on A efficiency. CMP = 20 mg %. No addition ○—○ ; glycerophosphate = 200 mg % ●—●.

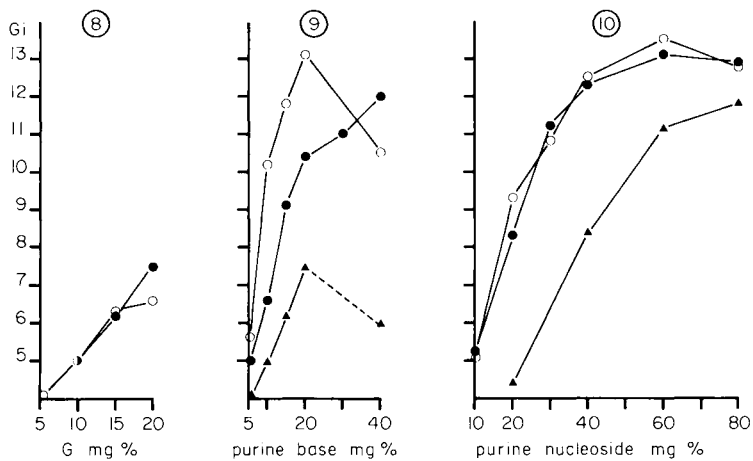


FIG. 8. — Effect of glycerophosphate on G efficiency. CMP = 20 mg %. No addition ○—○ ; glycerophosphate = 200 mg % ●—●.

FIG. 9. — Dietary efficiency of purine bases. CMP = 20 mg % ; glycerophosphate = 200 mg % ; A ○—○ ; HX ●—● ; G ▲—▲.

FIG. 10. — Dietary efficiency of purine nucleosides. CMP = 20 mg % ; glycerophosphate = 200 mg % ; AR ○—○ ; IR ●—● ; GR ▲—▲.

supply (200 mg %). On a molecular weight basis, A was the most efficient purine compound and G the least efficient. If the G concentration was increased to more than 20 mg % precipitation ensued and larval growth was impaired. Guanosine (GR) was less efficient than AR or inosine (IR), GMP was slightly less efficient than AMP or inosine 5'-monophosphate (IMP), but more efficient than GR. Differences among efficiencies are more important for bases than for nucleotides.

Besides growth rate, the induction of abdominal gonopodes by reduced purine:pyrimidine ratios was also used to measure the dietary purine efficiency. As seen in table 2, the most efficient inducers of abdominal gonopodes were purine compounds which were found to be the least efficient in meeting growth requirements.

When the purine requirement of *Artemia* is met by AMP, the quantitative CMP requirement for optimal growth rate increases with increasing the AMP

Table 2

Dietary efficiency of purine compounds measured by the percentage of animals bearing abdominal gonopodes.

CMP = 20 mg % glycerophosphate 200 mg %	Bases 10 mg %			Nucleosides 20 mg %			Nucleotides 20 mg %		
	G	HX	A	GR	IR	AR	GMP	IMP	AMP
% of animals bearing abdominal gonopodes	80	57	6	100	16	20	62	33	54

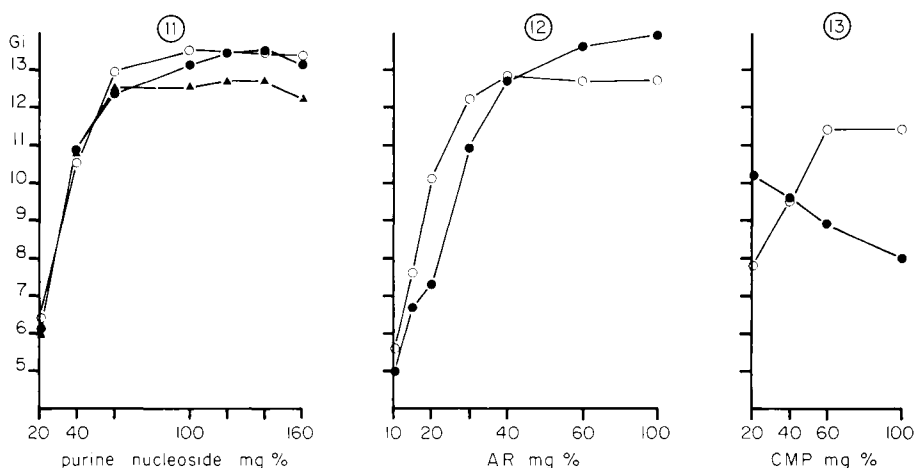


FIG. 11. — *Dietary efficiency of purine nucleotides.* CMP = 20 mg % ; glycerophosphate = 200 mg % ; AMP —○—○ ; IMP —●—● ; GMP —▲—▲.

FIG. 12. — *Effect of glycerophosphate in relation to AR concentration in CMP-rich media.* CMP = 100 mg %. No addition —○—○ ; glycerophosphate = 200 mg % —●—●.

FIG. 13. — *Effect of glycerophosphate in relation to the CMP concentration in A-deficient media.* A = 10 mg %. No addition —○—○ ; glycerophosphate = 200 mg % —●—●.

supply (Hernandorena, 1979). When the purine requirement is met by A or by AR instead of by AMP and the pyrimidine requirement by CMP, the metabolic fate of CMP must depend on the phosphate concentration of the diet. With no extra phosphate the growth rates of larvae reared in purine-rich media would be limited if part of the CMP supply was used to provide the base or the nucleoside with phosphate. With an extra phosphate source, growth should not be limited ; on the contrary, the growth rates of larvae reared in purine-deficient CMP-rich media would be limited by an extra phosphate source. Our results (fig. 12) demonstrate this in larvae reared with CMP constant at 100 mg % and increasing AR concentrations with or without additional glycerophosphate. The growth rate was limited by glycerophosphate supplementation when the larvae were reared in nutritive media containing 10 to 40 mg % AR and increased in nutritive media containing more than 40 mg % AR. With an AR concentration equal to or less than 15 mg %, abdominal gonopodes were induced with or without extra phosphate. With AR concentration equal to or higher than 30 mg %, no abdominal gonopodes were induced. With a 20 mg % AR concentration, abdominal gonopodes were induced if CMP was not used as a phosphate donor, that is if CMP was concurrently supplied with glycerophosphate. With no extra phosphate supply, morphogenesis was normal (table 3).

The same conclusion was true when the purine requirement was met by A. When larvae were reared in adenine-deficient media (A = 10 mg %) with no extra phosphate, the growth rate increased with increasing the CMP supply ; CMP served as a phosphate donor, and morphogenesis was normal. If CMP was concurrently supplied with an extra phosphate source, the growth rates of larvae reared in nutritive media containing excess CMP decreased (fig. 13). The percentage of larvae bearing abdominal gonopodes increased when the CMP supply was augmented (table 4).

Table 3

Influence of glycerophosphate on the percentage of animals bearing abdominal gonopodes. CMP constant at 100 mg %.

glycerophosphate	AR mg %			
	10	15	20	30
no addition	78	46	0	0
200 mg %	75	43	22	0

Table 4

Influence of glycerophosphate on the percentage of animals bearing abdominal gonopodes. Adenine constant at 10 mg %

glycerophosphate	CMP mg %			
	20	40	60	100
no addition	0	0	0	0
200 mg %	6	7	50	67

Discussion

Little is known about the processes which control the anamorphic pattern of development of the *Anostraca* and, as stressed by Fryer (1983), dietary studies in *Artemia* can provide clues to understand how this pattern is established and controlled. Developmental and evolutionary considerations have been developed recently (Hernandorena, 1985).

The present results show that purine bases (except guanine) and purine nucleosides can satisfy the purine requirement of *Artemia*, if concurrently supplied with additional phosphate. Inorganic phosphate as well as organic phosphate in the form of glycerophosphate, CMP or UMP may be used as phosphate donors; they supply the bases and the nucleosides with the phosphate required for their efficient utilization. Identical growth rates (≈ 12.8) are achieved by larvae reared in nutritive media containing 60 mg % adenosine and either 100 mg % CMP, 20 mg % CMP plus 100 mg % glycerophosphate, 20 mg % CMP plus 25 mg % KH_2PO_4 , or 20 mg % CMP plus 30 mg % $\text{Na}_2\text{HPO}_4 \cdot 12 \text{H}_2\text{O}$. Inorganic phosphate is thus more efficiently utilized than organic phosphate. But higher growth rates (≈ 13.5) can be achieved with a 60 mg % adenosine concentration if the phosphate supply is increased using glycerophosphate or CMP but not when using inorganic salts due to precipitation.

It was previously shown that inorganic phosphate is directly taken up and incorporated into acid-soluble organic phosphorous compounds by *Artemia* adults (California strain) reared with undefined food (Kobayashi *et al.*, 1972). The present results show that organic phosphate is also used. McLaughlin and Zahl (1959) demonstrated that nucleotides can serve as phosphate donors in marine algae. Alkaline phosphatases firmly bound to the algal cell surface split AMP and glycerophosphate at the same rate as glucose-6-phosphate, indicating that a similar uptake mechanism operates for all three compounds (Zuengler and Perras, 1965). Alkaline phosphatases found in *Artemia* guts (Bianchi and Pirodda, 1968) could play such a role.

Bases are salvaged via ribosyltransferases. Since adenine is more efficient than hypoxanthine, the activity of adenine phosphoribosyltransferase is possibly higher than that of hypoxanthineguanine phosphoribosyltransferase (HGPRT). The reduced efficiency of guanine as compared to hypoxanthine cannot be explained by a reduced HGPRT activity since the same enzyme is used to salvage both guanine and hypoxanthine. It cannot be explained either by a reduced GMP-reductase activity since guanosine and guanylic acid are more efficiently metabolized than guanine. It is possible that in *Artemia* as in *Drosophila* the major fate of guanine is the catabolic pathway; the low solubility of guanine may also affect its uptake (Johnson *et al.*, 1980).

Adenosine and inosine are respectively equally efficient and more efficient than guanosine. In view of our results on the ammonia excretion rates of *Artemia* (Hernandorena and Kaushik, 1983), adenosine may first be deaminated to inosine.

The fact that inosine 5'-monophosphate can satisfy the purine requirement as well as adenosine 2'-3'-monophosphate (mixed isomers) suggests that nucleotides are converted to nucleosides by non-specific phosphatase, regardless of the position of the phosphate (2', 3', 5'), rather than by specific nucleotidases.

The purine compounds found to be least efficient in meeting growth requirements are the most efficient inducers of abdominal gonopodes. These are guanine among the bases, guanosine among the nucleosides and guanylic acid among the nucleotides ; differences in molecular weight (table 1) cannot explain the observed discrepancies (table 2). The relationship between growth rate and morphogenetic change is not known. Supernumerary gonopodes were induced for the first time on *Artemia* larvae reared in an adenylic acid-deficient medium containing a standard pyrimidine supply. The adenylic acid deficiency reduced growth and survival rates (Hernandorena, 1970). Pyrimidine deletion restored normal morphogenesis in larvae reared in an adenylic acid-deficient medium. Pyrimidine deletion had no effect on growth rate but increased survival rate (Hernandorena, 1977). It was further shown that excess cytidylic acid increased the adenylic acid concentration threshold inducing abdominal gonopodes (Hernandorena, 1979). It became evident that a purine:pyrimidine imbalance was involved. The finding that cytidylic acid can be used by *Artemia* larvae to increase the dietary efficiency of purine bases and nucleosides served to strengthen the idea of the specificity of the purine:pyrimidine imbalance. If part of the cytidylic acid supply is used to provide adenine or adenosine with phosphate, abdominal gonopodes are not induced on larvae reared in a purine-deficient cytidylic acid-rich medium. If an identical cytidylic acid supply is fed concurrently with an extra phosphate source, abdominal gonopodes are induced. It appears that when larvae are reared in a glycerophosphate-deficient medium, cytidylic acid is deviated from its « normal » metabolic fate and utilized as a phosphate donor. This « normal » metabolic fate could be the production of deoxyuridylate, the substrate of thymidylate synthetase, because pyrimidinedeoxyribonucleosides have been found to be the most efficient inducers of abdominal gonopodes when given in excess in relation to the purine supply (Hernandorena and Kaushik 1985).

The uniqueness of *Artemia*, in its incapacity to synthesize the purine ring *de novo* makes it possible to divorce purine biosynthesis from thymidylate biosynthesis. This cannot be done in most organisms, including insects, because folic acid is involved in both purine and thymidylate biosynthesis. Among insects, a requirement for nucleic acids or components thereof is virtually restricted to Diptera in which all degrees of needs from nonrequirement to absolute essentiality, is documented. Requirements are usually satisfied by nucleotide mixtures (Dadd, 1977). Apart from growth inhibitory effects, morphogenetic effects due to purine : pyrimidine imbalances have not been reported. The use of *Artemia* in basic nutritional research on the purine requirement offers many advantages and a special point of interest in developmental biology.

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Résumé. *Implication du phosphate sur l'effet morphogénétique des purines chez Artemia (Crustacé : Anostraca).*

Contrairement aux nucléotides, les bases et les nucléosides puriques ne sont efficacement métabolisées par les larves d'*Artemia* que si le milieu nutritif est enrichi en phosphate. Le phosphate inorganique et le phosphate organique sous forme de glycérophosphate ou de nucléotides pyrimidiques sont utilisés. L'étude du besoin alimentaire d'*Artemia* en dérivés puriques présente un intérêt particulier dans le domaine de la biologie du développement, car les larves élevées dans des milieux nutritifs déficients en purine et riches en acide cytidylique développent des appendices génitaux surnuméraires sur les segments abdominaux normalement apodes. Les meilleurs inducteurs d'appendices abdominaux, la guanine, la guanosine et l'acide guanylique sont les dérivés puriques les moins aptes à satisfaire les besoins de la croissance. La relation entre le taux de croissance et l'effet morphogénétique reste à déterminer. La spécificité du rapport purine : pyrimidine dans le déclenchement de l'expression de l'information génétique qui code la morphogenèse des appendices abdominaux surnuméraires est mise en évidence par la non-induction de l'effet morphogénétique chez des larves élevées dans des milieux nutritifs déficients en purine et riches en pyrimidine si l'acide cytidylique est utilisé comme donneur de phosphate.

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