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Localization and Properties of Arginase in the Developing Quail Embryo, Coturnix Coturnix Japonica

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LOCALIZATION AND PROPERTIES OF ARGINASE IN THE DEVELOPING QUAIL EMBRYO, *Coturnix coturnix japonica*

by

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VITA

Peter Houtman, son of Johanna and Hendrik Houtman, was born in Groningen, The Netherlands, on July 19, 1955. He immigrated to the United States on February 2, 1957.

He received his elementary education in Oak Park, Illinois, and attended Oak Park River Forest High School, Oak Park, Illinois. He graduated from high school in 1974. After graduation, he attended Triton College until the summer of 1976.

He then transferred to Loyola University of Chicago, Illinois. In May, 1978, he received his Bachelor of Science. In May, 1978, he began his research under the guidance of Dr. Mark Goldie, which has continued to the present time.

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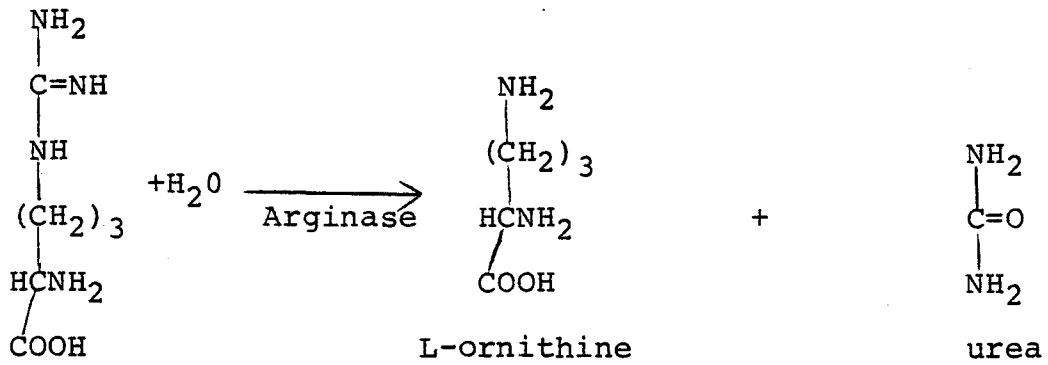
INTRODUCTION

L-arginine aminohydrolase (E.C. 3.5.3.1.) catalyzes the irreversible cleavage of arginine to form L-ornithine and urea.



L-arginine L-ornithine urea

With respect to their nitrogenous waste products, vertebrate animals are placed into three groups: ammoniotelic, ureotelic, or uricotelic. Ammoniotelic animals, such as most fishes, excrete ammonia as their principal nitrogenous end product. Ureotelic metabolism, in which urea is the principal nitrogenous waste product, is present in mammals, adult anurans, turtles, and elasmobranches. The third type of major excretory end product is uric acid, which is formed by birds, and reptiles.

Of the three principal nitrogenous waste products, ammonia is the most toxic, urea is less toxic, and uric acid is the least toxic. One of the factors which is associated with the excreted nitrogenous end product is the animals

osmotic environment. Animals which live in a watery environment, such as fish, excrete ammonia which dissolves in the aqueous environment. Birds which have a uricotelic metabolism, excrete uric acid. This excretion of uric acid is advantageous to the bird because uric acid is non-toxic and relatively insoluble, this promoting the retention of water. When the bird embryo begins to develop, the water content in the egg is high compared to the embryo. The principal nitrogenous waste product can be either ammonia or urea. As the embryo develops there is an increase in the nitrogenous waste products in the egg. If the nitrogenous waste products are toxic, the embryo would soon die. Therefore there must be a decrease in toxicity of the nitrogenous waste products if the growing embryo is to survive. The production of ammonia and urea should therefore decline as the embryo develops, and the amount of uric acid should increase. If this concept is true, the presence or absence of arginase may be of considerable developmental importance, either to excretion of urea or to some other process.

From the liver of many mammals (man, dog, pig, cat, rabbit, rat, guinea-pig, ox, calf, and horse), two arginase isozymes are resolved by DEAE-cellulose chromatography. They are designated as A_1 and A_3. Both have a molecular weight of about 130,000 (Porembska et al., 1971). A_1 is the main isozyme in man, dog, cat, rat, and rabbit, whereas A_3

predominates in horse, ox, calf, and pig. Porembska (1973) has also shown that mammalian hepatic arginase is probably a tetrameric protein. Upon treatment with 8 M-urea, the arginase dissociates into four inactive polypeptide chains, each with a molecular weight of about 30,800 (Hirsh-Kolb et al., 1970). In the kidney of ureotels there appear to be two isozymes (A_1 and A_4), whose molecular weights are the same as those in the mammalian liver (about 130,000). The isozyme A_4 represents about 90% of the activity in the kidney.

Uricotelic arginases have molecular weights about 280,000, and much lower affinities for arginine (Km's are approximately 100 mM). Reddy and Campbell (1970) have suggested that uricotelic arginase has an octomeric structure.

The substantial difference between Michaelis-Menton constants of ureotelic arginases from mammalian livers (Km less than 10 mM) and those of arginases from kidneys of uricotelic animals (Km approximately 100 mM) presumably reflects the physiological adaptation of mammals to excrete urea as a major excretory waste product. Therefore the arginases found in the kidneys of birds may be essentially unused enzymes.

The purpose of this investigation is to assay the activity and the properties of arginase in the 3,4, and 5 day old quail embryos (Coturnix coturnix japonica). Arginase

activity was assayed in the head, thorax, and tail as well as the whole embryo, to see if the level of activity remained constant or declined during three to five days of development. If there is a decline in the arginase activity in the developing embryo, is this decline uniformly distributed, or is the decline localized in a specific region of the embryo? The results of this investigation were compared to those of the chick embryo (Goldie, 1959), to determine if the specific arginase activity declines as the quail develops.

The Km value and intracellular localization of arginase were also investigated in the quail embryos at 3, 4, and 5 days of incubation, as well as the newly hatched quail. Any changes of Km values may indicate the presence of isozymes of arginase. In this investigation the mitochondria were also isolated from the 3,4, and 5 day embryos, and from the newly hatched quail liver to assay the arginase activity of these organelles in an effect to determine if mitochondria differentiate in terms of this enzyme. Arginase from the chicken kidney and liver has been localized in the inner mitochondrial matrix (Traniello et al., 1974).

REVIEW OF RELATED LITERATURE

There are three primary pathways in which arginine is involved: protein synthesis, the formation of urea and ornithine in the presence of arginase, and the production of quanidoacetic acid in the presence of glycine and glycine transamidase.

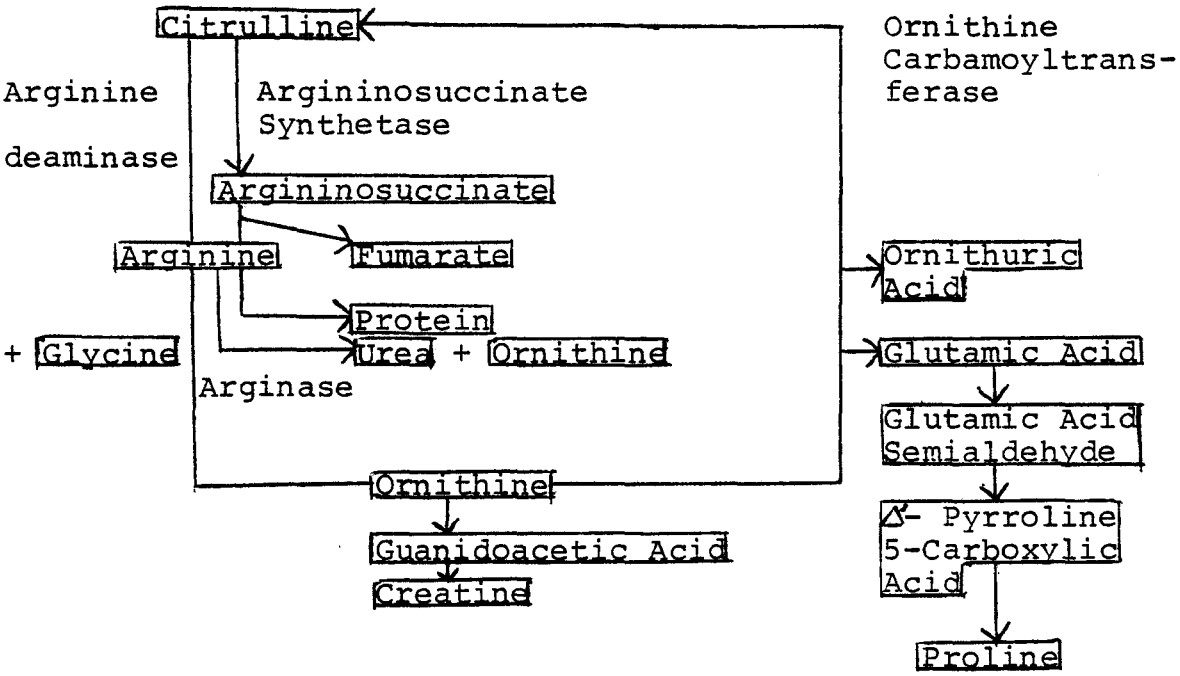


FIGURE 1. Arginine Metabolism

(Modified from figure 1, Smith and Lewis (1963) and figure 25-2, Lehninger (1975))

Arginase activity is high generally in the homogenate in the livers of mammalian species as compared to the kidney of the same species. In the adult male rat the arginase activity has been shown to be about 30 times greater than in the kidney of the same male rat, (Kayson and Strecker, 1973). Kayson and Strecker, (1973) have also shown that there are other differences, among which is inhibition of the liver arginase by high salt concentrations; the kidney arginase is not. Arginase activity has also been found in the intestines of the rat (Fujimoto et al., 1976), as well as in the mammary gland in the rat (Glass and Knox, 1973; Yip and Knox, 1972). The arginase in the rat intestine is believed to be involved in epithelial cell proliferation; it provides a supply of ornithine for synthesis of spermine and spermidine (Fujimoto et al., 1976). The mammary arginase is probably involved in proline synthesis, since when lactating, the mammary gland converts a large amount of arginine to proline (Fig. 1.). This proline is secreted as part of the milk proteins (Glass and Knox, 1973). The conversion of arginine to proline in the lactating rat is supported by the work of Yip and Knox (1972), and this conversion is known to occur in the udder of goats.

The arginase activity has been assayed in fresh water teleosts (Cvancara, 1969). He has shown that the level of arginase activity in the livers of the fresh water tele-

osts, varies depending on the species. Carnivorous species have relatively high levels of hepatic arginase activity, while those species, which are primarily herbivorous or omnivorous, exhibited relatively low levels of liver arginase activity. Since the diet influences the level of arginase activity in fresh water teleosts, Cvancara (1969) suggests that the urea production in carnivorous teleosts may result from the degradation of purines and the catabolism of dietary arginine.

The level of arginase activity in birds is not the same from species to species. There is a relatively high level of arginase activity in the livers of carnivorous birds such as kingfishers, herons, and gulls, and the lowest arginase activity are found in the livers of chickens and pigeons, which are herbivorous birds (Brown, 1966).

Arginases from the ureotelic mammals differ from the arginases of the uricotelic animals in several ways (Mora et al., 1965). The arginases differ with respect to their Km's, inhibition by an excess of substrate, antigenicities and molecular weights. The arginases that are present in ureotelic mammals (man, monkey, cat, dog, pig, and ox) cross-react with rabbit antibody prepared against rat-liver arginase, whereas arginase from uricotelic animals (birds, and reptiles) do not cross-react with the same antibodies (Mora et al., 1965).

Arginase activity has been demonstrated in livers of adult and embryonic birds (Lemonde, 1959; Goldie, 1959; Brown, 1966; Kadowaki et al., 1976). Birds however lack carbamoyl phosphate synthetase (Brown, 1966), an enzyme required for the complete urea cycle. Brown (1966) also has shown that birds have less than 5×10^{-12} micrograms of DNA per somatic cell, yet birds do have liver arginase. This lower amount of DNA per somatic cell may have been linked with the loss of one or more enzymes in the urea cycle which were not closely linked in the genetic sequence to arginase. Goldie (1959) reported that the specific activity (mg. Urea N per Embryo per Hour) of arginase increases in the different parts of the developing chick embryo proportional to increased accumulation of DNA. Eliasson (1967b) considers the increase in arginase synthesis of amino-acid deficient cells, to result from augmented translation of messenger RNA, rather than from an increase in the rate of transcription of genes specific for arginase.

Amino-acids can either increase the arginase activity, have no effect on arginase activity, or even depress the arginase activity. The amino-acids that inhibit arginase activity in the sheep liver (Rao et al., 1973) and rat small intestine (Fujimoto et al., 1976) were ornithine, lysine, leucine, isoleucine, valine and proline. The mode of inhibition varies depending on the amino-acid. Valine

and leucine exhibited mixed inhibition when incubated with Mn^{++}. When leucine was not incubated with Mn^{++} the amino-acid displayed competitive inhibition of the protein. Isoleucine showed competitive inhibition at high substrate concentrations, and non-competitive inhibition at low substrate concentrations. Eliasson and Strecker (1966) showed that in Chang's liver cells lysine, leucine and valine caused an increase in arginase activity. Leucine or valine had no effect on growth of the liver cells. Eliasson (1967a) also showed that when the concentration of lysine is increased from 0.4mM to 6.0 mM the arginase activity increases more than 100%. Retardation in growth was also shown in chicks fed with an excess of lysine (Austic and Nesheim, 1970, Austic and Scott, 1975). This growth depression could be prevented by arginine. When a large excess of lysine is fed, arginine is observed in excretory products (Kadivirel and Kratzer, 1974). This urinary loss of arginine only becomes significant when the lycine levels are greater than 2% of the diet (Jones et al., 1967). The amount of arginine excreted may be as high as 10-15% of the arginine intake (Jones et al., 1967).

Eliasson and Strecker (1966) suggest that the arginase level is regulated by a feedback control mechanism in which ornithine or urea causes a depression of the synthesis of the enzyme. Eliasson and Strecker (1966) also suggests

the regulatory mechanism found in Chang's liver cells may be similar to that of catabolite repression found in micro-organisms. Austic and Scott (1975) report that in the chick, lysine competes competitively with arginine in the renal tubules, thus reducing in the efficiency of arginine retention. The lysine arginine antagonism in the chicks seem to involve at least three metabolic interactions; increased renal arginase activity, a depression of glycine transamidase activity, and competition of lysine and arginine for transport across the renal tubules (Austic and Scott, 1975).

The transport of arginine into the mitochondria may be a key factor in the regulatory mechanism. The dietary level of potassium affects the arginine metabolism, and this effect could be related to the transport of arginine across the mitochondrial membrane (Kadowski et al., 1976) in the chicken kidney. Grazi et al., (1975) suggests that the penetration of arginine in the kidney and liver occurs only in respiring mitochondria.

The effect of hormones on arginase activity on both ureotelic and uricotelic species have been demonstrated by several investigators (Grazi et al., 1972; Rao and Kanungo, 1974; Traniello et al., 1975; Grazi et al., 1975). Cortisol and insulin when injected into chickens, the livers showed a rapid increase in arginase activity (Grazi et al., 1972), due primarily to an appearance of a different arginase iso-

zyme, not to a change in the activity of the pre-existing arginase. Rao and Kanungo (1974) investigating the role of cortisone in young and old rats found that this hormone increased the arginase activity only in the young adrenalectomized rats. Rao and Kanungo speculated that the arginase is a similar molecular species in both the young and in the old rats. Rao and Kanungo suggest that the gene involved does not undergo any structural changes as the rat becomes older, but that the inducibility of the enzyme is changed by cortisone levels.

Rao and Kanungo (1974) have also shown that cortisone was not the only hormone which has an influence on arginase. Thyroxine stimulates activity of hepatic arginase in the prenatal rat, Greengard et al., (1970) reported that thyroxine and glucocorticoids increase arginase activity in the liver of the rat during fetal and late suckling period. Thyroxine has been hypothesized (Gorbam, 1943) to be the primary stimulus for the prenatal formation of arginase in the rat, since the thyroid gland begins to function on about the 16th day of gestation, and since injection of thyroxine increases arginase activity. Glucocorticoids probably are the stimuli for the thyroid growth. During the late suckling period an increase in arginase activity occurs at the same time the hypophyseal-adrenocortical axis begins to function. The increase in arginase activity can be brought about prenatally

by hydrocortisone, and adrenalectomy inhibits the increase of arginase activity which occurs normally during the 3rd week of prenatal life (Greengard et al., 1970). Several factors may be involved in the increase of activity of arginase when a hormone is administered. There may be an increase in transcription or translation of a particular messenger RNA, or a decrease in the breakdown of arginase, or even the synthesis of a molecule which causes a change in the conformation of arginase in such a way that the catalytic activity increases without any changes in the net synthesis of the apoenzyme (Rao and Kanungo, 1974).

In the liver and kidney of uricotelic species, two isozymes are present (Porembska et al., 1971; Reddi/Knox/Herzfeld, 1975; Herzfeld and Raper, 1976; Baranczyk-Kusma et al., 1976; Hirsch-Kolb et al., 1970; Farron, 1973). In the liver of ureotelic animals, one of two isozymes predominates. In the dog, cat, rabbit and man isozyme A_1 predominates, whereas in the horse, ox, calf and pig A_3 predominates. In the kidney, isozyme A_4 accounts for 88 to 97% of the total activity in all of these animals, the isozyme A_1 plays the lesser role (Porembska et al., 1971).

Arginase in the liver of rats is thought to be a basic protein (Schimke, 1966). The arginase found in the rat intestine is believed to be a neutral or slightly acidic protein. This neutral or slightly acidic arginase is less

stable than the basic protein because Mn^{++} is bound more weakly to the enzyme complex (Fujimoto et al., 1976). Herzfeld and Raper (1976) showed on bidirectional electrophoresis on polyacrylamide gels that there is three distinct bands of arginase activity, which corresponds to isozymes A_1 and A_4 in the kidney and isozyme A_1 in the rat liver.

The primary differences between ureotelic arginases and uricotelic arginases appear in their affinities to the substrate and in their molecular weights. The Km value of the chicken liver arginase is approximately 100 mM (Grazi and Magri, 1972), while Mora et al., (1965) reported chicken arginase Km values as high as 200 mM. These large values are indicative of low affinity of the enzyme for the substrate. Ureotelic arginase has a Km value of approximately 10 mM (Mora et al., 1965) while Carlisky (1972) reported a Km value of bullfrog liver arginase to be 20 to 50 mM; the affinity of arginase is greater than the uricotelic arginase. The molecular weight of arginase of the rat liver is 120,000 (Hirsh-Kolb et al., 1970; Fujimoto et al., 1976; Baranczyk-Kuzma et al., 1976), this is about 50% of the molecular weight of uricotelic species which is reported as 276,000 by Mora et al (1965) and 252,000 by Muszynska et al., (1972). Viellie and Bretburd et al., (1972) demonstrated that the arginase of rabbit liver splits into four subunits of 38,000 when treated with 0.25% sodium dodecyl sulphate at pH 10 and

is an oligomeric structure. This is in agreement with Harell and Sokolovsky (1972) who have shown that beef liver arginase splits into four subumits of molecular weights of 30,000. Since uricotelic arginase has twice the molecular weight of ureotelic arginase it is reasonable to assume that the uricotelic arginase may be composed of eight units of 30,000 daltons (Reddy and Campbell, 1970).

Maximum arginase activity is achieved with the addition of divalent ions (Brown, 1966). The order of effectiveness of various divalent metal ions to activate bird hepatic arginase ($Mn^{++} > Cd^{++} > Co^{++} > Ni^{++}$) is similar to that of the bullfrog liver arginase ($Cd^{++} > Mn^{++} > Co^{++} > Ni^{++}$), Carlisky, 1972). Harell and Sokolovsky (1972) has reported that one molecule of beef liver arginase binds four atoms of Mn^{++}. Hirsh-Kolb et al. (1971) also reported that the four ions that bind to one molecule of arginase.

Kadowaki et al. (1976) reported that in the chicken kidney, arginase is located in the mitochondrial matrix. Grazi et al. (1975) have also shown that chicken kidney and liver arginase is located on the external side of the inner mitochondrial membrane (Kaysen and Strecker, 1973).

MATERIALS AND METHODS

I. Care of Adults and Eggs, Coturnix coturnix.

Japanese quails (Coturnix coturnix japonica) were maintained in wire cages. Each cage contained at least one female and one male. Quails received Purina Game Bird Chow (Layena) and tap water and ad libitum. The room temperature was kept at 26+2° C, while the illumination was continuous, for maximum egg production. Each morning eggs were collected, and either stored in a refrigerator at 13° C or incubated immediately in an incubator (Jamesway) at 37°+2°C. Eggs were never stored longer than seven days at 13°C. When the eggs were incubated, they were incubated for 3,4,5, days, or to hatching, depending on the experimental design.

II Preparation of Homogenate

At the end of the incubation period the eggs were opened, and the embryos were freed of all membranes. They were next placed into Howard's saline solution at 4°C to reduce cellular damage. The wet weight of the pooled embryos was determined, and a 20% homogenate of embryos in 0.15M KCl (wet weight/volume) was prepared in an ice bath. Aliquots of 0.5 mM $MnCl_2*4H_2O$, an enzyme cofactor, were added to the 0.15M KCl to increase the arginase activity.

Duplicate samples (0.1 ml.) of the homogenate was added to 0.1 ml. of the assay solution, containing 0.25M

Glycine-in NaOH - 0.25 M Arginine, or to 0.1 ml. of the control solution containing 0.25 M Glycine - in NaOH. Both incubating solutions were maintained at pH 9.5, and 4°C. The enzymatic reaction was stopped by the addition of 10% perchloric acid solution to a volume of 1.0 ml. Each centrifuge tube contained 0.1 ml. homogenate, 0.1 ml. incubating solution, and 0.8 ml. perchloric acid at the end of incubation. The incubation of the enzymatic reaction was carried out at 38°C. in a Dubnoff Metabolic Shaking incubator for 0, 5, or 10 minutes.

After the addition of the 10% perchloric acid solution the suspention was centrifuged in a IEC Clinical Centrifuge at 1640 X G for ten minutes. The precipitated protein pellet was analyzed by the method of Hartree (1972), while the supernatant was quantitatively tested for its urea content (Wybenga et al., 1971).

III. Protein and Urea Assay

Protein was determined according to the Hartree method (1972).

The urea was determined according to the Wybenga et al. technique (1971).

The procedure and reagents for the protein assay and the urea assay are listed in the appendix.

IV. Preparation of Embryonic Parts

The embryos were prepared in Howard's saline for homogenization and incubation. The parts of the embryo were defined as follows:

Head; anterior to the forelimbs
Thorax; forelimbs to hindlinbs
Tail; posterior to the hindlimbs

In each experiment duplicate samples were analyzed for every time interval (0, 5, and 10 minutes), as described in Part II.

V. Determination of Km

The Km values for the 3, 4, 5 day old quail embryos, for the liver of the newly hatched quail and for the mitochondrial fraction of the 5 day old quail embryo were calculated by the Lineweaver-Burk method, by varying the concentration of arginine. The time of the enzymatic incubation was 5 minutes. The procedure for the determination of urea and for protein were the same as described in the appendix.

VI. Localization of Arginase

Intracellular localization of arginase was determined for the 3, 4, 5 day old embryos and the liver of the newly hatched quail. A 20% homogenate (weight/volume) of embryos in 0.25 M Sucrose-Tris-HCl, pH 6.65, was centrifuged in a Sorval R C 2-B refrigerated centrifuge ($4^{o}C$) for 30 minutes at 1,000 x G to isolate the nuclei and cells. The supernatant was centrifuged for an additional 30 minutes at 10,000

x G to isolate the mitochondria. The 20% homogenate sample was analyzed for its arginase activity as described earlier.

EXPERIMENTAL DATA

I. Arginase Activity in the Whole Embryo From Three to Five days of Development.

A linear regression was performed from data from three, four and five day old embryos, to determine the linearity of the data and the best fit line for each embryonic age. After the linearity was determined, the specific arginase activity was calculated.

Figure II is a bar graph comparing the specific arginase activity of the embryonic ages (3, 4, and 5 days) of development. The standard deviations are indicated for the specific arginase activity at each age.

The three day embryo had the highest level of arginase activity. As the embryo developed, the level of specific arginase activity declined. At five days, the specific arginase activity is approximately 50% that of the three day old embryo.

TABLE I. - SPECIFIC ARGINASE ACTIVITY IN THE WHOLE EMBRYO

Days of Development	Mean	Standard Deviation	Variance	No.of Experiments
3	0.0039	0.0010	6.022x10-7	3
4	0.0031	0.0004	1.143x10-7	7
5	0.0017	0.0001	1.36x10-8	5

There is a significant different difference between the mean values between the three day old quail embryo and the four day old quail embryo ($t= 2.38$, D.f.=8, $P.>.025$). There is also a significant different difference between the four day quail embryo and the five day quail embryo ($t=8.78$, D.f.=10, $P.>.0005$).

FIGURE II

SPECIFIC ARGINASE ACTIVITY IN THE WHOLE EMBRYO FROM THREE TO FIVE DAYS OF DEVELOPMENT

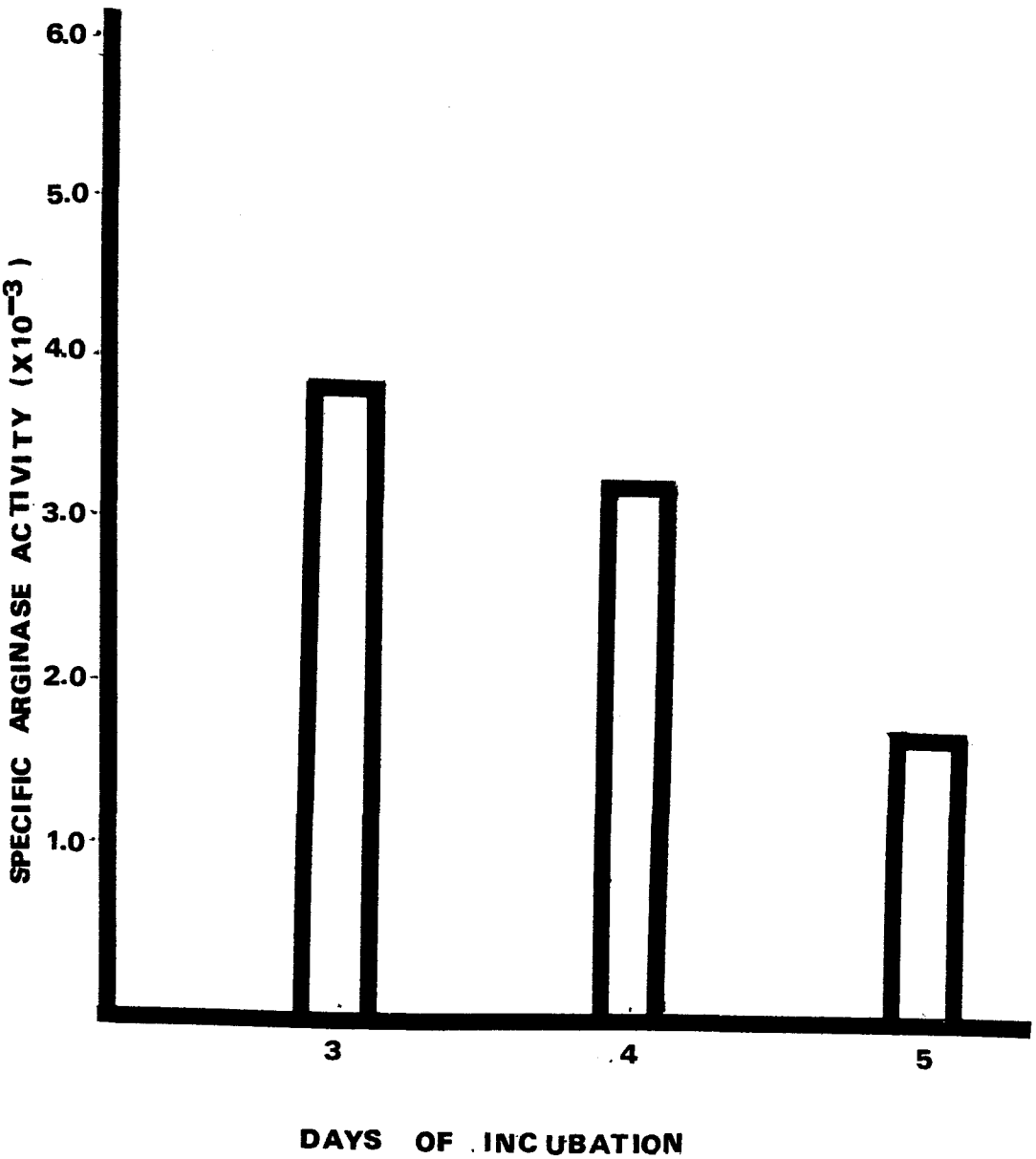


Figure II. Specific arginase activity is defined as the micromoles of urea formed per micrograms homogenate protein in five minutes, pH 9.5, at 38°C.

II. Arginase Activity of Embryonic Parts

A linear regression was calculated for the arginase activities in the head, thorax, and tail tissues of the three, four and five day old embryos. The values for the zero-minute readings were subtracted from all values to correct for endogenous arginine.

Figure III is a bar graph comparing the specific arginase activitity of the various parts in the three, four, and five day old quail embryos. The standard deviations are plotted for the specific arginase activity (Table II).

FIGURE III

SPECIFIC ARGINASE ACTIVITY

IN EMBRYONIC PARTS

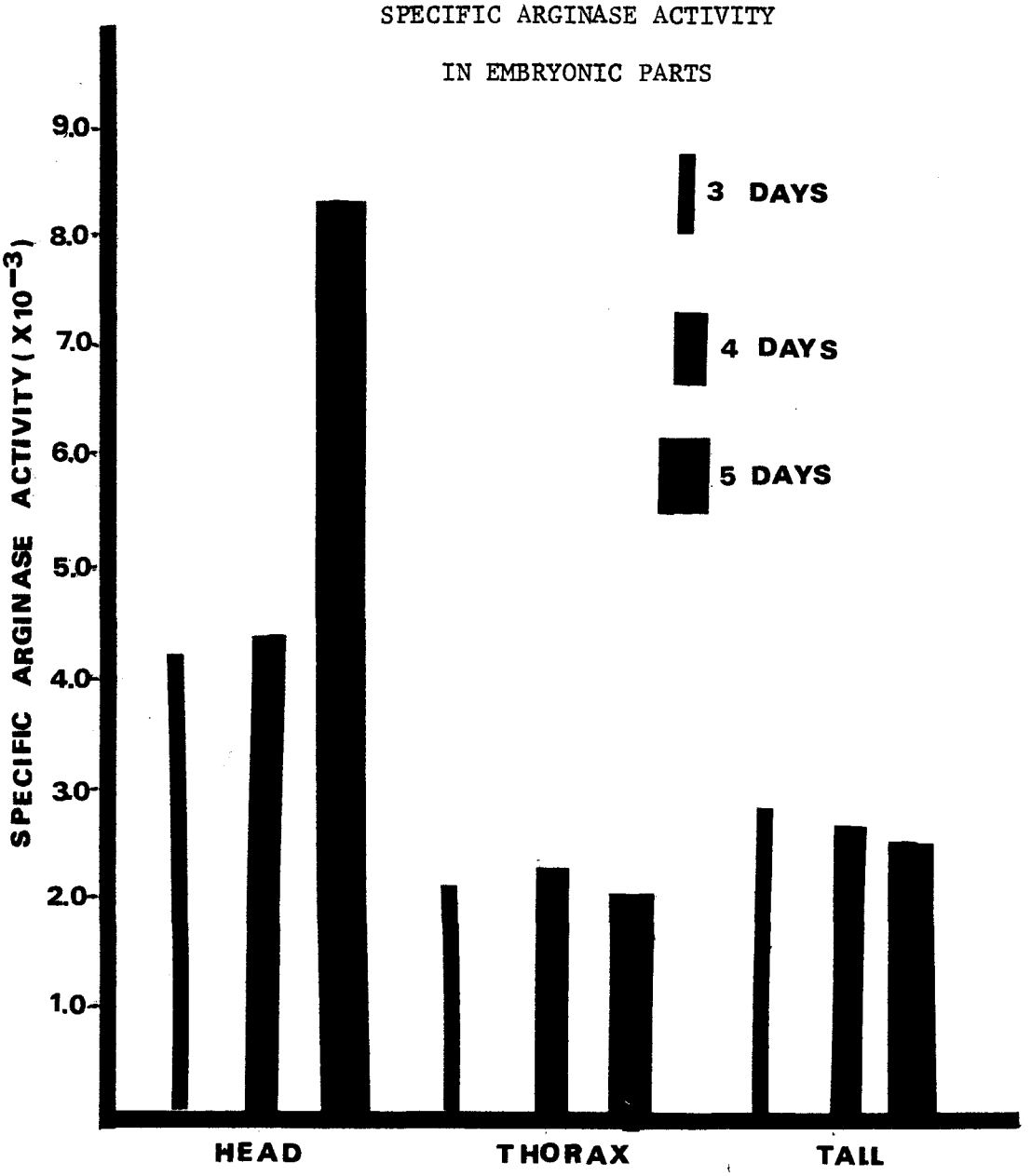


Figure III. Specific arginase activity is defined as the micromoles of urea formed per micro grams homogonate protein in five minutes, pH 9.5, at 38°C.

The specific arginase activity is higher in the head than in the other parts analyzed (Table II). The activities in the head of three and four day embryos are approximately the same, but in the head of the five day old embroy there is a two-fold increase. The arginase activities in the thorax and tail are relatively constant between three and five days of development.

TABLE II. - SPECIFIC ARGINASE ACTIVITY IN EMBRYONIC PARTS

Days of Development	Mean	Standard Deviation	Variance
3 Day Head	0.0042	0.0008	5.55x10-7 N=6
4 Day Head	0.0044	0.0010	8.72x10-7 N=10
5 Day Head	0.0084	0.0010	8.067x10-7N=6
3 Day Thorax	0.0019	0.0007	4.192x10-7 N=6
4 Day Thorax	0.0023	0.0007	3.967x10-7 N=6
5 Day Thorax	0.0021	0.0003	9.58x10-8 N=6
3 Day Tail	0.0029	0.0007	3.71x10-7 N=8
4 Day Tail	0.0027	0.0009	5.75x10-7 N=4
5 Day Tail	0.0027	0.0006	3.289x10-7 N=6

A significant difference was found between the mean values, for the three day head and for the five day head ($t=8.05$, D.F.$=10$, $P.<0.005$). There is also a significant difference between the three day thorax and the four day tail ($t=2.14$, D.F.$=10$, $P.>0.25$), and between the three day head and the four day tail ($t=3.10$, D.F.$=8$, $P>0.01$). No significant difference was found between the mean values between the three day thorax and the four day tail.

III. Determination of Km Values

The Km values of the three, four, five day old quail embryos, and in the liver of the newly hatched quail were determined by a Lineweaver-Burk plot.

Table III compares the Km values for arginase in the three, four, five day old quail embryos and the liver of the newly hatched quail. The standard deviations are also given.

The Km value for arginase of the three day old quail embryo was lower than in the four and five day old embroys, and as well as in the newly hatched quail. The Km values in the four and five day old quail embryo, and in the liver of the newly hatched quail are approximately the same.

TABLE III

Km VALUES FOR ARGINASE IN

Coturnix coturnix japonica

Age of quail	Mean Km	Standard Deviation	Variance
3 day embryo	0.0229 M	0.0013	9.025x10-7 N=2
4 day embryo	0.0726 M	0.0244	3.972x10-4 N=3
5 day embryo	0.0648 M	0.0394	1.032x10-3 N=3
5 day embryo mitochondrial fraction	0.0898 M	0.0283	4.000x10-4 N=2
Liver of a newly hatched quail	0.0920 M	0.0016	1.323x10-6 N=2

There is a significant difference in the mean value of the Km between the three day quail and the four day quail ($t=3.34$, D.F.=3. $P.<0.025$). There is no significant difference in the mean values between the four day and five day quail ($t=0.3573$, D.F.=4, $P.>0.35$), between the five day quail and the five day mitochondrial fraction ($t=0.956$, D.F.=3, $P>0.020$), and between the five day quail and the liver of a newly hatched quail ($t=1.136$, D.F.=3, $P.>0.15$).

IV. Localization of Intracellular Arginase Activity

Intracellular localization of specific arginase activity was determined for the three, four, five day old quail embryos and the liver of the newly hatched quail.

A linear regression was to determine the linearity of the best fit line for each experiment. After the linearity was calculated, the specific activity of arginase was calculated.

Figure IV is a bar graph comparing the specific arginase activity of the nuclear and cellular fraction with the mitochondrial fractions from the three, four and five day old quail embryos, and from the liver of the newly hatched quail.

SPECIFIC ARGINASE ACTIVITY OF

INTRACELLULAR FRACTIONS

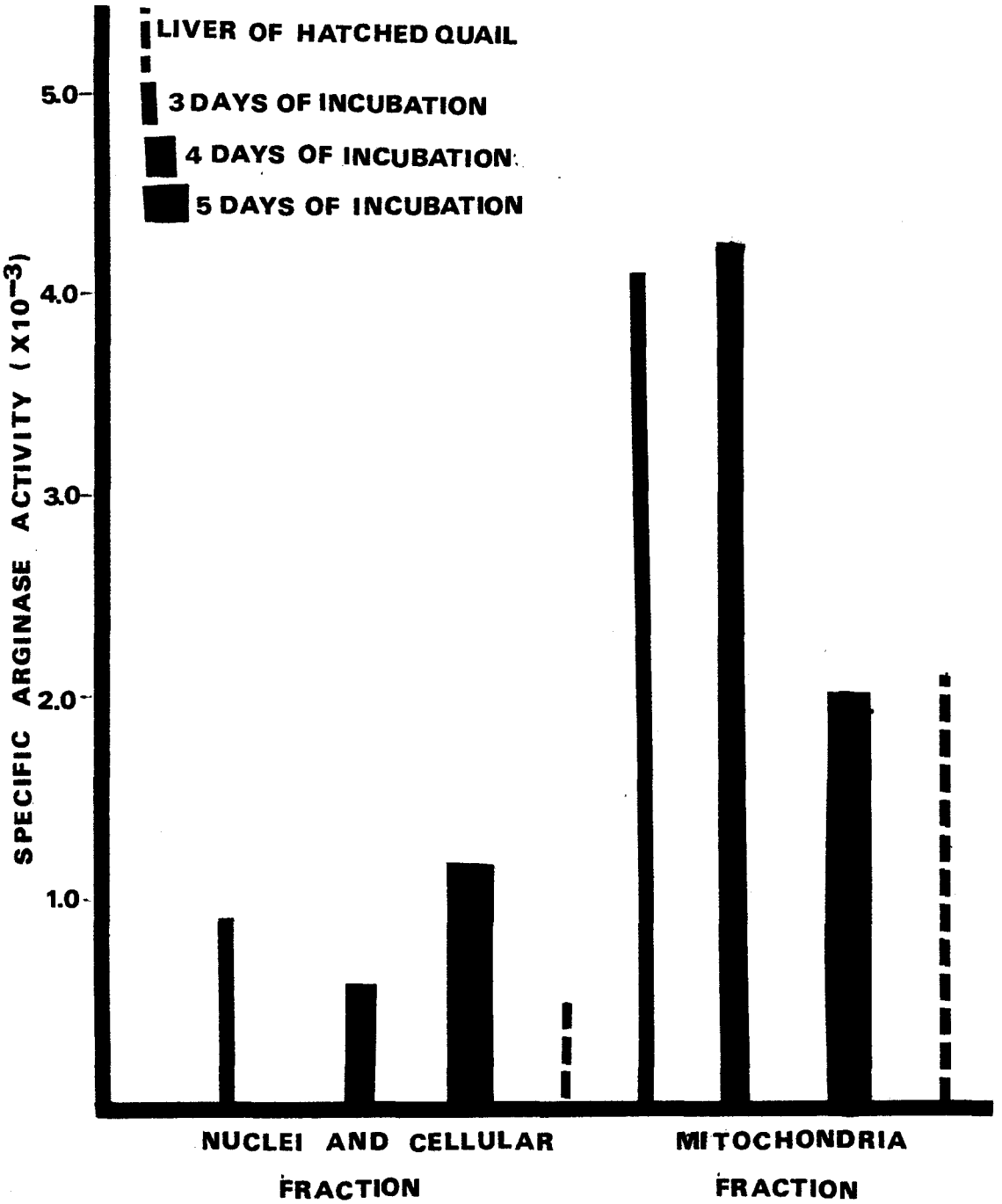


Figure IV. Specific arginase activity is defined as moles of urea formed per micrograms homogenate protein in five minutes, pH 9.5, at 38°C.

The highest specific arginase activity is in the mitochondrial fraction at all ages. The specific arginase activities in the three and four day old quail embryo are about four times higher in the mitochondrial fraction than in the nuclear and cellular fraction. The specific activity in the five day quail embryo and in the liver of the newly hatched quail is approximately twice as high in the mitochondrial fraction than in the nuclear and cellular fraction. In the sucrose fraction there was no measureable specific arginase activity

TABLE IV. - ARGINASE ACTIVITY OF INTRACELLULAR FRACTIONS

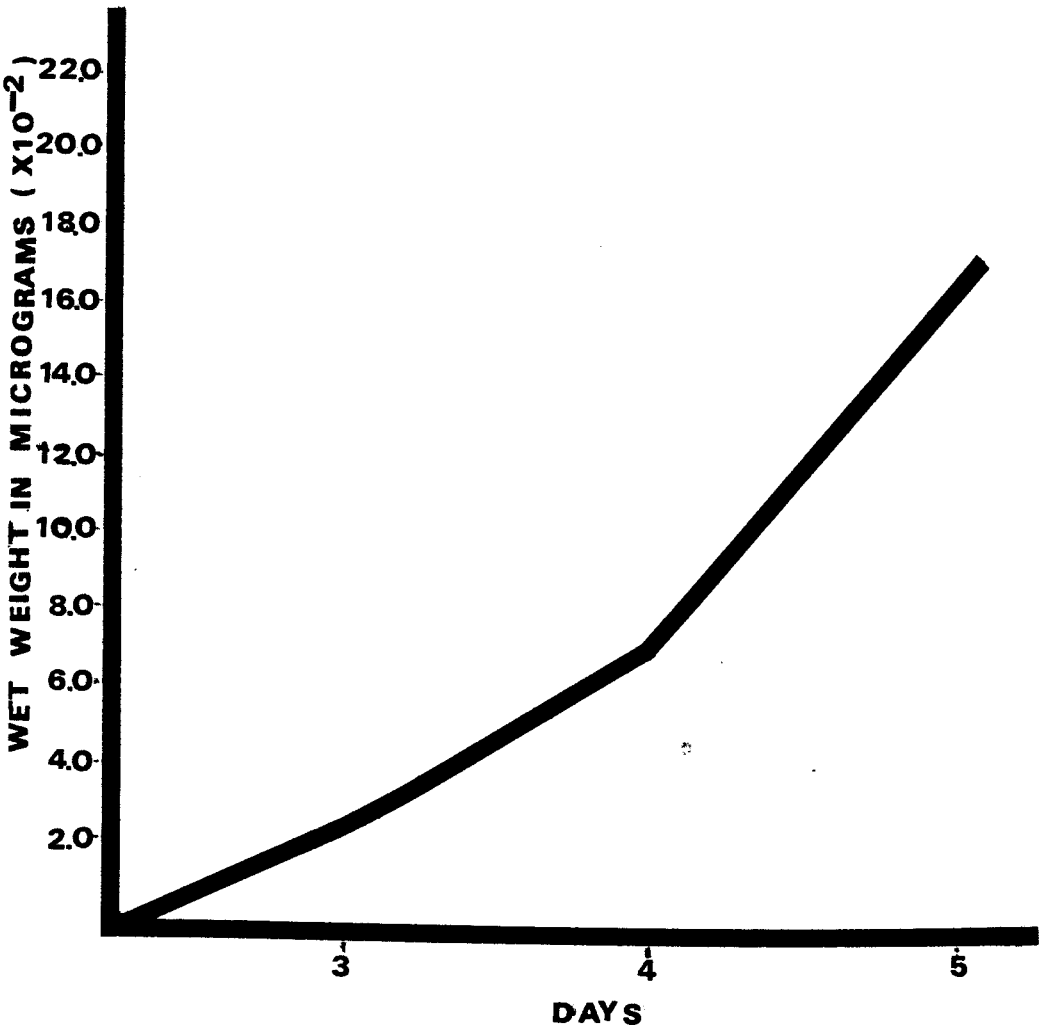
	Mean specific Activity x10-3	S.D.	Variance
3 day embryo, nuclei and cellular fraction	0.927	0.665x10-4	2.20x10-9
4 day embryo, nuclei and cellular fraction	0.613	0.340x10-3	5.74x10-8
5 day embryo, nuclei and cellular fraction	1.395	0.488x10-3	1.19x10-7
liver of newly hatched quail, nuclei and cellular fraction	0.503	0.205x10-4	2.10x10-10
3 day embryo, mitochondrial fraction	4.234	0.47x10-4	1.65x10-8
4 day embryo, mitochondrial fraction	4.31	0.32x10-3	5.05x10-8
5 day embryo, mitochondrial fraction	2.076	0.165x10-3	1.34x10-8
liver of newly hatched quail, mitochondrial fraction	2.169	0.14x10-3	1.02x10-8

Note ∴ N=2 in all cases

There is a significant difference between the mean values, between the five day mitochondrial fraction and the four day mitochondrial fraction ($t=12.49, D.F.=2, P.>0.0025$). There is no significant difference between the mean values, of the five day nuclear fraction and in the liver of the newly hatched quail ($t=3.65, D.F.,=2, P.<0.05$).

FIGURE V

GROWTH CURVE OF THE Coturnix coturnix japonica EMBRYO



In Table V the specific arginase activity of the Coturnix coturnix japonica embryo is compared to the values of the chick embryo, as reported by Goldie (1959).

TABLE V.

ARGINASE ACTIVITY IN THE CHICK EMBRYO FROM GOLDIE (1959) AND FROM THE PRESENT STUDY

Age (days)	Specific Arginase Activity (Mg. Urea N per Embryo Per Hour)	
	Goldie	This Study
3	0.005	0.0107
3.5	0.017	.....
4	.....	0.0198
4.5	0.047	.....
5	.....	0.0362

The present study shows that in the quail embryo, there is a higher arginase activity at three days of incubation, yet by 5 days of incubation the arginase activity has fallen below the arginase activity of the chick embryo.

V. Growth curve of the Coturnix coturnix japonica embryo to five days.

The wet weights of the quail embryos have been measured from three to five days of development. The weight increases linearly for the first four days of development. Between the fourth day and the fifth day there is more rapid increase in the wet weight. (Figure V.).

DISCUSSION

From the experimental results (see figures II to IV), it can be seen that the specific arginase activity can be measured in the developing quail embryos from three to five days of development. The results obtained correlate with Goldie (1959) with the respect that the specific arginase activity in the whole embryo decreases from day three to day five.

The specific arginase activity of the Coturnix coturnix japonica embryo is compared to the values of the chick embryo, as reported by Goldie (1959).

The present study shows that in the whole quail embryo, there is a higher arginase activity at three days of incubation, yet at five days of incubation the arginase activity has fallen below the arginase activity of the chick embryo.

Goldie (1959) and Fisher (1957) report that the arginase activity is uniformly distributed between the head, thorax and abdomen of the chick embryo during the first six days of incubation. This investigation indicates that during the fifth day of incubation the arginase activity in the head is significantly higher than in the three or four day old heads. The specific arginase activity is uniform between the tail and the thorax at each of the days investigated.

The rise in the specific arginase activity in the

five day head may be due to the differentiation of the quail brain, or to the presence of an isozyme of arginase.

The Michaelis-Menten constant (Km) was determined by the usual Lineweaver-Burk plot of 1/V vs. 1/S. The results give a value of 22.9 mM in the three day embryo, 72.6 mM in the four day embryo, 89.8 mM in the five day embryo, and 92.0 mM in the liver of the newly hatched quail. The Km values of the chicken liver is approximately 100 mM (Grazi and Magri, 1972), while Mora et al. (1965) reported chicken arginase values as high as 200 mM. Carlisky et al. (1972) reported the Km value of bullfrog liver arginase is approximately 20 50 50 mM. Therefore the Km value in the three day quail embryo (22.9 mM) is more characteristic of the ureotelic arginase, and the Km values of the four day quail embryo (72.6 mM), five day quail embryo *89.8mM), and the liver of the newly hatched quail (92.0 mM), are more typical of the uricotelic arginase (Km approximately 100 mM). The difference in the Km values show that the arginase in the three day quail embryo has a greater affinity for arginine than the arginase found in the four and five day quail embryo, or liver of the newly hatched quail. This may be due to the compartmentization of arginase in the cytosol rather than in the mitochondria.

There are many other possible explanations for the substantial difference in the Km values. These include the

availability of arginine, the binding of the manganous ions, or amino-acids which inhibit arginase activity, such as ornithine, lycine, leucine, isoleucine, valine, and proline (Fujimoto et al., 1976).

The localization of intracellular arginase has been investigated. Kadowaki et al. (1976) reported that in the chicken kidney, arginase is located in the mitochondrial matrix. This is confirmed by Grazi et al. (1975) that arginase is located in the mitochondrial fraction in both the kidney and the liver of the chicken. From the experimental results given here (Figure IV) it can be seen that the arginase activity is higher in the mitochondrial fraction as compared to the nuclear and cellular fraction. Therefore, this investigation supports the findings of Kayson and Strecker (1973), Grazi et al. (1975), and Kadowaki (1976) that the arginase is located in the mitochondria.

If the fractions (mitochondrial, and nuclei and cellular fraction) are added together, theoretically the value obtained should equal the specific activity in the whole homogenate of the same day. In the five day embryo, the arginase activity in the nuclei and cellular fraction plus the mitochondrial fraction equals 0.0035 units. This is twice the specific arginase activity found in the five day old quail embryo. The total arginase activity (100%)

is recovered in the five day fractions. There is a 55% recovery in the fractions of the three day old quail, and there is 76% recovery of the total specific arginase activity in the fractions of the four day quail embryo.

Brown (1966) reports that there is a relatively high level of arginase in the liver of carnivorous birds such as kingfishers, herons, and gulls, and the lowest arginase activities are found in the livers of chickens, and pigeons, which are herbivorous. Since carnivorous birds probably evolved before the herbivorous birds in evolution, the lower arginase activity in the herbivorous birds may be due to an alteration in the genome associated with their evolutions.

SUMMARY

1). The specific arginase activity decreases from three to five days of development in homogenates of the quail embryo.

2). The specific arginase activity is uniform in the head, thorax, and tail of the three and four day old embryo. In the five day old embryo the specific arginase activity is approximately the same between the thorax and tail, but in the head there is a two-fold increase.

3). The Michaelis-Menten constant (Km) was found to be consistent with the uricotelic arginase Km (approximately 100 mM) in the following developmental stages:

1. Four day old whole embryo.
2. Five day old whole embryo.
3. Liver of the newly hatched quail.
4. Mitochondrial fraction of the five day old quail embryo.

In the three day quail embryo however, the Km value is similar to that of the uriotelic arginase (approximately 10 mM).

4). The specific arginase activity is highest in the mitochondrial fraction as compared to the nuclear and cellular fractions.

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APPENDIX

PROTEIN ASSAY

This technique is a modification of the procedure described by Hartree (1972). Equipment and solutions including standards for the assay are listed in Table I.

The protein pellets were brought to 8 ml. with 1N NaOH. A 0.25 ml. or a 0.5 ml. sample is diluted to 1 ml. with water; this included the blanks and standards. 0.9 ml. of reagent "A" is added and mixed. The solution was allowed to stand at room temperature for ten minutes. After a minimum of 10 minutes, 0.1 ml. of "B" was added and mixed. Again the solution stood at room temperature for a minimum of 15 minutes. After the 15 minutes, 3.0 ml. of "C" is added very rapidly, and left at room temperature for 10 minutes before spectrophometric readings were taken. The optical density was then read at 650 nm. in a Beckman DU spectrophotometer.

In Figure I the standard curve for protein, absorbance is plotted (optical density, O.D.) against concentration. The points on the standard curve show the best fit points by using the mean absorbance of the protein concentration.

TABLE I

Solutions and Equipment for the Protein Assay

Solutions:

Reagent A:
2 g sodium potassium tartrate, 100 g Na_2C0_3. 500 ml. 1 N NaOH diluted to 1 liter with water

Reagent B:
2 g sodium potassium tartrate, 1 g $CuSO_4 . 5H_2O$ - dissolve in 90 ml. water, add 10 ml. 1 N NaOH

Reagent C:
Folin phenol reagent, 1 part - 15 parts water, pH = 0.15 = 0.18 1N acidity, prepared just before used.

Standard Protein solution:
The following standard solutions used to prepare the standard protein curve from crystaline bovine serum albumin dissolved in water.

400 micrograms of crystaline bovine serum albumin/1 ml.
375 "
350 "
325 "
300 "
275 "
250 "
225 "
200 "
175 "
150 "
125 "
100 "
75 "
50 "
25 "

Note: all reagents were obtained from Sigma Chemical Company, St. Louis, Mo.

Equipment:

Beckman DU Spectrophotometer
Test tubes
Timer (electric or wind-up)
50 ml. beakers
100 ml. beakers

1 ml. pipettes
5 ml. pipettes
10 ml. pipettes

FIGURE I

PROTEIN STANDARD CURVE

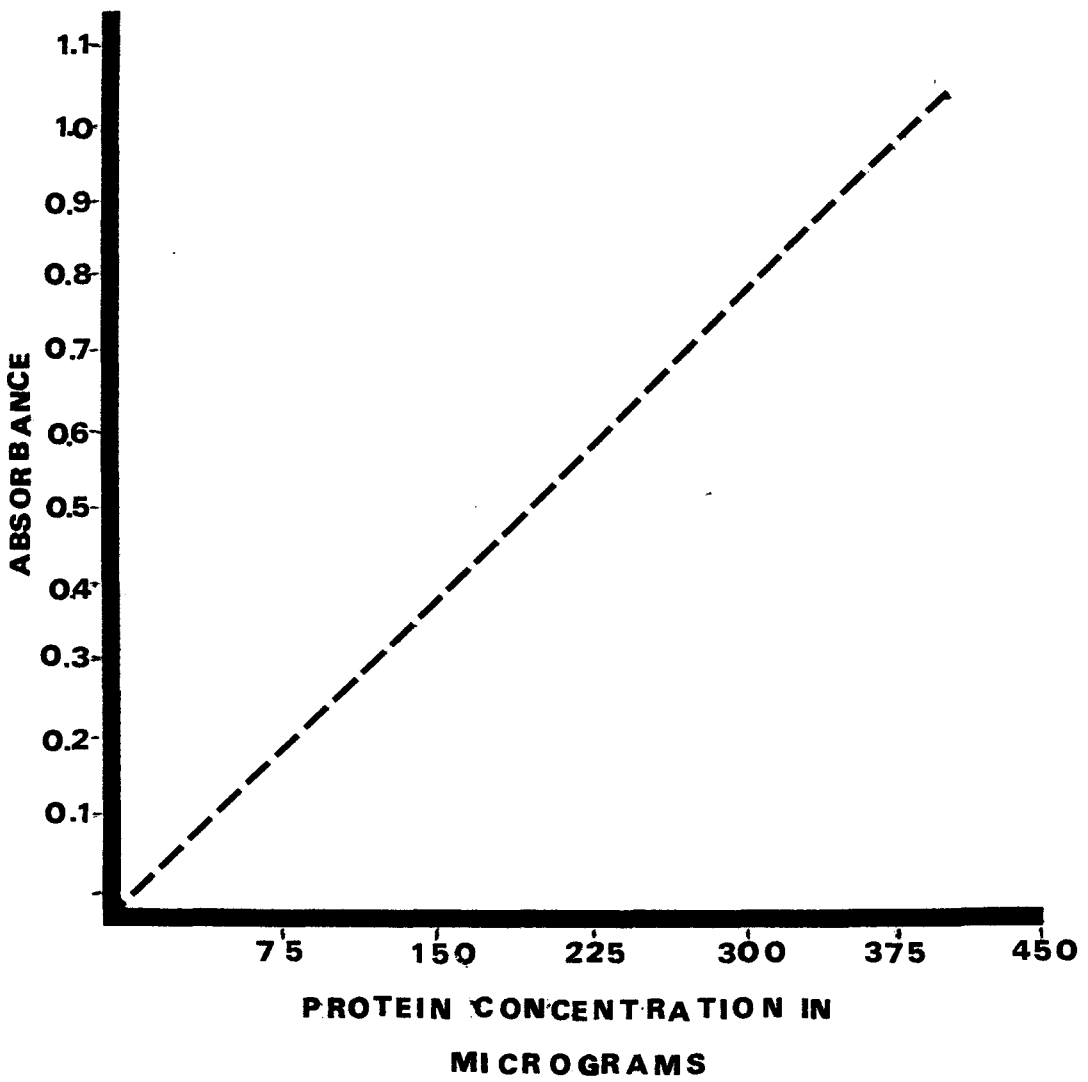

TABLE II

LINEAR REGRESSION VALUES FOR PROTEIN STANDARD CURVE

X	THEORETICAL Y	ACTUAL Y
25 micrograms	0.063	0.063
50 "	0.126	0.125
75 "	0.188	0.188
100 "	0.251	0.251
125 "	0.313	0.313
150 "	0.376	0.376
175 "	0.439	0.439
200 "	0.501	0.502
225 "	0.564	0.564
250 "	0.627	0.627
275 "	0.689	0.689
300 "	0.752	0.753
325 "	0.814	0.815
350 "	0.877	0.877
375 "	0.940	0.940
400 "	1.002	1.000

Slope = 0.0025048235

Y' = 0.00325

X' = 0.129749

Correlation
Coefficient = 0.9999

UREA ASSAY

The supernatent portion of the centrifuged homogenate was analyzed for the urea content by the Wybenga et al. technique (1971). In each experiment duplicate samples were analyzed at each time interval (0, 5, and 10 minutes).

Equipment and solutions including standards for the assay are listed in table III.

From the supernatent a 0.5 ml. sample is placed into a test tube. 0.5 ml. of water is added to a total volume of 1 ml. 0.5 ml. of 2% biacetyl monoxime is added to each solution, and 5.0 ml. of urea reagent is added. This mixture is heated at 100 C. for 15 minutes, then immersed in cold water for 5 minutes. Before spectrophometric readings were made with a Beckman DU spectrophotometer at 540 nm a blank with reagent alone was measured.

Figure II is the standard curve for urea, absorbence (optical density, O.D.) vs. concentration. The points on the standard curve for urea represent the best fit points by using the mean absorbance of the urea concentration. The standard deviation of the experimental values are also shown.

TABLE III

SOLUTIONS AND EQUIPMENT FOR THE UREA ASSAY

Solutions:

Urea Reagent:
Add concentrated H_2SO_4 (44 ml.) and 85% H_3PO_4 (66 ml.) to H_2O (100 ml.), cooling the mixture and adding thiosemicarbazide (50 mg.) and hydrated $CdSO_4$ (2 g.), dissolving each successively, and 10 ml. of aqueous urea (26 mg. per liter): finally, the solution is diluted to 1 liter with water. The solution is stored in an amber bottle in the refrigerator.

2% Biacetylmonoxime:
This aqueous solution was prepared and stored at room temperature.

Standard Solutions:
The following standard solution used to prepare the standard urea curve made from 0.0025 M standard urea.

1.500 micro moles of urea / ml.
1.375 "
1.250 "
1.125 "
1.000 "
0.875 "
0.750 "
0.625 "
0.500 "
0.375 "
0.250 "
0.125 "
0.000 "

Note: The crystalline urea was obtained from J.T. Baker Chemical Company, Phillipsburg, N.J.

Equipment:
Beckman DU spectophotometer
Test tubes
Timer (electric or wind-up)
50 ml. beakers
100 ml. beakers
1 ml. pipettes
5 ml. pipettes
10 ml. pipettes
Heating device, such as a hot-plate

FIGURE II

UREA STANDARD CURVE

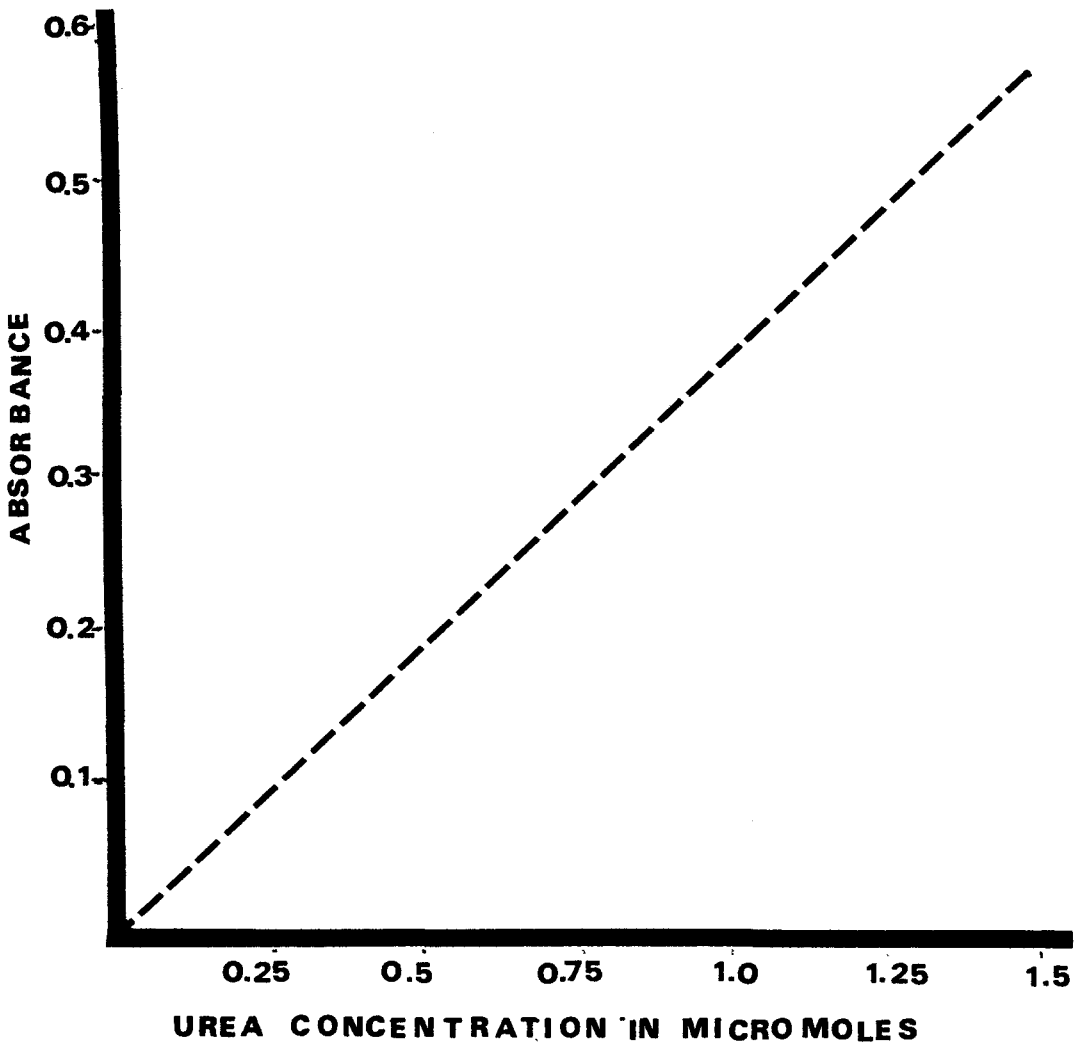

TABLE IV

LINEAR REGRESSION VALUES FOR UREA STANDARD CURVE

X	THEORETICAL Y	ACTUAL Y
0.000 micromoles	0.0076	0.0000
0.125 "	0.0524	0.0450
0.250 "	0.0972	0.0875
0.375 "	0.1421	0.1500
0.500 "	0.1869	0.1950
0.625 "	0.2317	0.2325
0.750	0.2765	0.2800
0.875 "	0.3215	0.3325
1.000 "	0.3661	0.3800
1.125 "	0.4109	0.4225
1.250 "	0.4558	0.4435
1.375 "	0.5006	0.4860
1.500 "	0.5454	0.5400

Slope = 0.3585274725

Y' = 0.0076043956

X' = 0.02100779

Correlation Coefficient=0.9984

T-TEST FOR INDEPENDENT SAMPLE MEANS

The t-test is used to test the difference between two sample means to determine whether if one is significantly larger or smaller than the other.

Sample:

"A" Arginase activity in five day head	"B" Arginase activity in three day head
0.0077	0.0048
0.0081	0.0047
0.0096	0.0039
0.0076	0.0026
0.0097	0.0045
0.0077	0.0039
N=6	N=6
Mean = 0.0084	Mean = 0.0042
Variance = 8.067×10^{-7}	Variance = 5.55×10^{-7}

$$t = \frac{\text{Mean "A"} - \text{Mean "B"}}{\sqrt{1/N_A + 1/NB \times \left[\frac{(N \text{ "A"} - 1)\ (\text{Var. "A"} + (N \text{ "B"} - 1)(\text{Var. "B"})}{N \text{ "A"} + N \text{ "B"} - 2}\right]}}$$

$$t = \frac{0.0084 - 0.0042}{\sqrt{2/6 \times \left[\frac{(6)\ (8.067 \times 10^{-7}) + (6)\ (5.55 \times 10^{-7})}{10}\right]}}$$

$$t = 8.05$$

Next, the t value is sound in the probability tables. Since the Df = (N of "A" + N of "B") -2, the Df in this case is 10. The probability is < 0.005, therefore the t value of 8.05 is significant, and hence there is a significant difference between the arginase activity in the five day quail head and the three day quail head.

APPROVAL SHEET

The thesis submitted by Peter Houtman

has been read and approved by the following committee:

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The final copies have been examined by the director of the thesis and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the thesis is now given final approval by the Committee with reference to content and form.

The thesis is therefore accepted in partial fulfillment of the requirements for the degree of Master of Science.

April 23, 1979
Date

Mark Goldie
Director's Signature