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THE PIGMENTS IN THE DORSAL SKIN OF FROGS

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ABSTRACT.—The five pigments **1–5** and a colorless substance **6** were present in the dorsal skin of frogs of nine selected species belonging to Rhacophoridae, Ranidae, Hylidae, and Bufonidae and were identified as pterin-6-carboxylic acid, xanthopterin, isoxanthopterin, *erythro*-biopterin, 6-hydroxymethylpterin, and guanine, respectively. Another pigment **7** was specifically present in the skin of genus *Rhacophorus* and was deduced to be a pteridine derivative composed of five molecules of pterin-6-carboxylic acid [**1**].

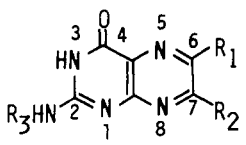
Frogs possess a characteristic body color, such as blue-green, red-brown, green-brown, yellow-brown, or green. Melanin, carotenoids, pteridines, guanine, and so forth are characterized as substances responsible for body coloration of the frogs (1). Some pteridine derivatives have been found in the skin of *Rhacophorus schlegelii, Rana catesbeiana,* and *Bufo bufo japonicus* by paper chromatography and electrophoresis (2,3). However, the characterization of the pigments in the skin of frogs has been reported for only *Rana esculenta* (4) and *Bufo vulgaris* (5). Recently, the presence of a violet chromatophore was found in the dorsal skin of genus *Rhacophorus* (6), but a pigment in this chromatophore has not been characterized as yet. The present paper describes the characterization of substances responsible for body coloration of frogs of nine selected species, the pigment compositions in their dorsal skin, and the presumed structure of the pigment present in the violet chromatophore. These nine species of frogs are representative of Rhacophoridae, Ranidae, Hylidae, and Bufonidae, which are widely distributed in Japan.

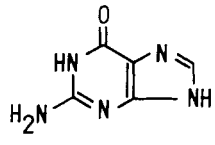
RESULTS AND DISCUSSION

CHARACTERIZATION OF THE SUBSTANCES RESPONSIBLE FOR BODY COLORATION.—After removal of lipids from the fresh dorsal skins by extraction with Me_2CO followed by Et_2O, the residual skins were extracted with EtOH. The EtOH extract was partitioned between $CHCl_3$ and H_2O to give a mixture of five pigments **1–5** and a colorless substance **6** in the upper aqueous phase. These pigments were separated from each other by gel filtration followed by hplc. The residual skins freed of the lipids, pigments **1–5**, and substance **6** were extracted with HCO_2H to give a black-violet pigment **7**.

The pigment **1** was identified as pterin-6-carboxylic acid on the basis of coincidence of the uv, ir, and ms spectra with those of authentic material. Other minor pigments **2** and **3** were identified as xanthopterin and isoxanthopterin, respectively, by comparison of their chromatographic behaviors and uv and ir spectra with those of authentic materials. The uv, ir, ms, and ^{1}H-nmr spectra of pigment **4** indicated that the pigment is probably a pterin derivative with a dihydroxypropyl group on C-6. The chemical shifts and coupling constants of the proton signals of its acetate **8** coincided with those of the signals of 2-*N*-acetyl-1′,2′-*O*-acetyl-L-*erythro*-biopterin (7). Thus, the pigment **4** was elucidated to be *erythro*-biopterin. The pigment **5** was identified with 6-hydroxymethylpterin on the basis of coincidence of its uv, ir, ms, and ^{1}H-nmr spectra with those of the synthetic compound. A colorless substance **6** was identified as guanine on the basis of coincidence of the ir and ms spectra with those of authentic material.

The ir spectrum of the black-violet pigment **7** showed bands assigned to an amido group (1697 cm^{-1}), a conjugated C=N group (1653 cm^{-1}), and a conjugated C=C linkage (1600, 1557, and 1484 cm^{-1}), which were similar to the corresponding bands





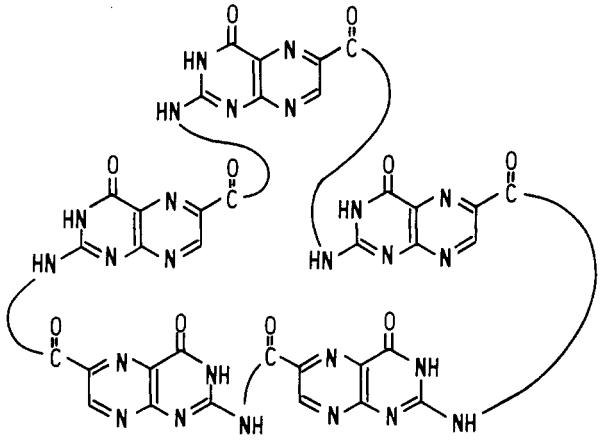
1 R_1=COOH, R_2=R_3=H
2 R_1=OH, R_2=R_3=H
3 R_1=H, R_2=OH, R_3=H

4 R_1=-CH(OH)-CH(OH)-Me, R_2=R_3=H

5 R_1=CH_2OH, R_2=R_3=H

8 R_1=-CH(OAc)-CH(OAc)-Me, R_2=H, R_3=Ac

6



Proposed structure of **7**

in the ir spectra of pterin-6-carboxylic acid [**1**], *erythro*-biopterin [**4**], and 6-hydroxymethylpterin [**5**]. This similarity indicated that the pigment **7** is a pteridine derivative. Pigment **7**, on hydrolysis with 1 M NaOH at either 27° or 100° under either N_2 or air or in vacuo and then on acidification, yielded pterin-6-carboxylic acid [**1**]. The molecular weight of **7** was determined by means of Toyopearl HW-40 gel filtration and was found to be approximately 1000. No prominent ion suggestive of the molecular weight and partial structure appeared in the eims, cims, fdms, or fabms of **7**. Accordingly, **7** was found to be composed of five molecules of **1**. However, the ^{1}H-nmr spectrum showed only a singlet signal at δ 8.30, which corresponds to a 7-H proton signal of **1**. This indicated that pteridine nuclei of five molecules of pterin-6-carboxylic acid [**1**] are constructed as being magnetically equivalent. Thus, the black-violet pigment **7** probably is in a cyclic structure as shown in a proposed structure **7**.

DISTRIBUTION OF THE SUBSTANCES RESPONSIBLE FOR BODY COLORATION.—The substances present in the dorsal skin of the frog were separated into three pigment groups: carotenoids, pteridines **1–5**, guanine **6**, and the black-violet pigment **7**. Although guanine is colorless, this substance had been described to be present in a pigment cell (called either reflecting platelet, iridophore, or leucophore) of vertebrates and concerned with body coloration (8–10). Therefore, this colorless substance was treated as a pigment in this paper.

Table 1 gives the content of the three pigment groups in the dorsal skin of the frogs of nine selected species, i.e., *Rhacophorus arboreus* (Y. Okada et Kawano), *Rh. schlegelii* (Günther), *Hyla arborea japonica* (Günther), *B. bufo japonicus* (Schlegel), *Rana nigromaculata* (Hallowell), *R. catesbeiana* (Shaw), *Rana japonica* (Günther), *Rana ornativentris*, (Werner) and *Rana tagoi* (Y. Okada). While only a trace amount of carotenoids was

TABLE 1. Composition of Three Pigment Groups in the Dorsal Skin of the Nine Frogs.

Frog	Body color	Content (mg/g skin)		
		Carotenoids	Pteridines and guanine	Black-violet pigment
Rhacophorus arboreus	blue-green	0.1	3.2	0.7
Rhacophorus schlegelii	blue-green	tr[a]	1.1	0.3
Hyla arborea japonica	green	tr	0.2	—
Bufo bufo japonicus	yellow-green	tr	0.4	—
Rana nigromaculata	green-brown	tr	0.2	—
Rana catesbeiana	green-brown	tr	0.6	—
Rana japonica	red-brown	0.1	1.5	tr
Rana ornativentris	red-brown	tr	1.3	tr
Rana tagoi	brown	tr	0.3	—

[a]Trace amount (<0.1).

present in the skin of all the frogs, pteridines and guanine were predominantly distributed in their skin. On the other hand, the black-violet pigment **7** was specifically present in the skin of the genus *Rhacophorus*, and **7** is probably responsible for coloration of the new violet chromatophore characteristic of the dorsal skin of the genus *Rhacophorus* (6). This is in coincidence with the fact that, when a section of the dorsal skin of *Rh. arboreus* is treated with HCO_2H, the elution of a black-violet pigment is microscopically observed. Pigment **7** possibly plays a role in the protection of the frog body from ultraviolet radiation, in place of melanin in other frogs, because a melanophore is not present in the skin of the genus *Rhacophorus* (6).

COMPARISON OF THE PIGMENT COMPOSITION.—Figure 1 shows a comparison of the compositions of pigments **1–6** and black-violet pigment **7** in the skin of the nine frogs. The pigment composition of *Rh. arboreus* (A) was almost identical with that of *Rh. schlegelii* (B). These pigment compositions differed markedly from the compositions of the other frogs. The pigment composition of *R. nigromaculata* (E) was similar to that of *R. catesbeiana* (F) and also the composition of *R. japonica* (G) to that of *R. ornativentris* (H). Although frogs E–I belong to the same family, the pigment composition of *R. tagoi* (I) differed from that of frogs E–H. Thus, the pigment composition of frogs E–I was divided into three patterns. *H. arborea japonica* (C) and *B. bufo japonicus* (D) showed a characteristic pigment composition. Accordingly, the pigment compositions in the dorsal skins of the frogs of the nine species were found to be divided into six patterns. The above nine species are representative of Rhacophoridae, Ranidae, Hylidae, and Bufonidae, which are widely distributed in Japan. These nine species were found to be divided into six chemotypes on the basis of their pigment composition, as follows: (a) *Rh. arboreus* and *Rh. schlegelii*; (b) *R. nigromaculata* and *R. catesbeiana*; (c) *R. japonica* and *R. ornativentris*;(d) *R. tagoi*; (e) *H. arborea japonica*; (f) *B. bufo japonicus*.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURE.—Tlc was performed on cellulose F plates (Merck). Uv spectra were obtained on a Shimadzu UV-240 spectrometer. Ir spectra were recorded on a JEOL JIR-40X FT-IR spectrophotometer for the pigments **1–5** and the black-violet pigment **7**, and on a JASCO IRA-1H for guanine [**6**] and an acetate **8**. ^{1}H-nmr spectra were obtained on a Hitachi R-22 spectrometer for **1**, **4**, **5**, and **8**, and on a JEOL JMN-GSX 400 for **7**. TMS was used as an internal standard. Mass spectra were obtained on a Shimadzu QP-1000 mass spectrometer and recorded by the direct inlet method at 70 eV.

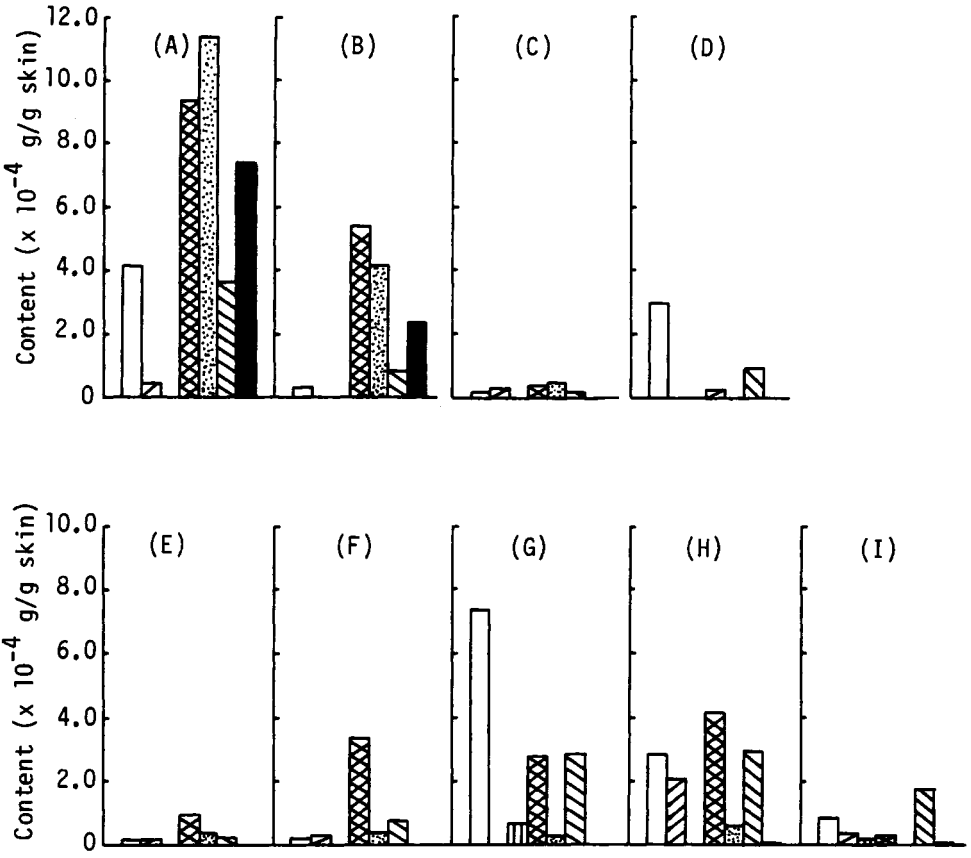


FIGURE 1. Comparison of the pigment composition in the skin of the nine frogs. (A) *Rhacophorus arboreus*; (B) *Rhacophorus schlegelii*; (C) *Hyla arborea japonica*; (D) *Bufo bufo japonicus*; (E) *Rana nigromaculata*; (F) *Rana catesbeiana*; (G) *Rana japonica*; (H) *Rana ornativentris*; (I) *Rana tagoi*.

Hplc was performed on a Radial Pak C18 with 1% aqueous NH_4OAc as a mobile phase. The flow rate was 1.0 ml/min, and the absorption was measured at 270 nm.

FROG MATERIALS.—"Adult" frogs or toads were used for this investigation. Eight species, *Rh. arboreus* (103 specimens), *Rh. schlegelii* (30 specimens), *R. nigromaculata* (39 specimens), *R. japonica japonica* (69 specimens), *R. ornativentris* (8 specimens), *R. tagoi* (8 specimens), *H. arborea japonica* (10 specimens), and *B. bufo japonicus* (5 specimens), were wild individuals collected in the suburbs of Hiroshima City, Japan, but *R. catesbeiana* (8 specimens) had been bred. Each of the species possesses a characteristic body color as follows: *Rh. arboreus* and *Rh. schlegelii* are blue-green; *R. nigromaculata* and *R. catesbeiana*, green-brown or gray; *R. japonica japonica* and *R. ornativentris*, red-brown; *R. tagoi*, brown; *H. arborea japonica*, green (changes to gray or brown with a change in the environment); and *B. bufo japonicus*, yellow-brown.

EXTRACTION AND SEPARATION OF SUBSTANCES.—A typical procedure was as described below. After removal of lipids from the fresh dorsal skins (17.604 g) of *Rh. arboreus* by extraction with Me_2CO (50 ml) followed with Et_2O (50 ml), the skins (9.500 g) were extracted with 75% EtOH (95 ml) at 80° for 90 min. The EtOH solution was partitioned between $CHCl_3$ (300 ml) and H_2O (50 ml) to give a mixture solution of **1**–**6** as the upper aqueous phase. The mixture (56 mg) of these substances, after lyophilization, was subjected to Bio gel P-2 gel filtration with H_2O followed by hplc to give **1** (1 mg), **2** (<1 mg), **3** (<1 mg), **4** (2 mg), **5** (2 mg), and **6** (1 mg). The lipids (137 mg) obtained were subjected to cc on Si gel (hexane/EtOAc, 0–80%) to give a mixture (2 mg) of some carotenoids, triacylglycerides and diacylglycerides (14 and 28 mg), free fatty acids (58 mg), and cholesterol (28 mg). The mixture of carotenoids was subjected to hplc analysis [Radial Pak C18 column, uv detector at 436 nm, MeCN-THF (75:25) as eluent at 2.0 ml/min]. Seven peaks (1.8%, 15.3%, 3.1%, 8.5%, 14.6%, 10.0%, and 46.7%) appeared in the hplc, and

one (46.7%) of them was identified with β-carotene by co-hplc with authentic material. Triacylglycerides and diacylglycerides were identified on the basis of the ir and ^{1}H-nmr spectra. Fatty acids of these glycerides were identified by means of gc and gc-ms of their methyl esters obtained from the glycerides by hydrolysis with 0.5 M NaOH followed by methylation with CH_2N_2. Free fatty acids were identified by gc and gc-ms of methyl esters resulting from methylation with CH_2N_2. Cholesterol was identified on the basis of the ir, ms, and ^{1}H-nmr spectra.

The residual dorsal skin (10.881 g), after extraction of **1–6** with 75% EtOH as above, was further extracted three times with 200 ml of 90% HCO_2H. The HCO_2H solution, after concentration in vacuo to 200 ml, was then subjected to Bio gel P-10 gel filtration followed by Bio gel P-4 gel filtration with 90% HCO_2H. Dilution of the red-orange eluate with H_2O gave 10 mg of a precipitate. This precipitate was then purified by reprecipitation from 28% NH_4OH to give 8 mg of **7** as an amorphous powder. Its purity was checked by tlc analysis with four kinds of solvents, such as 90% HCO_2H-MeOH-concentrated HCl (80:15:5), 90% HCO_2H-H_2O (4:1), 0.1% NaOH-H_2O (4:1), and 28% NH_4OH. Following a procedure similar to that used for the skins of *Rh. arboreus*, the fresh dorsal skins (6.282 g) of *Rh. schlegelii* were treated to give carotenoids (trace), a mixture (7 mg) of **1–6**, and a black-violet pigment (1 mg). The uv and ir spectra and chromatographic behaviors of the pigment coincided with those of **7**. The spectral data of **7** were as follows: uv λ max (90% HCO_2H or concentrated H_2SO_4) 500 and 480 nm; ir ν max (KBr) 1697 and 1653 (CON and C=N), 1600, 1557, and 1484 cm^{-1} (C=C); ^{1}H nmr (99% HCO_2H-d_2) δ 8.30 (s, 7-H).

PTERIN-6-CARBOXYLIC ACID [**1**] (2-AMINO-6-CARBOXY-4(1H)-PTERIDINONE).—Uv λ max (0.1 M NaOH) 365 (ε = 7900), 263 nm (15,800) [lit. (11)], (0.1 M HCl) 330–300 (ε = 5000), 260 (6300), 255 nm (6300) [lit. (12)]; ir ν max (KBr) 1737, 1653, 1568, 1532, 1502 cm^{-1}; ms *m/z* 207 $[M]^+$, (2), 163 (100), 136 (5), 135 (8), 122 (37), 109 (15), 94 (25), 93 (28); ^{1}H nmr (99% HCO_2Hd_2) δ 8.30 (2).

XANTHOPTERIN [**2**] (2-AMINO-6-HYDROXY-4(1H)-PTERIDINONE).—Uv λ max (0.1 M NaOH) 390 (ε = 5000), 255 nm (12,600), (0.1 M HCl) 355, 260, 230 nm [lit. (13)]; ir ν max (KBr) 1683, 1660, 1590, 1540 cm^{-1}.

ISOXANTHOPTERIN [**3**] (2-AMINO-7-HYDROXY-4(1H)-PTERIDINONE).—Uv λ max (0.1 M NaOH) 340 (ε = 4000), 253 (6300), 220 nm (10,000) [lit. (14)], (0.1 M HCl) 340, 286, 215 nm [lit. (15)]; ir ν max (KBr) 1663, 1592, 1558, 1453 cm^{-1}.

erythro-BIOPTERIN [**4**] (2-AMINO-6-(1′,2′-DIHYDROXYPROPYL)-4(1H)-PTERIDINONE).—Uv λ max (0.1 M NaOH) 362 (ε = 5800), 255 nm (18,600) [lit. (16)], (0.1 M HCl) 320 nm (ε = 6900) [lit. (11)]; ir ν max (KBr) 1747, 1684, 1621, 1542, 1516 cm^{-1}; ms [lit. (17)]; ^{1}H nmr (1 M NaOH/D_2O) δ 1.18 (3H, d, *J* = 7 Hz), 4.23 (1H, dq, *J* = 6, 6 Hz), 8.64 (1H, s).

6-HYDROXYMETHYLPTERIN [**5**] (2-AMINO-6-HYDROXYMETHYL-4(1H)-PTERIDINONE).—Uv λ max (0.1 M NaOH) 362 (ε = 4800), 255 nm (14,800) [lit. (18)], (0.1 M HCl) 320 nm (ε = 4100); ir ν max (KBr) 1726, 1684, 1621, 1542, 1511, 1484 cm^{-1}; ms *m/z* $[M]^+$ 193 (29), 177 (100), 164 (57), 162 (80), 147 (12), 136 (29); ^{1}H nmr (1 M NaOH/D_2O) δ 4.51 (2H, s), 8.96 (1H, s).

GUANINE [**6**].—Ir ν max (Nujol) 3200, 3100, 1690, 1670, 1560 cm^{-1}; ms.

2-*N*-ACETYL-1′,2′-*O*-ACETYL-*erythro*-BIOPTERIN [**8**].—The pigment **4** (2 mg) was acetylated with Ac_2O in pyridine to afford an acetate **8**: ir ν max (film) 3150, 3000, 2920, 1740, 1680, 1620, 1560, 1490, 1450 cm^{-1}; ^{1}H nmr [lit. (7)].

HYDROLYSIS OF **7**.—The pigment **7** was stirred in 1 M NaOH for 24 h at room temperature in an atmosphere of N_2. The reaction was traced at hourly intervals for 24 h by uv spectral measurement, hplc, and tlc (iPrOH-7% NH_4OH, 2:1). Two new maxima at 263 and 365 nm gradually increased with a decrease in the initial absorption maxima at 415 and 520 nm. These new maxima coincided with those of **1**. Hplc and tlc indicated the formation of one product that showed a chromatographic behavior identical with that of a sodium salt of **1**. When the initial violet color changed to pale yellow in 24 h, the solution was acidified to pH 4 with 1 M HCl to separate a supernatant from a small amount of black-violet precipitate by centrifugation at 1500 *g*. The supernatant, after concentration in vacuo and then on preparative hplc, gave **1**, which was identified by comparison of its uv and ir spectra and chromatographic behavior. The black-violet precipitate was the unchanged **7**. In a similar manner, the other reactions were performed with 1 M NaOH at 27° under air and at 100° either under air or in vacuo. Pigment **7** was insoluble in concentrated HCl and dilute acids. Therefore, **7** was not hydrolyzed at all even when it was heated with 6 M HCl at 110° in a sealed tube.

DETERMINATION OF THE MOLECULAR WEIGHT OF **7**.—The molecular weight of **7** was determined by gel filtration on a Toyopearl HW-40 (F) column (1.5 × 80 cm), as follows. The void volume (Vo) of the

column was measured by use of bovine serum albumin (mol wt 67,000). A 90% HCO_2H solution (1 ml) of a mixture of **7** and standard materials (protamine sulfate, average mol wt 4100; pterorhodin, mol wt 386) was applied to the column equilibrated with the same solvent. The column was eluted with the same solvent at flow rate 11 ml/h at 23°. The elution volumes (Ve) of protamine sulfate and pigment **7** and pterorhodin were determined by Bio rad protein assay and by absorbance measurement at 600 nm, respectively. The relative retention volumes (Ve/Vo) of protamine sulfate, pigment **7**, and pterorhodin were 1.43, 1.74, and 1.86, respectively. From the plot of the relative retention volumes (Ve/Vo) against the logarithm of mol wt, the mol wt of **7** was found to be approximately 1000.

ANALYSIS OF **1–6** IN THE SKINS OF FROGS.—Following the procedure similar to that in the case of *Rh. arboreus*, the fresh dorsal skins of *H. arborea japonica* (0.600 g), *B. bufo japonicus* (7.126 g), *R. nigromaculata* (6.530 g), *R. catesbeiana* (11.541 g), *R. japonica* (8.757 g), *R. ornativentris* (0.462 g), and *R. tagoi* (0.288 g) were treated to give mixtures of some carotenoids and of six substances **1–6**. The yields of these substances from the fresh skin tissues are given in Table 1. Each of the mixtures of the substances was dissolved in 10 ml of 2.8% NH_4OH and then subjected to hplc. Substances in these mixtures were identified by co-hplc with authentic materials. The content of **1–6** in each mixture was determined from standard curves, which were obtained as an equation of the regression line by the method of least squares by hplc.

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