Nitrogen Changes During the Development of Eggs of *Melanoplus bivittatus*

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Melanoplus bivittatus 알〔卵〕 發生過程에 있어서의 窒素의 消長

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SUMMARY

The studies could be summarized as follows:

1. Standard Rf values were made as Table Ⅱ for the identification of amino acids.
2. Amounts of free amino nitrogen in eggs were increased about 0.03% (Table Ⅳ) while the amounts of total nitrogen (Table Ⅲ) were increased lower percentage during the ten days of development.
3. Three unknown substances were found newly during the development.
4. Eight ninhydrine positive amino acids were present, in the fresh embryonic eggs, but the numbers increased to eighteen after fifty days of development.
5. Through the development, there were large amounts of glutamic acid, aspartic acid and alanine.
6. After fifty days development, the amount of amides, such as asparagine, glutamine and alanine were increased very large which were very small amount before it and the amount of asparagine were more than that of glutamine.
7. Tryptophane, Cystine, Histidine were detected after 50 days of development. This is very remarkable fact.

INTRODUCTION

The development of the embryo in eggs of *Melanoplus bivittatus* from laying to diapause and through diapause to emergence undoubtedly undergoes numerous and very pronounced dynamic changes of stored nutrients. The development theoretically can be divided into two different phases; respiration and growth.

The rate of respiration can be considered as an objective measure of metabolism in general, but this by itself does not offer the satisfactory evidence for actual changes in composition of egg contents during the course of development. Our study is concerned with total metabolic changes of nitrogenous compounds in eggs. It is believed that this problem can be satisfactory approached by tracing the changes by means of proper biochemical analytical methods. The methods which will be used during the study are Micro Kjeldahl method, Van Slyke method, and Paper chromatography.

EXPERIMENTAL METHODS AND MATERIALS

Maintenance and Rearing of *M. bivittatus*: *M. bivittatus* are a convenient source of live material for different studies. Some of the hardy species, especially *M. bivittatus* or *M.* differential is very adaptable to laboratory conditions and can be reared with ease. General outlines for rearing and maintenance are found elsewhere.

Two species, *M. bivittatus* and *M. mexicanus* were reared for the purpose of comparing their aerobic metabolism. Both species appear to be easily maintained and reproduced in captivity if some necessary precautions are taken.

Maintenance: The insects were reared in the

wooden cages, measureing 24×21×15 inches, having screens on two sides, top and bottom. The screen at the bottom serves for separation of feces and debris. Two sides are equipped with glass windows, movable vertically in grooves. The windows and the door at the top served for feeding, cleaning, or for collecting the eggs and insects. A single cage was suitable for housing 200-300 adult *M. bivittatus*. In order to prevent overcrowding should be aboided. The cages were cleaned daily and disinfected with an alcoholic solution of potassium hydroxide and rinsed subsequently with hot water whenever the old stock was used up. Illumination by sunlight was insufficient; therefore, incandescent lamps located at each cage and fluorescent lamps above the cages were left on continuously.

Temperature and Humidity: Temperature held between 20 and 30°C is suitable; However, it was observed that vitality, growth, and laying of eggs was better at the temperatures close to 30°C or slightly over. *M. mexicanus*, in particular, appeared to favour above 30°C temperatures. Humidity unless very high, does not seem to be an important factor during development but becomes important at the time of laying.

Feeding: In the winter, vegetable trimmings, largely lettuce leaves, were the main source of food.

The diet was supplemented with grain seedlings and carrots. Dry alfalfa and clover leaves were also eaten readily by *M. bivittatus*. In the late spring and summer, fresh green food was fed consisting of grasses, alfalfa, and other plants collected from the field. Fresh food was supplied twice a day.

Green food stuffs were always washed to avoid nematode parasites, then hung on a piece of wire and suspended overhead in the cage. The *M. bivittatus* seemed to favour food which was hung to that placed on the bottom of the cage. Moreover, contamination of food with feces was prevented.

Laying: It was noted that increasing the relative humidity by placing the water in shallow vessels close to the cages or by spilling the water on the floor favours laying. The female lays the eggs in moist sand placed in a container about two inches deep. Indirect light is sought by the female when laying; therefore, the container was placed in half shadow. The eggs were collected daily in order to ensure uniform conditions for development storage and hatching. The most convenient way to separate eggs from the sand is to empty the container on the wire screen of a suitable mesh and shake gently.

Hatching: The eggs, freshly collected, were stored at room temperature (25±3°C) in moist but not wet sand till diapause. The emergence of nymphs from non-diapausing eggs (after about 3-4 weeks) gives the most reliable assurance that the stage of diapause has been reached. The diapausing eggs are subsequently stored for at least thirty days in the refrigerator at 0-4°C temperature. When returned to room temperature the eggs hatch after 5-6 days; the emergence of hoppers from properly diapaused eggs is rapid.

Lower temperatures and wet storage prolong the time of hatching. The diapausing egg can be stored under refrigeration for a long time without impairing the hatch ability providing the temperature and moisture are controlled.

Preparation of Homogenate

Procedure:

1. Cut off the top of egg with a razor blade.
2. Press out squeeze inner material of egg with finger and place into homogenizer (Potter Elvehjem type) about 400mg. The homogenizer tube should be immersed in ice cold water while the contents of the eggs are being collected. Cooling of the tube is necessary as preparation of the sample takes a long time about one hour and cooling prevents denaturation of the proteins.
3. Weigh the eggs' contents
4. Add water of same volume of material of eggs' contents in the homogenizer tube and grind for one minute slightly at about 1000 r. p. m.

Average weight of a egg is about 5 mgs. and one egg contains about 1 mg. material. About 120 mg. of material was required for each complete run. This amount yielded enough 5% homogenate

for 10 flasks and 20 mg. for the determination of nitrogen. Therefore, 120 eggs were required and one day was involed in separation and preparation of homogenate.

5. Put the homogenate in the refrigerator (0°C-4°C) for two hours, remove and again grind for one minute slightly.

6. Replace the homogenizer tube in the refrigerator for another two hours.

7 After grind again slightly for two minutes.

8. Repeat the above procedure 5 times and allow the homogenate to stand in the refrigerator overnight.

9. Grind the homogenate again and then empty it into a calibrated centrifuge tube and dilute to 3 ml. with redistilled water.

10. Take 2 ml. of diluted homogenate and put it 10 ml. of volumetric flask for determination of amino nitrogen and total nitrogen.

11. Then, centrifuge the 1 ml. aliquots of the homogenate for 30 minutes at 2500 r.p.m. speed.

12. Then, take the middle layer sample by micro pippete and spot it on the filter paper for determination of free amino acids.

Preparation of Standard Paper Chromatogram of Pure Amino Acids (1)

1. Spot 10 μg (10μml) of known each amino acid with micropipette near the corner of Whateman No. 1 paper. The tiny drop spreads out on the dry paper to a circular spot which is allowed to dry for a short period.

2. Solvent

Ist solvent: n-Butanol: acetic acid: water=40:10:50 (Slotta, 1951)

2nd solvent: Phenol 80 g. and water 20ml.

Use in the presence of HCN ($NaCN+H_2O$)

The paper prepared are suspended so that its upper end hangs over the edge of a trough and dips into the suitable first solvent contained there in. When the solvent front hat almost reached the end of the paper of the of the lengthwise, the latter are removed from the trough and after marking the position of the solvent front, is allowed to dry at oven 80°C. (Be carefull the temperature of dry oven.)

3. When dried, run the other 2nd solvent across the paper. time is required about 24-26 hrs.

4. Dry paper again, then spray with indicator (1), and dry in the oven to develop colour of amino acids until the spot becomes clearly visible. (about20 minutes, be carefull the temperature about 80°C.)

Indicator: 0.2% solution of ninhydrin containing 4% acetic acid.

5. Measure the Rf value of these known amino acids from their position. By using these relative position, the unknown amino acid will be identified.

Determination of Amino Nitrogen of Egg Contents by Van Slyke Apparatus

A. Principle of the Method: The method employed with this apparatus depends on the fact that primary aliphatic amino nitrogen is evolved by the reaction with nitrous acid. The simple aliphatic alpha-amino acids react quantitatively with nitrous acid in about 4 min., at room temperature.

The time for complete reaction of beta, gamma, delta and epsilon amino acids increases directly with the distance of the amino from the carboxyl group (Gunn and Schmidt, 1922). About 30 min. is required for the complete reaction with lysine, arginine, cystine, glysine, histidine, tryptophane, and serine yield higher than the theoretical amount of nitrogen, particularly at elevated temperature, (Schmidt, 1929). Air most be displaced from the apparatus before the reaction takes place and this is acomplished by blushing the system, and finnaly fillig it, with nitric oxide resulting from decomposition of some of the nitrous acid which is to be used for reaction with the amine. The nitrous acid for this prepared in the apparatus by mixing sodium nitrite with acetic acid.

$$NaNO_2+CH_3COOCH=CH_3COONa+HNO_2$$

and $3HNO_2=H_2O+2NO+HNO_3$

After the air has been forced the apparatus and displace by nitric oxide the sample is introduced and the reaction with the amino nitrogen is allowed to go to completion.

$$R.NH_2+HNO_2\rightarrow R.OH+N_2+H_2O$$

The entire volume of gas in the apparatus is transfered to the absorption chamber where all

the nitric oxide is absorved in alkaline permanganate solution.

$$NO+KMnO_4 \rightarrow KNO_3+MnO_2$$

The volume of the remaining gas is measured in a gas burrette at atmospheric pressure and room temperature. The weight of nitrogen in the sample is calculated from these data by applying gas-law principles or, more conveneintly, by employing the conversion factor in the table factors prepaered by Van-Slyke.

B. Optimum Conditions for Van Slyke Apparatus: The most suitable amount of nitrogen for Van Slyke apparatus was calculated from theory to be 0.935 mg of nitrogen in the amino acids of the sample.

Total volume of gas burette of the Van Slyke apparatus is 3 ml. So, optimum volume of nitrogen that is (1.5+x)ml in gas burette. Therefore must be x <(3−1.5)ml

C. Reagents: (All reagents were analytical grade.)

Alkaline permanganate solution

Sodium nitrite solution

Octyl alcohol

D. Procedure.

1. Pour sufficient glacial acetic acid into the charging reservoir to fill the deaminizing chamber (about 3 ml) and pour the sample in to the reservoir.

2. Add sodium nitrite solution through the charging reservoir until the deaminizing chamber is filled with solution and a small amount liquid remains in the reservoir.

3. After sufficient gas has been generated to force most of the solution back into the charging reservoir, turn stopcock to allow the gas to escape and the liquid to return to the deaminizing bulb.

4. When the gas has again forced almost all of the liquid back into the charging reservoir, turn stopcock to allow the gas to escape, and approximately 4 ml of the solution to be forced once more in to the charging reservoir.

5. Immediately lower the levelling bulb, and carefully turn stopcock permitting exactly one or two ml of the sample to flow into the deaminizing chamber.

6. Agitate for 5 minutes.

7. Permit the excess acid nitrite solution to force all the gas from the diaminizing bulb into the gas burette.

8. Raise the leveling bulb as high as possible, allow the gas to be forced into the absorber as soon as the water has filled the capillary to the base of stopcok.

9. Agitate one to three minutes.

10. After read the volume of nitrogen in the burette and record, the baromertric pressure and the temperature in the vicinity of the measuring burette must be noted and recorded.

11. To test for completeness of reaction, repeat the procedure of the nitrogen evolving reaction.

12. A duplicate determination may be made after discarding the nitrogen from the burette by raising the leveling bulb.

13. It is necessary to run a blank determination following the precedure exactly as in the case of sample analysis except that the sample is replaced by an equal volume of water.

The blank reading should not exceed 0.06 to 0.12 ml.

Determination of Total Nitrogen

Nitrogen was determined in duplicate for all homogenates and other preparations by a Micro Kjeldahl method. (3, 4)

Reagents: Digestion mixture: Sulphuric acid (sp. gr. 1.84) and orthophosphoric acid (sp. gr. 1.56) 2:1; catalysts used were: copper sulphate, and selenium dioxide, 10g of each per liter of the digestion mixture.

Potassium hydroxide: 300g/l

Boric acid solution: 4%

Standard hydrochloric acid solution: 0.01N.

Indicator; Brom-cresol green and methyl red, 1% solution of each in ethanol, mixed in equal quantities.

A volume of 0.2 ml. of the 5% homogenates was employed as a suitable size of the sample, corresponding to approximately 0.3 mg. of nitrogen.

The accuracy of the method was checked against standard glycine solutions. The recovery of nitrogen averaged 98.6±0.7%. The nitrogen contents six

samples of the same homogenate compared with fresh tissue (100%) averaged 98.8±3.1% recovery. The deviation is most likely due to the fact that homogenate is not truly homogeneous and uniform sampling is difficult.

RESULTS AND DISCUSSIONS

Embryonic eggs were analysed by two dimensional paper chromatography, micro-Kjeldahl method, and Van-Slyke method for nitrogenous compounds as a part to the study no the amino acids metabolism.

The results obtained and discussions are as follows: In the fresh eggs, 9 ninhydrine positive free amino acids were present at the early development, but the number increased to 18 amino acids on 50 days old.

There were 3 unknown substances which formed newly during the development. After 30 days development, the amount of amides, such as asparatic acid and glutamine were increased large which were very small amount at the early days of development.

Those were accumulated more in 50 days old eggs than 10 days old, and the amount of glutamic acid were more than of asparatic acid.

Through the development, there were large amount of glutamic acid, asparatic acid, valine, tyrosine, isoleucine, glycine, arginine and alanine which seem to be concerned in transamination reaction through the development. dl-Threonine, homocystine mono-hydrochloride, histidine and leucine increased to considerable amount during development. This is very remarkable fact as those amino acid were reported to be concerned in transamination reaction.

Asparagine was not observed, it seems that the all asparagine were decomposed into the asparatic acid and ammonia by the asparaginase as well as Vickery (6) reported.

It is considered that the amount of glutamic

Table I. Free amino acids present in *M. bivittatus* eggs during the development

	Period of development in days.						
	1	10	20	30	40	50	60
Alanine	+	+	+	+	+	+	+
Arginine	+	+	+	+	+	+	+
Asparatic acid	−	+	+	+	+	+	+
Asparagine	−	−	−	−	−	−	−
Cysteine	−	−	−	−	−	−	−
DOPA	−	−	−	−	−	−	−
Glutamic acid	+	+	+	+	+	+	+
Glutamine	−	−	+	+	+	+	+
Glycine	+	+	+	+	+	+	+
Cystine	+	−	−	−	+	−	−
Histidine	−	−	−	−	+	+	+
Homocystine monohydrochloride	−	−	−	−	+	+	+
Isoleucine	+	+	+	+	+	+	+
Leucine	−	−	−	−	+	+	+
l-Lysine monohydrochloride	−	−	−	+	−	−	−
Phenylalanine	−	−	−	−	−	−	−
dl-Threonine	−	−	+	+	+	+	+
dl-Serine	−	−	+	+	−	+	+
Tryptophane	−	−	−	−	−	+	+
Tyrosine	+	+	+	+	+	+	+
Valine	+	+	+	+	+	+	+
Unknown A	−	−	−	−	+	+	+
Unknown B	−	−	+	+	+	+	+
Unknown C	−	−	−	−	+	+	+

\+ Indicates the presence of free amino acid.
− Indicates those free amino acids that were not found. 10 to 12 mg of extracts were applied on the paper (0.4~0.5 mg of N_2)

acid were more than of glutamine because of the glutamine decomposed by the glutaminase in the earlier stage. Glutamine could be decomposed into glutamic acid and ammonia by the glutaminase.

Bonner (2) reported that amidase has the enzymes of two kinds: gultaminase and asparaginase.

In the later stage, the increase of the numbers of amino acids (Table Ⅱ), amino nitrogen (Table Ⅳ) and total nitrogen (Table Ⅱ) were depend on decomposition of reserved protein, moreover it was considerable that the increasing was synthesis by the transamination.

Vitanen (7) was detected the eleven free amino acids in green peas during the germination and Ganguli (5) was detected the twenty-one free amino acids during the red (Indian) bean during the germination, then our studies were detected the eighteen amino acids during the development, especially tryptophane was detected.

Table Ⅱ. Standard Rf values of amino acids by two dimentional paper chromatography using Whatman No. 1 paper

At room temperature.
descending method.

Phenol solvent			Butanol-acetic acid solvent	
Distance of solvent front from starting point	32.6		45.7	
	Distance of substance	Rf	Distance of substance	Rf
Alpha-alanine	19.1	0.58	11.2	0.24
Phenyl alanine	27.2	0.83	26.4	0.57
Serine	10.6	0.32	7.5	0.16
Threonine	15.0	0.46	10.2	0.22
Histidine monohydrochloride	19.8	0.67	5.2	0.10
Lysine monohydrochloride	15.3	0.46	3.8	0.08
Homocystine	11.0	0.33	6.6	0.14
Isoleucine	27.7	0.84	27.3	0.59
Glutamine	18.0	0.55	6.9	0.15
Cysteine	7.1	0.21	3.0	0.06
Distance of solvent front from starting point	33.1cm		47.8cm	
Cystine	7.00	0.22	3.1	0.07
Arginine monohydrochloride	20.2	0.61	6.5	0.13
Methionine	25.5	0.77	22.1	0.46
Leucine	26.6	0.80	29.6	0.61
β-Alanine	21.3	0.64	13.8	0.28
Alanine	25.0	0.75	22.2	0.46
Tyrosine	18.0	0.54	19.5	0.40
Tryptophane	24.2	0.73	24.7	0.51
Glutamic acid	7.1	0.21	10.8	0.22
Asparatic acid	3.0	0.09	7.8	0.16
Glycine	12.6	0.38	9.5	0.19
Proline	29 7	0.90	4.8	0.10
DOPA	9.5	0.30	9.2	0.21

Table Ⅲ. Total nitrogen of egg contents of *Melanoplus bivittatus*

Period of development in days	average % of Total nitrogen (weight)
1	3.94
10	3.96
20	3.98
30	4.00
40	4.02
50	4.03
60	4.04

Table Ⅳ. Amino nitrogen of free amino acides of egg contents of *M. bivittatus*

Period of development in days	Average % of amino nitrogen
1	0.10
10	0.12
20	0.15
30	0.19
40	0.21
50	0.23
60	0.25

REFERENCES

1. Block, R. J., Durrum, E. L., Zweig, G. : A Manual of Paper Chromatography and Pape[r] Electrophoresis, Academic Press Inc., N. Y. (1955)
2. Bonner: Plant Biochem., Sec., 4
3. Cole, J. O & Parks, C. R.; Ind. Eng. Chem., Anal. Ed., 18:61 (1945)
4. Collias, E. C., McShan, W. H. & Lilly, J. H.; J. Cell. Comp. Physial., 40.507 (1952)
5. Ganguli, N. C.: Naturwiss., 40, 624 (1954)
6. Vickery, H. B., Pucher, G. W.: J. Biol. Chem., 150, 197(1943)
7. Virtanen, A. I.: Acta. Chem. Scand., 8, 358 (1954)

摘　　要

1. 標準 Rf 數値의 表 Table I)를 作成하였다.
2. Fresh egg에서는 Ninhydrin에 陽性인 아미노酸이 여섯개였으나 50日 經過後에는 17個(Table I)로 增加하였고 또 그 量도 大量으로 增加하였다.
3. Total Amino Nitrogen의 量은 10日間에 약 0.03% 餘增加하였다(Table Ⅳ 參照).
4. Total nitrogen의 量은 큰 變動을 보이지 않았으나 極少量의 增加가 있었다(Table Ⅲ).
5. 3個의 未知物質을 그本質을 確認하지 못하였으나 그중의 Glutamic acid 附近에 呈色된 것은 fresh egg에서는 전혀 없거나 極微量이 있는 것 같다.
6. Asparagine의 出現은 發見치 못하였으나 (Table I) Asparatic acid는 크게 나타났다.
7. Glutamine과 Glutamic acid는 모두 크게 나타났으나 Asparatic acid가 더 크게 나타났다.
8. Tryptophane의 出現을 보았다.
9. Aniline, Threonine, Cystine, 은 初期에는 少量이었으나 漸次 增加하였다.