Studies on the Metabolism of the Amphibian Embryo

2. Patterns of Amino Acids and Nitrogen End Products

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兩棲類 發生卵의 物質代謝에 關한 硏究

2. Amino Acid Pattern과 Nitrogen End Products에 關하여

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摘　　要

韓國産 도롱농(*Hynobius leechii* BOULENGER) 發生胚의 窒素化合物의 代謝를 硏究하고저 發生段階에 따른 蛋白質構成「아미노」酸 및 遊離「아미노」酸의 定性的 分析과 窒素 排泄物(ammonia, urea, uric acid, creatine and/or creatinine, total nitrogen)의 定量分析을 未受精卵, 卵割胚, 胞胚, 囊胚, 神經胚 및 孵化直前의 胚에 對하여 行하였다.

1. 發生段階에 따른 發生胚內 蛋白質構成「아미노」酸은 全 段階를 通하여 一樣하게 18種이 檢出되었고, 遊離「아미노」酸은 17 내지 23種 檢出되었으며, 全體的으로 遊離「아미노」酸 分布相에 큰 변화가 없었다.

2. 發生段階에 따른 窒素排泄物 分析의 結果는 다음과 같다. Ammonia와 urea의 胚內 含量은 未受精卵에서 부터 囊胚까지 점차 減少하며 囊胚 以後 孵化直前胚까지 급격히 增加한다. Uric acid의 含量은 全 段階를 통해 微少하였으며 creatine and/or creatinine은 本 實驗에서는 檢出되지 않았다.

3. 發生段階에 따른 發生胚內 ammonia 含量에 對한 sodium azide의 영향은 濃度에 따라 一定的인 特異性을 가지고 있었다.

INTRODUCTION

Patterns of nitrogenous excreta of the amphibian embryo have been studied within a definite category and the results obtained thus far are generally not in accord with each other. Bialascewicz and Mincovna (1921) reported that the amounts of urea and ammonia excreted from the embryo of *Rana pipiens* increased with time during development, while Brachet (1939) observed them unaltered in the same material. Ishihara (1956) also reported the same result as Brachet's in the embryo of *Bufo vulgaris*. Arima (1961) observed not only the same result with that of Brachet in the embryo of *Rhacophorus schlegelli* and *Rana nigromaculata*, but a slight increase of ammonia content at the time of heart-beating of *Rhacophorus*. He pointed out that the main nitrogenous end product was not excreted in the form of urea but of ammonia in both of the above animals. Kang, Ha and Han (1961) detected the amino acids related closely to urea-formation; namely, ornithine, arginine, and citrulline at neurula stage of *Hynobius* and supposed that the urea-cycle would begin to operate at this stage.

Meanwhile, the effect of metabolic inhibitors on the production of ammonia seemed to be closely correlated with protein-decomposition. Ishihara (1956) found an increase of ammonia excretion from the embryo of *Bufo vulgaris* treated with $1x10^{-4}M$ and $1x10^{-1}M$ of azide solutions and a decrease in $1x10^{-2}M$. This effect seemed to be dualistic.

The present paper deals with the patterns and amounts of nitrogen end products and with the effect of sodium azide on the production of ammonia of the embryo of *Hynobius leechii* BOULENGER during its course of embryonic development. Since the amino acid pattern of the embryo seems, at least partly, to reflect the pattern of nitrogen end products, free and protein-bound amino acids of the embryo were also analysed qualitatively.

MATERIAL AND METHODS

Unfertilized eggs and embryos of *Hynobius leechii* BOULENGER at five developmental stages; cleaving, blastula, gastrula, neurula and prehatching were used. The stages were determined under a dissecting microscope according to Shumway (1940).

Paper chromatographic analyses of amino acids were carried out by the procedures described elsewhere (Ha and Lee, 1961). Protein hydrolysis was done at 110°C for 24 hours with 6*N* HCl.

For the analyses of nitrogen end products, 100 eggs or embryos freed of their jelly coats and perivitelline membrane were placed in 10 ml. of Holtfreter's solution (pH, 6.8) at room temperature for 24 hours, after which they were transferred to another 10 ml. of fresh Holtfreter's solution and immediately homogenized in a glass homogenizer for an hour. The homogenate was then centrifuged at 1,300 x *G* for 30 minutes and supernatant was used for the analyses of ammonia, urea, and total nitrogen in the embryo by Conway's microdiffusion division method (Conway, 1962) in which $1 \times 10^{-3}N$ HCl and $2.14 \times 10^{-3}N$ $Ba(OH)_2$ were used for ammonia, $1 \times 10^{-3}N$ HCl and $1.67 \times 10^{-5}N$ $Ba(OH)_2$ for urea, and 1/150 *N* HCl and $3.57 \times 10^{-5}N$ $Ba(OH)_2$ for total nitrogen. In the analysis of total nitrogen, the sample was decomposed with the mixture of $CuSO_4$ and SeO_2 by the method of Borsook and Dubnoff(1939).

Uric acid was quantitatively determined by Folin's direct colorimetric method with an electrophotometer precipitating proteins with tungstate-sulfate solution. Creatine and/or creatinine were analysed by Edwards and Whyte's method (1958) modifying Jaffe's with an electrophotometer precipitating proteins with sodium-tungstate solution.

To observe the effect of azide on ammonia-production, embryos were treated with sodium azide of the concentrations of $1 \times 10^{-1}M$, $1 \times 10^{-2}M$, $1 \times 10^{-3}M$, and $1 \times 10^{-4}M$. Other procedures for the determination of ammonia in azide-treated embryos were the same as described above.

RESULTS

1. Amino acids in embryos at developmental stages. The qualitative distribution patterns of amino acids contained in the embryos are shown in table 1. Table 1 shows that the pattern of protein-bound amino acids is practically unchanged during embryonic development with 18 amino acids and one unidentified substance. This fact seems to suggest that there is no essentially profound change in the metabolism of protein during the early development, although there may be a variation in the pool of amino acids or their sequence.

From table 1, however, it is apparent that there is some, though not very significant, variation in the patterns of free amino acid distribution in the embryo according to stages. Arginine and ornithine which are known to be related closely to the urea formation are present throughout all stages examined. Citrulline, on the other hand, is detected only at neurula and prehatching stages. Glutamic acid is found in the embryos of all stages, being the most abundant at gastrula and neurula stages.

2. Nitrogen end products in the embryo. The results of quantitative analyses of nitrogen end products in embryo at developmental stages are shown in table 2. It shows that the amount of ammonia decreases slowly from the unfertilized egg (13.190 μg/10 emb.) to gastrula stage (7.133 μg/10 emb.), after which it increases gradually up to

Table 1. Protein-bound (P) and free (F) amino acids in embryo at developmental stages.

Amino acid \ Stage	P						F					
	U	C	B	G	N	H	U	C	B	G	N	H
Leuc./Isoleuc.	+++*	+++	+++	++	+++	++	+	++	+++	++	+++	++
Phenylalanine	++	+	++	+	++	+			+	++	+++	+++
Methionine/Val.								+	++	++	+++	+++
Methionine	++	+	+	+	++	+						
Valine	++	+	++	+	++	++						
Tyrosine	+	+	++	++	+	+			+			+++
β-alanine	+	+	+	+	+	+	++	+++	+++	++	++++	+++
Alanine	++	++	++	++	++	++	+	++	++	++	+++	+++
Proline	++	++	+	++	++	++	++	+	++	+	+	++
Prol. OH	+	+	+	+	+	+						+
Threonine	+	+	+	+	+	+	+	+	++	+	+	++
Glutamic acid	+	++	++	++	++	++	++	+	++	++	++++	++
Glycine	+++	+	+	+	+	+	++	+	++	++	++	++
Serine	++	++	+	+	++	+	++	+	++	++	++	++
Arginine	++	++	+	++	++	+	+	+	+	+	++	+++
Asparagine							+			+	++	+
Aspartic acid	++	++	++	++	++	++	+	++	++	++	+++	+++
Lysine	+	+	+	++	+	++	+	++	++	++	++	++
Histidine							++	+	++	++	+++	+++
Cystine	+	+	+	+	+	+	+	+	++	+	+	++
Ornithine							++	++	+++	++	++	++++
Citrulline											+	+
Tryptophan							+	++	+			+
Unknown.	+	−	+	+	+	+	+	+	+	+	+	+

* + signs show relative amount of amino acids detected.

Stages are designated throughout this paper as follows: U, unfertilized egg; C, cleavage; B, blastula; G, gastrula; N, neurula; and H, pre-hatching.

prehatching stage (24.544 μg/10 emb.). The ammonia content at prehatching stage is twice as much as that of unfertilized eggs which, in turn, is almost the same value with that of neurula stage and becomes twice as much as those of gastrula and blastula stages. Table 2 also shows that urea content begins to decrease at blastula stage and reaches its minimal amount at gastrula, after which it increases up to prehatching stage.

Table 2. The amounts(μg/10 embryos) of NH_3, urea, uric acid, creatine/creatinine and total nitrogen in embryos at developmental stages.

Stage \ N-end prod.	NH_3	Urea	Uric acid	Creatine/creatinine	Total nitrogen
U	13.190 ± 0.752	31.040 ±4.550	2.259 ±0.549	—	116.670 ±14.177
C	9.775 ± 0.747	31.230 ±7.198	2.649 ±0.378	—	117.140 ±28.018
B	7.460 ± 0.239	23.120 ±5.532	2.964 ±0.171	—	118.340 ±23.195
G	7.133 ± 0.841	8.760 ±3.834	4.436 ±0.306	—	154.340 ± 8.485
N	13.874 ± 3.224	7.280 ±4.494	3.270 ±0.123	—	136.840 ±14.731
H	24.544 ± 0.974	59.350 ±5.762	4.358 ±0.229	—	118.140 ± 5.119

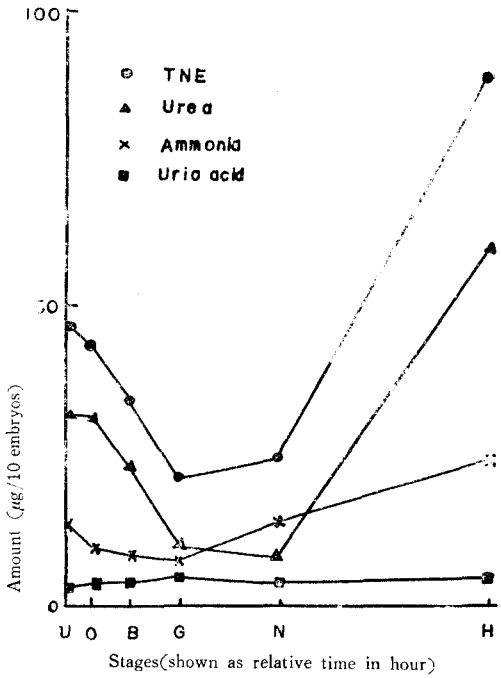

Fig. 1. The amounts of N-end products. TNE represents the sum of a nitrogen excreta(Total nitrogen excreta).

The amount of uric acid is so small as compared with the other nitrogen end products that its change during development can not practically bring any influence to the over-all change in nitrogen excretion. Uric acid content keeps increasing from the stage of unfertilized eggs to gastrula stage, after which becomes almost unaltered. At gastrula stage, uric acid shows its maximal amount while ammonia and urea contents show their minimal amounts.

No measurable creatine and/or creatinine are detected in the embryo in this experiment.

Table 3. Percentage of each nitrogen end product to total excreta

Stage \ N- end prod.	NH_3	Urea	Uric acid	Creatine and/or creatinine	Total excreta
U	28.37	66.77	4.86	0	100
C	22.39	71.54	6.07	0	100
B	22.24	68.92	8.84	0	100
G	35.09	43.09	21.82	0	100
N	56.80	29.81	13.39	0	100
H	27.81	67.25	4.94	0	100

In order to generalize the results of N-end products, their amounts are calculated and shown as percentage of total amount of nitrogen excreta in table 3. Uric acid represents the lowest percentage among all N-end products, while urea and ammonia occupy 65–30% and 20–30%, respectively, at all stages except at gastrula and neurula stages. Total amount of nitrogen excreta shows a U curve, its minimal value being at gastrula, as revealed in figure 1.

The amount of total nitrogen in the embryo is constant during development with a slight increase at gastrula stage.

3. The effect of azide on ammonia content in embryo. Ammonia content in the azide-treated embryo results in its decrease as compared with control, as shown in table 4. $1 \times 10^{-1}M$ of azide, the highest concentration used, has lower inhibiting action on ammonia production in embryo than lower concentration. In general, $10^{-3}M$ of azide shows the greatest inhibiting action on ammonia production in embryo than lower concentration.

Table 4. The amounts of NH_3 in control and azide-treated embryos at developmental stages.

Stage \ Treatment	Control	Azide $1\times10^{-4}M$	Azide $1\times10^{-3}M$	Azide $1\times10^{-2}M$	Azide $1\times10^{-1}M$
U	13.190 ±0.752	5.609 ±1.300	5.660 ±0.783	8.100 ±1.349	7.164 ±1.030
C	9.775 ±0.747	5.529 ±0.424	5.183 ±0.499	6.668 ±1.091	9.474 ±0.679
B	7.460 ±0.230	7.602 ±0.131	6.230 ±0.620	6.595 ±0.618	9.969 ±0.048
G	7.133 ±0.841	6.469 ±0.574	5.798 ±0.822	6.870 ±0.386	8.096 ±0.422
N	13.874 ±3.224	7.355 ±1.049	4.750 ±0.881	7.580 ±0.736	10.214 ±0.163
H	24.544 ±0.974	5.499 ±0.740	9.557 ±0.797	6.956 ±0.874	8.893 ±0.846

In general, $10^{-3}M$ of azide shows the greatest inhibiting action, followed by $10^{-4}M$, $10^{-2}M$ and $10^{-1}M$ in decreasing order. The ammonia-contents in cleaving egg and gastrula treated with $1 \times 10^{-1}M$ of azide have almost the same value with that of control, while blastula exceeds control (blastula, 9.969± 0.048; control, 7.460±0.239; $P<0.001$). Azide of every concentration examined has in general the weakest effect on blastula and gastrula. The effect of azide on ammonia production in embryo involves dualistic specificity in accordance with the concentration.

DISCUSSION

In the present experiment the distribution of free amino acids in embryos of *Hynobius leechii* were found to be almost unchanged during embryonic development.

This is not in accord with the results of Kang, Ha and Han (1961) who reported much smaller number of free amino acids at gastrula and neurula stages of *Hynobius leechii*. This discrepancy seems to arise from the different procedures employed for amino acid extraction. The method used by Kang, Ha and Han (1961) in free amino acid extraction might cause the loss of several kinds of amino acids which are supposed to exist in very low concentration. In fact, Kavanau (1953) has reported that 12 amino acids were present in very low concentrations at gastrula stage of sea-urchin eggs. Lowe (1954) also obtained the similar result as Kavanau's in *Rana pipiens*. Lowe (1954), on the other hand, indicated a considerable change of amino acid distribution among stages; for instance, between stages 22-24 and stage 34 in *Amblystoma*, and concluded that catheptic enzymes might be activated to make available larger amounts of amino acids from yolk for the embryo during this period. At present the exact pattern of amino acid distribution, both quantitative and qualitative, in the course of embryogenesis should wait for further studies.

That three amino acids related closely to urea formation were all detected at neurula and prehatching stages, which is partly in line with the result of Kang, Ha and Han (1961), seems to suggest that, from neurula stage, urea in the embryo is formed by the reaction of urea cycle, though no attempt was made to the determination of arginase activity in this work.

In the present data, urea contents in unfertilized and cleaving eggs are much larger than those of gastrula and neurula. At gastrula stage, not only urea but also ammonia is present in the least amounts among other stages examined. This seems to suggest a metabolic characteristics of the gastrula. According to Lø vtrup (1959), the energy for gastrula is sourced mainly from carbohydrate but not from protein. The lower concentrations of amino acids at gastrula may also reflect the small amounts of ammonia and urea in this stage. The observation of Kutsky *et al.* (1953) that total non-protein glutamic acid decreased sharply at mid-gastrulation followed by an increase to late gastrulation of *Rana pipiens* may also provide with the basis for the above assumption.

In the present study the ammonia contained in the embryo was found to increase from gastrula to prehatching stages. The amount of ammonia excreted from the embryo, however, was found to be practically unaltered during the course of the development in the present study. Brachet (1939), Ishihara (1956) and Arima (1961) also reported that the amount of ammonia excreted from the amphibian embryos did not change during the embryogenesis. From these findings it can be supposed that the amount of ammonia produced in the embryo increases with age and there is an intensive accumulation of the ammonia in the embryo since its excretion does not increase with age.

Uric acid, which is so small in amount throughout all the stages, seems not to be produced from ammonia as in case of the adult of aves or reptile but arise from the purine bases as an end product by breakdown of a part of nucleic acids in the undifferentiated cells and primitive tissues. Uric acid seems obviously not to participate in nitrogen excreta in the embryo.

As described previously, the effect of azide on the production of ammonia is less at blastula and gastrula stages than at other stages. According to Ha and Park (1963) the rate of oxygen consumption increased at the gastrula of *H. leechii* remarkably by the addition of carbohydrates, such as glucose, and succinate as substrates, and decreased strikingly by $1 \times 10^{-3}M$ KCN at this stage. This result seems to suggest that the effect of azide on ammonia production seems to reflect the possible characteristics of protein and carbohydrate metabolism at these stages in connection with the decrease of ammonia and urea-contents. The dualistic effect of azide on the ammonia production in *Hynobius* embryos shows the same result with that of *Bufo* obtained by Ishihara(1956).

SUMMARY

For the study of nitrogen metabolism during embryogenesis of amphibian embryos, protein bound and free amino acids were qualitatively detected and N-end products, including ammonia, urea, uric acid, creatine and/or creatinine and total nitrogen, were quantitatively analysed in the unfertilized eggs and embryos of *Hynobius leechii* BOULENGER at five

developmental stages.

1) The same 18 amino acids were found in protein bound form and 17 to 23 in free in the eggs and the embryos at five developmental stages. The distribution of free amino acids was found to be practically unchanged during development.

2) The analyses of N-end products in embryo during development resulted in the followings. Ammonia and urea decreased progressively in amount from the unfertilized to gastrula stages, from which their amounts continued to increase up to prehatching stage. The content of uric acid was so small throughout the developmental stages. Creatine and/or creatinine were not detected in embryos in the authors' experiment.

3) The effect of sodium azide on the content of ammonia in embryos was dualistic.

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