Creatine Regulation in the Embryo and Growing Chick

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1. The absence of creatine was demonstrated enzymically in the hen's-egg yolk and in the albumin contrary to former reports. 2. A comparison of the results obtained by enzymic and colorimetric methods to measure creatine is presented. 3. Creatine phosphate was not detected in the yolk extracts. 4. The content of free arginine enzymically assayed was $15.7\mu\text{mol}$ in the yolk and $3.38\mu\text{mol}$ in the albumin. Arginine amounts to practically all of the guanidine compounds in the yolk and one-half of those in the albumin. 5. No glycine amidinotransferase activity was found in the egg-yolk homogenates. 6. The heart of the chick embryo does not receive creatine from the egg and the creatine kinase activity present in this organ starting from the 27th hour of incubation suggests that the enzyme is a constitutive one working probably as an adenosine triphosphatase in a way similar to the kinase isolated from rabbit skeletal muscle. 7. Liver glycine amidinotransferase activity appeared clearly after day 5 of incubation. The specific activity reached a maximum at day 12 and then declined; however, the activity per total mass of liver increased steadily during all the prenatal period. Concomitantly with this steady increase a rise in the creatine content of the whole embryo was observed. An analogous increasing relationship between total liver amidinotransferase activity and liver creatine content was also detected during the postnatal period. 8. Repression of amidinotransferase by creatine cannot be accepted as occurring under physiological conditions since an inverse relationship between the two parameters was not observed. 9. Repression of liver amidinotransferase is observed only when pharmacological concentrations of the exogenous creatine are present in the chick liver.

The presence of creatine kinase (EC 2.7.3.2) activity in the chick embryo heart was detected from the 27th hour of incubation (Eisenberg & Ramírez, 1963); however, chick heart is unable to synthesize creatine throughout the embryonic period (Ramírez, Calva & de la Cruz, 1963). Moreover, although the two enzymes involved in the creatine biosynthetic pathway (Borsook & Dubnoff, 1941) have been found in chick liver (Walker, 1960), the hepatic primordium appears some hours after the cardiac contractile activity starts, i.e. at the end of day 2 (Romanoff, 1960), and glycine amidinotransferase (EC 2.1.4.1), the first enzyme implicated in creatine synthesis, becomes evident in this organ after 5 days of incubation.

The amidinotransferase is repressed either by feeding the chick with creatine or by injection of the compound into the egg (Walker, 1960, 1965, 1966; Walker & Walker, 1962). This repression is independent of age and can be reversed by restoration to normal conditions (Walker, 1960, 1965; Ramírez *et al.* 1963). Guanidinoacetate methyltransferase (EC 2.1.1.2), the second enzyme of

the creatine pathway, is not affected by creatine under the same conditions. Since Walker (1965) proposed glycine amidinotransferase as the key enzyme for the control of creatine biosynthesis in animals in a way similar to that observed in bacterial systems, it was decided to analyse further this suggestion because of its uniqueness in higher organisms.

The postulated negative-feedback control of creatine biosynthesis through amidinotransferase repression occurs either when very large amounts of creatine are administered to the animals (Walker & Walker, 1962; Ramírez *et al.* 1963) or under pathological conditions such as those occurring in excessive tissue destruction, muscle wasting or starvation (Walker, 1960; Fitch, Hsu & Dinning, 1960, 1961; Ungar & Van Pilsun, 1966b). Walker & Wang (1964) completely repressed the liver amidinotransferase activity after the injection of $220\mu\text{mol}$ of creatine into the 6-7-days-incubated eggs. On analysis of the liver tissue 6 days after the injection, they detected $3.8\mu\text{mol}$ of creatine/g of liver, which is slightly more than 10 times

the physiological amount ($0.35\mu\text{mol}/\text{total yolk}$; Romanoff, 1967). Therefore the lag period preceding the appearance of amidinotransferase in the liver of normal chick embryo might not be either necessarily or sufficiently explained even by assuming that all the creatine reported in the whole egg would be concentrated in this organ.

The creatine values mentioned in previous work were obtained by colorimetric assays, but Gerber, Gerber, Koszalka & Miller (1962) proved the presence of inhibitory factors for colour development in animal tissue extracts. Consequently the values reported for creatine in the chick embryo, especially at the low concentrations prevailing under physiological conditions (Sendju, 1927; Walker & Wang, 1964), should be questioned.

The present work investigates (a) whether the heart receives creatine from the egg before amidinotransferase can be detected in the embryonic liver, to clarify the significance of the presence of creatine kinase in cardiac tissue as early as the nine-somites stage; (b) whether the creatine contained in the embryo represses the liver amidinotransferase synthesis during the first 3 days of development of the hepatic primordium; and (c) whether the creatine present in the liver during the pre- and post-natal stages is sufficient to act as a repressor. Enzymic methods for determining creatine and arginine were used to avoid the inhibition and non-specificity of the colorimetric techniques.

MATERIALS AND METHODS

A Hy-Line variety of White Leghorn (*Gallus gallus*) eggs (J. Dickinger Hatcheries, México, D.F., México) were stored before use at nearly 18°C for no longer than 10 days after laying. Incubation was carried out in a single stage 252-B Jamesway or in a Petersime no. 4 machine. New-born chicks were divided into two groups (1) chicks fed with a control diet consisting of Purina Chick Startena Powder (Ralston Purina, México, D.F., México) and (2) chicks fed with a creatine-rich diet consisting of 2% (w/w) creatine mixed with Startena Powder.

Extracts were prepared from crude egg yolk or albumin, whole embryo or liver of growing chicks at the ratio of $1\text{g}/8\text{ml}$ of 5% (w/v) trichloroacetic acid. Each mixture was centrifuged for 15 min at 0°C and 7000g in a PR-2 International Centrifuge and the pellet discarded. The supernatant was adjusted to pH approx. 12 with 8M -NaOH and kept at 4°C for at least 12 h to precipitate all the remaining proteins. The pH was finally adjusted to 7.4 with 4M -HCl and the volume was made up with double-glass-distilled water according to the weight of the tissue.

Homogenates for glycine amidinotransferase determinations were prepared as described elsewhere (Walker, 1960; Ramírez *et al.* 1963) from egg yolk, embryonic disc and liver from embryos or growing chicks. Fresh preparations were used although the enzymic activity was stable below 0°C for at least 1 month.

Creatine and creatine phosphate were determined

colorimetrically by the method described by Ennor (1957). Commercial α -naphthol was purified by double steam-distillation from $\text{m-H}_2\text{SO}_4$.

Total guanidine compounds were measured by the procedure of Rosenberg, Ennor & Morrison (1956).

Creatine was assayed enzymically by the method of Bernt, Bergmeyer & Möllering (1963) based on the work of Tanzer & Gilvarg (1959). The pH of the m-glycine-NaOH buffer was 9.65 to give a final pH of 9.0 in the incubation mixture. A creatine standard sample was run with each set of measurements and the decrement of extinction at 340nm was registered in a Unicam SP.800 recording spectrophotometer. A sensitivity of approx. 5nmol and a reproducibility of approx. 10nmol of creatine were obtained. Arginine was determined by the procedure of Oginsky (1957) and Greenberg (1955) but modified in some details. As a buffer, m-glycine-NaOH , pH 9.65, was used instead of arginine. Commercial arginase, activated in the presence of maleate- MnSO_4 solution, was not further diluted and 0.25ml of this preparation (8 units) was added to the mixture containing 0.50ml of buffer and 0.75ml of the solution to be assayed. The final pH of the incubation mixture was 9.5.

The assay for glycine amidinotransferase previously used by Walker (1960) and Ramírez *et al.* (1963) was modified as follows. The reaction was started by the addition of 0.20ml of the homogenate and stopped with 0.70ml of water and 0.28ml of 30% (w/v) trichloroacetic acid after 0, 30 and 60 min incubation. An 0.50ml portion of the clear supernatant was mixed with 0.16ml of water, 0.66ml of $\text{m-potassium phosphate buffer}$, pH 7.0, 0.10ml of acetone and 0.10ml of 1% (w/v) sodium pentacyano-aminoferrate. Extinction was read at 480nm in a Beckman DU spectrophotometer after 10 min. A standard graph with hydroxyguanidine sulphate solution (Walker & Walker, 1959) was prepared in each analysis. An extinction value of 0.700 for $120\mu\text{g}$ of the guanidine compound was obtained under the described conditions. Specific activity is expressed as μmol of hydroxyguanidine formed/h per g wet wt. at 37°C and pH 7.4 (Walker, 1960).

RESULTS

Egg. The determination of creatine by Ennor's (1957) colorimetric procedure in four eggs gave an average of $0.38\mu\text{mol}$ in the albumin and $2.97\mu\text{mol}$ in the yolk. Creatine phosphate was not found in the yolk extract even when conditions for hydrolysis of the phosphorylated form were followed. By contrast with the colorimetric assay, creatine was not found either in the albumin or in the yolk extracts by the enzymic method, in spite of the analyses being carried out on an extract prepared with several eggs and concentrated by freeze-drying. The presence of inhibitors was ruled out on the basis of the 100% recovery of known amounts of creatine added to the initial trichloroacetic acid mixtures. This result also proves the stability of creatine throughout the procedure. Finally, no glycine amidinotransferase activity was detected in the yolk homogenates.

Some other guanidine compounds could be responsible for the creatine values obtained with Ennor's (1957) procedure (Dubnoff, 1957). However, since neither creatine nor amidinotransferase were detected in the yolk homogenates it seemed very unlikely that guanidinoacetate could be present in the egg, so it was decided to look for free arginine. The enzymic assay of arginine gave an average of 15.7 (10.0–27.2) $\mu\text{mol/yolk}$ and 3.38 (3.14–4.33) $\mu\text{mol/total albumin}$. The percentage of free arginine in the total amount of guanidino compounds determined by the method of Rosenberg *et al.* (1956) was measured. Known amounts of arginine were separately determined by the procedures described by Rosenberg *et al.* (1956) and Greenberg (1955) and the content of arginine in the egg was measured enzymically in the yolk and in the albumin extracts. These values were converted into guanidino compound extinction values and subtracted from the extinction values obtained for total guanidino compounds in the same extracts (Rosenberg *et al.* 1956). Thus arginine represented an average of 85% of guanidino compounds in the

yolk and 48% in the albumin. The remaining amidine colour-forming compounds have not been isolated or identified.

Embryo. Creatine was clearly shown in the extracts of whole chick embryos after day 6 of incubation, by either the enzymic or the colorimetric procedures, in contrast with its absence from non-incubated eggs. The amount of creatine, creatine phosphate and total guanidino compounds, all expressed as creatine, increased in the embryo throughout the incubation period (Fig. 1). To attempt to explain the difference between the amounts of creatine measured enzymically and by the colorimetric procedures of Ennor (1957) and Rosenberg *et al.* (1956), free arginine was sought but it was not detected in any extract. In addition, when both the value obtained as total guanidino compounds and that of creatine determined colorimetrically were referred separately to the corresponding value of creatine measured enzymically, the calculated ratio for each one was almost the same for all the extracts prepared during the incubation period. This constancy suggests that the discrepancy in comparing the three values for the same sample is due to the analytical method applied, as no other guanidine compound but creatine is involved in the measurements.

The hepatic glycine amidinotransferase specific activity showed an increase from zero to a maximal value around day 12 of incubation and, in contrast

Fig. 1. Creatine content in the whole chick embryo. □, Total guanidino compounds expressed as creatine; Δ, creatine assayed colorimetrically and ○, enzymically; ●, creatine phosphate, measured as creatine after hydrolysis of the extract; ▲, all of the above values overlap in this region of the graph.

Fig. 2. Ontogenesis of the glycine amidinotransferase specific activity in the liver of the chick embryo. Assays were done as described in the Materials and Methods section. Each point represents the average of four determinations and the bars indicate the range of values. The last point includes liver from chicks killed between 12h before and 12h after hatching.

Fig. 3. Liver amidinotransferase specific activity in chicks receiving either a normal (—) or a 2% creatine-rich (---) diet at least 5 days before the assay. Bars have the same meaning as in Fig. 2.

Fig. 4. Change in the creatine content determined enzymically in livers from growing chicks. —, Normal diet; ---, 2% creatine-rich diet. The livers were those used for results appearing in Fig. 3.

with the statement by Walker & Walker (1962), the specific activity near hatching did not decrease to very low values (Fig. 2). In this stage of incubation our results were obtained from the livers of chicks killed between 12h before and 12h after hatching.

Chick. The liver glycine amidinotransferase specific activity of normally fed chicks increased

Fig. 5. Specific activity of liver amidinotransferase as a function of creatine content determined enzymically in the liver of chicks fed with a creatine-rich diet: ○, 6h; ●, 12h; △, 5 days; ▲, 10 days; □, 40 days; ■, 51 days after hatching.

sharply from hatching to day 10. The values at this time-limit are the highest recorded during the embryonic and the post-hatching periods studied. After this maximum, as found by Ramírez *et al.* (1963), the enzymic activity decreased to an average value of 8 units at day 50 (Fig. 3). This value is similar to that of the activity plateau observed in the liver of the embryo at the end of the incubation period (Fig. 2). In contrast, chicks receiving the creatine-rich diet at least 5 days before the assay showed a remarkably low amidinotransferase activity (Fig. 3), thus verifying former findings (Walker, 1960; Ramírez *et al.* 1963).

The enzymically measured creatine content of the liver from chicks fed on a creatine-rich diet had two increments, one at the beginning and another at the end of the 50-day period (Fig. 4). In contrast, the normal fed chicks showed a lower and almost constant hepatic concentration of creatine except for a slight increase from day 5 to day 10 (Fig. 4). Lastly, in creatine-fed animals, an inverse relationship was established between the hepatic amidinotransferase activity and the liver content of creatine (Fig. 5).

DISCUSSION

Chick embryo heart does not receive creatine from the egg, since, contrary to the findings of

Table 1. *Total liver amidinotransferase activity and creatine content of the whole chick embryo*

Incubation time (days)	Liver* (g)	Liver amidinotransferase activity		Creatine/ embryo† (μ mol)
		Specific‡	Total	
12	0.112	14	1.57	20
20	0.370	8	2.96	80

* Values taken from Ramírez *et al.* (1963) for the same strain of chicks.

† Values from present results are expressed as described by Walker (1960).

‡ Values determined enzymically.

Sendju (1927), such a compound could not be demonstrated in the entire egg by the enzymic assay employed in the present work. These results suggest that heart creatine kinase is a constitutive enzyme at the early stages of chick-embryo development. However, before the significance of such an enzyme in the chick can be properly discussed, it must be recalled that Sasa & Noda (1964) demonstrated the coexistence of adenosine triphosphatase and creatine kinase activities within the same molecule in rabbit skeletal-muscle preparations. It is probable that the genome of heart cells at the nine-somites stage selectively directs the synthesis of this kind of enzyme (Eppenberger, Eppenberger, Richterich & Aebi, 1964) in the absence of creatine but in the presence of ATP (Romanoff, 1967). This explanation fits well into the developmental terms derived from theories based on selective protein synthesis (Gorini & Horowitz, 1965; Strehler, Hendley & Hirsch, 1967).

On the other hand, the hypothesis of repression to explain the lack of chick liver amidinotransferase in the hepatic primordium during the first 3 days of its development seems unlikely in view of the absence of creatine from the whole embryo at that time (Fig. 1). Moreover, the results on the ontogenesis of the liver amidinotransferase show a steady increase of specific activity from 0 to an average of 13 units in spite of the increase of the total embryo creatine from day 6 to day 12 of incubation; afterwards the activity decreased until day 16, when an average value of 8 units was reached and maintained after hatching while the embryonic creatine content increased rapidly in the last two-thirds of the incubation time (Figs. 1, 2 and 3). Our values for the enzyme activity just before and after hatching disagree with that (2 units) reported by Walker & Walker (1962) in their control animals. This discrepancy might be due to the fact that in the present work the number of chicks used was four for each experimental point (Fig. 2) whereas Walker & Walker (1962) did not state the number of animals used. In spite of the

decline of amidinotransferase specific activity of the liver observed from the day 12 of incubation, the total hepatic activity actually increases (Table 1) because the liver grows in total mass irrespective of its lower differential growth rate (Dumm & Levy, 1949). A rise in the total amidinotransferase activity implies that more guanidinoacetate is produced and, since Walker & Walker (1962) showed that embryonic chick liver can convert labelled guanidinoacetate into creatine at the same rate at day 12 or day 18 of incubation, the total guanidinoacetate methyltransferase activity is also higher in the last one-third of the incubation. The increase in the total liver activity of the two enzymes involved in the creatine pathway explains the observed increase in the embryonic creatine content (Fig. 1). Consequently, the theory of regulation by negative feedback cannot be supported any more. In summary, it is very unlikely that the feedback repression of amidinotransferase operates during the physiological development of the chick embryo: thus the synthesis of this transferase appears to be controlled by a mechanism independent of the creatine content.

It can be assumed that increasing amounts of the creatine synthesized in the liver are taken up by cardiac and skeletal muscles since both the creatine kinase activity (Eisenberg & Ramírez, 1963; Reporter, Königsberg & Strehler, 1963) and the creatine phosphate content increase steadily from day 10 of incubation (Fig. 1) (Herrmann & Cox, 1951).

Speculatively one is tempted to explain the decrease in the specific activity of liver amidinotransferase after day 12 as being due to an increase in the ornithine content in the same compartment, produced apparently by the previous increase of this transferase activity. This standpoint is supported by the findings of Ratner & Rochovansky (1956) who observed inhibition of this enzyme activity with high concentrations of ornithine in the assay system. The lack of inhibition reported by Walker & Walker (1962) with 220 μ mol of

ornithine injected to an egg several days before the assay could be explained if this compound were in another compartment producing glutamate for protein synthesis.

As far as the repression of glycine amidinotransferase by other guanidine compounds is concerned a question arises: is this hepatic enzyme repressed by just 16 μmol of free arginine found in the entire egg yolk at the first stages of the embryo development? It seems very unlikely, since only 33% of the liver activity was repressed 1 week after 220 μmol of arginine was injected into a 6-day-old embryo (Walker & Walker, 1962).

In the group fed on the control diet, the increase in creatine content per g of liver at about day 10 (Fig. 4) seems to coincide with the increase in the liver amidinotransferase specific activity (Fig. 3). Afterwards, the creatine content was constant and too low (0.8 $\mu\text{mol/g}$) to explain the decrease of the enzymic specific activity observed after day 10 (Fig. 3) since, as described by Ramírez *et al.* (1963), more than 2 μmol of creatine/g of liver is necessary to cause a 70% decrease of the enzymic activity. Repression again appears as an unlikely mechanism to control the activity of this enzyme in the liver of the chick during the postnatal period.

As reported by Walker (1960) and Ramírez *et al.* (1963) creatine-fed birds, in contrast with normal animals, showed higher liver creatine content and lower amidinotransferase activity (Fig. 5). Such an inverse relationship has been explained on the basis of a repression mechanism (Walker, 1960).

All these facts provide evidence that modulation of enzymic activity, which in turn exerts control over developmental events, is not simple. In the chick and probably in most vertebrates there are two different mechanisms involved in the control of creatine metabolism: (a) end-product repression of the liver glycine amidinotransferase, but only at pharmacological or pathological concentrations of creatine within the affected organ and irrespective of age or of conditions of life (Walker, 1960, 1965; Ramírez *et al.* 1963), and (b) regulation of this enzyme by other mechanisms not yet clearly understood, but surely more complicated than the simple negative-feedback regulation observed in lower organisms and involving compounds such as substrates or hormones at physiological concentrations (Krisko & Walker, 1966; Ungar & Van Pilsum, 1966a,b; Schimke, 1967; Van Pilsum & Ungar, 1968).

To learn more about this kind of regulation, model systems involving cultivated organs or clones of cells must be studied with precisely controlled parameters.

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