was shown that the subfornical organ, situated in the roof of the third ventricle and *outside* the blood-brain barrier, is extremely sensitive to the dipsogenic effect of A II [J. B. Simpson and A. Routtenberg, *Science* **181**, 1172 (1973)]. Second, it was found that A II, applied intracerebrally at the predilection site in the preoptic area, elicits drinking only when the brain cannula passes through the lateral ventricle and thus permits the entry of injected A II through the punctured blood-brain barrier into the ventricular system (A. K. Johnson, paper presented at the Eastern Psychological Association meeting in Washington, D.C., 1973), providing direct access of A II to the subfornical organ. There is thus no longer any need to postulate passage of peripherally injected A I or A II across the blood-brain barrier in the explanation of their dipsogenic activity.

19. We thank Dr. Giancarlo Guideri for suggestions during the course of this study.

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Color Changes, Unusual Melanosomes, and a New Pigment from Leaf Frogs

Abstract. *Melanosomes of phyllomedusid frogs are unusually large and are composed of an amorphous matrix of thick fibers. Their hitherto undescribed dark red pigment is neither phaeomelanin nor eumelanin, but seems to be related to melanins. Melanophores of at least one of these species,* Agalychnis dacnicolor, *exhibit color change in direct response to illumination, and it is suggested that these chromatophores are innervated.*

Melanophores of all vertebrate classes are remarkably consistent in structure and composition. They contain melanin pigments which show little chemical variation between taxonomic groups and which are found in melanosomes that, in organisms as unrelated as fishes and man, are much alike in origin, development, shape, and size (*1*). The first deviation from this consistent pattern was observed in the dermal melanophores of the phyllomedusid leaf frog, *Agalychnis dacnicolor*, which contains melanosomes that, at more than 1.00 nm in diameter, are twice as large as the usual organelle (*2*). Moreover, these melanosomes are of a dark red color and consist of an electron-dense core that is surrounded by a large concentric matrix of dense fibers.

In order to ascertain whether the red pigment might be the first example of the presence of phaeomelanin in a poikilotherm, skins were removed from the dorsal surface of adult *A. dacnicolor* collected in Sinaloa, Mexico. The skins were preserved in 80 percent ethanol and were subsequently extracted. The most satisfactory results were obtained with 80 to 85 percent dimethyl sulfoxide (DMSO) which produced an extract of an intense red color which when diluted with water formed a red precipitate. The pigment material so precipitated could be purified further by redissolving it in DMSO and by subsequent reprecipitation. The ultraviolet absorption spectrum of the pigment (in DMSO) revealed absorption maximums at 558, 520, 492, 358, 350, 336, and 263 nm, which were little affected by addition of acid or alkali. However, in basic media, for example, 0.1*N* NaOH, the dark red pigment proved to be unstable and was rapidly converted to a yellow product that has an absorption maximum at 400 nm (Fig. 1). On the basis of these preliminary observations, it is evident that the properties of the dark red pigment differ markedly from those of either eumelanin or phaeomelanin; thus, we are dealing with a new type of chromatophore pigment.

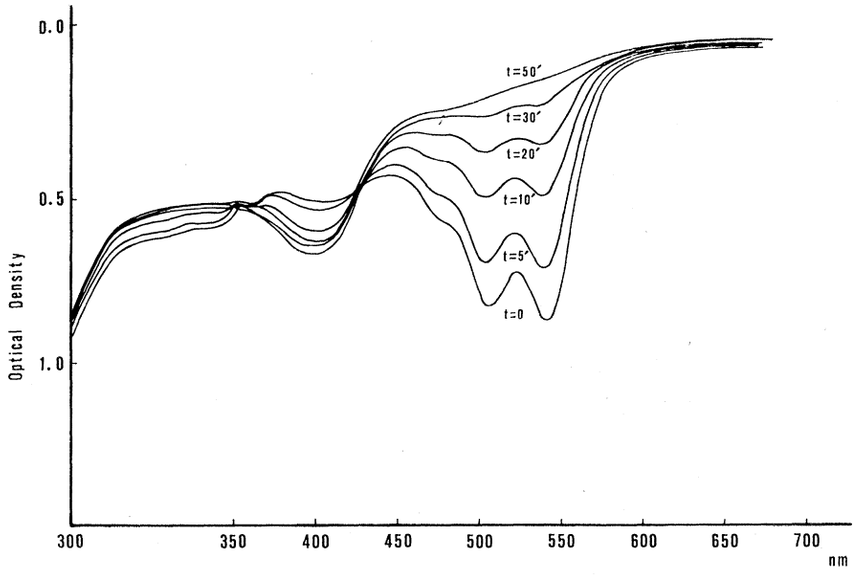

Fig. 1. Time-dependent changes (0 to 50 minutes) in the ultraviolet spectrum in 0.1*N* NaOH of the red pigment from *A. dacnicolor* as it is converted to a yellow product having an absorption maximum at 400 nm.

It is most likely that this new pigment is in some way related to melanins because it is localized in cells that, in terms of morphology, function, and position in the dermal chromatophore unit, are undeniably melanophores (*3*). Accordingly, skins removed from the dorsal surface of *A. dacnicolor* were incubated in Ringer solution containing [^{3}H]dopa and [^{3}H]tyrosine, both labeled in the 2, 3 positions of the side chain, in order to learn whether these melanin precursors were selectively taken up by the skin. In a series of experiments, pieces of dorsal skin from adults of *A. dacnicolor* and from *Rana pipiens*, which served as a control, were washed in amphibian Ringer solution for 3 hours. The skins were then cut into pieces of identical size and incubated for 20, 30, 60, or 90 minutes in fresh Ringer solution which contained either [^{3}H]dopa, [^{3}H]tyrosine, or [^{3}H]-alanine (DL-[3-^{3}H]alanine), the latter serving as a nonspecific control. The radioactivity of the incubation medium for each of the compounds tested was 40 to 50 μc/ml. At the end of the incubation periods the skins were extracted with 10 percent ice-cold trichloroacetic acid for 30 minutes, dehydrated, and analyzed in an LS-200 liquid scintillation spectrometer (Beckman) for radioactivity incorporated into the acid-insoluble skin residue. It was revealed that dopa and tyrosine were quickly incorporated by both *A. dacnicolor* and *R. pipiens* and that uptake increased linearly with time. Skins of *A. dacnicolor* selectively incorporated dopa and tyrosine as indicated by the results of

the following typical experiment. After 20 minutes of incubation the radioactivity incorporated into acid-insoluble material was determined. The recorded values were then normalized to correct for the differences in the specific radioactivities of the specific precursors used. The normalized radioactivity was : [^{3}H]-alanine, 11 count/min; [^{3}H]tyrosine, 550 count/min; and [^{3}H]dopa, 14,000 count/min. Similarly, the radioactivity after 60 minutes of incubation was: [^{3}H]alanine, 59 count/min; [^{3}H]tyrosine, 870 count/min; and [^{3}H]dopa, 40,000 count/min. It seems justified to conclude from these experiments that tyrosine and dopa are selectively taken up by the melanophores and are utilized in the synthesis of the new pigment. It would seem that while the unknown pigment is neither eumelanin nor phaeomelanin, it is somehow related to the melanins.

In the course of maintaining and observing several hundred adult *A. dacnicolor* in the laboratory, it was realized that at a given time and under normal daylight illumination about 80 percent of the animals are bright green in color, whereas the colors of the remaining 20 percent range from light tan to reddish brown. When part of the dorsal surface of these darker individuals is masked, as in experiments in which letters or figures of black plastic are placed on the skin, the area under the mask turns bright green and the rest of the surface remains tan or brown. This color change phenomenon is not due to contact with the skin or to heat generated under the mask, for the localized "greening" occurs equally well when the mask does not touch the skin and air is allowed to flow freely between the skin surface and the mask. When one picks up such frogs it is observed that areas on the limbs or flank that covered each other as the frog sat dormant also are green. If the frog becomes sufficiently excited from being handled, its brown areas may begin to turn green immediately and in a matter of minutes it may become as green as the usual green individual. It is concluded that the skin of *A. dacnicolor* is locally sensitive to changes in illumination and that this sensitivity provides the basis for the local color change that occurs during masking. Whether the pigment cells themselves serve as light receptors or whether other light receptors are present in the skin is unknown. As an additional conclusion, it seems likely that direct innervation of either chromatophores or of immediately adjacent areas of the skin is the basis for the extremely rapid color change that occurs as brown individuals become green during excitement. This response is much faster than the "excitement pallor" of many other amphibians (*4*), and the possibility that it is mediated by direct innervation of chromatophores is suggested because the response seems to occur more rapidly than can be accounted for by a blood-borne agent.

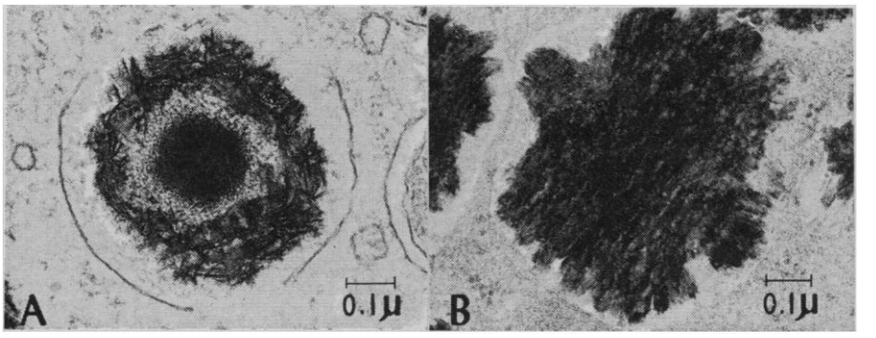

Fig. 2. Melanosomes in dermal melanophores of *A. dacnicolor* (A) and *A. callidryas* (B). The melanosomes of the two species are each larger than the typical vertebrate melanosome, and that of *A. dacnicolor* is normally larger than that of *A. callidryas*. Each melanosome is composed of a fibrous mass enclosed by a limiting membrane, but that of *A. callidryas* lacks the electron-dense kernel typical of the *A. dacnicolor* melanosome.

The physiological experiments described herein have been performed exclusively on *A. dacnicolor*; however, preliminary ultrastructural examinations have been performed on two other phyllomedusid species, *A. moreleti* and *A. callidryas*. We have observed that both of these species contain the same dark red pigment that is found in *A. dacnicolor* and we know that melanophores of adult *A. callidryas* contain melanosomes which very much resemble those of *A. dacnicolor*, in size, form, and color (Fig. 2). While *A. callidryas* melanosomes contain a fibrous matrix much like that of *A. dacnicolor*, they are slightly smaller and lack the electron-dense kernal that is typical of the melanosome of the latter. The fact that the new red pigment and the unusually large melanosome are found in several phyllomedusid species, and seem to be restricted to this group, has interesting phyllogenetic implications which should become even more important when more individuals of this family and other related families are studied. The knowledge obtained from such studies promises to contribute to our understanding of the evolution of both pigment cells and amphibians.

The presence in *Agalychnis* of a new type of melanophore pigment in an unusual melanosome warrants further study, as does the discovery that the skin of *Agalychnis*, in a remarkably precise way, is directly sensitive to changes in illumination. Possibly these three facts are interrelated. The possibility that chromatophores of adult *Agalychnis* are innervated is a major consideration, for unlike the situation in certain fishes and reptiles, it has never been clearly demonstrated that amphibian chromatophores are innervated.

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References and Notes

1. J. T. Bagnara and W. R. Ferris, in *Biology of the Normal and Abnormal Melanocyte*, T. Kawamura, T. B. Fitzpatrick, M. Seiji, Eds. (Univ. of Tokyo Press, Tokyo, 1971), pp. 57–76; J. T. Bagnara, in *Pigmentation: Its Genesis and Control*, V. Riley, Ed. (Appleton-Century-Crofts, New York, 1972), pp. 171–1 → J. D. Taylor and J. T. Bagnara, *Am. Zool.* **12**, 43 (1972); J. T. Bagnara and M. E. Hadley, *Chromatophores and Color Change* (Prentice-Hall, Englewood Cliffs, N.J., 1973).
2. J. D. Taylor and J. T. Bagnara, *J. Ultrastruct. Res.* **29**, 323 (1969).
→ J. T. Bagnara, J. D. Taylor, M. E. Hadley, *J. Cell Biol.* **38**, 67 (1968).
4. G. H. Parker, *Animal Colour Changes and Their Neurohumours* (Cambridge Univ. Press, London, 1948).
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