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Original article

Development of a new simplified nutritive medium for the axenic culture of *Artemia*

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Summary — A new artificial nutritive medium has been developed for the axenic culture of *Artemia* enabling the production of adults in < 1 wk. The techniques for its preparation have been detailed. Its utilization is recommended for the standardized production of experimental animals.

***Artemia* / axenic culture / nutrition / development / purine**

Résumé — Mise au point d'un nouveau milieu nutritif synthétique pour l'élevage axénique d'*Artemia*. *Un nouveau milieu nutritif synthétique permettant la production d'adultes en moins d'une semaine a été mis au point pour l'élevage axénique d'*Artemia. *Son mode de préparation est détaillé. L'utilisation de ce milieu est recommandée pour la production standardisée d'animaux expérimentaux.*

Artemia / *élevage axénique* / *nutrition* / *développement* / *purine*

INTRODUCTION

A remarkable feature of *Artemia* nutrition, determined with the Utah strain larvae reared axenically using the artifical medium "100" as defined by Provasoli and d'Agostino (1969), is the essentiality of its purine requirement (Hernandorena 1972, 1974, 1977, 1979, 1985, 1987). This unusual requirement is the result of an incapacity to synthesize the purine ring *de novo* (Clegg *et al*, 1967; Warner and McClean, 1968). The study of *Artemia* dietary purine requirement is of special interest in developmental biology because, as shown for the first time 20 yr ago (Hernandorena, 1970), larvae reared in purine-deficient nutritive media develop into adults with supernumerary appendages on otherwise apodous abdominal segments. Recognition of the genital character of the supernumerary abdominal appendages required serial aseptic transfers of growing larvae to new media (Hernandorena, 1972). A major step towards the understanding of the developmental strategy of *Artemia* was made with the definition of a new simplified and more efficient artifical nutritive medium described in this paper.

MATERIAL AND METHODS

Animals

Cysts from the Utah strain were axenized according to the technique described by Provasoli and Shiraishi (1959). Disinfected cysts were incubated in screw-cap tubes containing 10 ml of sterile sea water. Nauplii, hatched 24 h after cyst hydration, were transferred in groups of 5 into each screw-cap culture tube containing 10 ml of the autoclaved nutritive medium. Transfers were performed with sterile Pasteur pipettes using a transfer hood equipped with a germicidal lamp as *per* Provasoli *et al* (1959). Twenty to 100 larvae were reared for each experimental condition. Their growth-rate was measured by the numerical growth index defined by Provasoli and d'Agostino (1969) and by the size of adult individuals anesthesized with chloroform. Hatching and culturing processes took place at 25 ± 0.5 °C with a 10 l–14 D photoperiod, except for 1 experiment performed at 30 ± 0.5 °C.

Media composition

The original medium "100" developed by Provasoli and d'Agostino (1969) was composed of a liquid phase containing 17 mineral salts, organic phosphate, 5 nucleic acid components, 8 vitamins, 6 amino acids, 2 sugars, a pH buffer and a fine particulate phase consisting of albumin, starch and cholesterol.

Simplification of the liquid phase was achieved with medium A by replacing mineral salts with aged sea water and omitting amino acids, sugars and organic phosphate. The nucleic acid components were replaced by RNA. The other ingredients were similar to those of medium "100".

The vitamin mixture included in medium "100" as in medium A contained putrescine which was omitted from media B and C.

The preparation of albumin particles included in medium "100" and medium A involved several time-consuming steps. An attempt was made to replace albumin by casein particles. Advantage was taken of the process of autoclaving to coagulate precipitated casein enabling the production of fine protein particles which sedimented less heavily than albumin particles. The quality of the casein particles formed during autoclaving depended on the pH of the medium. The glycylglycine concentration of medium "100" was not sufficient to prevent pH changes during autoclaving. The pH drop was reduced when the pH before autoclaving was adjusted to pH 7.6 and when the glycylglycine concentration increased to 200 mg per 100 ml of media.

In medium "100", Utah larvae are known to grow to adults with cholesterol as the only lipid source. Our own trials showed that in media containing casein, larvae grew and survived only when lecithin was supplied in addition to cholesterol (medium B). The beneficial effect of lecithin on larvae fed casein was subordinate to the supplementation of an antioxidant as lecithin is very prone to oxidation. The most critical component of medium B was found to be lecithin as contrary to other components, it had a sharp optimal zone (2 mg per 100 ml media). In medium C, lecithin was replaced by egg yolk which showed a wider optimal zone.

As liver extracts are considered to contain essential factors for oogenesis (Provasoli and d'Agostino, 1969), these were also included in medium C.

Preparation of media

Medium "100" and medium A were prepared according to the techniques described by Provasoli and Shiraishi (1959) and Provasoli and d'Agostino (1969).

Lecithin included in medium B was prepared as follows: 200 mg egg lecithin (Sigma ref P-8640) and 100 mg of butylated hydroxytoluene (BHT) (Sigma ref B-1378) were dissolved in 10 ml ethanol; this stock solution was kept at –10 °C in 1-ml aliquots. One ml of the alcoholic solution was slowly introduced into a tube containing 10 ml of hot distilled water. A fine precipitate was formed. The alcohol was evaporated by keeping the tube in a bath of boiling water. Ten ml of the suspension contained the amounts of lecithin and BHT required to prepare 1 000 ml of medium B.

The preparation of medium C is detailed below. The nutrients are listed in the order in which they were included under constant stirring to

prepare 1 000 ml of media. They were supplied as particles and solutes added to sea water in the proportion of 800 ml sea water per 1 000 ml of medium. With the stock solutions at hand, the preparation of the medium was completed in 1 h.

Casein

Casein soluble in alkali Merck ref 2241 was utilized. Eight g casein was suspended in 200 ml sea water. Twenty ml NaOH 1 N and 40 ml HCl 1 N were added; the casein precipitated. The solution was stirred constantly for 30 min before adding RNA.

RNA

Torula yeast RNA Sigma ref 6625 was utilized. Four g RNA was dissolved in 15 ml NaOH and added to the medium.

Glycylglycine

Glycylglycine-free base, Sigma ref G-1002, was utilized. Two g glycylglycine was dissolved in 300 ml sea water and added to the medium.

Starch

Insoluble rice starch, BDH ref 30263, was utilized. Three g starch was suspended in 100 ml sea water and brought to the boil, then allowed to cool. This was added to the medium.

Cholesterol

Cholesterol A grade, Calbiochem ref 2281, was utilized. 250 mg cholesterol was dissolved in 25 ml 95% ethanol. This stock solution was kept at room temperature. Two ml of the alcoholic solution was slowly introduced into a tube containing 10 ml hot distilled water. A fine precipitate was formed. The alcohol was evaporated by keeping the tube in a bath of boiling water and then added to the medium.

BHT

Butylated hydroxytoluene, Sigma ref B-1378, was utilized. 100 mg BHT was dissolved in 10 ml 95% ethanol. This stock solution was kept at room temperature and renewed twice monthly. One ml of the alcoholic solution was slowly introduced into a tube containing 10 ml hot distilled water. A fine precipitate formed. The alcohol was evaporated and added to the medium.

Egg yolk

Dried chicken egg yolk, Sigma ref E-0625, was utilized. 500 mg egg yolk was suspended in 100 ml sea water and added to the medium.

Liver

Powdered liver extract, Sigma ref 202-1, was utilized. 1.5 g was dissolved in 100 ml sea water and added to the medium.

Vitamins

The vitamins mixture was prepared from stock solutions of individual vitamins stored in the refrigerator. Ten ml of the vitamin mixture contained the amounts of vitamins required to prepare 1 000 ml of medium (table I). It was added to the medium, which was then complete.

pH was adjusted to 7.6 ± 0.05 and the vol to 1 000 ml with distilled water. Ten-ml aliquots were put into 100 screw-cap tubes and autoclaved at 120 °C for 20 min. They were homogenized after autoclaving using a Bioblock "maximix".

RESULTS

The development of the final medium C was a stepwise process requiring numerous experiments. The main steps towards simplification of the original medium "100" resulted in the formulation of media A and B (table II). The growth rates achieved by

Table I. Composition of the vitamin mixture included in nutritive media.

	Medium "100" Medium A	*Medium B Medium C*
Thiamine HCl	2.4	1.2
Riboflavin	0.6	0.3
Nicotinic acid	4.8	2.4
Ca pantothenate	8	4
Pyridoxine HCl	0.2	0.1
Biotin	0.12	0.06
Folic acid	1.4	0.7
Putrescine	0.4	

Concentrations expressed in mg per 100 ml of media.

larvae reared in the different nutritive media were significantly improved as compared to those of larvae reared in medium "100" (fig 1). The survival rates to adulthood of larvae reared in medium "100", A, B or C approximated 100%. The sizes achieved by adult individuals reared in the different media are given in table III. When reared in medium C, the larvae developed into adults in 6 d at 30 °C and in 8 d at 25 °C. The females were always longer than the males, but their size varied more than that of the males. Reproducing adults were obtained in 9 d at 25 °C. The males started grasping females which showed the first sign of oogenesis by d 9. They produced their first offspring at d 16.

DISCUSSION

The basic knowledge accumulated by the pioneer work of Provasoli *et al* on the nutritional requirements of *Artemia*, and my own experience in the axenic culture of this crustacean served in designing simplified and more efficient nutritive media, which are recommended whenever the production of experimental animals under standardized and reproducible conditions is required.

The production of *Artemia* adults using medium "100" requires 23 d with 2 aseptic transfers of animals to new media (Prova-

Table II. Composition of nutritive media.

Medium A		*Medium B*		*Medium C*	
Egg albumin	20	Casein	400	Casein	800
Starch	100	Starch	300	Starch	300
Cholesterol	0.8	Cholesterol	2	Cholesterol	2
RNA	200	RNA	400	RNA	400
		Lecithin	2	Egg yolk	50
		BHT	1	BHT	1
				Liver	150
8 vitamins in glycylglycine	2 ml * 100	7 vitamins in glycylglycine	1 ml * 200	7 vitamins in glycylglycine	1 ml * 200
Sea water	80 ml	Sea water	80 ml	Sea water	80 ml
Water to pH 7.4	100 ml	Water to pH 7.6	100 ml	Water to pH 7.6	100 ml

* See table I. Concentrations expressed in mg per 100 ml of media.

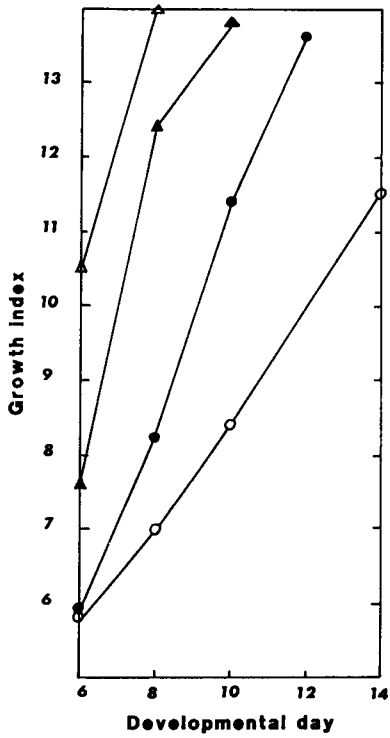

Fig 1. Growth rates of *Artemia* reared in medium C, Δ—Δ; medium B, ▲—▲; medium A, ●—●; medium 100, O—O. Ordinate: growth index; abscissa: developmental day.

soli and d'Agostino, 1969). The nutrients included in medium "100" support growth up to 14 d. By then, the medium is exhausted and the animals do not grow unless transferred to a new medium (Samain *et al,* 1985). Transfer increases the risk of bacterial contamination. The production of *Artemia* adults using medium C requires 6 d at 30 °C and 8 d at 25 °C and no transfer, the aseptic work being limited to the initial step of the culture. The utilization of the new artificial medium reduces the technical difficulty involved in handling animals under aseptic conditions.

The attainment of high growth rates for these filter-feeders depends not only on the quantity but also on the quality of the particulate nutrients. Provasoli and d'Agostino (1969) stated that: "casein, which is well balanced for all insects so far investigated, is ineffective or toxic for *Artemia*; several samples were tried with similar results." Their negative results were obtained with powdered or heat-sterilized casein. The present results show that casein, when properly precipitated before autoclaving, is an efficient source of amino acids, provided lecithin or egg yolk is also supplied. D'Abramo *et al* (1985) found that the requirement of juvenile lobsters for lecithin depended on the quality of the protein source in the diet. Results with *Artemia* confirmed this. The beneficial effect of egg yolk on larvae fed casein was subordinate to the supplementation of an antioxidant. This point illustrates a major problem in devising efficient nutritive media which preserve the quality of the nutrients during the incubation period.

The new medium included Torula yeast RNA to satisfy the purine requirement of *Artemia.* Larval growth and survival rates increase with the increasing dietary RNA concentration (Hernandorena, 1990a). In order to grow normally, *Artemia* larvae require an ample dietary RNA supply. When reared in RNA-deficient media, they develop into abnormal adults. The induced developmental anomalies are strain and salinity-dependent and correspond to homeotic-like transformations of the genital and abdominal segments (Hernandorena, 1991). In spite of its crudeness, the new artificial nutritive medium can be used to study the developmental biology of *Artemia.*

The use of an artificial nutritive medium enables experimental designs to be carried out to study the effect of environmental conditions (temperature, salinity, nutrition)

Table III. Size (mm) of *Artemia* reared in different nutritive media.

Designation of media		100	A	B	C	
Rearing temperature		25°	25°	25°	25°	30°
Developmental day	6					♂6.9 ± 0.2 (7) ♀7.7 ± 0.3 (7)
	8				♂7.2 ± 0.2 (28) ♀7.9 ± 0.4 (22)	
	10			♂6.5 ± 0.2 (20) ♀7 ± 0.4 (24)		
	12		6.2 ± 0.4 (40)			
	14	5.3 ± 0.2 (20)				

No of animals measured in parentheses.

on *Artemia* development. Under xenic or even monoxenic culture conditions, environmental factors would alter the nutritional value of living food algae. To avoid the cumbersome task of growing food algae to rear *Artemia*, many attempts have been made to replace the algae by inexpensive foods suchs as Spirulina, Cerophyl (dehydrated cereal leaves), yeast, defatted rice bran, soybean, lactoserum (Douillet, 1987) and by mixed diets consisting of single cell protein yeast and micronized waste products (Lavens *et al*, 1987), as well as by Germalyne, a commercial dietetic nutrient (Verriopoulos *et al*, 1987). Data show that growth and survival rates are not only reduced but highly variable. As stressed by d'Agostino (1980), the success of these xenic cultures is strictly dependent on the instantaneous establishment of a microflora fortuitously harmless to *Artemia* and supplemental to possibly nutrient-deficient diets. Unless sufficient care is taken, xenic conditions often lead to heavy bacterial contamination, resulting in high mortality rates (Douillet, 1987). With nutrient-rich diets, the constraint of axenic culture conditions is unavoidable. The major benefits derived from such conditions appear to be high efficiency and reproducibility. The inclusion of natural sea water in the new artificial medium is of no importance as the provision of macro and micro nutrients is not limited. However, natural sea water can be replaced by artificial sea water. One of the major perspectives opened by the utilization of the new medium is that of the production of animals suitable for ecotoxicological tests.

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