Biochem. J. (1968) **110**, 425
Printed in Great Britain

Studies on the Advent of Ureotelism

FACTORS THAT RENDER THE HEPATIC ARGINASE OF THE MEXICAN AXOLOTL ABLE TO HYDROLYSE ENDOGENOUS ARGININE

BY R. PALACIOS, REBECA TARRAB* AND G. SOBERÓN
Departmento de Biología Molecular, Instituto de Investigaciones Biomédicas, Universidad Nacional Autónoma de México, Apdo. postal 70228, *México* 20, *D.F., Mexico*

(*Received* 18 *June* 1968)

1. A study was undertaken of the conditions that might operate in the synthesis and hydrolysis of arginine by axolotl liver homogenate to test a previous postulate that liver arginase of the non-metamorphosed Mexican axolotl is not able to hydrolyse arginine formed from citrulline and aspartic acid, though it can split exogenous arginine, and also that an enhanced capacity to hydrolyse endogenous arginine plays a major role in the advent of ureotelism observed during the metamorphosis of the axolotl. 2. It was found that the arginase from axolotl liver is very unstable under the conditions followed, contrary to what is observed in rat liver. 3. Axolotl arginase is able to hydrolyse endogenous arginine if preserved. 4. Mn^{2+} protects the enzyme and renders it able to split endogenous arginine. 5. It is suggested that the metal ion produces a change of conformation of the enzyme that, being stable, is capable of hydrolysing the amino acid, or that the new conformation is appropriate for interaction with the sites of arginine synthesis.

In an early study of the urea-biosynthesis enzymes in the liver of the neotenic Mexican axolotl no activity of the arginine synthetase system (argininosuccinate synthetase and argininosuccinase, assayed together) could be detected (Soberón, Flores, Mora & Torres, 1959). It was later established that this was because the assay system measured only the urea formed in the incubation medium and the axolotl liver homogenate was found to have a high arginase activity. It was rather surprising to find that arginine accumulated in spite of the presence of arginase as determined by its ability to hydrolyse added arginine (Mora, Martuscelli, Ortíz-Pineda & Soberón, 1965*a*). It was also found in many cases that, when the metamorphosis of the Mexican axolotl was initiated by the administration of tri-iodothyronine, the arginase already present became able to hydrolyse the endogenous arginine formed from citrulline and aspartic acid even though no increase in total enzyme activity was observed (Mora *et al.* 1965*a*). In addition, purified arginase acquires this capability (Soberón, Ortíz-Pineda & Tarrab, 1967) and it appears that this results from treatment of the protein with ethanol. Indeed, during the purification procedure described by Schimke (1964) the arginase activity obtained after each step was tested for its ability to hydrolyse endogenous arginine and it was found that the precipitate after addition of 3 vol. of cold ethanol was able to hydrolyse endogenous arginine. Rat liver homogenate and purified arginase from rat liver behave similarly but purified arginase from *Neurospora crassa* does not (Soberón *et al.* 1967).

The above findings may have considerable biological significance, since *N. crassa* in the presence of citrulline but without ammonia in the growth medium accumulates arginine and possesses a high induced activity of arginase, which, paradoxically enough, does not hydrolyse its substrate; the addition of ammonia causes the rapid hydrolysis of the arginine accumulated (Castañeda, Martuscelli & Mora, 1967).

It has been postulated that the main change produced by induced metamorphosis of the Mexican axolotl, responsible for the switch from ammonotelism to ureotelism, is the acquired capacity of arginase to hydrolyse endogenous arginine (Mora *et al.* 1965*a*; Soberón *et al.* 1967). If such is the case it could be assumed that, for various reasons, endogenous arginine cannot be hydrolysed in the non-metamorphosed axolotl.

The present paper deals with the study of the conditions operating in the hydrolysis of endogenous and exogenous arginine by liver homogenates from the Mexican axolotl before and after metamorphosis. It was found that a rapid denaturation of arginase might be of importance in explaining its inability

* Present address: Department of Microbiology, University of Illinois Medical Center, Chicago, Ill. 60680, U.S.A.

to hydrolyse arginine and that Mn^{2+} is critical for preserving the enzyme in the system tested. The biological implications of these observations are discussed.

MATERIAL AND METHODS

Animals. The axolotls were collected from Lake Xochimilco near Mexico City. They belonged to the species *Ambystoma mexicanum*, which is neotenic. The animals were kept in the laboratory for a few days in chlorine-free water (changed every other day) before decapitation. They were fed with liver according to the procedure of R. R. Humphrey (personal communication). The liver was cut into thin strips and, blunt forceps being used for handling the pieces, each animal was given as much as it would take quickly when the food was offered. They were fed three times per week. The rats, Wistar strain, weighed 150–250g. They were fed with a commercial diet (Purina Chow) and were killed by decapitation.

Metamorphosis of the Mexican axolotl was induced by placing the animal in a plastic container with 10μg. of tri-iodothyronine/l. of water. Each container held up to six animals, with a fixed proportion of 500ml. of water per animal. The tri-iodothyronine solution was changed every day. The administration of the hormone lasted for 25 days; thereafter the animals were kept in chlorine-free water for 5–6 weeks. When most of the morphological changes of metamorphosis were achieved, the animals showing signs of pulmonary respiration, the level of water was kept low to permit air-breathing.

Tissue homogenates. After removal of the liver, it was washed with cold water and homogenized for 2min. in 0·1% cetyltrimethylammonium bromide solution in a Potter–Elvehjem homogenizer. All further manipulations were carried out at 4°. The homogenate was centrifuged at 5000*g* for 10min., the supernatant saved and the precipitate extracted again with 0·1% cetyltrimethylammonium bromide solution. The mixture was centrifuged at 5000*g* for 10min., the precipitate discarded and the supernatant pooled with the first one. Both were made up to volume so that 10ml. of homogenate was obtained/g.

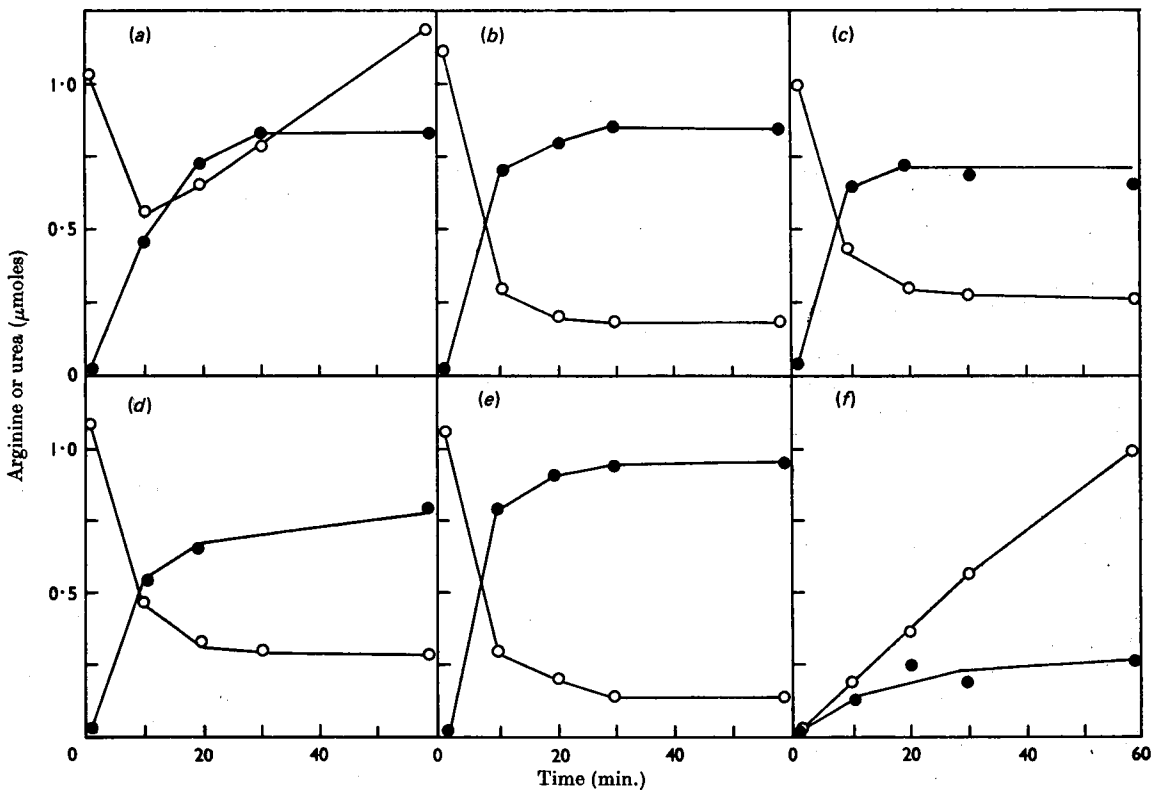

Fig. 1. Influence of each of the components of the arginine synthetase system on axolotl arginase activity. The incubation system contained 40mg. of tissue, 50μmoles of potassium phosphate buffer, pH 7·0, 5μmoles of L-citrulline, 5μmoles of L-aspartic acid, 5μmoles of ATP and 5μmoles of $MgSO_4$, final volume 1·0ml.; the temperature of incubation was 37°. At zero time 1μmole of arginine was added and arginine (○) and urea (●) were measured at the indicated times. (*a*) Complete system; (*b*) citrulline omitted; (*c*) aspartic acid omitted; (*d*) ATP omitted; (*e*) citrulline, aspartic acid and ATP omitted; (*f*) arginine omitted.

of tissue. All the experiments were carried out immediately after the preparation of the homogenates.

Enzyme assays. Arginase (L-arginine ureo-hydrolase, EC 3.5.3.1) activity was determined under two different conditions: (*a*) with 2mg. of homogenized tissue, 100μmoles of L-arginine, 0·25μmole of $MnCl_2$ and 25μmoles of glycine–NaOH buffer, pH 9·5, final volume 1·0ml.; the temperature of incubation was 37°; (*b*) with 40mg. of homogenized tissue, 1μmole of L-arginine, 50μmoles of potassium phosphate buffer, pH 7·0, without Mn^{2+}, final volume 1·0ml.; the temperature of incubation was 37°. In both cases the activity was followed by measuring urea formation as a function of time. When 1μmole of arginine was utilized the enzyme rate was also followed by arginine disappearance. The capacity to synthesize arginine and the further conversion of this into urea was followed by measuring arginine and urea formation as function of time in a system containing 40mg. of homogenized tissue, 50μmoles of potassium phosphate buffer, pH 7·0, 5μmoles of L-citrulline, 5μmoles of L-aspartic acid, 5μmoles of ATP and 5μmoles of $MgSO_4$, final volume 1·0ml.; the temperature of incubation was 37°.

Urea and arginine determinations. The incubation systems were stopped with 5·0ml. of 0·5M-$HClO_4$ for determination of urea and with either 5·0ml. of 5% (w/v) trichloroacetic acid or 5·0ml. of 1% (w/v) picric acid for determination of arginine. Urea was assayed colorimetrically by using isonitrosopropiophenone (Archibald, 1945). Arginine was determined by the Sakaguchi method as modified by Van Pilsum, Martin, Kito & Hess (1956) in a sample of the trichloroacetic acid extract. When it was desirable to increase the sensitivity for the determination of arginine or to eliminate glucose (experiment described in Fig. 3), which interferes with the Sakaguchi reaction, the amino acid was adsorbed on Amberlite CG-120 resin before removal of the picric acid with AG2 (X8) resin and measured in the 0·2N-NaOH eluate.

Chemicals. Amino acids were purchased from Calbiochem (Los Angeles, Calif., U.S.A.). Isonitrosopropiophenone and ATP were from Sigma Chemical Co. (St Louis, Mo., U.S.A.). Inorganic salts were from Merck, Sharp and Dohme Inc. (Rahway, N.J., U.S.A.). Hexokinase preparation was from Fluka A.-G. (Buchs, Switzerland).

RESULTS

To determine the factors that may interfere with the ability of the liver arginase from the non-metamorphosed Mexican axolotl to hydrolyse endogenous arginine, a systematic study was made of the effect of each of the components of the arginine synthetase system on the capacity for arginine synthesis, on the conversion of arginine into urea and ornithine, and on the hydrolysis of exogenous arginine. An absolute requirement for L-citrulline, L-aspartic acid and ATP was found for the production of arginine. ATP inhibited the production of arginine, as already established by Brown & Cohen (1959) for *Rana catesbeiana*. The citrulline and aspartic acid concentrations usually employed in the incubation system did not produce any abnormal effect. Although citrulline slightly inhibited the capacity of arginase to hydrolyse

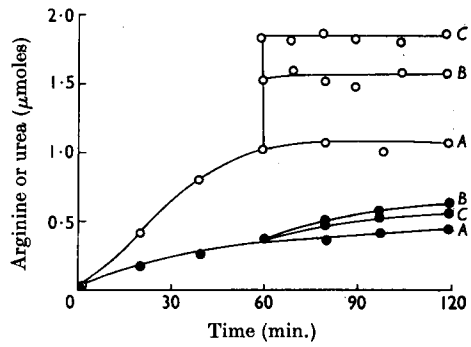

Fig. 2. Fate of exogenous arginine in the presence of accumulated endogenous arginine in axolotl liver homogenate. At zero time, incubation for the arginine synthetase activity was started. The incubation mixture contained 40mg. of tissue, 50μmoles of potassium phosphate buffer, pH 7·0, 5μmoles of L-citrulline, 5μmoles of L-aspartic acid, 5μmoles of ATP and 5μmoles of $MgSO_4$, final volume 1·0ml.; the temperature of incubation was 37° (curves *A*). At 60min. 0·5μmole (curves *B*) or 0·8μmole (curves *C*) of arginine was added. Arginine (○) and urea (●) were measured at the indicated times.

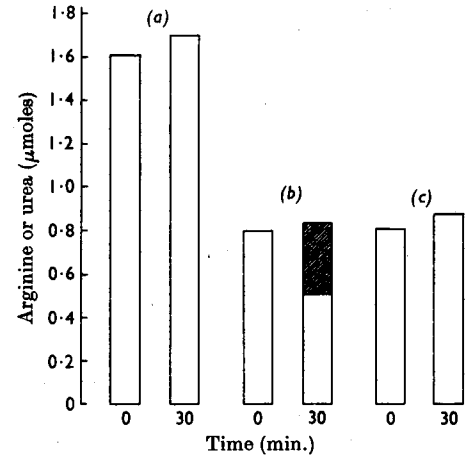

Fig. 3. Capacity of axolotl liver arginase to hydrolyse endogenous arginine. The incubation system contained 40mg. of tissue, 50μmoles of potassium phosphate buffer, pH 7·0, 5μmoles of L-citrulline, 5μmoles of L-aspartic acid, 5μmoles of ATP and 5μmoles of $MgSO_4$, final volume 1·0ml.; the temperature of incubation was 37°. At 90min. 2mg. of glucose and 1mg. of partially purified hexokinase were added to consume ATP; at 105min. an equal volume of homogenate in potassium phosphate buffer (40mg. of tissue and 50μmoles of potassium phosphate buffer/ml.) was added to two separate portions of the original incubation mixture, one freshly prepared and kept in the cold (*b*) and the other preincubated for 30min. to destroy arginase activity (*c*). Arginine (□) and urea (■) were measured at the indicated times. Results for the original incubation mixture are also shown (*a*).

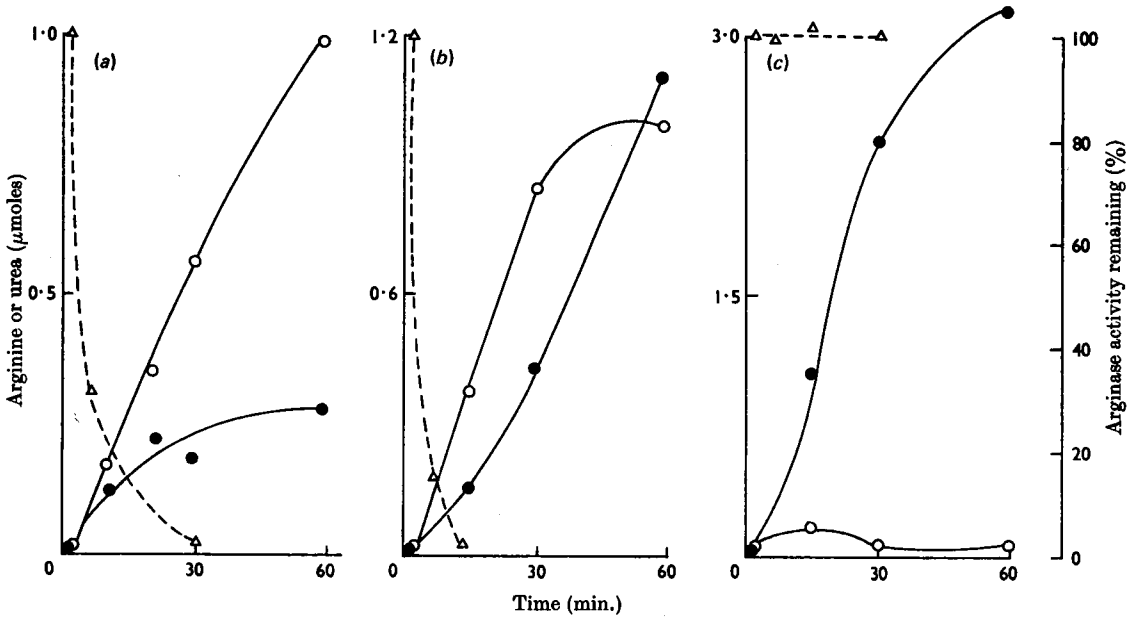

Fig. 4. Relation between stability of liver arginase and its capacity to hydrolyse endogenous arginine in the non-metamorphosed (*a*) and the metamorphosed (*b*) axolotl, and in the rat (*c*). The incubation mixture for the assay of arginine synthetase activity contained 40 mg. of tissue, 50 μmoles of potassium phosphate buffer, pH 7·0, 5 μmoles of L-citrulline, 5 μmoles of L-aspartic acid, 5 μmoles of ATP and 5 μmoles of $MgSO_4$, final volume 1·0 ml.; the temperature of incubation was 37°. Arginine (○) and urea (●) were determined at the indicated times. For the simultaneous assay of arginase activity decay, 40 mg. of tissue and 50 μmoles of phosphate buffer in 1·0 ml. were preincubated at 37° for the indicated times and then the remaining activity was determined by measuring arginine disappearance and urea formation 5 and 10 min. thereafter, in an incubation system containing 40 mg. of tissue, 50 μmoles of phosphate buffer, pH 7·0, and 1 μmole of arginine. Arginine and urea were measured and the stability is expressed as percentage of the initial activity (△).

exogenous arginine under the conditions of the system tested (Fig. 1), this could not be responsible for the inability to hydrolyse endogenous arginine, in view of the high arginase activity present in the tissue.

The possibility that endogenous arginine was confined to a given compartment where arginase did not have access could be supported if it could not readily diffuse, whereas exogenous arginine could move freely. When the capacity of the arginine to diffuse was challenged by dialysing the preparations against 0·05 M-potassium phosphate buffer, pH 7·0, at 0°, endogenous arginine diffused out of the dialysis bag and no striking differences were observed between the rates of diffusion of endogenous and exogenous arginine.

Since endogenous arginine diffused out of the dialysis bag it must be able to move from the site of its formation, as it probably does in the incubation medium of the arginine synthetase system, hence making the existence of the postulated compartment unlikely. However, it is possible that the conditions of the experiment disrupted such a compartment. Another possibility is that the molecule synthesized from citrulline and aspartic acid is arginine somehow modified so that it could not be attacked by the arginase present in the preparation. The compound extracted with trichloroacetic acid has been proved to be arginine (Mora *et al.* 1965*a*), but it had not been ruled out that the treatment with acid could convert the 'modified' arginine into arginine. Because of this possibility, endogenous arginine collected in the cold outside of the dialysis bag was tested with axolotl liver and rat liver homogenates, and it was hydrolysed in both cases.

The possibility that arginase from axolotl liver might be able to distinguish between endogenous and exogenous arginine could be tested by the use of labelled metabolites. Indeed, radioactive citrulline should produce radioactive endogenous arginine to which non-labelled exogenous arginine could be added and, if this were preferentially hydrolysed, the specific radioactivity of the arginine and urea extracted would vary as hydrolysis proceeds, as would the total radioactivity incorporated into these molecules. Conversely, the

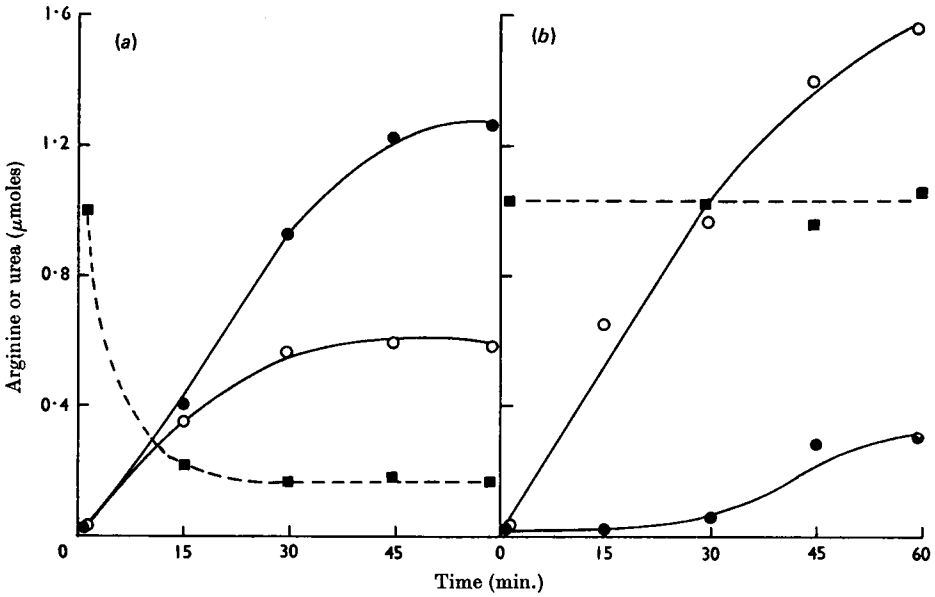

Fig. 5. Effect of preincubation on the capacity of liver arginase from the metamorphosed axolotl to hydrolyse endogenous and exogenous arginine. The ability to hydrolyse endogenous arginine was tested by measuring arginine (○) and urea (●) as a function of time in a system containing 40mg. of tissue, 50μmoles of potassium phosphate buffer, pH 7·0, 5μmoles of L-citrulline, 5μmoles of L-aspartic acid, 5μmoles of ATP and 5μmoles of $MgSO_4$, final volume 1·0ml.; the temperature of incubation was 37°. The capacity to hydrolyse exogenous arginine was determined by measuring arginine disappearance (■) as a function of time in a system containing the same as above, except that ATP was omitted and 1μmole of arginine was added at zero time. (*a*) Liver homogenate not preincubated; (*b*) liver homogenate preincubated for 30min. at 37°.

experiment could be carried out by using non-labelled citrulline and radioactive exogenous arginine. To establish the conditions for this experiment it was necessary to determine more precisely the kinetics of endogenous arginine formation and of exogenous arginine disappearance. While this was being carried out it was observed that when the exogenous arginine was added 60min. after the start of the incubation of the arginine synthetase system it remained unchanged (Fig. 2).

It was then proved that preincubation of the homogenate during 60min. completely destroys the arginase activity, which, as shown in Fig. 1, is highly active when the incubation is started. Arginase decays regardless of the presence or absence of each of the components of the arginine synthetase system.

These findings suggested that the inability to hydrolyse endogenous arginine could be explained by instability of the arginase. Fig. 3 shows that fresh homogenate added to a system where endogenous arginine has been allowed to accumulate readily hydrolysed it. Under the same experimental conditions the arginase activity from rat liver was completely stable. Since endogenous arginine was readily split into urea and ornithine by rat liver homogenate containing a stable arginase but arginine accumulated if axolotl liver homogenate containing an unstable arginase was used instead, one is tempted to conclude that arginase should be stable in the metamorphosed axolotl, where endogenous arginine is frequently hydrolysed. However, the situation is not so clear-cut as this, since we have found some metamorphosed axolotl liver that will readily produce urea from endogenous arginine, in spite of the fact that arginase activity is unstable (Fig. 4). On the other hand, preincubation of arginase to cause loss of activity also caused endogenous arginine to accumulate (Fig. 5).

The stability of the liver arginase from axolotl does not depend to a great extent on pH (Fig. 6). Rat liver arginase is fairly stable over the same pH range. The half-life of the axolotl arginase varied from 10 to 12min. at the different pH values explored. When the enzyme activity remaining after preincubation was assayed at the same pH in an incubation system containing 0·1M-arginine and 0·25mM-manganese chloride straight lines were obtained by plotting urea formation as a function

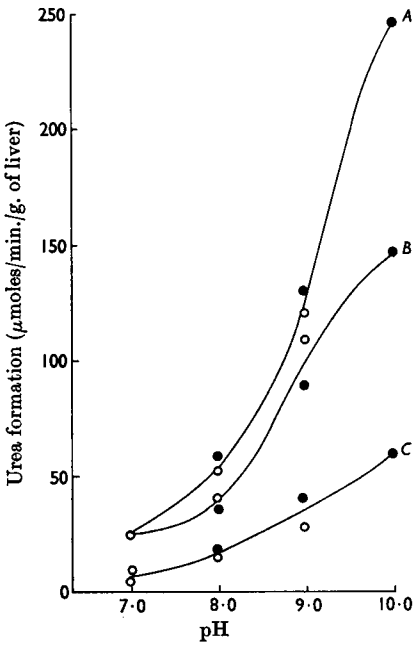

Fig. 6. Effect of pH on the stability of axolotl liver arginase. Homogenized tissue (40mg.) was incubated at 37° for 0min. (curve *A*), 10min. (curve *B*) and 20min. (curve *C*) in 1ml. of solution containing 10μmoles of tris–HCl (○) or glycine–NaOH (●) buffer at the pH value indicated. For the determination of the enzyme activity remaining, a sample containing 4mg. of tissue was transferred to 1·0ml. of solution containing 25μmoles of the same buffer, 100μmoles of arginine and 0·25μmole of $MnCl_2$. The pH of the assay system was adjusted to the same value as before addition of the sample, and urea was measured after 10 and 20min. of incubation.

of time. This would be difficult to get during a 20min. assay incubation unless there were something in the incubation system that protected the enzyme from decay. In the absence of Mn^{2+}, arginase activity was very low when axolotl liver homogenate was used. A concentration of 2·5μM was found to be effective for eliciting enzyme activity and 0·25mM or higher evoked full response. In rat liver a sizable arginase activity was obtained in the absence of added Mn^{2+} and this was increased to a maximum activity with 0·25mM-Mn^{2+} (Fig. 7). Mn^{2+} was able to prevent the decay of arginase during preincubation at pH7·0 and pH9·5. However, the decay of arginase was not reversed by the addition of Mn^{2+}. Arginine also increases the life of the enzyme, although for the expression of the activity remaining after preincubation addition of Mn^{2+} is necessary (Fig. 8).

From the above findings, it was readily possible to test whether Mn^{2+} could render the enzyme able to hydrolyse endogenous arginine. That such is the case is clearly shown by Fig. 9.

DISCUSSION

The purpose of the present paper was to clarify the reason why the arginase of the Mexican axolotl was unable to hydrolyse the arginine produced from citrulline and aspartic acid. This was necessary since it had been postulated that such acquired capacity could play a major role in the advent of ureotelism during the metamorphosis of the Mexican axolotl induced by the administration of tri-iodothyronine.

We are now justified in stating that the arginase from the Mexican axolotl can indeed split endogenous arginine. The experimental results reported here have failed to show either a modification of the arginine molecule synthesized or the compartmentalization of the same that could account for the amino acid not coming in contact with the arginase.

On the other hand, it was found that, contrary to what is observed in the rat, the liver arginase from the Mexican axolotl is very unstable and decays rather rapidly under the conditions that have been followed to form arginine from citrulline, aspartic acid and ATP as well as the conversion of arginine into urea and ornithine. When fresh homogenate, in which there was certainty that arginase was preserved, was added to an incubation medium where endogenous arginine was allowed to accumulate, it was readily hydrolysed; however, if the same homogenate was preincubated to destroy the arginase activity, arginine remained unchanged. The problem that immediately arises is whether enzyme stability is the only factor responsible for its capacity to split endogenous arginine, as could be inferred from the experiment shown in Fig. 5. That such might not necessarily be the case is illustrated by Fig. 4, where a liver homogenate from a metamorphosed axolotl continuously formed urea in spite of the fact that no arginase could be detected after 15min. of incubation.

Nevertheless, it is clear that Mn^{2+} has a striking effect on both the stability of the arginase molecule and its capacity to hydrolyse endogenous arginine. It could be inferred that the latter effect is a direct consequence of the former, although it could also happen that the metal ion in becoming associated with the arginase introduces a change in its conformation that renders it stable in the system explored and perhaps by acquiring a convenient form becomes associated with the arginine-synthesizing site. It should be recalled that a rather minute activity of arginase purified from axolotl liver is capable of hydrolysing endogenous arginine

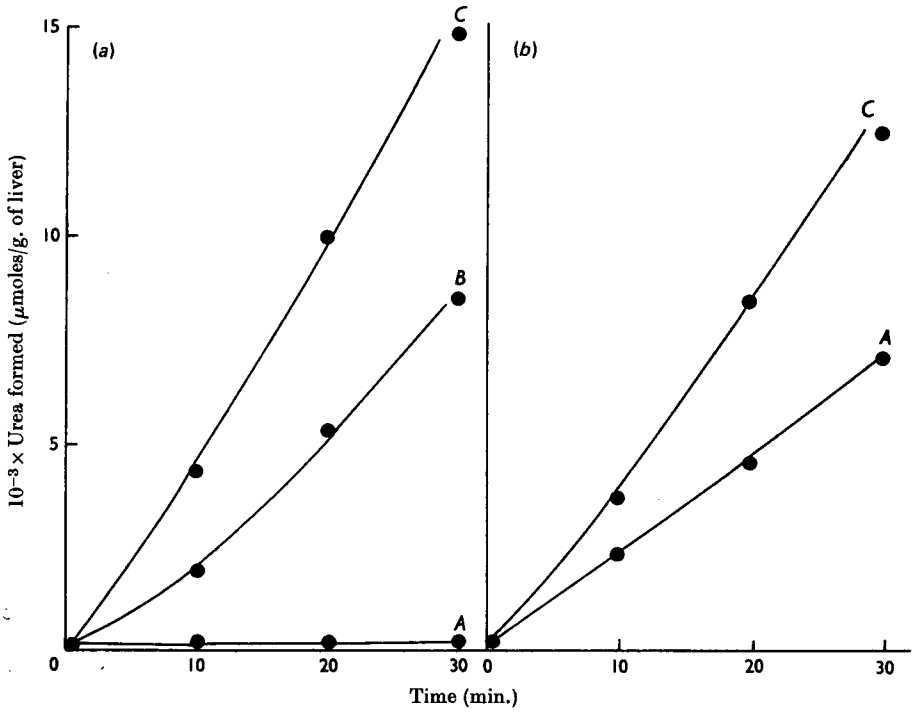

Fig. 7. Effect of Mn^{2+} on the arginase activity of axolotl (*a*) and rat (*b*) liver homogenates. The incubation system contained 2mg. of tissue, 25μmoles of glycine–NaOH buffer, pH 9·5, and 100μmoles of arginine previously adjusted to pH 9·5, final volume 1·0ml.; the temperature of incubation was 37°. $MnCl_2$ was either omitted (curves *A*) or added to give a final concentration of 2·5μM (curve *B*) or 0·25uM (curves *C*). Urea formation was determined at the indicated times.

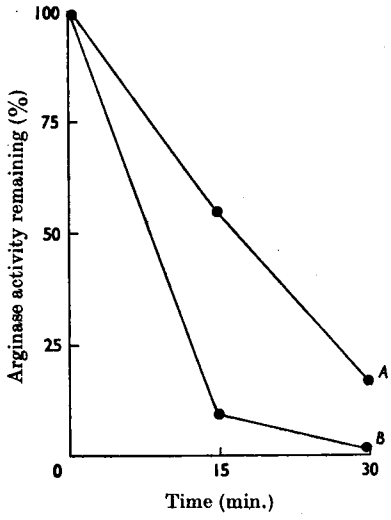

Fig. 8. Effect of arginine on the decay of axolotl liver arginase. The incubation system contained 4mg. of tissue and 25μmoles of glycine–NaOH buffer (pH 9·5)/ml. The temperature of incubation was 37°. For curve *A* the incubation system contained in addition 100μmoles of arginine previously adjusted to pH 9·5. The enzyme activity was measured at the indicated times by determining the urea formation 10min. after the addition of $MnCl_2$ to give a final concentration of 0·25mM. Correction was made for the small amount of urea produced before the addition of Mn^{2+}. For curve *B* enzyme activity was assayed by adding at the indicated times the amino acid and the $MnCl_2$ at the concentration stated above. Urea was also measured after 10min. of incubation.

when added to a system originally unable to do so (Soberón *et al.* 1967).

The protection conferred on the enzyme by Mn^{2+} may have important biological significance. Indeed, it has been demonstrated by Tono & Kornberg (1967) that during sporulation of *Bacillus megaterium* a pyrophosphatase activity appears that on electrophoresis migrates differently from the enzyme present in the spore and in the vegetative form; the new form can be converted into the latter by addition of Mn^{2+}. In addition, Mn^{2+} is required for sporulation and accumulates in the spore to an extent influenced by its concentration

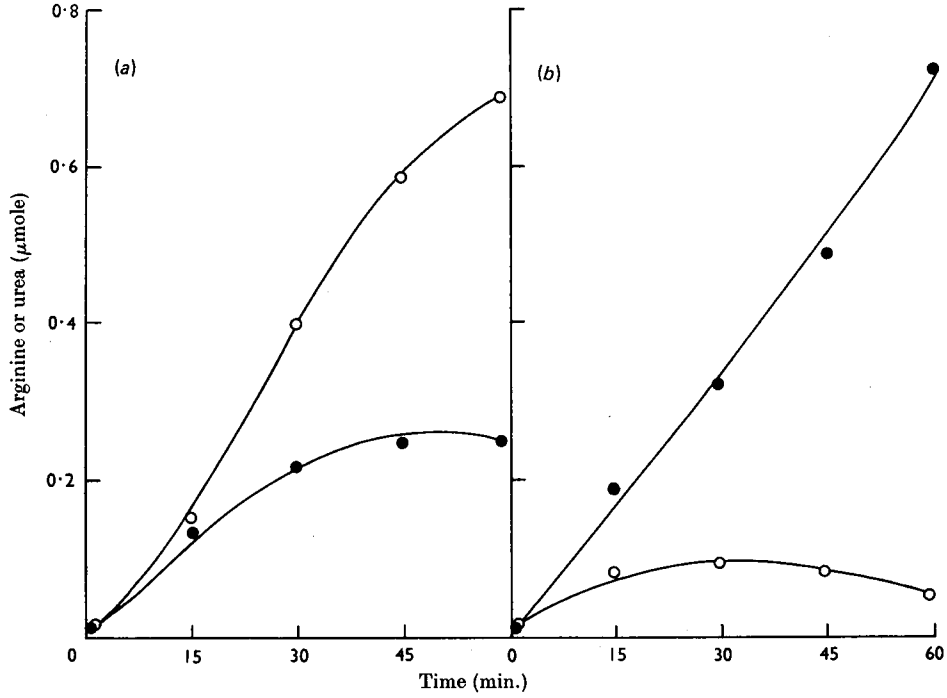

Fig. 9. Effect of Mn^{2+} on the capacity of axolotl liver arginase to hydrolyse endogenous arginine. The incubation system contained 40mg. of tissue, 50μmoles of potassium phosphate buffer, pH7·0, 5μmoles of L-citrulline, 5μmoles of L-aspartic acid, 5μmoles of ATP and 5μmoles of $MgSO_4$, final volume 1·0ml.; the temperature of incubation was 37°. Arginine (○) and urea (●) were measured at the indicated times. (*a*) Experiment run in the absence of Mn^{2+}; (*b*) $MnCl_2$ added to give a final concentration of 0·25mM.

in the culture medium (Slepecky & Foster, 1959). Also relevant is the finding of Ramaley & Bernlohr (1966) that during the sporulation of *Bacillus licheniformis* there is a great increase in arginase activity with a concomitant utilization of arginine to produce ornithine and glutamic acid, necessary for the synthesis of bacitracin, which is augmented during this period. In addition, arginase from *N. crassa*, which participates in the catabolism of arginine (Cañedo, Martuscelli & Mora, 1967; Castañeda *et al.* 1967), is highly activated by Mn^{2+} (J. Mora, personal communication). It might well be that during the metamorphosis of the Mexican axolotl there is an increase in the concentration of Mn^{2+} in the liver cells.

The different behaviour of rat and axolotl liver arginases deserves further investigation. We have previously reported that the rat enzyme is not inhibited by *p*-chloromercuribenzoate at pH9·5, gives a single peak on sucrose-density-gradient analysis and is found by differential centrifugation to be attached to particles, whereas axolotl arginase gives two peaks in the sucrose-density-gradient analysis, is inhibited by 30–40% by *p*-chloromercuribenzoate at the same pH and remains in the supernatant (Mora, Tarrab, Martuscelli & Soberón, 1965*b*; Soberón *et al.* 1967). By learning more of the characteristics of the two proteins, one could gain insight into the evolution of ureotelism. Relevant to this context is the fact that two types of arginase have been described (Mora *et al.* 1965*b*; Mora, Tarrab & Bojalil, 1966).

The more critical question at this point is to determine what metabolic change is responsible for the advent of ureotelism during metamorphosis of the Mexican axolotl. It certainly does not appear to be the same mechanism as that described in the metamorphosis of the tadpole in *Rana catesbeiana*, where a great increase in the activities of all the urea-cycle enzymes has been reported (Brown, Brown & Cohen, 1959). Neither has a selective increase in carbamoyl phosphate synthetase been found, as was described for aestivating *Xenopus laevis* (Balinsky, Choritz, Coe & Van Der Schans, 1967). We had previously suggested that an enhanced capacity to hydrolyse endogenous arginine

might play an important role in the establishment of urea biosynthesis in the Mexican axolotl. However, it is proved in the present paper that axolotl liver arginase is certainly able to split endogenous arginine.

This work was supported by Grant GB6170 from the National Science Foundation, U.S.A. The authors are grateful to Professor Philip P. Cohen and to Dr Jaime Mora for their encouraging criticism throughout this work and to Dr Balinsky for reviewing the manuscript. Recognition is given to the technical assistance provided by Miss Rocio Salceda, Miss Mercedes Baca and Mr Carlos Huitron. The advice of Dr R. R. Humphrey in the handling of the axolotl is much appreciated.

REFERENCES

Archibald, R. M. (1945). *J. biol. Chem.* **157**, 507.

Balinsky, J. B., Choritz, E. L., Coe, C. G. L. & Van Der Schans, G. S. (1967). *Comp. Biochem. Physiol.* **22**, 59.

Brown, G. W., jun., Brown, W. R. & Cohen, P. P. (1959). *J. biol. Chem.* **234**, 1775.

Brown, G. W., jun. & Cohen, P. P. (1959). *J. biol. Chem.* **234**, 1769.

Cañedo, L., Martuscelli, J. & Mora, J. (1967). *Monogr. nat. Cancer Inst.* no. 27, p. 273.

Castañeda, M., Martuscelli, J. & Mora, J. (1967). *Biochim. biophys. Acta*, **141**, 276.

Mora, J., Martuscelli, J., Ortíz-Pineda, J. & Soberón, G. (1965*a*). *Biochem. J.* **96**, 28.

Mora, J., Tarrab, R. & Bojalil, L. F. (1966). *Biochim. biophys. Acta*, **118**, 206.

Mora, J., Tarrab, R., Martuscelli, J. & Soberón, G. (1965*b*). *Biochem. J.* **96**, 588.

Ramaley, R. F. & Bernlohr, R. W. (1966). *Arch. Biochem. Biophys.* **117**, 34.

Schimke, R. T. (1964). *J. biol. Chem.* **239**, 3808.

Slepecky, R. & Foster, J. W. (1959). *J. Bact.* **78**, 117.

Soberón, G., Flores, G., Mora, J. & Torres, J. (1959). *Gac. Med. Mex.* **89**, 955.

Soberón, G., Ortíz-Pineda, J. & Tarrab, R. (1967). *Monogr. nat. Cancer Inst.* no. 27, p. 283.

Tono, H. & Kornberg, A. (1967). *J. Bact.* **93**, 1819.

Van Pilsum, J. E., Martin, R. P., Kito, E. & Hess, J. (1956). *J. biol. Chem.* **222**, 225.