Biochem. J. (1965) **96**, 28

The Regulation of Urea-Biosynthesis Enzymes in Vertebrates

By J. MORA, J. MARTUSCELLI, JUANA ORTIZ-PINEDA and G. SOBERÓN
Departamento de Bioquímica, Instituto Nacional de la Nutrición, México, D.F., México

(*Received* 30 *November* 1964)

1. Carbamoyl phosphate synthetase, ornithine transcarbamoylase, the arginine-synthetase system and arginase were measured in the livers of ammoniotelic, ureotelic and uricotelic animals. The chelonian reptiles, whose nitrogen excretory patterns vary according to the habitat, and the Mexican axolotl, a neotenic species, were also studied. 2. The levels of the activities of the first three enzymes mentioned correlate with the amount of nitrogen excreted as urea. 3. The terrestrial turtle, which excretes mainly uric acid, maintains a high arginase activity but has very low levels of the activities of the other three enzymes. 4. The first three enzymes of the urea cycle vary in the phylogenic scale in a co-ordinated manner, which suggests that they are under the same regulatory mechanism. 5. Urea formation from endogenous arginine *in vitro* has a low efficiency in the Mexican axolotl. 6. The induction of metamorphosis in the Mexican axolotl by the administration of L-tri-iodothyronine, which causes a shift from ammonio-ureotelism to complete ureotelism, is accompanied by an increase mainly in carbamoyl phosphate synthetase and also by an improvement in the efficiency of hydrolysis of endogenous arginine *in vitro* to give urea. 7. The results obtained by differential centrifugation of the urea-cycle enzymes in rat and Mexican-axolotl livers are presented. The location requirements for the integration of a metabolic cycle are discussed.

Regulatory mechanisms in bacteria involve changes either in enzyme activity or in enzyme synthesis. Moreover, metabolically linked enzymes vary in a co-ordinated form (Jacob & Monod, 1961). It has been implied that similar mechanisms control the cellular activity of multicellular organisms, and there are a few indications that this may be the case (Walker & Walker, 1962; Schimke, 1962*a*; Pitot & Peraino, 1963), but the results, in general, are not encouraging. One of the main difficulties involved in this type of study in adult animals is the fact that phenotypic changes are greatly minimized (Schimke, 1963).

An approach to this problem might be to study the natural mutants that have been selected during the course of evolution, i.e., the different species, the activities of a group of metabolically linked enzymes. The enzymes of urea biosynthesis were selected for this work because they have been well characterized and their appearance can be traced back in evolution (Brown & Cohen, 1960). The species chosen included clearly defined ureotelic animals where the urea enzymes are present (Brown & Cohen, 1960) and well-confirmed uricotelic animals where most of the corresponding enzymes are absent (Cohen & Brown, 1960). The chelonian reptiles were selected because their nitrogen excretory pattern comprises ammonio-ureotelism and uricotelism, according to their habitat (Moyle, 1949), and the unique opportunity of studying the disappearance of a well-established metabolic cycle in a single biological Order is offered. In addition, a neotenic species, the Mexican axolotl, which is mainly ammoniotelic (Munro, 1953; Soberón, Flores, Mora & Torres, 1959), was also included.

EXPERIMENTAL

The animals were obtained from commercial and private sources. They were classified by conventional criteria. Some of the Mexican axolotls were induced to metamorphose by the intraperitoneal administration of 100μg. of L-tri-iodothyronine (Glaxo Laboratories Ltd., Greenford, Middlesex). They were killed 6–7 weeks after. Fresh livers here homogenized at 4° in a Potter–Elvehjem homogenizer and the enzyme assays were carried out immediately after the preparation of the samples. The activities of carbamoyl phosphate synthetase (ATP–carbamate phosphotransferase, EC 2.7.2.2) and ornithine transcarbamoylase (carbamoyl phosphate–L-ornithine carbamoyltransferase, EC 2.1.3.3) were determined by the procedures described by Brown & Cohen (1959). Carbamoyl phosphate synthetase was determined in the presence of an excess of ornithine transcarbamoylase, which was purified 35-fold by submitting an acetone-dried powder of rat liver to chromatography on DEAE-cellulose and specifically eluting the enzyme with 0·5M-ornithine solution, pH7.

The carbamoyl phosphate used as substrate was prepared by the method of Metzenberg, Marshall & Cohen (1960). All other substrates were purchased from the California Corp. for Biochemical Research, Los Angeles, Calif., U.S.A. Argininosuccinate synthetase [L-citrulline–L-aspartate ligase (AMP), EC 6.3.4.5] and argininosuccinase (L-argininosuccinate arginine-lyase, EC 4.3.2.1) were measured as a single activity (arginine-synthetase system) by the production of arginine (Van Pilsum, Martin, Kito & Hess, 1956) or urea (Brown & Cohen, 1959), after extraction with a 0·1% solution of cetyltrimethylammonium bromide (technical grade; Eastman Kodak Co., Rochester, N.Y., U.S.A.). Arginase (L-arginine ureohydrolase, EC 3.5.3.1) was measured by the Brown & Cohen (1959) method but in water homogenates; K_m and V were determined in each liver extract from activity–substrate concentration curves. All determinations were performed in duplicate. The enzyme activities are expressed as μmoles of product produced/hr./g. wet wt. of tissue. An analysis was performed on stones found in the cloaca of the species *Gopherus flavomarginatus*; they were composed of uric acid (more than 90%), which was characterized by a positive murexide test and the absorption spectrum after crystallization ($\lambda_{max.}$ at 285–290 mμ).

The differential centrifugation of the livers of rat and Mexican axolotl was performed as described by Novikoff & Heus (1963).

Table 1. *Activities of urea-cycle enzymes in the liver of different species*

B.L.D., Below limit of detection.

Common name	Binominal name	Enzyme activity (μmoles of product/hr./g. wet wt.)				Percentage of N excreted	
		Carbamoyl phosphate synthetase	Ornithine trans-carbamoylase	Arginine-synthetase system	Arginase	Urea	Uric acid
Rat	*Rattus norvegicus*	260	18000	72·0	56400	90	
		275	21600	78·0	50400	(Schimke, 1962*b*)	
		247	18000	72·0	46800		
Mouse (Ajax DBA strain)	*Mus musculus*	180	14500	66·0	79000		
		180	14500	67·0	76800		
		165	13000	60·0	68500		
Frog	*Rana montezumae*	225	18000	90·0	80000	90	
		237	18000	90·0	96000	(Brown *et al.* 1959)	
		211	9450	42·0	51000		
		211	9450	42·0	51000		
Semi-aquatic turtle	*Pseudemys scriptae*	140	7570	46·0	45600	50	
		144	8100	43·0	60000	(Moyle, 1949)	
		120	7500	43·0	55000		
		95	6500	40·0	50000		
Semi-aquatic turtle	*Kinosternon hirtipes*	133	8900	45·0	37800	50	
		128	6850	35·0	38000	(Moyle, 1949)	
		139	8750	—	—		
		124	7500	—	—		
		292	20200	90	57600		
Mexican axolotl	*Ambystoma mexicanum*						
	Non-metamorphosed	81	5670	30·9*	78000	20–40	
		81	5940	30·9*	37000	(Munro, 1953)	
	Metamorphosed	225	8100	41·25*	52200	90	
Terrestrial turtle	*Gopherus flavomarginatus*	9·0	276·0	10·0	28000	(Munro, 1953)	Great amount
	Gopherus berlandieri	27·0	900·0	18·0	29600		(Present paper)
Lizard	*Ctenosaura pectinata*	B.L.D.	36·0	1·5	1800		85 (Brown *et al.* 1959)
Chicken (White Leghorn)	*Gallus domesticus*	B.L.D.	B.L.D.	B.L.D.	140		85 (Brown *et al.* 1959)
					120		
					144		
Rattlesnake	*Crotalus molossus nigrescens*	B.L.D.	B.L.D.	B.L.D.	115		85 (Brown *et al.* 1959)
					84		
					102		
					56		

* These values represent the total arginine and urea produced in the incubation system (see the text).

RESULTS

Activities of urea-cycle enzymes in the liver of different species. The results of the assays performed with the livers of the animals studied are given in Table 1. Individual determinations are included. Statistical treatment of the data for a given species was not attempted, since the purpose of this work is to establish the relation between the enzyme activities present in each animal.

In most cases there is a good correlation between the presence or absence in the liver of the first three enzymes of urea biosynthesis, as well as their approximate content, and the nitrogen excretion pattern. Thus rats and mice and some frogs excrete about 90% of their nitrogen as urea (Brown, Brown & Cohen, 1959), and they have the higher enzyme activities found; semi-aquatic turtles excrete 50% of their nitrogen as urea (Moyle, 1949) and they have about half of the enzyme concentration of the completely ureotelic animals. The Mexican axolotl, a neotenic species, eliminates 20–40% of its nitrogen as urea (Munro, 1953; Soberón *et al.* 1959; Cragg, Balinsky & Baldwin, 1961) and the activities of its enzymes are in accordance with this fact. The terrestrial turtles that excrete mainly uric acid have very low activities of the first three enzymes but, surprisingly, they retain some arginase activity, although it is lower than that of the other chelonian reptiles. The activity of carbamoyl phosphate synthetase in the liver of this species was the same in the presence and in the absence of an excess of purified ornithine transcarbamoylase, and no activity was detected when ornithine was omitted from the incubation system. In the other uricotelic species carbamoyl phosphate synthetase, ornithine transcarbamoylase and the arginine-synthetase system are below the limits of detection of the assay procedures used, except for the lizard, which has traces of ornithine transcarbamoylase. More concentrated homogenates and longer incubation periods were used to assay these species to ascertain a real lack of a given activity. In the metamorphosed Mexican axolotl and in some frogs the stated correlation holds only for carbamoyl phosphate synthetase (see the Discussion section). The findings with chicken liver agree well with the report of Tamir & Ratner (1963) and those for the other species with the data of Cohen & Brown (1960).

A low arginase activity was detected in the livers of uricotelic animals (with the exception of the terrestrial turtle, which, as indicated above, has a considerable enzyme content). The enzyme in the livers of uricotelic species, with the exception of the terrestrial turtle, differs from that in the liver of ureotelic species in its K_m for L-arginine (Table 2). In addition, it was consistently found that the arginase from ureotelic animals was inhibited by substrate, whereas such was not the case with the arginase from uricotelic animals.

Evidence that the differences between the K_m values of arginase from ureotelic and uricotelic species is not a consequence of the assays being carried out in crude homogenates is that the K_m for crystalline arginase from ox, sheep and horse livers is 7mM (Bach & Killip, 1961). Other results (J. Mora, R. Tarrab, J. Martuscelli & G. Soberón, unpublished work) indicate that the K_m remained about the same after purification of the chicken-liver arginase, and this arginase is a different protein from that present in the livers of ureotelic animals.

The Mexican axolotl was of particular interest

Table 2. *K_m values of liver arginase in different species*

Species	K_m for arginine (mM)
Rat	20–40
Mouse	20–40
Frog	20–40
Axolotl	16
Turtle (*Pseudemys*)	13–25
Turtle (*Kinosternon*)	6–13
Turtle (*Gopherus*)	40
Lizard	100–200
Chicken	100–200
Rattlesnake	100–200

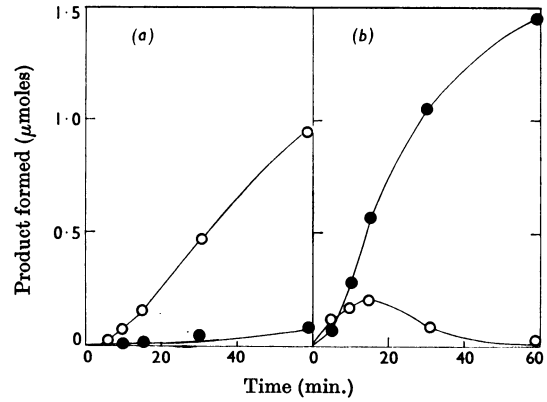


Fig. 1. Conversion of endogenous arginine into urea in the livers of the Mexican axolotl (*a*) and the rat (*b*). The incubation system contained 40mg. wet wt. of liver tissue, 5μmoles each of L-citrulline, aspartic acid, ATP and $MgSO_4$ and 50μmoles of potassium phosphate buffer, pH 7·0, in a final volume of 1ml. Arginine (○) and urea (●) were determined at the indicated times.

because previously we had reported that the arginine-synthetase system was not detectable in its liver, a rather unexpected finding, since some of its nitrogen is excreted as urea (Soberón *et al.* 1959). Further studies proved that the enzyme could be detected only when assayed by arginine production but not by the formation of urea, contrary to what is observed in ureotelic animals. This is illustrated in Fig. 1, where the formation of arginine and urea by Mexican-axolotl-liver homogenate is compared with rat-liver homogenate under similar conditions. That arginine and no other guanidino compound capable of giving the Sakaguchi reaction is the product of the incubation system was demonstrated by paper chromatography [in butan-1-ol–formic acid–water (63:20:17, by vol.); R_F values: arginine, 0·11; product of the incubation system, 0·11]; that the arginine is being formed by the system described by Ratner (1955) can be inferred from the fact that there is an absolute requirement for citrulline, aspartic acid and ATP to form arginine.

Table 3 presents a comparative survey with a greater number of axolotls and with the rat. Batches A and B, which differ in the time of collection from the lake, show different capacities for urea and arginine production under the conditions of the assay for the arginine-synthetase system; however, the total activity as determined by the addition of the arginine and urea produced is quite similar in both batches.

The diminished capacity of the Mexican axolotl to produce urea cannot be explained by a low arginase content under the conditions of the assay, since its activity, as judged by the ability to hydrolyse exogenous arginine, is comparable with that present in the rat, which is highly efficient in urea formation (Table 3).

An attempt to induce metamorphosis by the intraperitoneal administration of L-tri-iodothyronine was only partially succesful, since only three animals out of ten survived long enough to allow their study. One of them, the most completely metamorphosed, judged from the external appearance, showed a capacity for urea formation, under the assay conditions of the arginine-synthetase system, similar to that of frogs, which do not have co-ordinated activities of carbamoyl phosphate synthetase and ornithine transcarbamoylase (see the Discussion section and Fig. 6). The other frogs, which have these enzymes co-ordinated, show an efficiency of urea production from endogenous arginine similar to that manifested by the rat (Fig. 2). In this respect it is highly relevant that the increased capacity for urea formation developed by the metamorphosed animal is probably not due to a greater arginase content, since this activity did not increase as measured under optimum conditions (Table 1) as well as under the conditions of the arginine-synthetase assay system (Table 3).

When arginase is inhibited by isoleucine (Hunter & Downs, 1945) in rat-liver homogenate, as tested in the arginine-synthetase assay system, urea formation is decreased and arginine accumulates, a situation that simulates the behaviour of axolotl (Fig. 3). The addition of isoleucine to liver homogenate of non-metamorphosed axolotl did not produce

Table 3. *Arginine-synthetase-system and arginase activities in the livers of the non-metamorphosed Mexican axolotl and the rat*

The conditions of the system are the same as in Fig. 1.

	Enzyme activity (μmoles of product/hr./g. wet wt.)		
	Arginine-synthetase system		Arginase (under conditions of arginine-synthetase assay)
Species	Urea formation	Arginine formation	
Non-metamorphosed axolotl			
Batch A	0	26·3	108
	0	23·7	
	0	25·7	
	0	30·0	
Batch B	7·5	21·2	
	11·4	19·2	175
	17·6	14·7	
	19·0	11·9	
	19·0	11·9	
Metamorphosed axolotl (from batch A)	31·0	10·2	87·5
Rat	60·0	0·0	117

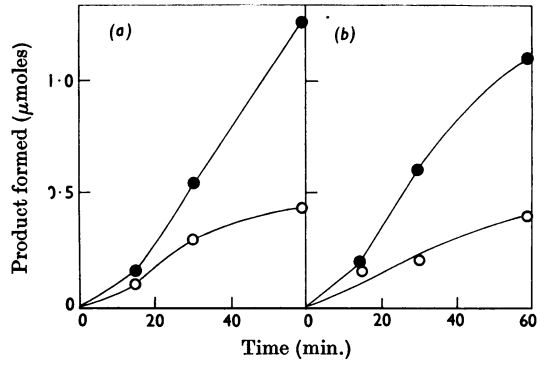


Fig. 2. Conversion of endogenous arginine into urea in the livers of the metamorphosed Mexican axolotl (*a*) and some frogs (*b*). The frog utilized in this experiment belongs to the group where carbamoyl phosphate synthetase and ornithine transcarbamoylase were not co-ordinated (see the text). The conditions were as indicated for Fig. 1. Arginine (○) and urea (●) were determined at the indicated times.

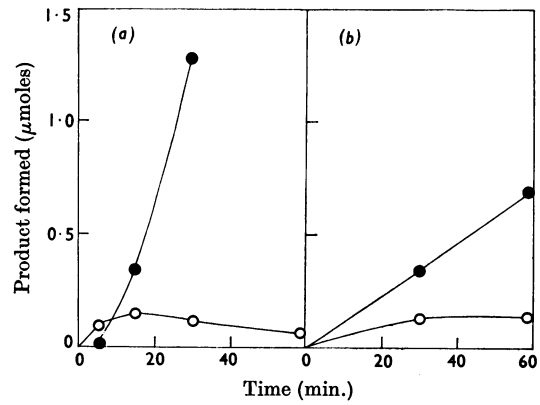


Fig. 4. Conversion of endogenous arginine into urea by the livers of ureotelic (*a*) and uricotelic (*b*) turtles. The conditions were as indicated for Fig. 1. Arginine (○) and urea (●) were determined at the times indicated.

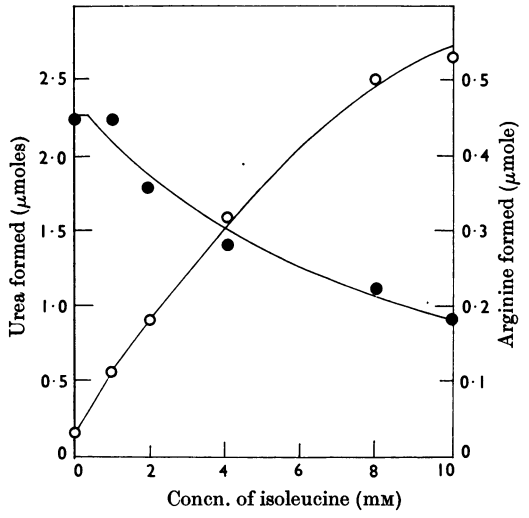


Fig. 3. Production of urea and arginine by rat-liver homogenate in the presence of an arginase inhibitor. The incubation time was 60min. Other conditions were as indicated for Fig. 1. Arginine (○) and urea (●) were determined in the presence of the indicated concentrations of isoleucine.

any substantial change (10% inhibition of the arginine-synthetase system). If the arginine-synthetase incubation system containing axolotl liver is stopped by the addition of trichloroacetic acid and this compound extracted with ether, the arginine produced becomes available to the freshly added axolotl-liver homogenate. Attempts to achieve the same effect by other means, e.g. heating, ultrasonic treatment, extraction with sodium deoxycholate, were unsuccessful; the detergent caused inactivation of the arginine-synthetase system. The capability of exogenous arginase to cause the appearance of urea from the endogenous compound formed is a further proof of the nature of the product formed from citrulline and aspartic acid.

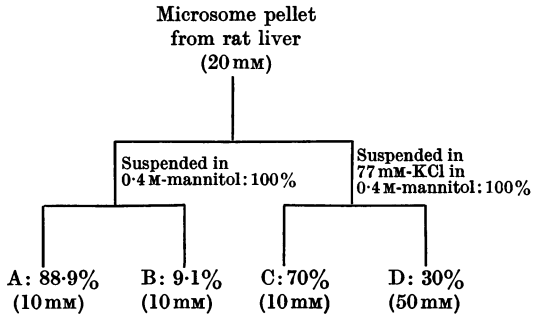
The arginase present in the liver of the uricotelic terrestrial turtle is able to hydrolyse endogenous arginine like other ureotelic chelonians, although the absolute amount of urea produced in a given time is less, a consequence of the lower enzyme content (Fig. 4).

Intracellular distribution of urea-cycle enzymes. The intracellular distribution of the urea-cycle enzymes was investigated in Mexican-axolotl and rat livers to study the physical relations of enzymes that participate in a metabolic cycle, and also to investigate whether the differences in the efficiency of urea production in the arginine-synthetase system could be explained by different location of the participating enzymes (Table 4). The enzyme location for rat liver confirms previous work (Cohen & Hayano, 1948; Ratner, 1955; Schimke, 1962*a*); of note is the finding of a sizable amount of carbamoyl phosphate synthetase and ornithine transcarbamoylase in the nuclear fraction. Mitochondrial contamination of this fraction, as demonstrated by microscopic studies, was less than 5%.

It is apparent that the urea-biosynthesis enzymes, with the exception of the arginine-synthetase system, are distributed in a different manner in the livers of the species being compared. Whereas in the rat carbamoyl phosphate synthetase and orni-

Table 4. *Differential centrifugation of liver homogenates from rat and non-metamorphosed Mexican axolotl*

	Distribution of activity (% of total)							
	Carbamoyl phosphate synthetase		Ornithine trans-carbamoylase		Arginine-synthetase system		Arginase	
Fraction	Rat	Axolotl	Rat	Axolotl	Rat	Axolotl	Rat	Axolotl
Nuclei	34	8·6	41	37·0	0	0	40	3·0
Mitochondria	66	44·0	53	32·0	0	0	19	1·0
Microsomes	0	0·0	2	0	0	0	37	3·0
Supernatant	0	47·0	4	31·0	100	100	4	93·0



Scheme 1. Release of the microsomal arginase by KCl. The precipitates (A and D) were resuspended in KCl solution free of mannitol. All fractions were assayed in the presence of the same amount of KCl except the original pellet. The numbers in parentheses indicate the K_m values, and the percentages refer to the distribution of enzyme activity.

thine transcarbamoylase are mainly located in mitochondria and nuclei, as defined by the procedure employed (Novikoff & Hens, 1963), in the axolotl an appreciable amount of these activities is free in the supernatant; even more striking, most of the arginase activity remains soluble, in contrast with the rat where the enzyme is bound to the particles and a great amount is linked to the microsome fraction.

The K_m value of the microsomal arginase (200 mM) was higher than that observed in the total water homogenate from rat liver, and also higher than that in the axolotl liver (Table 2). It was decided to determine the K_m of the enzyme released from the microsomes by potassium chloride (Rosenthal, Gottlieb, Gorry & Vars, 1956). Since sucrose interferes with the colour development with urea, mannitol was employed as suspending medium after separation of the microsomes in sucrose. The microsome pellet was divided in two parts; one was resuspended in 0·4 M-mannitol, the other in 77 mM-potassium chloride in 0·4 M-mannitol and the suspension centrifuged again at 114000*g* for 70 min. (Scheme 1). The free enzyme (B) and the enzyme released (C) have K_m values about 10 mM, even lower than the value originally found in total homogenate of ureotelic animals. The same value was also obtained when the microsomal enzyme was assayed in the presence of potassium chloride without removing the particles (A), which suggests liberation of the bound enzyme. The higher K_m observed in fraction (D) is probably due to the fact that some arginase is still attached to the microsomes.

DISCUSSION

The present findings throw light on the disappearance of a well-established metabolic cycle. The enzymes that participate in the Krebs–Henseleit cycle are present in ureotelic animals. The terrestrial turtle, although uricotelic, retains arginase from its ureotelic ancestors, indicating perhaps that it is in the process of evolution to a completely uricotelic pattern, characterized by the almost total absence of the first three enzymes and a very low activity of arginase, as manifested in the Classes Crocodilia, Aves and Squamata. This means that, in all probability, a further mutation in the terrestrial turtle with the complete disappearance of its arginase activity would not be a handicap for survival in their habitat. The fact that the activities of three enzymes linked in a multi-enzyme system simultaneously decreased in the terrestrial turtle suggests that they are regulated by the same mechanism. Further support for this assumption is that uricotelism, evolved from ureotelism, is accompanied by an arginine requirement (Oesterling, Womack & Rose, 1946; Holt, György, Pratt, Snyderman & Wallas, 1960); this indicates that, once the urea cycle has been established, ornithine transcarbamoylase and the arginine-synthetase system cannot be preserved for their original function, arginine biosynthesis. Again, arginine is a partially indispensable amino acid in ureotelism;

it is required by the rat (Oesterling *et al.* 1946) and by rapidly growing children (Holt *et al.* 1960). Moreover, HeLa cells, not engaged in urea biosynthesis, can still form arginine from citrulline, since they keep the arginine-synthetase system but do not have ornithine transcarbamoylase (Schimke, 1964).

A correlation exists between the activities found for carbamoyl phosphate synthetase, ornithine transcarbamoylase and the arginine-synthetase system among the different species studied, in spite of the wide range of the activities detected. This can be better appreciated with the help of Fig. 5. This correlation could be interpreted as a 'co-ordinated variation' according to the concepts advanced by Jacob & Monod (1961), who have postulated a model for a regulatory control at the genetic level. However, some frogs and the metamorphosed Mexican axototl fail to keep the stated relation, showing a high value of carbamoyl phosphate synthetase for the corresponding activity of ornithine transcarbamoylase. In this respect, in multicellular organisms it is likely that other levels of co-ordination should exist, since a given hormone can elicit the expression of a group of genes not necessarily physically linked (Clever, 1961).

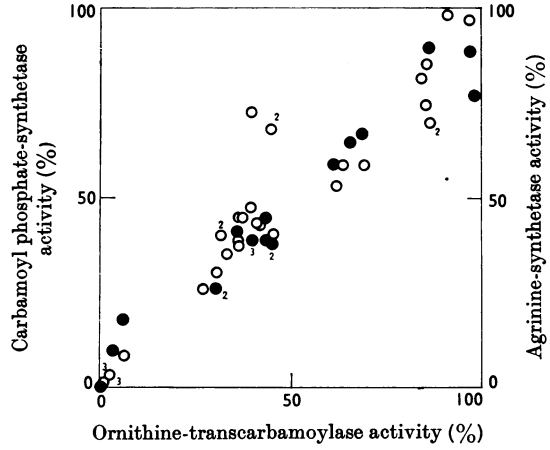


Fig. 5. Relation between the activities of carbamoyl phosphate synthetase, ornithine transcarbamoylase and the arginine-synthetase system among species with different nitrogen excretory patterns. The values are expressed as the percentages of the highest activities: 21000 units/g. wet wt. for ornithine transcarbamoylase, 300 units/g. for carbamoyl phosphate synthetase and 100 units/g. for the arginine-synthetase system. ○, Carbamoyl phosphate synthetase–ornithine transcarbamoylase plot; ●, arginine–synthetase system–ornithine transcarbamoylase plot. The numbers on the Figure denote numbers of overlapping points.

Carbamoyl phosphate synthetase, ornithine transcarbamoylase and the arginine-synthetase system vary in the same manner and rather closely under the influence of different physiological and experimental conditions, e.g., the metamorphosis of the tadpole (Brown *et al.* 1959), the development of the pig and the rat (Kennan & Cohen, 1959), feeding with a low-protein or a high-protein diet (Schimke, 1962*a*, 1963; Rosado, Flores, Mora & Soberón, 1962; Flores, Rosado, Torres & Soberón, 1962), the ingestion of a protein of low biological value (Flores *et al.* 1962), starvation (Schimke, 1962*b*), adrenalectomy (Schimke, 1963; McLean & Gurney, 1963) and hypophysectomy (McLean & Gurney, 1963). Within a given species, the semi-aquatic turtle (*Kinosternon hirtipes*), there are different activities of urea-cycle enzymes and they also vary in a similar manner, perhaps indicating phenotypic changes. Although arginase also varied in the same direction it did not follow very closely the changes of the other enzymes.

The Mexican axolotl on metamorphosis changes from ammonio-ureotelism to complete ureotelism (Munro, 1953). We present two facts that could have some meaning with regard to the shift in the nitrogen excretion pattern. The first refers to the increase in carbamoyl phosphate synthetase, more noticeable than that observed for ornithine transcarbamoylase and the arginine-synthetase system, while arginase remains about the same (Table 1). The development of the frog, which also implies a change from ammoniotelism to ureotelism, is different in the sense that it is accompanied by an augmentation of all urea enzymes (Brown *et al.* 1959). However, the results obtained with the group of frogs assayed show that some of them behave as Brown *et al.* (1959) have described, whereas others closely resemble the metamorphosed axolotl. The second fact concerns the development of an increased efficiency of urea formation from endogenous arginine. The decreased efficiency of the non-metamorphosed axolotl could be explained by postulating that the arginine produced from citrulline and aspartic acid is not released to become available to the soluble arginase, as suggested by the experiment where it was freed by the addition of trichloroacetic acid. One might speculate whether the different location of the arginase in the non-metamorphosed axolotl, as compared with the rat, a completely ureotelic animal, has some significance in relation to the observed difference in such efficiency. The accumulation of arginine by rat-liver homogenate in the presence of isoleucine might be due, not only to the inhibition of arginase, but also to a liberation of the enzyme from the particles.

The presence of arginase bound to different particles can be explained as an artifact consequent on arginase being a basic protein that readily

attaches to particles containing nucleic acids (Rosenthal *et al.* 1956); nevertheless, it is completely soluble in the axolotl and the K_m is the same for both enzymes, provided that the one present in the rat liver is released. Therefore, and within the limitations of the procedure employed, the results given by the differential centrifugation represent a real situation in the cell. Thus the location of a fraction of arginase in the microsomes can be related to the excretion of urea. The connexion of carbamoyl phosphate synthetase and ornithine transcarbamoylase with mitochondria could be rationalized if it is assumed that citrulline can be utilized as a source of energy, a possibility enhanced by the finding of Kennedy, Raijman, Lizarralde, Tigier & Grisolia (1964) that ATP can be formed by carbamoyl phosphate and ADP, a situation that is operative *in vivo* in unicellular organisms (Knivett, 1954; Schimke & Barile, 1963).

For the functioning of a metabolic cycle the participating enzymes must not only be present but also properly located. The existence of the urea cycle in *Neurospora crassa* has been postulated because of the presence of arginase and the enzymes involved in arginine biosynthesis (Srb & Horowitz, 1944). Further, exogenous arginine, ornithine or citrulline can replace proline in a mutant that requires the latter for growth, although it can synthesize arginine (Vogel & Kopac, 1959), which indicates that arginase can utilize exogenous but not endogenous arginine. Wagner & Bergquist (1963) found that the synthesis of isoleucine requires, not only the enzymes that participate in the biosynthetic path, but their physical connexion.

In the rat, where the urea-biosynthesis enzymes constitute a metabolic cycle, apparently these enzymes are not physically connected, and this condition calls for some other device to make the cycle to function, i.e. there must exist a mechanism to make citrulline leak out of mitochondria for conversion into arginine, which should then be transferred to the microsomes to be split into urea, for excretion, and ornithine, which must pass back to the mitochondria for the synthesis of citrulline.

The authors are indebted to Professor Philip P. Cohen for his stimulating suggestions and to Dr R. Martin del Campo for his help in the classification of the biological species. This investigation was supported with a research grant given by the Rockefeller Foundation (RF 62005).

REFERENCES

Bach, S. J. & Killip, J. D. (1961). *Biochim. biophys. Acta*, **47**, 336.

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.

Brown, G. W., jun. & Cohen, P. P. (1960). *Biochem. J.* **75**, 82.

Clever, U. (1961). *Chromosoma*, **12**, 607.

Cohen, P. P. & Brown, G. W., jun. (1960). In *Comparative Biochemistry*, vol. 2, p. 161. Ed. by Florkin, M. & Mason, H. S. New York: Academic Press Inc.

Cohen, P. P. & Hayano, M. (1948). *J. biol. Chem.* **172**, 405.

Cragg, M. M., Balinsky, J. B. & Baldwin, E. (1961). *Comp. Biochem. Physiol.* **3**, 227.

Flores, G., Rosado, A., Torres, J. & Soberón, G. (1962). *Amer. J. Physiol.* **203**, 43.

Holt, L. E., György, P., Pratt, L. E., Snyderman, S. E. & Wallas, W. M. (1960). *Protein and Amino Acid Requirements in Early Life*, p. 32. New York: New York University Press.

Hunter, A. & Downs, C. F. (1945). *J. biol. Chem.* **157**, 427.

Jacob, F. & Monod, J. (1961). *J. molec. Biol.* **3**, 318.

Kennan, A. L. & Cohen, P. P. (1959). *Developmental Biol.* **1**, 511.

Kennedy, J., Raijman, L., Lizarralde, G., Tigier, H. & Grisolia, S. (1964). *Fed. Proc.* **23**, 476.

Knivett, V. A. (1954). *Biochem. J.* **56**, 602.

McLean, P. & Gurney, M. W. (1963). *Biochem. J.* **87**, 96.

Metzenberg, R. L., Marshall, M. & Cohen, P. P. (1960). *Biochem. Prep.* **7**, 23.

Moyle, V. (1949). *Biochem. J.* **44**, 581.

Munro, A. F. (1953). *Biochem. J.* **54**, 29.

Novikoff, A. B. & Heus, M. (1963). *J. biol. Chem.* **238**, 710.

Oesterling, M. J., Womack, M. & Rose, W. C. (1946). *J. biol. Chem.* **166**, 585.

Pitot, H. C. & Peraino, C. (1963). *J. biol. Chem.* **238**, PC1910.

Ratner, S. (1955). In *Methods in Enzymology*, vol. 2, p. 358. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.

Rosado, A., Flores, G., Mora, J. & Soberón, G. (1962). *Amer. J. Physiol.* **203**, 37.

Rosenthal, O., Gottlieb, B., Gorry, J. D. & Vars, H. M. (1956). *J. biol. Chem.* **223**, 469.

Schimke, R. T. (1962*a*). *J. biol. Chem.* **237**, 459.

Schimke, R. T. (1962*b*). *J. biol. Chem.* **237**, 1921.

Schimke, R. T. (1963). *J. biol. Chem.* **238**, 1012.

Schimke, R. T. (1964). *J. biol. Chem.* **239**, 136.

Schimke, R. T. & Barile, M. F. (1963). *J. Bact.* **86**, 195.

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

Srb, A. M. & Horowitz, N. H. (1944). *J. biol. Chem.* **154**, 129.

Tamir, H. & Ratner, S. (1963). *Arch. Biochem. Biophys.* **102**, 249.

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

Vogel, R. A. & Kopac, M. J. (1959). *Biochim. biophys. Acta*, **36**, 505.

Wagner, R. D. & Bergquist, A. (1963). *Proc. nat. Acad. Sci., Wash.*, **49**, 892.

Walker, M. S. & Walker, J. B. (1962). *J. biol. Chem.* **237**, 437.