STUDIES ON THE MECHANISM OF PATTERN FORMATION IN THE ELYTRA OF LADY BEETLES

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THE mechanism of the formation of various patterns in the wings of insects is a most intriguing problem from embryological, as well as cytological, genetical, and biochemical aspects.

In Lepidoptera, GOLDSCHMIDT (1932) has advanced a theory that the color pattern of wings was caused by the unequal rates of development of different parts. He has supposed that the color pattern would be changed as the rate of development was disturbed by a genic action.

In Coleoptera, GORTNER (1911) and TENENBAUM (1935) have speculated that the black pattern of the elytra of beetles was formed by the localization of melanin-chromogen. SUSSMAN (1949), WIGGLESWORTH (1950) and RICHARDS (1951) stated that the formation of the melanin pattern occurred according to the complex factors controlling the physiological status of epidermal cells developing in the region.

Harmonia axyridis Pallas, a species of lady beetle having four main variations with respect to pattern in the elytra, was used in our experiments. On the basis of the data on genetical analyses of HOSHINO (1936) and TAN (1946), it was assumed that these characters of pattern would be manifested by a set of genes belonging to multiple alleles.

Another type of lady beetle, *Coccinella bruckii* Mulsant, a closely related species, has no variation in the elytral pattern.

This present paper deals with the process of pattern formation, the analysis of the quantity of chromogen in the elytra before and after pigmentation, and also with the interaction between a few of the metabolic systems in epidermal cells of the elytra. From our data obtained by use of a chemical mutagen, nitrogen mustard, it is probable that the elytral pattern might be bestowed at the egg stage. The genic action in the manifestation of patterns has been also discussed from a viewpoint of biochemical genetics.

MATERIALS

Harmonia axyridis has a polymorphic elytral pattern which is due to a set of genes belonging to multiple alleles. In the natural populations of Japan, there are four main variations as follows: *conspicua* type (C-type), *spectabilis* type (S-type), *axyridis* type (A-type) and *succinea* type (s-type). The genes responsible for each character P^c, P^s, P^a and p show a dominance relationship designated as $P^c > P^s > P^a > p$. On the other hand, Coccinella sp. has no variation of the elytral pattern, but has a uniform color pattern of the type as shown in figure 1.

EXPERIMENTAL METHODS AND RESULTS

Observations of the process of pigmentation

The soft and unpigmented elytra of adults immediately after emergence generally have a uniform color of yellowish white. Pigmentation of the various parts of these elytra begins at the same time as the hardening of the cuticle, and approximately two hours later, the pattern is formed completely as shown in figure 2. The formation of elytral pattern in the F_1 hybrid between homozygous C-type (P^c/P^c) and s-type (p/p) was observed. Fifty minutes after emergence, the pattern of s-type overlapped the pattern of C-type in the course of pigmentation, and only the latter pattern was recognizable at the end of pigmentation. The elytral pattern of the hybrid, however,

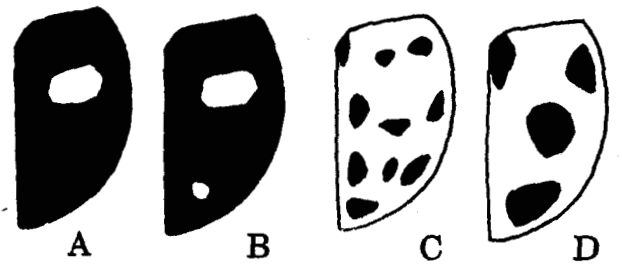

FIGURE 1.—The main variations of the elytral pattern in lady beetles. A: C-type. B: S-type. C: s-type. D: Coccinella sp.

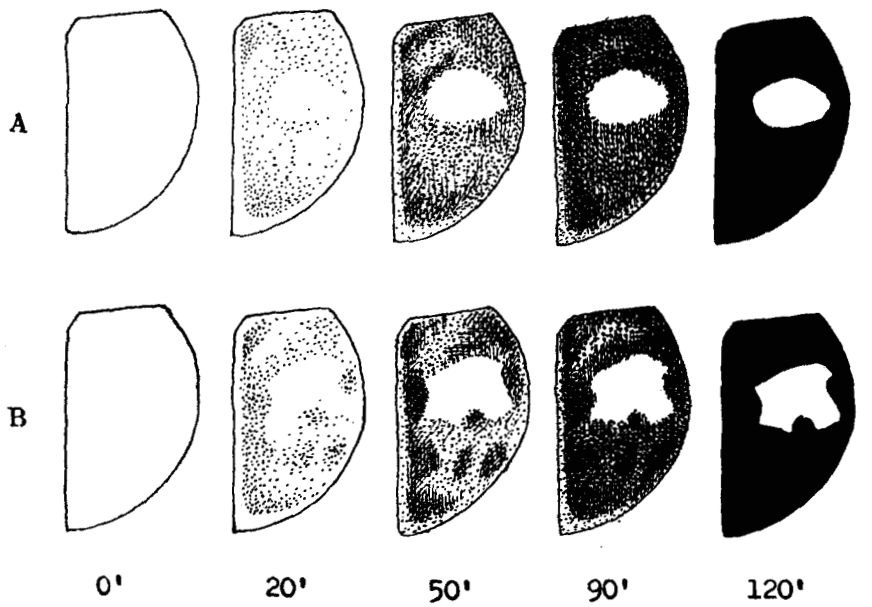

FIGURE 2.—The formation of the elytral pattern after emergence. A: C-type, homozygote P^c/P^c. B: C-type, heterozygote P^c/p.

generally had a number of depressions around the red colored region which showed no melanin.

TAN (1946) has found that the expression of the color pattern in the heterozygotes conformed to the rule of "mosaic dominance". This rule indicates that any portion of the elytra which has black pigment in the homozygote will also have black pigment in the heterozygotes in which the allele is present. The genic action of both P^c and p genes should be considered to have an equal bearing on the process of pigmentation.

Distribution and quantity of tyrosine, the substrate of melanin, in the elytra

Large quantities of tyrosine, the substrate of melanin, were extracted with 80 percent ethanol from the soft, unpigmented elytra which were pulverized immediately after emergence of the adult. The soft, unpigmented C-type elytra were separated into two parts by cutting them perpendicularly to the mid-line immediately posterior to the presumptive red portion. It was difficult to extract free tyrosine from the hard, black elytra. The tyrosine bound to protein could not be readily extracted with 80 percent ethanol, but was released by hydrolysis with 5N sodium hydroxide, at 110 $\sim$ 130°C, for 15 $\sim$ 20 hours. The quantity of tyrosine was measured by the following procedures: the elytra suspended in 80 percent ethanol were homogenized for six minutes by a Waring blender and centrifuged. The supernatant was evaporated to dryness and dissolved in buffer solution (pH 4.6, total vol. 5 ml). The precipitate was hydrolyzed with 5N NaOH, neutralized with acetic acid and adjusted to pH 4.6, and centrifuged. The precipitate was washed with buffer solution and the supernatant was combined with the washings of the precipitate giving a total volume of 10 ml. The two buffer solutions were adsorbed on the column (7 $\times$ 180 mm) of Amberlite IR-112 and eluted by M/10 citrate buffer (pH 4.6) containing 2 percent benzylalcohol (v/v).

TABLE 1

The quantity of tyrosine in the soft, unpigmented elytra (C-type)

	Dry weight of 100 elytra (mg)	Free tyrosine extracted with 80% ethanol (γ/mg)	Bound tyrosine released by hydrolysis with 5N NaOH 15 hrs, at 110°C (γ/mg)
Anterior region	46.0	6.8	30.0
Posterior region	32.0	5.9	30.3

TABLE 2

The quantity of tyrosine in the hard, black elytra

	Dry weight of elytra (mg)	Free tyrosine extracted with 80% ethanol (γ/mg)	Dry weight of elytra after extraction (mg)	Bound tyrosine released by hydrolysis with 5N NaOH: 15 hrs. at 110°C (γ/mg)
C-type	23.0	0.2	19.8	11.1
s-type	50.4	0.3	40.6	13.3
Coccinella	65.0	0.2	57.0	14.7

The effluent was separated by the automatic fraction collector constructed by T. SEKI (1953). To each fraction 5 ml of Na_2CO_3 5 percent solution and 1 ml of Folin phenol reagent were added, and after one hour, transmission of these colored fractions was measured by a photoelectric colorimeter. Tyrosine was generally found around the fifteenth fraction. The quantity of tyrosine in the elytra was calculated on the basis of the total color measurements (− log T) compared with the standards of tyrosine. Some of the histographs of the results of these experiments are presented in figure 3.

From the results shown in table 1, it was evident that the distribution of tyrosine in the soft unpigmented elytra was almost uniform and independent of the development of melanin pattern. Therefore, the general hypothesis, that the chromogen is localized at definite portions of the elytra, seems to be inadequate in this case. It was also

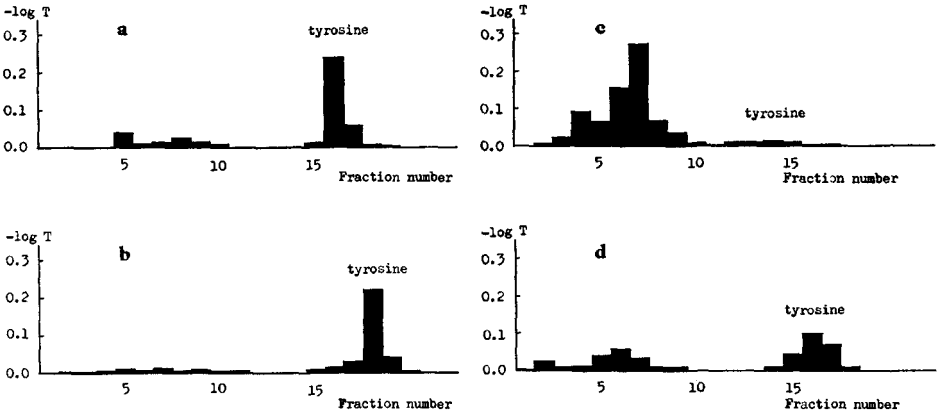

FIGURE 3.—Ion-exchange separation of tyrosine and other Folin positive substances (in C-type elytra). One fraction: 1.3 ml. a: Before pigmentation, extracted with 80% ethanol. b: Before pigmentation, hydrolyzed with 5N NaOH. c: After pigmentation, extracted with 80% ethanol. d: After pigmentation, hydrolyzed with 5N NaOH.

TABLE 3

Folin phenol reagent positive substances in various types of elytra (unit/mg)

Soft, unpigmented elytra	Extracted with 80% ethanol		Hydrolyzed with 5N NaOH: 15 hrs, at 110°C
	"A"	"B"	"C"
C-type	10.1	14.3	58.0
Hard, pigmented elytra	Extracted with 80% ethanol		Hydrolyzed with 5N NaOH: 20 hrs, at 130°C
	"A"	"B"	"C"
C-type	6.3	24.1	77.3
s-type	7.4	72.6	290.8
Coccinella	10.3	50.3	220.2

found that the content of the bound tyrosine in the light colored elytra was slightly higher than that in the black colored elytra (see table 2).

Substances colored by Folin phenol and uric acid reagents in the elytra and the body

In addition to tyrosine, several substances positive for the Folin phenol test were found in the fractions separated by use of a cation-exchange column. As shown in figure 3, the substances around the fourth and seventh fractions were designated as "A" and "B" substances, respectively; both were extracted with 80 percent ethanol.

TABLE 4

Folin phenol and uric acid reagent positive substances in the elytra and bodies of stored lady beetles

Elytra	Extracted with 80% ethanol (unit/mg)			Body	Extracted with 80% ethanol (unit/mg)		
	"A"	"B"	Uric acid in "B"		"A"	"B"	Uric acid in "B"
C-type	3.4	8.4	4.1	C-type	9.1	7.6	2.0
s-type	5.8	18.4	11.7	s-type	11.5	11.2	2.2

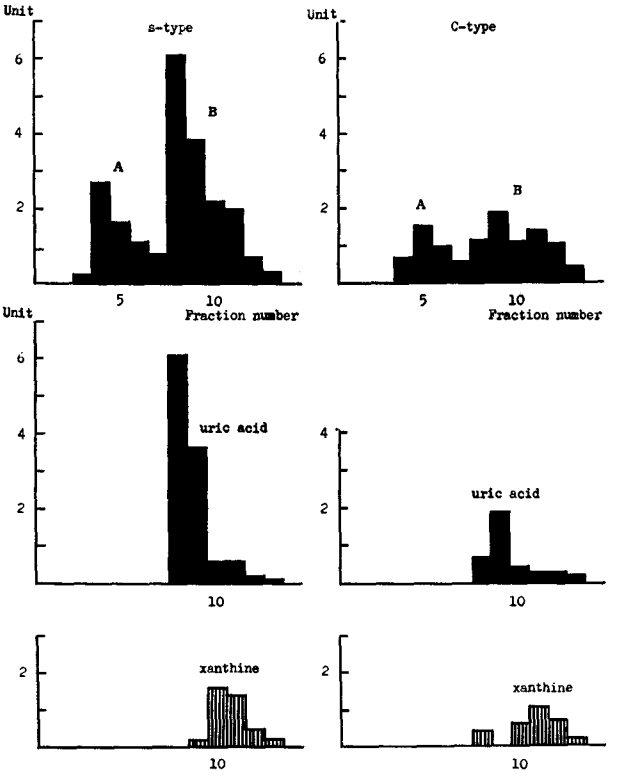

FIGURE 4.—Separation of "A" and "B" substances in the elytra of stored lady beetles by a cation-exchange column. Resin: IR-112, 200 mesh. Column size: 7 × 180 mm. Eluting solution: M/10 citrate buffer (pH 4.6) containing 2% benzylalcohol (v/v). One fraction: 1 ml.

The substance around the sixth fraction was called "C" substance and was hydrolyzed with sodium hydroxide. The content of these substances in various types of elytra is shown in table 3 by arbitrary units per microgram of dry weight of elytra. It was later found that the "B" substance was composed of uric acid and its precursor, xanthine. The quantities of uric acid and xanthine in "B" substance were separately obtained by determining the former by use of uric acid reagent.

The next experiment was carried out by using a number of previously stored lady beetles of C-type and s-type. The substances separately extracted with 80 percent ethanol from their elytra and bodies were treated by the same procedures as stated in the previous paragraph. The quantities of "A" and "B" and of uric acid in "B" substance were measured. The quantitative data and the histographs are shown in table 4 and figures 4 and 5.

From these results, a remarkable difference was noted between the "B" substances in the elytra and, to lesser extent, in the body of the two types. The elytra of s-type contained a larger quantity of uric acid than C-type, but in the body, the uric acid content of the two types was the same. "A" substance was presumably related to a polyphenol substance responsible for the tanning of cuticle. Further investigations will be undertaken on "A" and "C" substances.

Fluorescent substances in the elytra and body

Fluorescent substances were extracted from the elytra and bodies and purified as follows: the elytra were extracted four or five times with N/10 sodium hydroxide

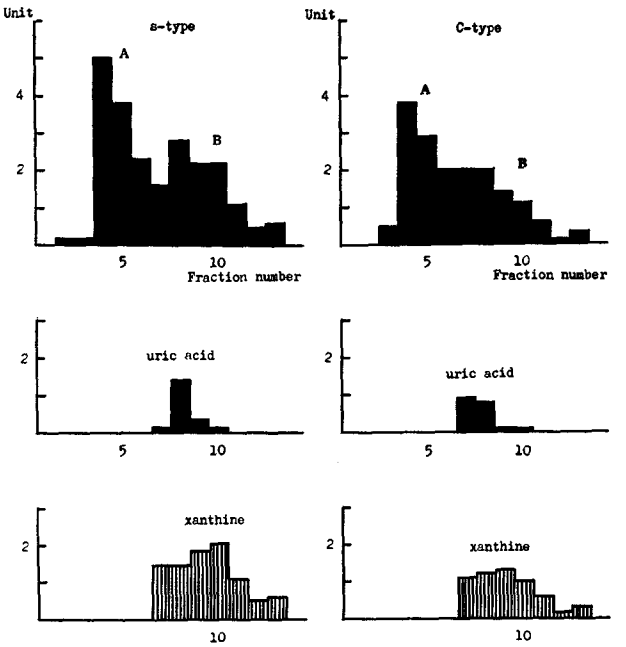

FIGURE 5.—Separation of "A" and "B" substances in the body of stored lady beetles by a cation-exchange column.

and the extracts were adjusted to pH 5 with acetic acid and adsorbed to Norit A. The fluorescent substances were eluted three or four times with a mixture of 50 percent ethanol and 5 percent ammonia (1:1) at 60°C, evaporated and then dissolved in 0.5 ml of N/10 sodium hydroxide. The aqueous solution thus obtained, as well as some synthesized pteridine derivatives and photodecomposed folic acid were paper chromatographized. Four percent citric acid solution, a mixture of n-butanol, glacial acetic acid and distilled water (4:1:1) and 3 percent ammonium chloride solution were used as developing solvents. Spots developed on the filter papers were detected under the ultraviolet rays. Several fluorescent spots were recognized and their Rf values were compared with those of the synthesized pteridines. The results are shown in table 5. Although several fluorescent substances were extracted from the elytra of Coccinella sp. and s-type, these substances, except for the spot no. 1 (table 5, part a), could not be detected in the extracts from the C-type elytra. The data of table 5 indicate that isoxanthopterin is contained in the red colored elytra in a larger quantity than other pteridines.

The fluorescent substance was readily extracted with acetone from the soft, unpigmented elytra immediately after emergence. The extracts were evaporated

TABLE 5

Rf values of the fluorescent substances extracted from the elytra and of several synthesized pteridines

Coccinella, s-type elytra				Synthesized pteridines		
Spot no.	Color	Rf.	Intensity*		Color	Rf.
			(a) Solvent: 4% citric acid solution: temp. 25°C			
1	purple	0.34	±	isoxanthopterin	purple	0.34
2	violet blue	0.53	+++	leucopterin	pale blue	0.34
3	violet blue	0.79	++	P-6-COOH†	sky blue	0.53
4	blue	0.94	+	P-6-CH_3	violet blue	0.53
			(b) Solvent: BuOH : AcOH : H_2O (4:1:1)			
1	?	0.09	+	leucopterin	pale blue	0.09
2	purple	0.21	++	P-6-COOH	sky blue	0.13
3	blue	0.47	+	isoxanthopterin	purple	0.21
				P-6-CH_2OH	sky blue	0.31
				P-6-CH_3	blue	0.47
			(c) Solvent: 3% NH_4Cl solution			
1	green	0.05	+	isoxanthopterin	purple	0.29
2	purple	0.29	+++	leucopterin	pale blue	0.32
3	?	0.47	±	P-6-CH_3	sky blue	0.47
4	sky blue	0.63	±	P-6-COOH	sky blue	0.47
5	yellow (?)	0.78	+	P-6-CH_2OH	sky blue	0.50

* Intensity: +++, strong; ++, moderate; +, weak; ±, very weak.

† P-6-COOH: 2-amino-4-hydroxy-6-carboxylic pteridine.
P-6-CH_3: 2-amino-4-hydroxy-6-methyl pteridine.
P-6-CH_2OH: 2-amino-4-hydroxy-6-ethyl pteridine.

and dissolved in 0.5 ml of distilled water. The aqueous solution was analyzed by paper chromatography. When developed by a mixture of n-butanol, glacial acetic acid and distilled water (4:1:1), it gave a sky-blue fluorescent spot with Rf about 0.1 under the ultraviolet rays. This fluorescent spot was segregated and eluted with distilled water from the paper chromatogram. The ultraviolet spectrum of the eluate showed the substance to be a type of pteridine having two maximums at 270 mμ and 360 mμ.

TABLE 6

Rf values of fluorescent substances from the elytra and bodies of lady beetles immediately after adult emergence

Spot no.	Color	Rf.	Identification
	(a) Extracted with 99% ethanol		
	(1) Solvent: BuOH : AcOH : H_2O (4:1:1)		
1	yellow	0.03	flavin (?)
2	sky blue	0.13	P-6-COOH
3	purple	0.22	isoxanthopterin
4	sky blue	0.38	P-6-CH_2OH
5	yellow	0.87	(?)
	(2) Solvent: 4% citric acid solution		
1	yellowish green	0.06	flavin (?)
2	?	0.09	(?)
3	?	0.23	(?)
4	purple	0.34	isoxanthopterin
5*	sky blue	0.55	P-6-COOH
	(b) Extracted with N/10 sodium hydroxide		
	(1) Solvent: BuOH : AcOH : H_2O (4:1:1)		
1*	blue (?)	0.09	P-6-COOH
2	purple	0.17	isoxanthopterin
3*	blue	0.36	P-6-CH_3
4	yellow	0.47	(?)
	(2) Solvent: 4% citric acid solution		
1	purple	0.32	isoxanthopterin
2*	blue	0.41	P-6-COOH
3	yellowish green	0.65	(?)
	(3) Solvent: 3% ammonium chloride solution		
1	purple	0.20	isoxanthopterin
2*	blue	0.40	P-6-COOH
3	yellowish green	0.43	(?)

* Fluorescent spots from the extracts of s-type lady beetle were larger in quantity than those from C-type lady beetle.

The elytra and the bodies of s- and C-type lady beetles immediately after adult emergence were extracted with acetone and the residues were further extracted with 99 percent ethanol and N/10 sodium hydroxide, respectively, in this order. Each of the extracts was treated as described at the beginning of this section and was analyzed by paper chromatography. The results on the paper chromatograms are shown in table 6.

From these results, it was considered that a larger quantity of the photo-decomposed pteridine of folic acid, e.g. P-6-COOH, was contained in the s-type than in the C-type lady beetles. In addition to this pteridine, isoxanthopterin and a certain riboflavin compound were also contained in the lady beetles when tested immediately after emergence.

The fluorescent substance, which was largely extracted with acetone from the soft, unpigmented elytra of Coccinella sp. was tested for its effect on melanogenesis. Since a large quantity of tyrosinase could not be obtained from the elytra of lady beetles, it was prepared from the body fluid of the mature silkworm (strain: Nichi 115, normal type). The acetone powder of the body fluid was suspended in about five parts of distilled water and stored for one hour in an ice box (0 $\sim$ 5°C). The soluble enzyme was obtained by centrifuging the homogenate. This experiment was carried out by using a conventional Warburg apparatus, with the following composition of the vessel contents made up to a final volume of 2.44 ml.

Main compartment: enzyme solution 1.0 ml., 1 M phosphate buffer (pH 7.2) 0.24 ml and the extracted fluorescent substance; finally made up to 2.04 ml by the addition of distilled water.

Side arm: tyrosine or dopa; 1 micromol each in 0.2 ml distilled water.

Center well: 20% KOH 0.2 ml.

The above reaction mixture was incubated at 30.0°C. The results are shown in table 7.

The fluorescent substance partially inhibited the activity of tyrosinase and the rate of the inhibition was more remarkable when dopa, instead of tyrosine, was oxidized. The amounts of oxygen consumed between tyrosine and dopa were about twice as much in the presence of the fluorescent substance as in its absence. From these data, therefore, it is considered that the inhibitory action of the substance is exerted on the melanogenetic process below dopa, and that the oxidation of tyrosine to dopa is rather accelerated by its presence.

TABLE 7

Effects of the fluorescent substance extracted from the elytra of Coccinella sp. on the activity of tyrosinase

	O_2 uptake (cmm)				Inhibition (%)
	30 min.	80 min.	110 min.	140 min.	
Tyrosinase + tyrosine	21.5	58.5	61.0	61.8	
Tyrosinase + tyrosine + fluorescent sub.	32.3	51.7	54.0	52.8	13
Tyrosinase + dopa	19.5	50.5	53.4	55.1	
Tyrosinase + dopa + fluorescent sub.	24.5	38.2	40.9	39.8	27

Metals related to the color of elytra

The soft, unpigmented elytra of C-type were separated into two parts by cutting them perpendicularly to the mid-line and equal quantities of each part were burnt to ashes by dry oxidation and dissolved with a small amount of dilute HCl or HNO_3. The ash solutions thus obtained were examined by paper chromatography. A mixture of acetone n-butanol HCl (10:4:2) was employed as the solvent. The chromatogram was sprayed with suitable reagents such as 0.1 $\sim$ 0.5 percent rubeanic acid dissolved in 98 percent ethanol, 1 percent potassium ethyl-xanthogenate dissolved in water, and kinds and amounts of metals were detected. The results are shown in figure 6. From the size of these spots, it was recognized that a large quantity of molybdenum was already present in the anterior part of the elytra, before hardening and darkening.

Then, the hard, pigmented elytra of C-type were divided along the lower margin of the red colored pattern and equal amounts of each part were subjected to wet oxidation with conc. HNO_3, H_2SO_4 and 60 percent perchloric acid. After the pH of the ash solutions was adjusted to pH 9.0 with ammonium citrate, the amounts of metals, e.g. molybdenum, copper and cobalt, were measured by the modified method of PARKS, HOOD, HURWITZ and ELLIS (1943).

From the data in table 8, it is evident that different amounts of molybdenum and cobalt are involved in each part after darkening and hardening of the cuticle. In view of these data on the different contents and distribution of the metals, it is

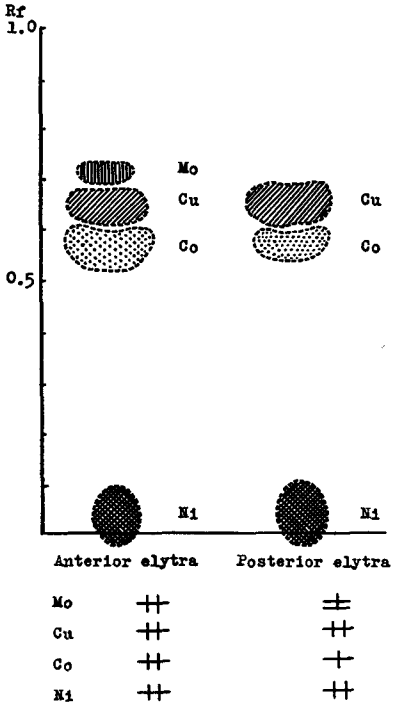

FIGURE 6.—Schematic representation of the paper-chromatographic patterns of metals from the soft, unpigmented elytra of C-type. Solvent: acetone n-butanol HCl (10 : 4 : 2).

assumed that the biological significance of these metals might be concerned with a preferential localization of the metallic enzymes in the elytra, e.g. molybdenum as a flavoprotein complex in the red colored portions of the elytra and copper as a tyrosinase distributed over the elytra in the process of hardening and pigmentation.

Effect of nitrogen mustard on the formation of elytral patterns

The eggs of lady beetles were smeared with nitrogen mustard which is known to be a mutagenic agent. Eggs, laid by the F_1 hybrids between the homozygous S-type

TABLE 8

The amounts of metals, molybdenum, copper and cobalt contained in the elytra of C-type

Type of metal	Anterior region γ/100 mg	Posterior region γ/100 mg
Mo	394	3
Co	79	45
Cu	87	92

TABLE 9

Action of nitrogen mustard at the various stages of development of lady beetles

Conc. of N.M. (%)	Time of smearing	No. of treated eggs	No. of hatched larvae	No. of adults	Percentage of hatched adults
1.0	immediately after laying	48	0	—	
	24 hrs after lay	48	0	—	
	48 hrs after lay	31	0	—	
	before pupation	102	102	1	1.0
0.2	immediately after laying	23	0	—	
	24 hrs after lay	43	0	—	
	48 hrs after lay	184	146	0	
0.1	48 hrs after lay	25	25	5	20.0
0.05	48 hrs after lay	48	48	14	29.2
Control (untreated)		74	74	33	44.6

TABLE 10

Occurrence of an unexpected elytral pattern in lady beetles following treatment with nitrogen mustard

Conc. of N.M. (%)	Homozygous S-type (P^s/P^s)		Heterozygous S-type (P^s/p)	Homozygous s-type (p/p)	Unexpected C-type
0.1	♀	0	1	1	0
	♂	2	1	0	0
0.05	♀	0	5	1	2
	♂	1	5	0	0
Control	♀	3	8	4	0
	♂	4	10	4	0

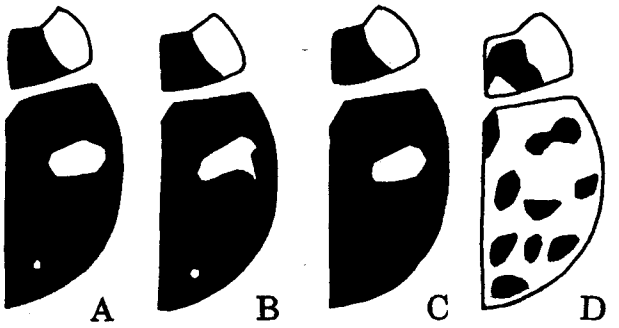

FIGURE 7.—The elytral and thorax pattern of the lady beetles in the experiment using nitrogen mustard. A: homozygous S-type (P^s/P^s). B: heterozygous S-type (P^s/p). C: unexpected C-type caused by the effect of nitrogen mustard. D: homozygous s-type (p/p).

and s-type of lady beetles, were used in this experiment. The deleterious action of nitrogen mustard at the various stages of development was observed as shown in table 9. All the eggs smeared with 0.2 percent nitrogen mustard died either at the egg or larval stage, but with 0.1 and 0.05 percent nitrogen mustard, some adults developed from the treated eggs. As shown in table 10 and figure 7, among these adult lady beetles, two individuals having an unexpected elytral pattern (C-type) were noted. The lady beetles with the unexpected pattern were observed to have the same pattern on their thorax and the anterior red colored pattern of their elytra as in the homozygous S-type lady beetle. Based on the evidence that the unexpected C-type pattern, possibly due to a somatic mutation from the S-type pattern, was obtained by the mutagenic effect of nitrogen mustard, it was assumed that the elytral pattern of an adult might be affected at the egg stage or approximately 48 hours after oviposition.

DISCUSSION AND CONCLUSION

PRYOR (1940) postulated that the cuticle of insects could be formed by the reaction between cuticle protein and oxidation products of phenol to yield sclerotin. A similar concept was extended by FRAENKEL and RUDALL (1947) who found that the amount of substrate, free tyrosine, increased in the body fluid of blowfly larvae before the hardening of pupal cuticle. RICHARDS (1951) speculated that tyrosine would be oxidized in the integument of arthropods as follows; tyrosine $\rightarrow$dopa $\rightarrow$ lactic deriv. $\rightarrow$ propionic deriv. $\rightarrow$ acetic deriv. $\rightarrow$ benzoic deriv. He also assumed that the formation of the brown and black pigment patterns could be ascribed to the participation of tyrosinase at least partially in this process, and that a local melanization would occur by the localization of the substrate, while the enzyme was distributed uniformly over the cuticle. The speculation that pigment patterns could be formed by the localization of chromogen, has been favored by a series of experiments of GORTNER (1911), TENENBAUM (1935) and DANNEEL (1943) etc. This concept was reviewed by SUSSMAN (1949) and WIGGLESWORTH (1950). In our experiment, the soft, unpigmented elytra of C-type immediately after emergence were used and it was observed that free and bound tyrosine were both contained in equal amounts in the anterior and the posterior parts regardless of melanin pattern. However, many workers have

stated that the pigment pattern is formed by a difference in tyrosinase activity. GRAUBARD (1933) compared the tyrosinase activities in the larvae and pupae of various body color races of Drosophila. SCHUURMAN (1937) assumed by using *Tenebrio molitor* that the formation of pigment might be due to the difference of enzyme actions. HARIZUKA (1947), ARUGA and KAWASE (1951) and OHNISHI (1954) advocated that the variation of melanin pigment controlled by genic action would be attributed to the difference of tyrosinase activity. WADDINGTON (1941) reported in his observation of various mutants of *D. melanogaster*, that in the pigmentation of adult cuticle, there were two processes of browning and blackening, i.e. one for the tanning and the other for the deposition of melanin. Furthermore, on the basis of his findings, he also believed that the pattern was constructed by partially concentrated blackening and that the mutants showed a remarkable difference in blackening pattern. We also have a concept that both processes could coexist in the cuticle of elytra and that its black pattern was concerned with blackening cuticle.

ARUGA, YOSHITAKE and ISHIHARA (1953) investigated the relationship between uric acid, pterins and melanin, and found that the amount of uric acid in the epidermal cells was inversely proportional to the amount of melanin in the cuticle. ITO (1953) suggested the significance of the relationship among uric acid, pterins and phenol oxidase. In the elytra of the lady beetle, we found about twice as much uric acid in the red part as in the black part. Considering that uric acid is produced from xanthine by xanthine oxidase, attention should be drawn to the interaction between xanthine oxidase and tyrosinase before passing on the relation of uric acid to melanin.

In an in vitro experiment, POLONOVSKI, GONNARD and BORIL (1951) showed that isoxanthopterin specifically inhibited the initial oxidation of dopa. From the viewpoint of physiological genetics, KIKKAWA (1953) has advanced an opinion that there is an intimate relationship among riboflavin, pterin and uric acid, and he has assumed that these substances are produced from a certain common precursor.

MATSUDA (1950) detected folic acid in the pupae of silkworm by a bioassay method and also found that a mutant "lemon" contained more folic acid than wild strains. By a similar method, we also detected folic acid in the pupae of lady beetles. According to the findings of LOWRY, BESSEY and CRAWFORD (1949), folic acid is believed to be decomposed by ultraviolet rays as follows: folic acid → 2-amino-4-hydroxy-6-formylpteridine → 2-amino-4-hydroxy-6-carboxylic pteridine → 2-amino-4-hydroxy pteridine. The last compound in the sequence is converted to isoxanthopterin by the catalytic action of xanthine oxidase. HIRATA, NAKANISHI and KIKKAWA (1950) found isoxanthopterin in the epidermis of the silkworm. ARUGA and YOSHITAKE (1954) recognized some pterins obtained by the decomposition of folic acid in similar material. In frog skin, HAMA (1951) found a fluorescent substance very similar to 2-amino-4-hydroxy-6-carboxylic pteridine. As shown in this paper, we have extracted fluorescent substances from the elytra and bodies of lady beetles. By using paper chromatography, some of these substances have been proved to correspond to P-6-COOH, P-6-CH_2OH, P-6-CH_3, and isoxanthopterin. It is interesting that a large quantity of isoxanthopterin was found in the elytra having little black melanin. It has been also shown that the pterin extracted from the elytra of Coccinella sp. inhibited the tyrosinase activity. This pterin may be

considered to be P-6-COOH on the basis of the result of paper chromatography. The inhibitory action of this substance is similar to that described by GONNARD and SVINÁREFF (1952).

Recently, KIKKAWA, OGITA and FUJITO (1954–55) have worked on the metabolic systems of tyrosine and tryptophane in animals and found that there existed a definite relationship between metals and color pigments. In the elytra of the lady beetle, it has been shown that the red colored part contains a remarkable quantity of molybdenum. Xanthine oxidase has been proved to be molybdoflavoprotein by MACKLER, MAHLER and GREEN (1954), and RICHERT and WESTERFELD (1954). From these findings mentioned above, it is conceived that the xanthine oxidase in the red colored part of elytra may have a high degree of activity.

In the review of copper metabolism in the invertebrates, DETHIER (1950) and MALLETTE (1950) referred to the fact that tyrosinase, an enzyme containing copper, was related to the hardening of cuticle and the formation of pigment pattern. DENNELL (1947) showed that the factor affecting the localization of pigment was the oxidation-reduction potential of the system in the integument of Sarcophaga larvae. This result may possibly account for the inhibitory action of the xanthine oxidase system on tyrosinase at a low rH value.

In view of the analytical data on the metals contained in elytra, it seems to be that the distribution of copper may be uniform in the process of hardening and pigmentation. At this point, it may be speculated that tyrosinase is partially inhibited by an increased activity of molybdoflavoprotein, which may be originally controlled by genic action. Figure 8 summarizes the above discussion and the possible mechanism of pigment pattern formation in elytra.

Our experiments using nitrogen mustard indicate that it is at the late egg stage that genic action controlling pigment pattern is manifested. According to GEIGY'S work reviewed by WIGGLESWORTH (1950), the epidermal structure of the adult blowfly appears to be determined in the early developmental stage, e.g. egg stage.

The idea that genotype controls the specificity of protein is the general concept of the physiology of gene action. A similar one may be affirmed in the formation mechanism of pigment pattern.

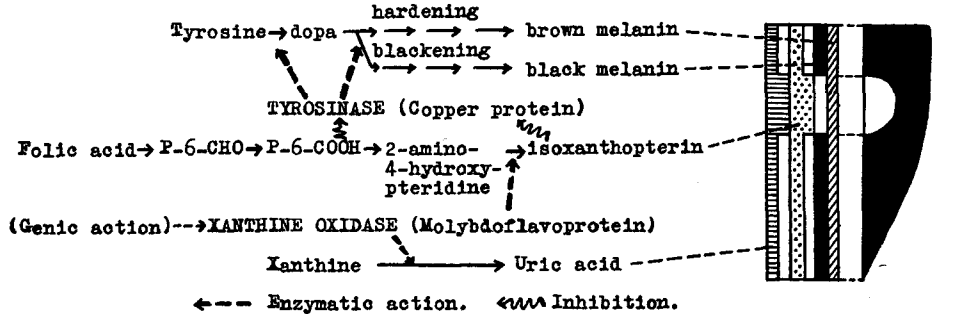

FIGURE 8.—The diagram illustrating the mechanism of pigment pattern formation in the elytra of lady beetle.

SUMMARY

1. The mechanism of the formation of elytral patterns in lady beetles, *Harmonia axyridis* Pallas and *Coccinella bruckii* Mulsant, was studied by means of genetical and biochemical methods. Several variant types of elytra (C-type, S-type, A-type and s-type) of *Harmonia axyridis* were presented as multiple alleles; the genic action controlling these characters was assumed.

2. Tyrosine, the substrate of melanin, was extracted from the soft, unpigmented elytra of C-type immediately after emergence, isolated by use of action ion-exchange resin and measured quantitatively. The amount of free tyrosine was increased before the hardening and darkening of the cuticle and its distribution over the elytra appeared to be independent of the formation of melanin pattern.

3. Uric acid was extracted from the hard, pigmented elytra and from the bodies. It was measured after being treated in the same manner as tyrosine. The content of uric acid was higher in the s-type elytra than in the C-type elytra. However, the melanin contents of both types were reversely proportional to those of uric acid. Same quantities of uric acid were extracted from the bodies of these two types.

4. The fluorescent substances were extracted and separated by paper chromatography. The photo-decomposed substances of folic acid such as the pteridines, e.g. P-6-COOH, P-6-CH_2OH, P-6-CH_3, and isoxanthopterin were detected. The fluorescent substance extracted from the soft, unpigmented elytra of Coccinella sp. with acetone had an inhibitory effect on tyrosinase and the behavior of its inhibition coincided with that of 2-amino-4-hydroxy-6-carboxylic pteridine. The isoxanthopterin was extracted from the elytra of s-type and Coccinella sp.; this substance was extracted from the elytra of C-type in lesser amounts.

5. The elytra of C-type were horizontally divided into two parts along the lower margin of the red colored pattern and equal amounts of each part were burnt to ashes by wet oxidation. The quantities of metals, e.g. molybdenum, copper and cobalt, were measured. The content of molybdenum was most remarkable in the red colored region, but the quantity of copper in each part was almost equal.

6. The eggs laid by the F_1 hybrids between the homozygous S-type and s-type lady beetles were smeared with 0.05 and 0.1 percent of nitrogen mustard. A few individuals identical with respect to having C-type elytra developed from these treated eggs. It was speculated that determination of the elytral pattern might be initiated in the egg about 48 hours after oviposition.

7. The interaction between the metabolic systems related to the process of pigmentation was discussed, and the mechanism of pattern formation was presented diagrammatically. The genic action yielding the pattern was assumed to be closely related to the determining factor in the localization of xanthine oxidase.

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