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Aspartate transcarbamylase in *Artemia* during early stages of development[1]

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Abstract

Kinetic properties of aspartate transcarbamylase (ATC) in crude extracts of *Artemia* were studied. The enzymatic kinetic is linear with respect to time for at least 1 hr with crude extract from cysts or the earliest development stages, but not with crude extract from older stages. ATC activity was assayed during early stages of development of two *Artemia* strains ; a relation has been sought between ATC activities and growth rates.

Introduction

Aspartate transcarbamylase (ATC) catalyses the first specific step to *de novo* synthesis of pyrimidine bases. Studies on this enzyme have indicated that its activity is correlated with growth in plant and animal tissues. ATC is particularly active in tissues which are growing rapidly (Calva *et al.*, 1959 ; Nordmann *et al.*, 1964 ; Kim and Cohen, 1965 ; Stein and Cohen, 1965 ; Herzfeld and Knox, 1972 ; Waymire and Nishikawara, 1972 ; Weichsel *et al.*, 1972). Because of the apparent relationship between rate of cell growth or cell division and ATC activity it seemed interesting to investigate the possibility to use this enzyme activity as indicator of growth rate in *Artemia*. This paper presents data on the enzymatic properties of ATC in different *Artemia* strains and preliminary results on the variation of ATC activity during the first stages of development in *Artemia*.

Material and methods

Origin of material

Experiments were performed with *Artemia* strains obtained from Metaframe (San Francisco Bay Brand Division Newark, California, USA), CIRNE (CIA Industrial Do Rio Grande Do Norte, Macau, Brazil), World Ocean (Shark Bay, Australia), Ege University (Bornova Izmir, Turkey), Ministry of Agriculture and National Resources of Cyprus (Nicosia, Cyprus) and the Great Salt Lake (Utah, USA).

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At day 0, the cysts were incubated in filtered natural seawater at 25, 22, or 16 °C. The hatched larvae were harvested and separated from the hatchery debris. Approximately 200 000 nauplii were put into 20 l of filtered natural seawater. Every day the water was changed and food was added (*Tetraselmis suecica*, 2.10^5 cells/ml of *Artemia* culture medium).

Preparation of the enzyme extract

ATC activity was assayed on cysts or animals homogenized in a Potter glass homogenizer with H_2O or different buffers under freezing conditions. Homogenates were made just before the ATC assay. All the assays were performed on crude extracts.

Estimation of enzyme activity

The assay for ATC was based on the radiochemical method of Bresnick and Mosse (1966). The assay medium had the following composition : 0.2 ml homogenate, 9 μmoles of L. aspartate (brougth to pH 9.5), 6.3 μmoles of carbamyl phosphate (brought to pH 9.5), and 120 μmoles of Tris HCl buffer in a total volume of 0.5 ml. Incubation was performed at 35 °C and the exact pH was measured in the assay medium at the incubation temperature. The reaction was stopped by the addition of 0.1 ml 1 N HCl after which the assay medium was cooled. After centrifugation, 0.5 ml of the supernatant layer was passed through a column of Merck Lewadit H^+ resin (60-150 mesh ASTM) in a glass tube (6 mm internal diameter and 6 cm length). After collection of the eluate, the column was washed with 3.5 ml distilled H_2O. The eluate and washings were combined, and a 1.0 ml aliquot was transferred to counting vials containing 10 ml Packard picofluor. The samples were counted in an Intertechnique liquid scintillation spectrometer (Model SL 33). A blank was run for each experiment, consisting of the components mentioned above, except that the enzyme preparation was replaced by distilled H_2O.

Estimation of proteins

Proteins were determined on samples using the automatic method of Samain and Boucher (1974).

Results

Preparation of the enzyme

Different buffers were tested to prepare homogenates ; the results are presented in Table I. The most active homogenate was the one in which phosphate buffer pH 7, had been used ; the activity was, however, more stable with distilled H_2O when the homogenates were kept at 18 °C. The activity of homogenates prepared with 0.15 M KCl had the same intensity as that of the homogenates prepared with distilled H_2O but it was less stable. Homogenates with distilled H_2O were therefore chosen for the following experiments. Cysts were incubated in filtered seawater (Millipore 0.3 μm) containing streptomycine (50 mg/l), penicillin (50 000 UI/l) and chloramphenicol (8 mg/l) ; the latter precaution was taken to ensure that the activity measured was the ATC of *Artemia* and not ATC of bacteria. The activity measured

TABLE I

Effect of different buffers used to prepare *Artemia* crude extracts on the activity and the stability of aspartate transcarbamylase (cysts incubated at 25 °C for 1 day)

Buffer	Activity cpm/mg/90 min	% Activity after freezing at -18 °C	
		24 hr	48 hr
Distilled H_2O	1.51×10^{-5}	99	99
	1.41×10^{-5}	96	99
	1.40×10^{-5}	97	98
Buffer Tris 0.2M pH 8.5	1.09×10^{-5}	86	84
	1.12×10^{-5}	87	80
	1.08×10^{-5}	80	81
Buffer Tris 0.2M pH 9	1.01×10^{-5}	76	72
	9.93×10^{-5}	76	78
	9.20×10^{-5}	83	76
Buffer Tris KH_2PO_4 0.04M pH 7	1.62×10^{-5}	83	69
	1.63×10^{-5}	74	71
	1.60×10^{-5}	81	77

after 24 hr incubation was similar to that measured on homogenate prepared from *Artemia* cultured in parallel but without antibiotics.

KINETIC PROPERTIES OF ATC IN *ARTEMIA* CYSTS AND LARVAE

With the cysts of the different strains the rate of formation of carbamylaspartate was linear with respect to time for at least 1 hr. The effect of pH on the enzymatic velocity is presented in Fig. 1. Maximum velocity was observed at around pH 9. At this pH the temperature optimum was 42 °C for a 30 min incubation (Fig. 2). With San Francisco larvae maximum velocity was also observed around pH 9 but the rate of formation of carbamylaspartate was not always linear. This non linear kinetic was also observed with the larvae of the other strains (Fig. 3); this was not due to too small concentrations of substrate because the reaction was proportional with the concentrations of the homogenate up to formation of about 1.5 μmoles of carbamylaspartate (Fig. 4). This quantity of carbamylaspartate corresponds to the utilization of 16% aspartate and 24% carbamylphosphate. Fig. 4 shows the result obtained with an homogenate of larvae of the San Francisco strain which exhibited a linear kinetic ; the same result was, however, also obtained with an homogenate of larvae of this strain which exhibited a non-linear kinetic.

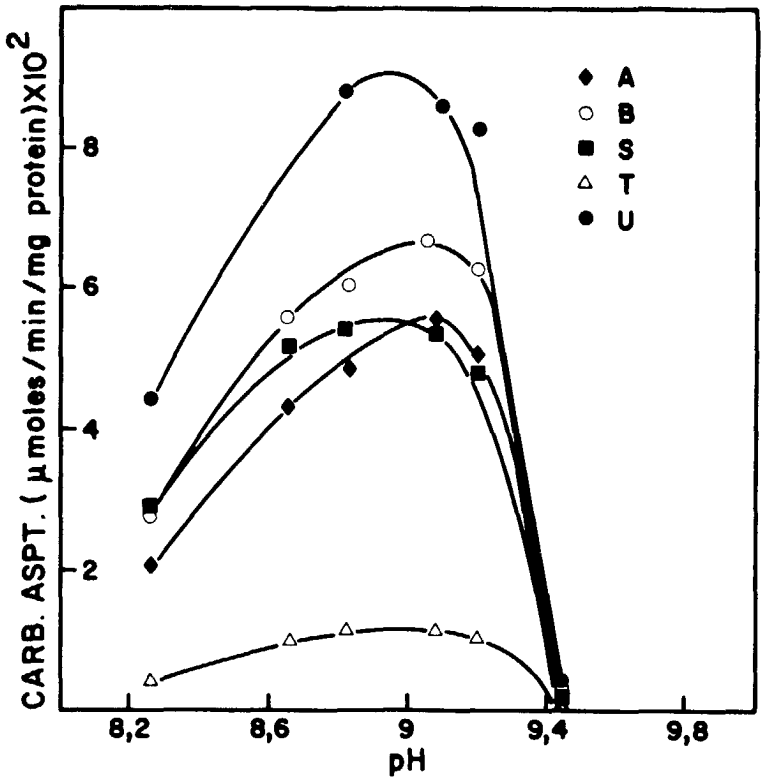

FIG. 1. Dependence of the enzymatic velocity upon pH. The reaction mixture was incubated for 30 min at 35 °C and contained the following compounds : carbamyl phosphate, 6.6 μmoles ; L. aspartate, 9 μmoles ; homogenate of cysts, 0.2 ml and buffers in a total volume of 0.5 ml. $NaHCO_3$ 0.2 M was used for pH 10 and tris 0.4 M for all other pH's to prepare carbamyl phosphate and L. aspartate pH of the extract was measured in the reaction mixture ♦ Australia, ○ Brazil, ■ San Francisco, △ Turkey and ● Utah strain.

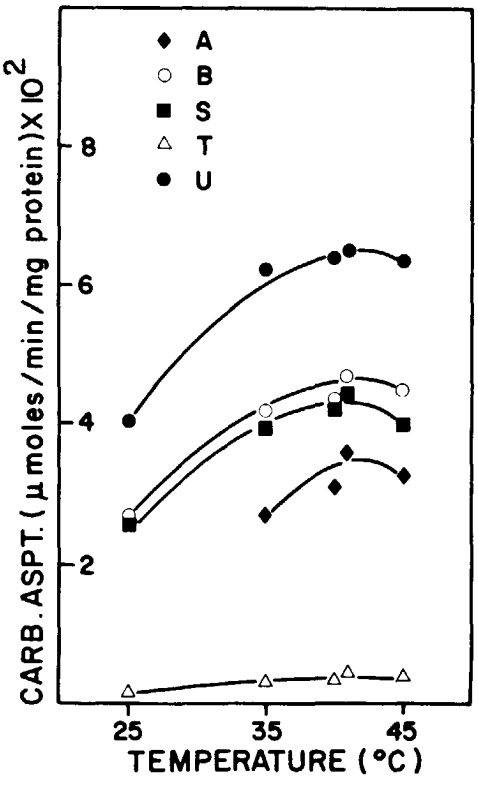

FIG. 2. Dependence of the enzymatic velocity upon temperature. The incubation conditions were similar to those indicated in the legend of Fig. 1. Tris 0.4 M pH 9.5 was used to prepare carbamyl phosphate and L. aspartate.

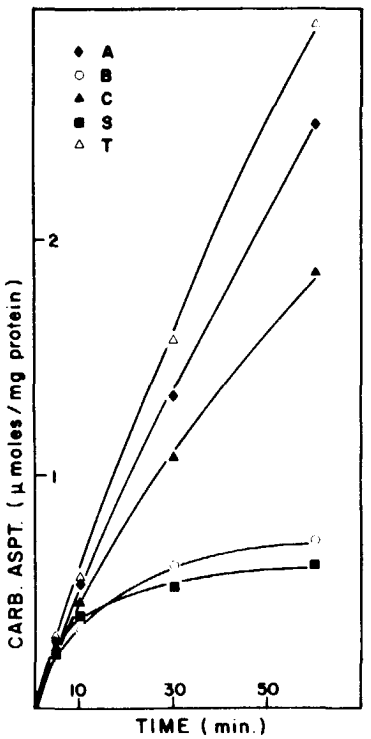

FIG. 3. Dependence of the velocity upon the time of incubation with homogenate of *Artemia* larvae of ♦ Australia, ○ Brazil, ▲ Cyprus, ■ San Francisco, and △ Turkey strains (cysts incubated for 48 hr at 25 °C). The reaction mixtures were similar to those indicated in the legend of Fig. 1. Tris 0.4 M pH 9.5 was used to prepare carbamyl phosphate and L. aspartate.

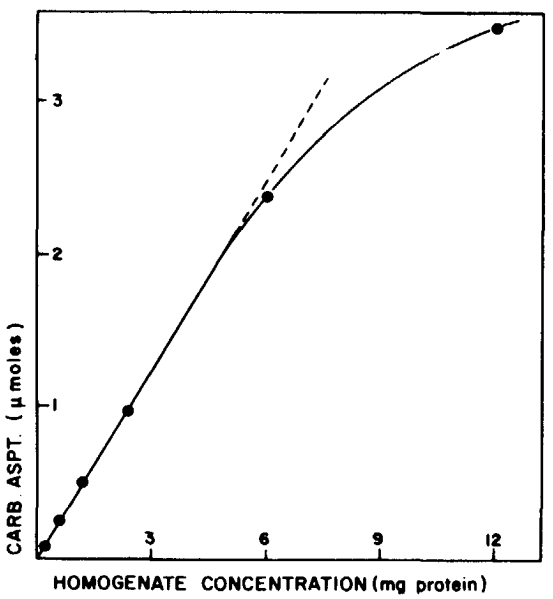

FIG. 4. Dependence of the velocity upon the homogenate concentration for larvae of the San Francisco strain (cysts incubated for 24 hr at 25 °C). The reaction mixture was similar to that described in the legend of Fig. 3 with the exception that the incubation time was 90 min and the temperature was 30 °C.

Fig. 5. shows a decrease of the activity with increase of the temperature with an homogenate with non-linear kinetic. This decrease was due to the increase of the instability of the activity with rise of the temperature (Fig. 6).

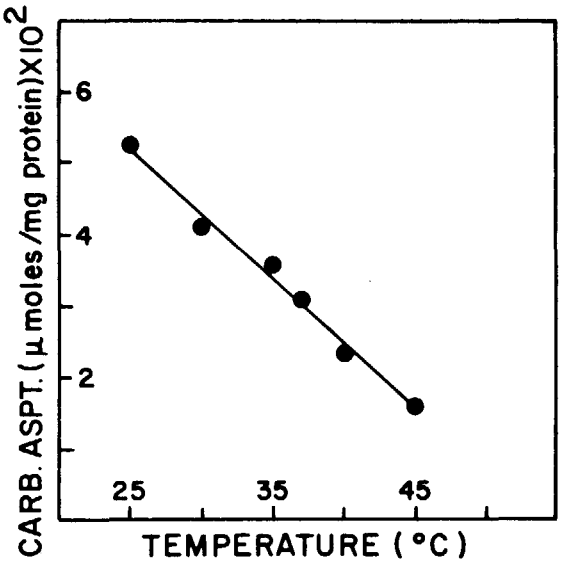

FIG. 5. Dependence of the enzymatic velocity upon temperature for an homogenate of larvae of the San Francisco strain (cysts incubated for 3 days at 25 °C). The reaction mixture was similar to that described in the legend of Fig. 2 with the exception that the incubation time was 90 min.

TESTS TO STABILIZE THE ATC ACTIVITY

Bresnick (1962) has shown that mercaptoethanol was necessary to stabilize the ATC from rat liver ; on the other hand Vassef *et al.* (1973) had found that UMP was effective as an *in vitro* stabilizer within physiological concentrations for ATC from *Chlorella*. 2.10^{-3}M mercaptoethanol and 4.10^{-3}M UMP were added but no stabilization was observed. 8.10^{-4}M EDTA and 1mM PMSF, a protease inhibitor widely used to avoid proteolytic artefacts, were without effect.

EFFECTS OF DIFFERENT SUBSTANCES

UMP which exhibited no effect at 4.10^{-3}M appeared to be inhibitory at higher concentrations. The addition of 5.10^{-2}M UMP resulted in 28% inhibition of the activity of an homogenate of San Francisco larvae which contained 15.8 mg proteins/ml. The activity of San Francisco strain ATC (homogenate containing 11 mg proteins/ml) was inhibited for 90% in the presence of $4.8\ 10^{-3}$M p. hydroxymercuribenzoate. The heavy metals were also effective as inhibitor. A 26% reduction in enzymatic activity was observed in presence of 10^{-4} M Cu^{2+} in a crude extract containing 2.5 mg proteins/ml and 18% at the same concentration with Zn^{2+}.

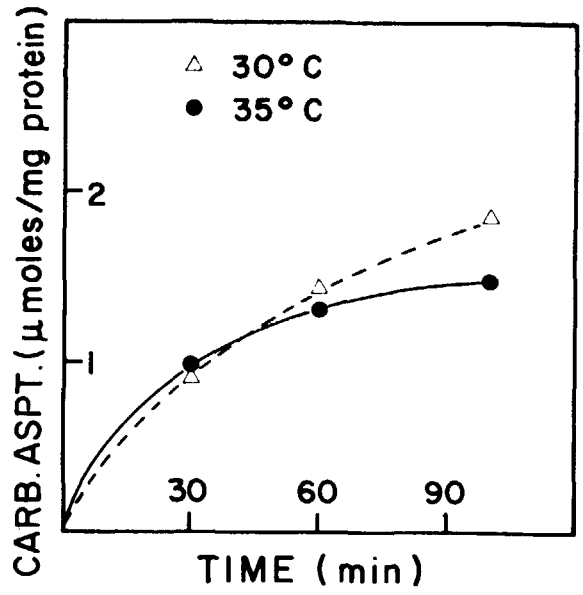

FIG. 6. Dependence of the velocity upon the time of incubation for an homogenate of larvae of the San Francisco strain (cysts incubated for 48 hr at 25 °C). The reaction mixture was similar to that described in the legend of Fig. 3. with the exception that the two temperatures tested were 30 °C and 35 °C.

ESTIMATION OF THE ATC ACTIVITY OF THE CRUDE EXTRACTS WHICH EXHIBIT NON-LINEAR KINETICS

It was difficult to determine the initial velocity of the enzyme reaction with the inactivation which occured during its catalytic reaction. The graphical method described by Stinshoff (1972) for arylsulphatase A was tested. With this method, linear relations were obtained between the reciprocal of the quantities of carbamylaspartate synthetised (1/P) and the indubation time (1/t). The curves obtained may be described by the following equation of Stinshoff (1972):

$$\frac{1}{P} = \frac{1}{P_{max}} + \frac{1}{Vo.t}$$

where P is the quantity of carbamylaspartate formed.

P_{max} is the maximum quantity of carbamylaspartate that the enzyme can synthetise under the conditions of incubation at infinite time.

Vo is the initial velocity of the enzymatic reaction.

It was therefore possible to estimate graphicaly P_{max} and Vo from this equation. The rate at which the enzyme is inactivated can also be estimated by determination of the time necessary for the reaction velocity to decrease to half its initial value. The above equation can be written in this manner :

$$P = \frac{P_{max}\ Vo.t}{P_{max} + Vo.t}$$

The differentiation quotient dp/dt is a function of the incubation time :

$$\frac{dp}{dt} = \frac{P^2_{max}.Vo}{(P_{max} + Vo.t)^2} = V$$

if $V = \frac{Vo}{2}$, the equation can be written :

$$\frac{Vo}{2} = \frac{P^2_{max}.Vo}{(P_{max} + Vo.\tau_{1/2})^2}$$

where $\tau_{1/2}$ is the half time of the enzyme. $\tau_{1/2}$ can be calculated from the following equation :

$$\tau_{1/2} = 0.414 \frac{P_{max}}{Vo}$$

Fig. 7 shows the application of the graphical method outlined above to calculate the activity of the larvae of different *Artemia* strains, the kinetics of which are presented in Fig. 3. The characteristics of the straight lines, slope (Vo) and the point of intersection with the reciprocal activity axis (P_{max}) were calculated by the method of the least squares. The maximum quantities of carbamyl aspartate that the homogenates could synthetize per mg of proteins (P_{max}), the specific initial velocities (Vo) and the half-lives ($\tau_{1/2}$) are listed in Table II. Differences appeared between the strains though the cysts had been incubated at the same time and under the same conditions (for 48 hr at 25 °C). The initial velocity was approximately equal for the Australia, Cyprus, and Turkey strains and higher with the Brazil and San Francisco strains. The crude extract from the San Francisco strain was about 1.7 times more active than that of the Australia strain. The differences were more important between the half-lives. ATC activity was more stable in crude extracts from the Turkey, Australia, and Cyprus strains than in those from the Brazil and San Francisco strains. In particular ATC of the Turkey strain had a $\tau_{1/2}$ 19 times longer than ATC from the San Francisco strain.

Influence of substrate concentrations

The influence of the two substrates was studied on the activity of a crude extract of San Francisco larvae which exhibited linear kinetics (cysts incubated for 1 day at 22 °C). Lineweaver-Burk plots obtained by varying the concentration of aspartate at a constant carbamylphosphate concentration, and vice versa, were linear. A system of parallel straight lines was obtained in each case (Fig. 8A,B). A replot of intercepts on the reciprocal activity axis versus the reciprocal of the substrate concentration gave a straight line. Figures insert in Fig. 8A,B show that the V_{max} is similar ($1.33.10^{-3}$ µmoles/min/mg of protein) for each curve and indicate the Km values of 7 mM and 1.6 mM for aspartate and carbamylphosphate respectively.

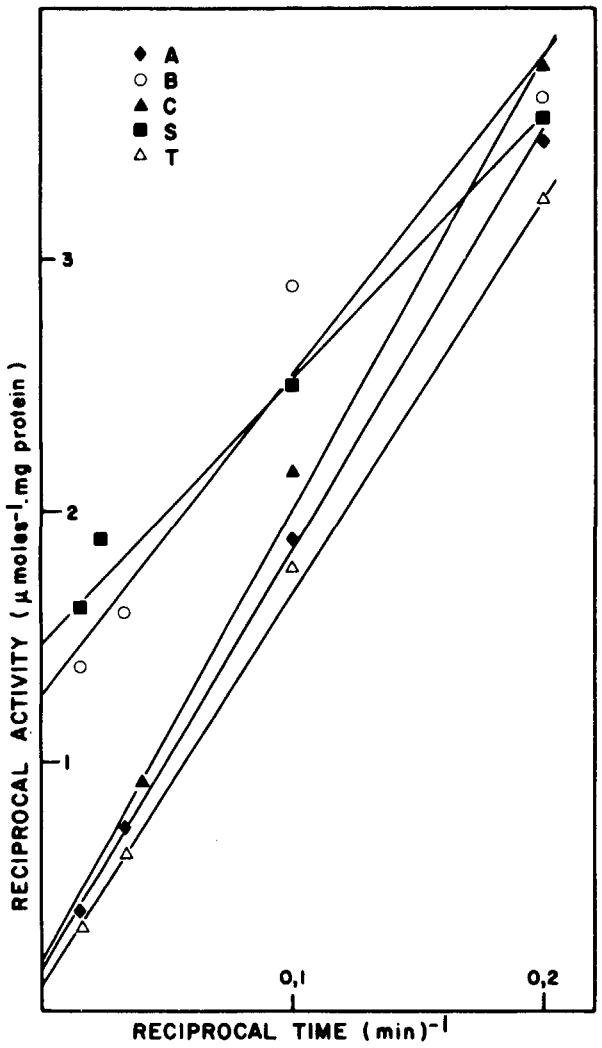

FIG. 7. Reciprocal plot corresponding to Fig. 3.

TABLE II

Parameters of the enzymatic reaction of aspartate transcarbamylase for different *Artemia* strains (cysts incubated at 25 °C for 2 days)

Strain	Vo Initial velocity (μmoles/min/mg protein)	Maximum P_{max} amount of carbamyl aspartate that can be formed (μmoles/min/mg protein)	$\tau_{1/2}$ Half life (min)
Australia	0.059	6.02	41.6
Brazil	0.079	0.78	14.0
Cyprus	0.055	4.80	36.3
San Francisco	0.096	0.68	2.9
Turkey	0.063	8.79	57.6

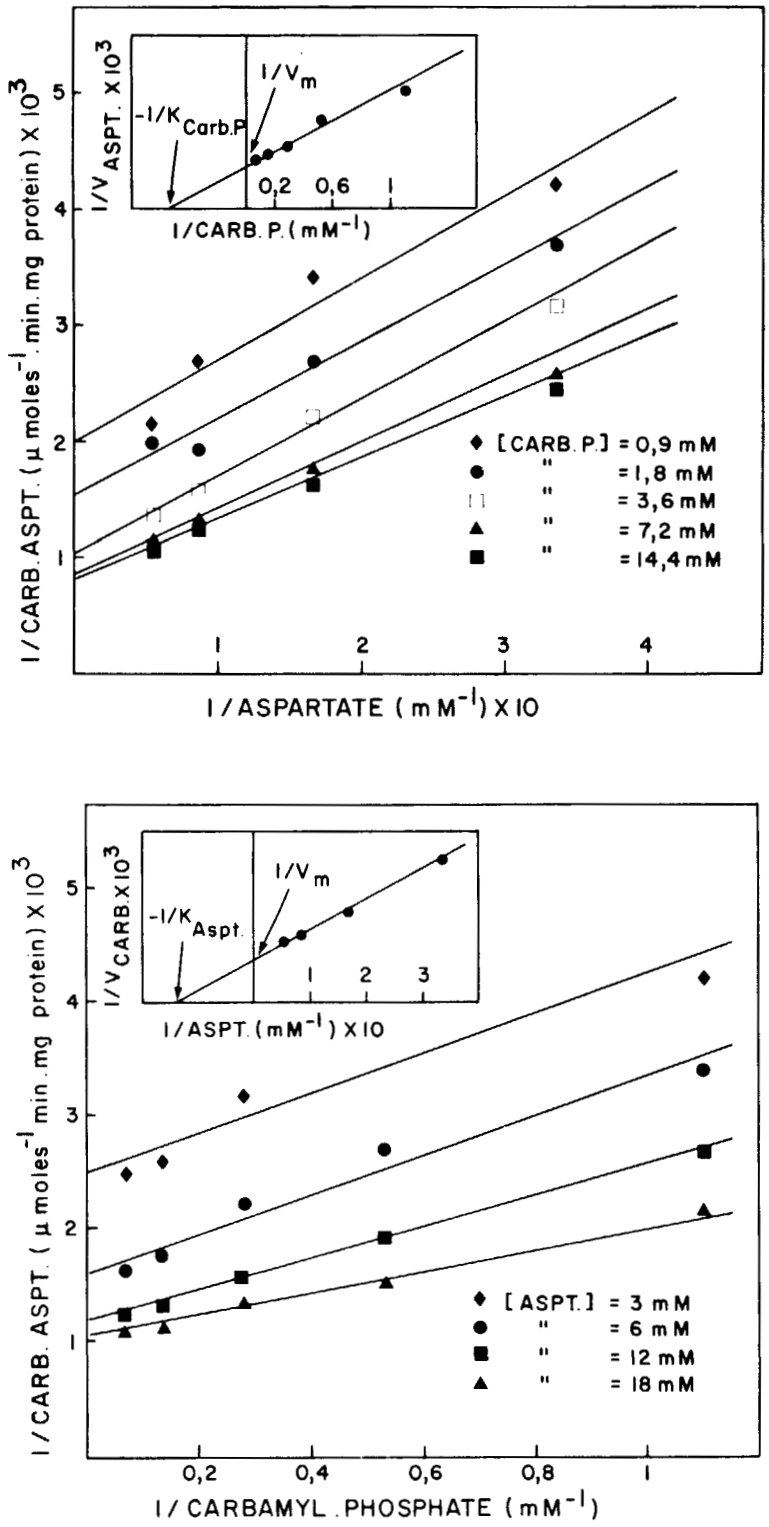

FIG. 8. Reciprocal plot corresponding to the dependence of the velocity upon the concentration of carbamyl phosphate. (A) and of L. aspartate (B) with larvae of the San Francisco strain (cysts incubated at 25 °C for 1 day). The reaction mixture was incubated for 90 min at 30 °C and contained the following : carbamyl phosphate, L. aspartate (the concentration of the two substrates varied as indicated in the figure), in a total volume of 0.5 ml-0.2 M $NaHCO_3$; carbamyl phosphate and L. aspartate were prepared at pH 9.5.

VARIATION OF THE ATC ACTIVITIES DURING THE EARLY STAGES OF DEVELOPMENT OF AUSTRALIA AND BRAZIL *ARTEMIA* STRAINS

Developmental patterns of specific activities of ATC (initial velocities per mg of proteins) in crude extract from Australia and Brazil strains incubated at 22 °C are compared in Fig. 9. Developmental patterns of the ATC of these two strains were very similar, with maximal activity after two days of incubation of the cysts. Thereafter the activity decreased rapidly. With the two strains kinetics were linear until day 2. The specific activities were approximately equal after the decrease, but during the first 2 days the activity in the Brazil strain was higher. In parallel, growth in length was determined after the hatching period which started between day 1 and day 2. During this experiment, the two strains grew exponentially since linear curves were obtained in semilogarithmic plot with $r = 0.996$ and 0.974 for the Brazil and the Australia strain respectively (Fig. 10). The specific growth rates estimated as the slope of the straight lines were 0.150 ± 0.004/day and 0.091 ± 0.007/day respectively. The Brazil strain thus grew 1.6 times faster than the Australia strain in the conditions of this experiment. A second experiment with the same two strains was performed at 15 °C. In this case, hatching began between day 2 and day 3 for the Brazil strain and day 3 and day 4 for the Australia strain. Kinetics of enzymatic reaction were linear until day 5 with the Brazil strain and until day 7 with the Australia strain. Fig. 11 shows the maximal activity at 5 days of incubation of the Brazil cysts and between 5 and 7 days of incubation of the Australia cysts. Thereafter, the activity decreased more slowly than in the experiment performed at 22 °C. Growth rate curves are presented in Fig. 12 ; straight lines were obtained

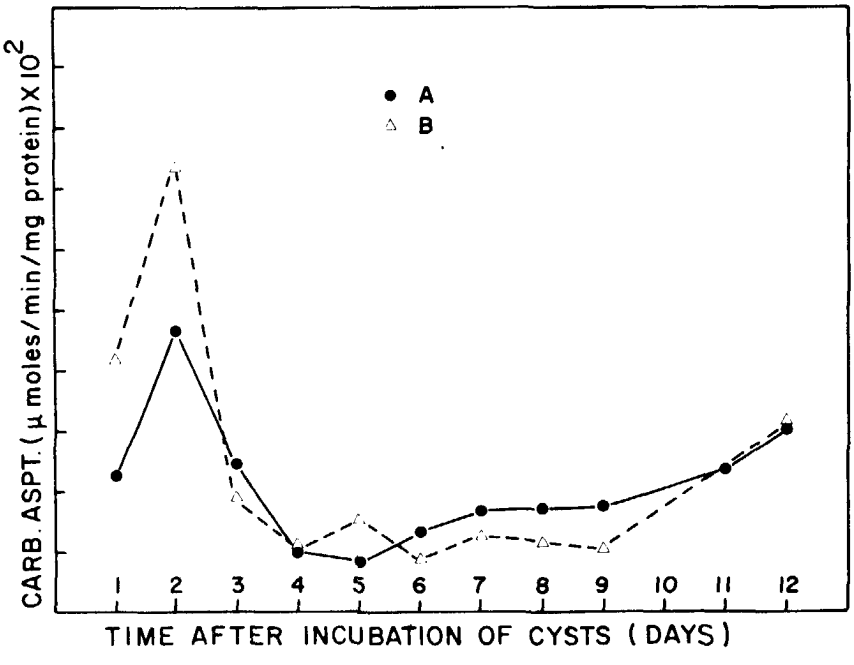

FIG. 9. Patterns of aspartate transcarbamylase activity during early stages of development of *Artemia* from ● Australia and △ Brazil strains incubated at 22 °C.

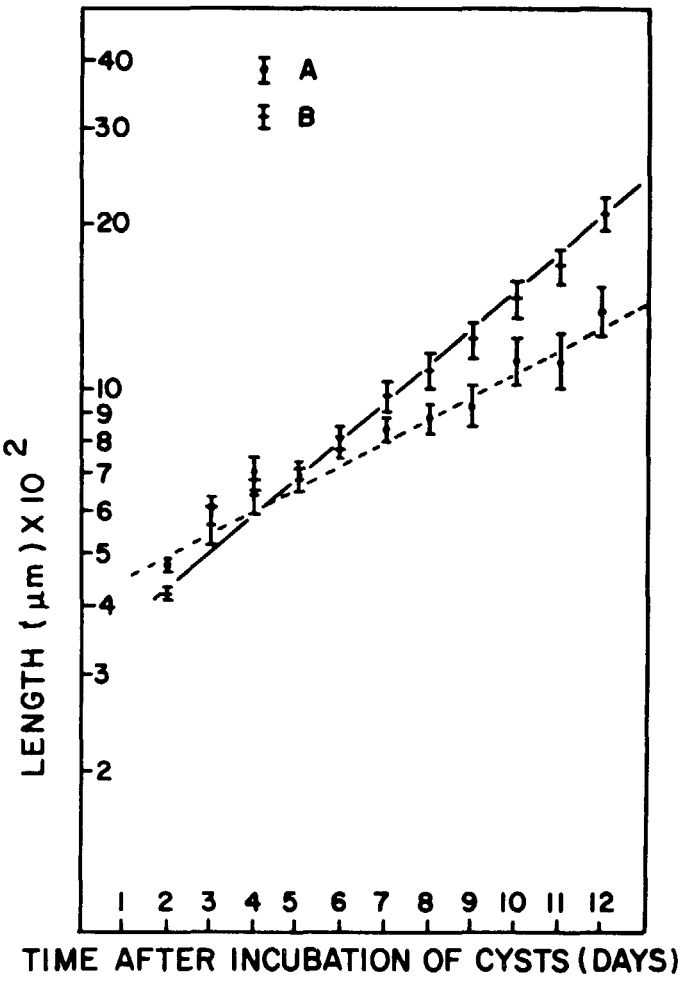

FIG. 10. Semi-logarithmic plot of the lengths of the *Artemia* from • Australia and — Brazil strains incubated at 22 °C during early stages of development.

which suggest specific growth rate values of 0.11 ± 0.03/day and 0.093 ± 0.003/day for the Brazil and the Australia strain respectively. The specific growth rate of the Brazil larvae cultured at 15 °C was smaller than when the strain was cultured at 22 °C but the maximum of the specific activity of the ATC had the same value around 7.2 10^{-2} μmoles/mg protein. In the case of the Australia strain, the specific growth rate was the same at 15 °C as at 22 °C and the maximum ATC activity (3.4 10^{-2} μmoles/min/mg protein) was smaller than at 22 °C (4.6 10^{-2} μmoles/min/mg protein). In the two strains at the two temperatures tested the specific activity increased after hatching and instability of the enzyme appeared with the subsequent decrease of the activity.

Discussion

Aspartate transcarbamylase from *Artemia* shows similarities with the enzyme from rat liver. Its pH optimum near 9 is similar to the 9.2 reported for rat liver enzyme. The *Artemia*

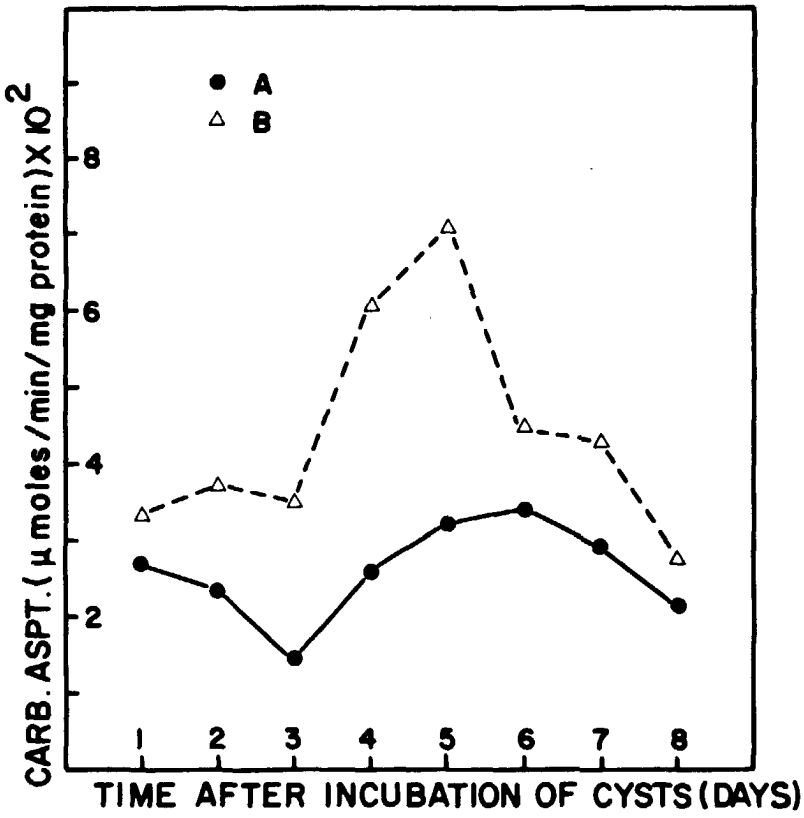

FIG. 11. Patterns of aspartate transcarbamylase activity during the early stages of development of *Artemia* from ● Australia and △ Brazil strains incubated at 15 °C.

enzyme is also inhibited by UMP, mercurial reagent (PHMB) and by heavy metals (Cu^{2+} and Zn^{2+}) (Bresnick, 1962 ; Bresnick and Mosse, 1966). Anomalous kinetics observed with crude extracts of larvae were not described with ATC from other origin. An unusual kinetic as found by Cygan and Zak (1966) with ATC of beef liver, but in this case the activity of the homogenates increased during the incubation. Different hypotheses can explain this non-linear kinetic obtained with *Artemia* larvae : degradation by proteolytic enzyme(s), enzymatic release of an inhibitor or inversely enzymatic disappearance of an activator during the incubation, or the substitution of the stable enzyme by an instable isoenzyme in *Artemia* during the earliest stages of development or the disappearance of a stabilizer. Osuna *et al.* (1977) showed that after hatching of *Artemia* nauplii a high increase of four proteolytic activities appeared in extracts from the larvae and that only one of these proteases was strongly inhibited by PMSF. Also it is not possible to eliminate that hypothesis of an artefact due to the presence of proteases to explain the appearance of instable ATC activity in extracts from larvae. Therefore it is necessary in our future studies to try to determine the origin of the non-linear kinetics of ATC to test the other protease inhibitors used by Osuna *et al.* (1977).

If decrease of the activity during the incubation was not described with ATC from other sources, on the other hand instability of the ATC activity has been observed in diluted homogenates of *Chlorella*. Vassef *et al.* (1973) could demonstrate that UMP was effective as an *in vitro* stabilizer within physiological concentrations for ATC of *Chlorella*.

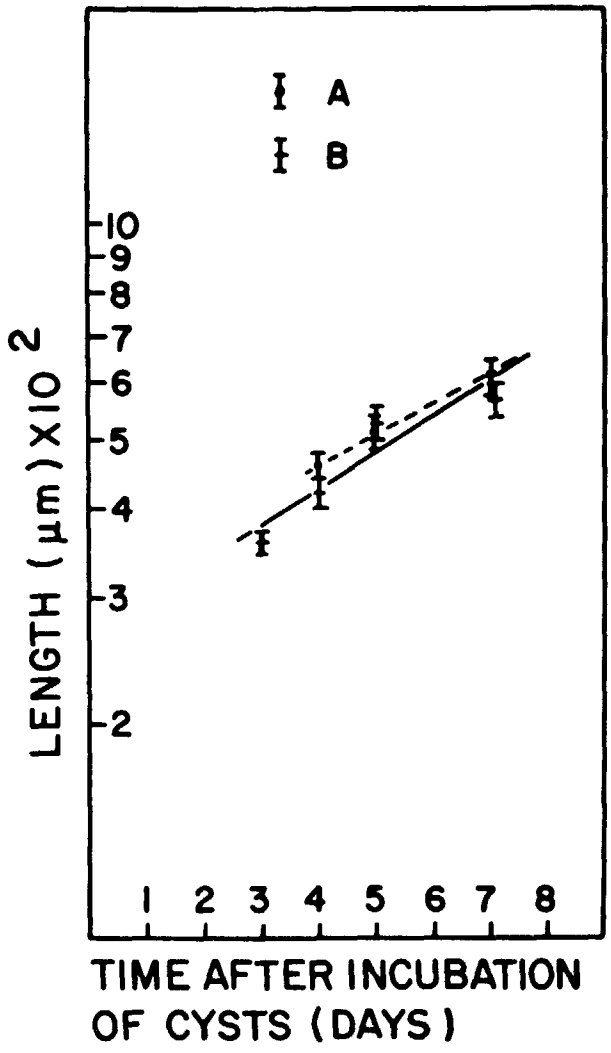

FIG. 12. Semilogarithmic plot of the lengths of *Artemia* from • Australia and — Brazil strains incubated at 15 °C during early stages of development.

The system of parallel straight lines obtained by varying the concentration of the two substrates with homogenate which exhibit linear kinetic suggest a non sequential ping-pong mechanism. This same system was obtained by Lue *et al.* (1976) with ATC from Baker's yeast, by Ong and Jackson (1972) with ATC from *Phaseolus aureus* and in our laboratory with ATC from mussel and scallop (Bergeron and Alayse-Danet, in preparation). Lue *et al.* (1976) and Ong and Jackson (1972) could not opt unequivocally for a ping-pong mechanism because the inhibitor effects obtained with the products of the reaction (phosphate and carbamylaspartate) on bakers' yeast ATC were consistant with an ordered BiBi mechanism where carbamylphosphate and then aspartate bind to the enzyme followed by release of carbamylaspartate and phosphate, in that order. Ordered BiBi mechanism had been also suggested in the case of the ATC from mouse spleen (Hoogenraad *et al.*, 1971) and the catalytic subunit of *Escherichia coli* ATC (Porter *et al.*, 1969).

Clegg and Golub (1969) showed that a fast *de novo* RNA synthesis could be detected from the beginning of the incubation of *Artemia* cysts ; the presence of active ATC in cysts corroborates this result. Decrease of the specific activity of ATC during the first stages of the development was also observed by Weichsel *et al.* (1972) and by Herzfeld and Knox (1972) with ATC from different organs of rat. Herzfeld and Knox (1972) could establish an equation for tumors exhibiting a wide range of different growth rates. From our results a relation has been sought between growth rates of two *Artemia* strains (Australia and Brazil) and their ATC activities. On an average during the experiment at 22 °C the two strains seemed to grow with a constant rate but at the same time the specific activity of the ATC varied. No relation appears since apparently one value of growth rate corresponds with several values of specific ATC activity. On the other hand specific growth rates calculated each day by the following formula :

$$\frac{\text{length}_{\text{day}_n} - \text{length}_{\text{day}_{n-1}}}{\text{length}_{\text{day}_{n-1}}}$$

were taken into consideration. A positive correlation appeared with the specific activity values measured the day before (day_{n-1}) and the growth rates determinations mentioned above. The correlation coefficients obtained are : for the Brazil strain $r = 0.809$ with $df = 6$; for the Australia strain $r = 0.626$ with $df = 7$ and if the two strains are taken in consideration $r = 0.624$ with $df = 15$. These sample correlation coefficients are different from zero respectively at the 2, 10, and 1 % level of significance. The differences which appear between the specific growth rates each day are important since they may explain the variation of the specific ATC activity.

In the experiment performed at 15 °C larvae of the Australia strain seemed to grow with the same average specific growth rate as at 22 °C which is certainly an artefact due to the small number of length values especially as the development in the cysts had slackened (hatching started between day 3 and 4 instead of day 1 and 2 at 22 °C). With this experiment it is not possible to look for a correlation because only one pair of values can be used with the Australia strain and two with the Brazil strain. The specific activity of ATC at its maximum level-particularly in the Brazil strain – was not significantly different from that at 22 °C thought the growth rate was weaker. These results are not inconsistent because at 15 °C the same quantity of enzyme is certainly less active.

A relation seems to exist between ATC activity and growth rate in *Artemia*. This interpretation of our experiments is based on the hypothesis that, on one hand the ATC activities were not significantly influenced during homogenization and, on the other hand the daily ATC and protein determinations were reproducible. New experiments have to be made to confirm such a relation with a wider range of growth rates and, if possible, after a way to stabilize the ATC activity during the experiments will have been found.

Summary

Aspartate transcarbamylase (ATC) of homogenates of cysts of different *Artemia* strains – Australia, Brazil, San Francisco (USA), Cyprus, and Utah (USA) – has a pH optimum around

9 and exhibits linear kinetics. Non-linear kinetics were observed in larvae and were dependent of the temperature of the culture medium.

The mathematical correlation between the reaction velocity and the incubation time, as used by Stinshoff (1972), could be applied and the initial velocity of the anomalous kinetics could be calculated.

The rate of decreasing of the enzymatic activity depends of the temperature of incubation. Initial velocity studies with homogenate containing stable ATC activity, and variation of the concentration of aspartate at a constant carbamyl phosphate concentration and *vice-versa*, revealed a set of parallel reciprocal plots suggesting a non sequential ping-pong mechanism.

The levels of ATC activity were found to be highest in the earliest stages of development. The specific ATC activity was significantly correlated with the specific growth rate calculated each day.

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