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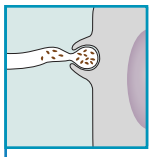
MELANOSOMES: BIOGENESIS, PROPERTIES, AND EVOLUTION OF AN ANCIENT ORGANELLE

Liliana D'Alba and Matthew D. Shawkey

Evolution and Optics of Nanostructures Group, Department of Biology, University of Ghent, Ghent, Belgium



D'Alba L, Shawkey MD. Melanosomes: Biogenesis, Properties, and Evolution of an Ancient Organelle. *Physiol Rev* 99: 1–19, 2019. Published September 26, 2018; doi:10.1152/physrev.00059.2017.—Melanosomes are organelles that produce and store melanin, a widespread biological pigment with a unique suite of properties including high refractive index, semiconducting capabilities, material stiffness, and high fossilization potential. They are involved in numerous critical biological functions in organisms across the tree of life. Individual components such as melanin chemistry and melanosome development have recently been addressed, but a broad synthesis is needed. Here, we review the hierarchical structure, development, functions, and evolution of melanosomes. We highlight variation in melanin chemistry and melanosome morphology and how these may relate to function. For example, we review what is known of the chemical differences between different melanin types (eumelanin, pheomelanin, allomelanin) and whether/how melanosome morphology relates to chemistry and color. We integrate the distribution of melanin across living organisms with what is known from the fossil record and produce hypotheses on its evolution. We suggest that melanin was present in life forms early in evolutionary history and that melanosomes evolved at the origin of organelles. Throughout, we discuss the (sometimes gaping) holes in our knowledge and suggest areas that need particular attention as we move forward in our understanding of these still-mysterious organelles and the materials that they contain.

I. INTRODUCTION

Melanin is a generic name for a group of dark biological pigments with unusual combinations of properties (130). Optically, melanin has one of the highest refractive index values known for any biological material (~1.8–2.0) and a broad absorption spectrum. This enables strong interactions with, and control of, wavelengths in the electromagnetic spectra from gamma rays to ultraviolet (UV), to visible and infrared. Thus melanin protects against damaging UV rays (44, 136) and, in the fungus *Cryptococcus*, against gamma radiation (31, 66). Furthermore, it enhances material strength of structures from chelicerate jaws (93) to squid beaks (97) to avian feathers (10). Nanostructures composed of melanin produce structural colors ranging from UV to near-infrared (141). It is involved with the immune response of numerous organisms (39, 89). Neuromelanin is found associated with nerve cells and could aid in positioning and/or navigation through sensing of electromagnetic fields (9, 123, 126, 166). Melanins thus have diverse functions, helping to explain their widespread presence in living organisms from bacteria to mammals **(FIGURE 1)**.

In most cases, melanin pigments are produced in intracellular organelles called melanosomes (130) within cells termed melanocytes (or melanophores). These melanosomes are then deposited throughout the integument and other organs. Melanin's properties are dependent on its hierarchical organization, from monomer building blocks to coordinated metals to morphology of melanosomes, to the organization of melanosomes over a larger length scale by supramolecular assembly (130). Thus chemistry, morphology, and organization all play roles in the functions and evolution of melanin and melanosomes.

Our first goal in this paper is to review the hierarchical structure of melanosomes, from the chemistry of the melanin they contain to their morphology. We then describe how melanosomes grow and are deposited in tissues. Our next goal is to explore how these properties drive the phylogenetic distribution, diversity, and evolution of melanosomes and to highlight how the fossilization potential of melanosomes has recently enabled us to examine these patterns in the fossil record. Finally, we discuss outstanding questions and future directions in melanosome research,

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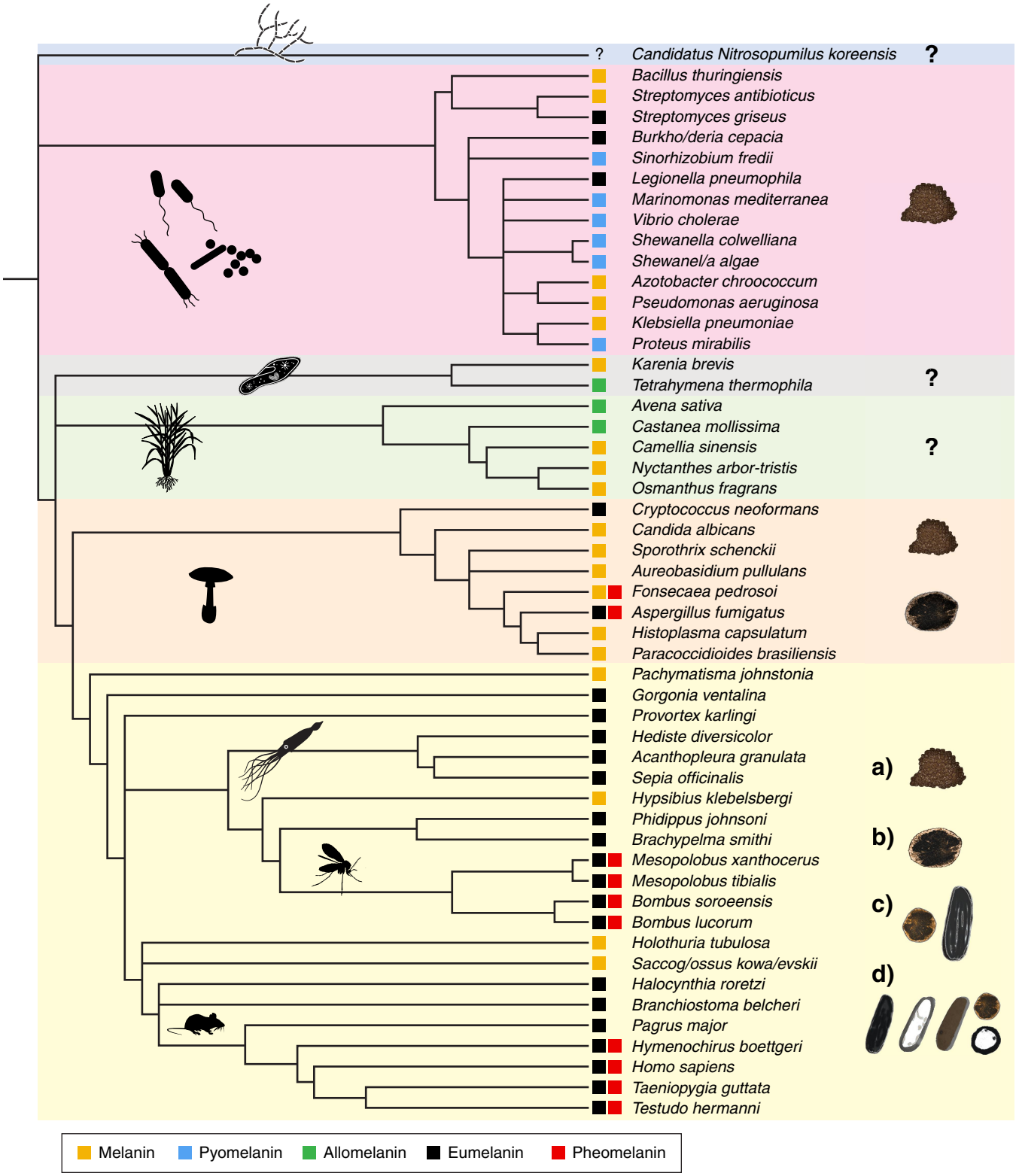

FIGURE 1. Distribution of melanins across a phylogeny of selected organisms from all kingdoms of life. The type of melanin known to be present is indicated at the tree tips, while the melanin morphology is indicated to the right of the species names. Melanin morphology in the Kingdom Animalia includes the following: *a*) granular (non-melanosomal; e.g., in the moth *Manduca sexta*; Curtis et al. 1984); *b*) round melanosomes (e.g., in *Sepia officinalis*; Schraermeyer 1994); *c*) round and elongate melanosomes (e.g., in human hair; Cesarini 1990); and *d*) elongate, flat, or hollow melanosomes in birds (e.g., African starlings; Ref. 88). Question marks indicate that presence or morphology is unknown. Phylogenetic tree was constructed in PhyLoT (https://phylot.biobyte.de) using the lineage information from NCBI taxonomy; the visualization of the tree was conducted in iTOL (version 4.0.3; Letunic and Bork 2016).

with particular emphasis on increased study of diverse natural melanins and melanosomes.

II. CHEMICAL STRUCTURE AND SYNTHESIS

On the basis of their structure, a general classification of melanins as they occur in Pro- and Eukaryota contains three groups (104, 113): Allomelanins, Eumelanin, and Pheomelanin **(FIGURE 2)**.

Allomelanins are a highly heterogeneous group of polymers in bacteria, fungi, and plants that develop through the oxidation and polymerization of 1,8-dihydroxynaphthalene (DHN), tetrahydroxynaphthalene (THN), and catechols via pentaketide synthases instead of tyrosinase **(FIGURE 2)**. Despite its widespread distribution, this is the least studied group of melanins. The biosynthetic pathways involve polyketide synthases (through the DHN pathway common in fungi; Ref. 154), polyphenol oxidases common in plants (164), or RppA and P450-mel to produce 1,4,6,7,9,12-

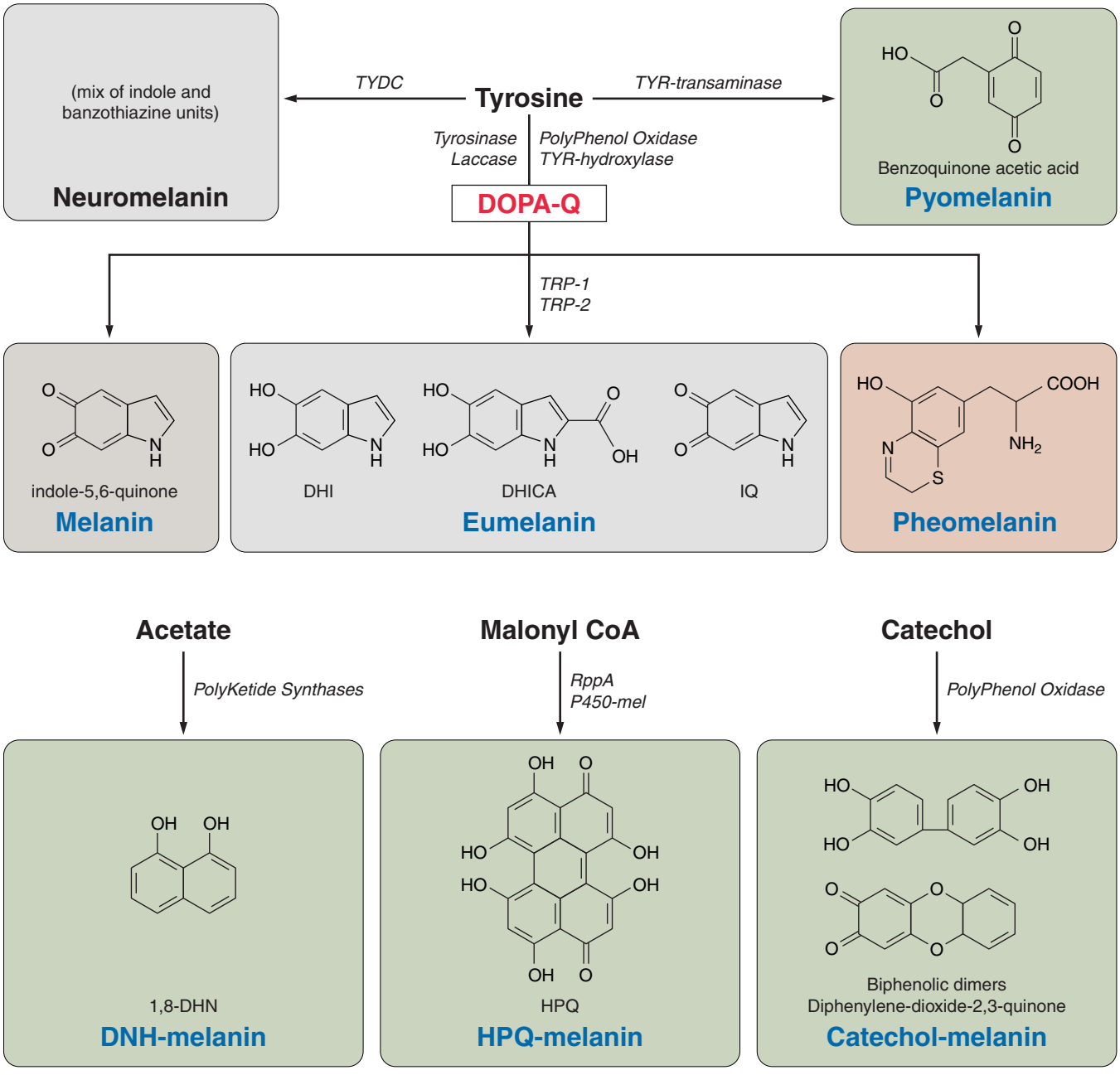

FIGURE 2. Simplified biosynthetic pathways of melanins by animals, plants, fungi, and bacteria. Melanin classification includes eumelanin, pheomelanin, and allomelanins (including pyomelanin, DHN-melanin, HPQ-melanin, and Catechol-melanin; shown in green insets). Melanins arise from different substrates including tyrosine in animals, plants, fungi, and bacteria; acetate in most fungi; malonylCoA in bacteria; and Catechol in plants. The main enzymes involved in each pathway are shown. The last precursor monomer in the formation of each melanin type is given for comparison purposes. [Modified from Singh et al. (134).]

hexahydroxyperylene-3,10-quinone (HPQ) or homogentisic acid (pyomelanin) synthesized by bacteria (45, 109). Generally, allomelanins lack nitrogen, consistent with the limitation for nitrogen that some of the producing organisms (e.g., plants) have in their environments or during growth. This means that many of the substrates used for the synthesis of allomelanins are nitrogen-free phenolic compounds like diphenols and cathechols (136).

Traditionally, animal melanin is classified as either black to brown eumelanin or yellow to reddish pheomelanin (112, 130). In our current understanding, eumelanin is composed of two major precursors: 5, 6-dihydroxyl indole (DHI) and 5,6-dihydroxyindole-2-carboxylic acid (DHICA). These melanin precursors and their oxidized forms are cross-linked through either chemical bonding or physical interaction (95). X-ray diffraction and high-resolution transmission electron microscopy show planar eumelanin protomolecules (oligomers of DHI and DHICA) of 13–20 Å are stacked into lamellae with a spacing of 3.7–4.0 Å (22, 30, 154). However, the exact chemical structure of eumelanin remains elusive, mainly due to its insolubility in most solvents, close binding with other cellular tissues, and an amorphous structure. These challenges have led researchers to study synthetic melanins, using experimental techniques such as Fourier transform infrared spectrometry (38), X-ray photoelectron spectroscopy (XPS) (36), mass spectroscopy (79), and solid-state nuclear magnetic resonance (20, 38, 87) as well as theoretical simulations. However, the chemical structures of synthetic eumelanin are highly dependent on the monomer type (e.g., dopamine, L-DOPA, or tyrosine), reaction conditions (e.g., temperature, pH, oxidants, and reaction time), and postsynthetic procedures in the synthesis (5, 6, 28, 67) Moreover, the chemical structure of synthetic eumelanin is stripped down and its similarity to natural eumelanin is not well known. Thus data based on synthetic melanin should not be interpreted as strictly representative of natural eumelanin, and a full understanding of the chemical structure of natural eumelanin remains elusive.

Less is known on the chemical structure of pheomelanin, which has been studied less than eumelanin since the 1960s (35, 100, 111, 152, 153) and typically using synthetic mimics. It seems to consist of two types of benzothiazine intermediates that are synthesized in the presence of sufficient cysteine **(FIGURE 1)**, specifically, its monomer thought to have a benzothiazine moiety with benzothiazole and isoquinilone moieties (131). Exactly how these monomers are connected to one another is still unknown but should be a target for future research.

One difficulty in the study of both eumelanin and pheomelanin is that pheomelanin is rarely found in isolation; the chestnut brown flank feathers of zebra finches *Taenopygia guttata* seem to be one exception (94). Indeed, although frequently considered separately, pheomelanin and eumelanin are almost always found in combination and are rarely reported in pure form (61, 94, 102). Thus (as in eumelanin) many studies have relied on synthetic pheomelanin, whose similarity to natural pheomelanin is unknown.

The ratio of eu- to pheomelanin in melanosomes is determined by tyrosinase activity and cysteine availability (130). This is because, while both eu- and pheomelanin production begin with the tyrosinase-catalyzed conversion of tyrosine to dopaquinone, pheomelanin production requires the presence of cysteine to form the intermediates 5-*S*-cysteinyldopa (5SCD) and 2-*S*-cysteinyldopa (2SCD). In addition, eumelanin production requires the activity of dopachrome tautomerase (TRP-2) and TRP-1 (DHICA oxidase) to catalyze the conversion of dopachrome to DHICA **(FIGURE 2)**. These enzymes are found in eumelanosomes, but not pheomelanosomes (see sect. V).

In addition to its fundamental structure, another important aspect of natural melanin chemistry is its ability to extract metals from the environment and chelate them to coordination sites within the polymer framework. Melanin's affinity for metals leads to accumulation of several metal ions (57). Such metal chelation has been shown to sequester bio-relevant metals (Na, Ca, Cu, Fe, Zn) as well as more toxic ones (Hg, Pb, Cr, Mn). Liu et al. (85) showed that Mg, Ca, Na, and K were the main metals in natural sepia melanin (in declining concentration). In situ tests reveal moderate affinity but high binding capacity for Ca and Zn (11, 124). In contrast, melanins have a high affinity for heavier metal cations like Fe^{3+}, Cu^{2+}, and Mg^{2+}, Ca^{2+} and Zn^{2+}, which form complexes with carboxylic acid groups. Cu^{2+} shows a preference for hydroxyl groups, and Fe^{3+} is more flexible and forms complexes with both hydroxyl and amine functionalities. Fe^{3+}, Cu^{2+} can cause damage to biological systems (by affecting Fenton reactions), and thus when melanin sequesters iron and copper it prevents their reduction and subsequent generation of oxidative stress (108, 165).

These catechol binding sites often serve a protective purpose, such as sequestering reactive metals, enhancing binding strength, altering absorption properties, and preventing harm to other biological systems (57, 133). Due to the catechol groups, melanin can bind with a variety of metal ions, such as Cu, Fe, Mg through metal coordination bonds, which helps to reduce metal toxicity in cells or form cross-linking networks to enhance mechanical properties. However, the mechanism of this protective role is still poorly understood. For instance, it is not known how the integrity of a melanosome changes upon metal chelation, or if some metals are more prone to degrading the polycatechol structure than others. Using in situ magnetic and spectroscopic information is one approach to track changes in melano-

some structure, metal coordination, and oxidation state under radical stress. Of particular interest are whether *1*) metals are clustered into superparamagnetic structures or sequestered as isolated cations and *2*) metals oxidation states are consistent with those produced in synthetic melanin mimics. This is because, as in the basic chemistry of melanin itself, much of what we know about metal binding of melanins comes from these mimics. Although we likely have a good understanding of the basic chemistry of melanins, a greater emphasis on study of natural material will enable greater certainty.

III. PROPERTIES

Melanin in nature has physicochemical properties that are atypical of biological pigments. Likely due to its dense, cross-linked polymeric structure, melanin has a high refractive index (RI). Because most direct measurements of RI assume transparency of the material being measured, researchers have primarily used indirect methods to measure RI of highly absorbant melanin. Stavenga and co-workers (137, 138) used polarizing interference microscopy and optical modeling to estimate the RI of melanin in damselfly wings and bird feathers as ~1.7–1.8. Xiao et al. (161) measured the RI of aquaeous synthetic melanin at a similar value. However, optical modeling assuming an RI of 2.0 has produced excellent matches between theoretical and empirical reflectance curves (e.g., Refs. 41, 160), so more direct measurements of diverse natural melanins are needed.

Melanins absorb light in the UV, visible, and near-infrared regions of the electromagnetic spectrum and protect their embedding tissues by scattering or dissipating light (17, 55). In some animals like cephalopods, controlling the melanin density and distribution in the integuments can lead to dynamic colors (47). For humans, exposure to UV enhances melanogenesis, leading to production of more melanin to protect the skin from UV damage. Melanin has been reported to transfer almost 90% of UV radiation to heat within a nanosecond or even faster (98). Cuttlefish use melanin as an anti-predator defense, while *Glycera* use it to strengthen their jaws (93). Eumelanin's high content of carboxyl groups facilitates a reversible binding of ions and their semiquinone units account for their ability to reduce and oxidize oxygen radicals (112). A particularly interesting property of eumelanin is that it behaves as a semiconductor, for example, converting light into heat (114).

Melanin molecules interact with environmental radiation and pollution causing configuration changes that are not fully understood yet but that might have important implications for the way in which they protect biological tissues. For example, absorption of solar radiation by melanin induces a transformation to an excited state, and some of those species live long enough to induce excited-state reactions (144). Excited melanin molecules transfer energy to molecular oxygen ($^3O_2$) forming singlet oxygen ($^1O_2$). It has been proposed that under certain conditions the excited species of melanin generate reactive oxygen species making it a potentially damaging molecule (23).

Despite multiple biological functions rising from broadband absorption of melanin, it is still not well understood how the molecular structure relates to the optical properties. Meredith and Sarna (95) demonstrated that melanin is chemically heterogeneous, consisting of numbers of different chemical species, and the superposition of absorption of all species at different wavelength leads to a large broadband absorption curve. Recently, Chen et al. (21) have found the excitonic couplings within melanin also affect the absorption and proposed that geometric disorder of melanin aggregates could also broaden the absorption spectrum in a similar way as the chemical disorder (21). However, identifying the chemical structure-function relationships of melanin should be a high priority.

IV. MELANOSOME DEVELOPMENT

Generally, melanin is synthesized in specialized cells called melanocytes, or in the case of insects, hemocytes (119). In all studied cases, melanin is composed of 30–50 nm nanoparticles, in which melanin monomers are cross-linked together and form stacking structures by pi-pi interactions. Melanin can be produced diffusely in intracellular and/or extracellular spaces of bacteria (110), or in granules at the surface of the cell wall in some fungi (18). In contrast, animal melanin is located almost exclusively within well-defined submicron melanosomes, specialized organelles derived from early endosomal membranes whose functions are to synthesize and store melanin. In a few exceptions, melanin-like material has been found deposited diffusely in the scales of butterflies (*Papilio Ulysses*; Ref. 128), mosquitoes (*Aedes aegypti*; Ref. 24), and hydrobiid snails (32).

All cells responsible for skin color in vertebrates are called chromatophores. Chromatophores receive different names depending on the pigment they contain. Melanophores (or melanocytes in humans) contain melanin and are the most common type of chromatophores, responsible for the black, brown, and brownish red coloration of vertebrates. Melanophores contain thousands of melanosomes that vary in size, shape, and composition depending on the type of melanin they contain. Eumelanosomes (containing predominantly eumelanin) are ellipsoidal and tend to be larger than pheomelanosomes, which are generally more spherical but also more irregularly shaped (86). It has been hypothesized that the irregular shape of pheomelanosomes is due to the lack of protein scaffolds responsible for leading the orderly deposition of melanin observed in eumelanosomes (63).

Clear differences exist in the biogenesis of eu- and pheomelanosomes starting with the type and ratio of mela-

nin they contain. All melanosomes contain tyrosinase, the enzyme that converts L-tyrosine to L-dopaquinone (DQ); however, after this first step of melanogenesis and if L-cysteine (Cys) is present in sufficient amounts within melanosomes, DQ and Cys interact to eventually produce pheomelanin **(FIGURE 2)**. In eumelanosomes exclusively, two additional membrane-bound enzymes form a multienzyme complex and help stabilize tyrosinase (68), these are tyrosinase-related protein-1 and -2 (TRP-1 and TRP-2 respectively; Refs. 69, 145). The production of eumelanin or pheomelanin is then modulated by the opposing action of alpha-melanocyte stimulating hormone (MSH) and agouti protein. A switch from the production of eumelanin to pheomelanin can occur though changes on the pH inside melanosomes, where eumelanogenesis progresses around neutral pHs but production of pheomelanin proceeds faster and even suppresses eumelanogenesis at acidic pHs (153).

Melanosomes are formed through a series of well-defined stages. The first stage in eumelanosomes (I) begins with a membrane-bounded vesicle that lacks pigment and is characterized by incipient proteinaceous fibrils present in the organelle's lumen. Fibrils are completely formed in stage II, and the melanosome adopts an ellipsoidal shape **(FIGURE 3)**. The protein PMEL17 (also known as gp100) is exclusively synthesized by melanocytes and represents the main structural constituent of fibrils (117). PMEL17 expression is strongly downregulated in cells synthesizing pheomelanins by agouti signaling (46, 69), which might explain why pheomelanosomes lack fibrils and have an irregular shape (63). PMEL17 has been used as a tracker of transferred melanin into keratinocytes (135).

In stages III and IV, electron-dense melanin is synthesized and progressively deposited on the fibrils until the internal structure of the melanosome is completely obscured at the end of stage IV. Unlike eumelanosomes, pheomelanosomes are always spherical during development and contain only amorphous proteinaceous material and granular melanin, but never clear protein filaments nor matrices (62, 63; **FIGURE 3**). In epidermal melanocytes, stage IV melanosomes are then transported to keratinocytes through at least four different, still unresolved, processes (see below).

A. Growth of Protein Scaffolds

During the formation of eumelanosomes, intraluminal fibrils that originate from the proteolytic processing of the protein PMEL accumulate and form a matrix that optimizes melanin polymerization, condensation, and storage (8; **FIGURE 3**). The PMEL amyloid-like fibrils begin to form between stages I and II of melanosome development, in multivesicular bodies (MVBs) and are fully formed into β-sheet like arrays in the melanosomes (60). The fibrils are closely associated with intraluminal vesicles (ILVs), and as melanosomes mature, the fibrils grow and the vesicles shrink (60; **FIGURE 3**). Several proteins form amyloid fibrils that are associated with pathologies like neurodegenerative diseases including Alzheimer's disease and Parkinson's disease. However, PMEL fibrils, although amyloidogenic, are physiological or nonpathogenic (43) and thus have been the subject of intensive recent research. Unlike pathogenic amyloid fibers, PMEL shows an ordered fibrillization most likely due to the presence of a lipoprotein named ApoE (147). This lipoprotein concentrates on the surface of ILVs and caps the fragments of PMEL; there it seems to guide the organized fibrillization of PMEL by using the ILVs as nucleating platforms (8; **FIGURE 3**). PMEL transcripts are expressed in different tissues in vertebrates (14); however, the expression of the PMEL protein in epidermal melanocytes has been found exclusively in mammals (7, 118) and birds (65), coinciding with the presence of elongate melanosomes in these groups.

Metal binding does not appear to affect the morphology of melanosomes, as it did not change granule morphology of sepia on metal binding, indicating that iron coordination does not occur at or near the surface of the melanin granule (85). Similarly, synthetic melanin granules doped with varying amounts of Fe(III) had consistent morphology (80). Thus melanosome shape seems to be almost entirely determined by the form of the protein fibrils.

B. Migration and Transfer From Melanocyte to Developing Tissue

The transfer of melanosomes from melanocytes to keratinocytes is the only known example of organelle movement between cells. Many efforts have been made to elucidate the pathway of this movement between epidermal melanocytes and keratinocytes in skin, feathers, or hair. These efforts had been mainly hampered by the lack of suitable assays that allow a rapid and effective way to evaluate transference events in vivo or in vitro. The vast majority of research in the field of melanin transfer has been done in mammals with a few exceptions on amphibians (1) and birds (40). Four mechanisms of transport of melanosomes between melanophores and keratinocytes were proposed a decade ago (146; **FIGURE 4**), and diverse experimental evidence in favor of each model supports the idea that different modes of transfer might not be mutually exclusive and might vary depending on the system. The first mechanism of melanosome transport (the cytophagocytosis model; **FIGURE 4*A***) was demonstrated by detecting and tracking the protein gp100 on the surface of developing melanosomes. Singh et al. (135) showed that keratinocytes can phagocytize entire melanocyte constituents (e.g., dendrite tips) as a way to internalize melanin (135).

In human skin, Scott et al. (125) documented that after the formation of filopodia from melanocyte dendrites, a line of individual melanosomes moves along filopodia, most likely

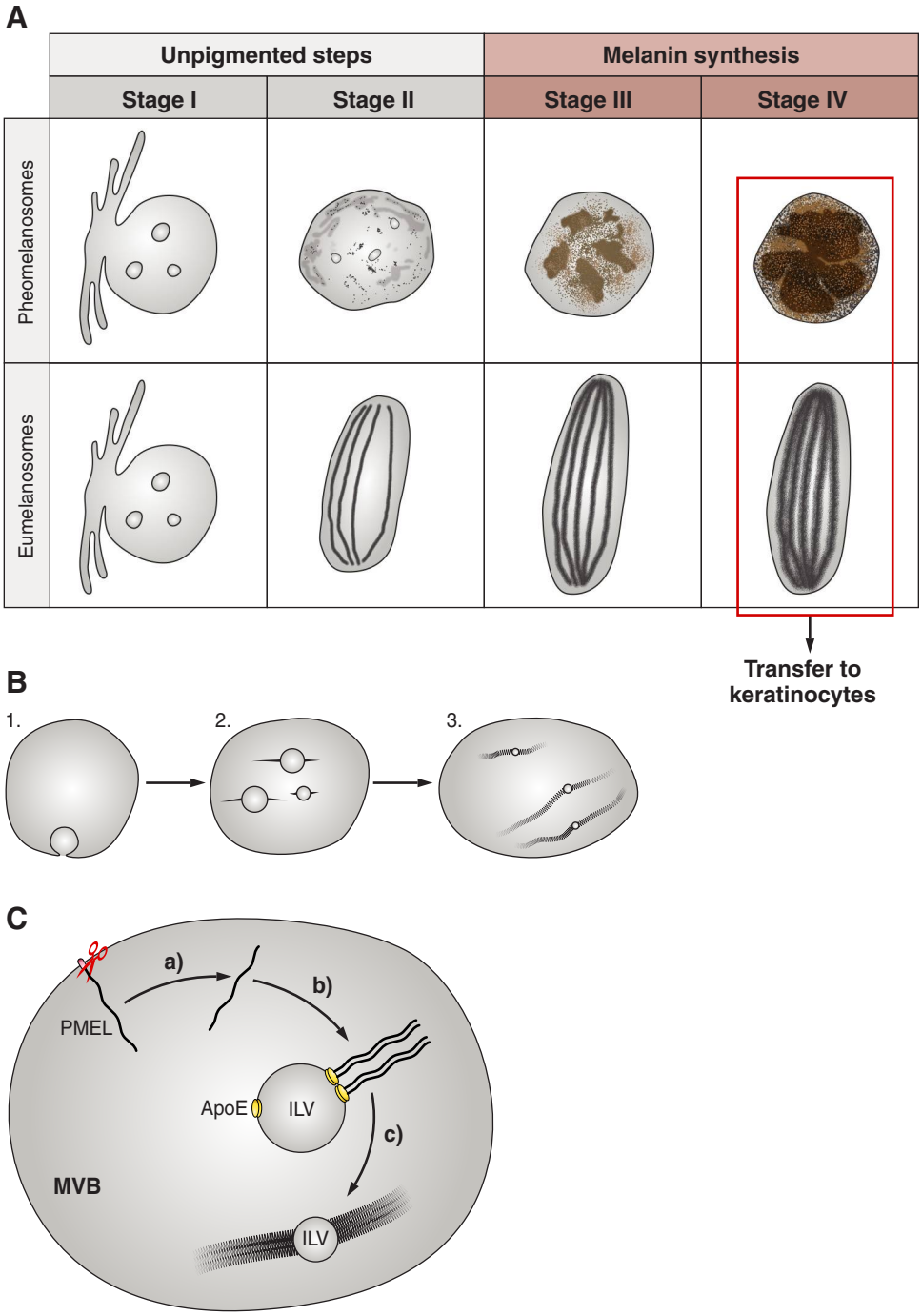

FIGURE 3. Different stages in the development of melanosomes and formation of protein scaffolding in eumelanosomes. *A*: stages of development of pheo- and eumelanosomes. Between stages I and II, PMEL fibrils form a well-defined scaffolding of protein sheets. In stages III and IV melanin synthesis takes place. In pheomelanosomes PMEL is downregulated; thus there is no protein scaffolding. Mature melanosomes in epidermis are then transferred to keratinocytes. *B*: melanosomes originate as multivesicular bodies (*1*). In eumelanosomes the protein PMEL is cleaved from the pre-melanosomal membrane and start to assemble in premelanosomes stage I (*2*). PMEL fibrils are organized in parallel sheets resulting in the elongation of the organelle (*3*). *C*: model of fibril formation. PMEL fragments are cleaved and released into the lumen of premelanosomes (*a*). Formation of fibrils happens in close association with intraluminal vesicles guided by the presence of ApoE on the ILV surface (*b*). Fibrillation occurs in a last step (*c*). ILV, intraluminal vesicles; MVB, multivesicular body; ApoE, apolipoprotein E. [Modified from Bissig et al. (8).]

via the presence of myosin Va, and cross this quickly formed channel into the keratinocyte; transfer was assumed to occur by fusion of the filopodium with the keratinocyte membrane (the fusion model; **FIGURE 4*B***). Later, Singh et al. (135) confirmed that filopodia directly donate melanosomes via the fusion of nanotubes with recipient keratinocytes **(FIGURE 4*B*)**.

More recently, Wu et al. (158) provided detailed electron micrographs of mice cell cultures supporting the idea that melanocytes package melanosomes into beads and then lose them in the periphery of the melanocyte, which in turn captures and phagocytizes these melanosome beads (model of vesicle transfer; **FIGURE 4*C***). Both accounts of the ontogeny of iridescent color in feathers described a mechanism of melanosome transfer that agrees with the model of vesicle transfer (40, 129). A fourth mechanism (the exocytosis model; Ref. 146; **FIGURE 4*D***) proposes that melanin units, free of its melanosomal membranes (termed melanocores; Ref. 159), are released into the extracellular space between melanocyte and keratinocyte. Subsequently, the melanocores are internalized by keratinocytes through phagocytosis and organized in clusters around the perinuclear area. A growing amount of evidence from elec-

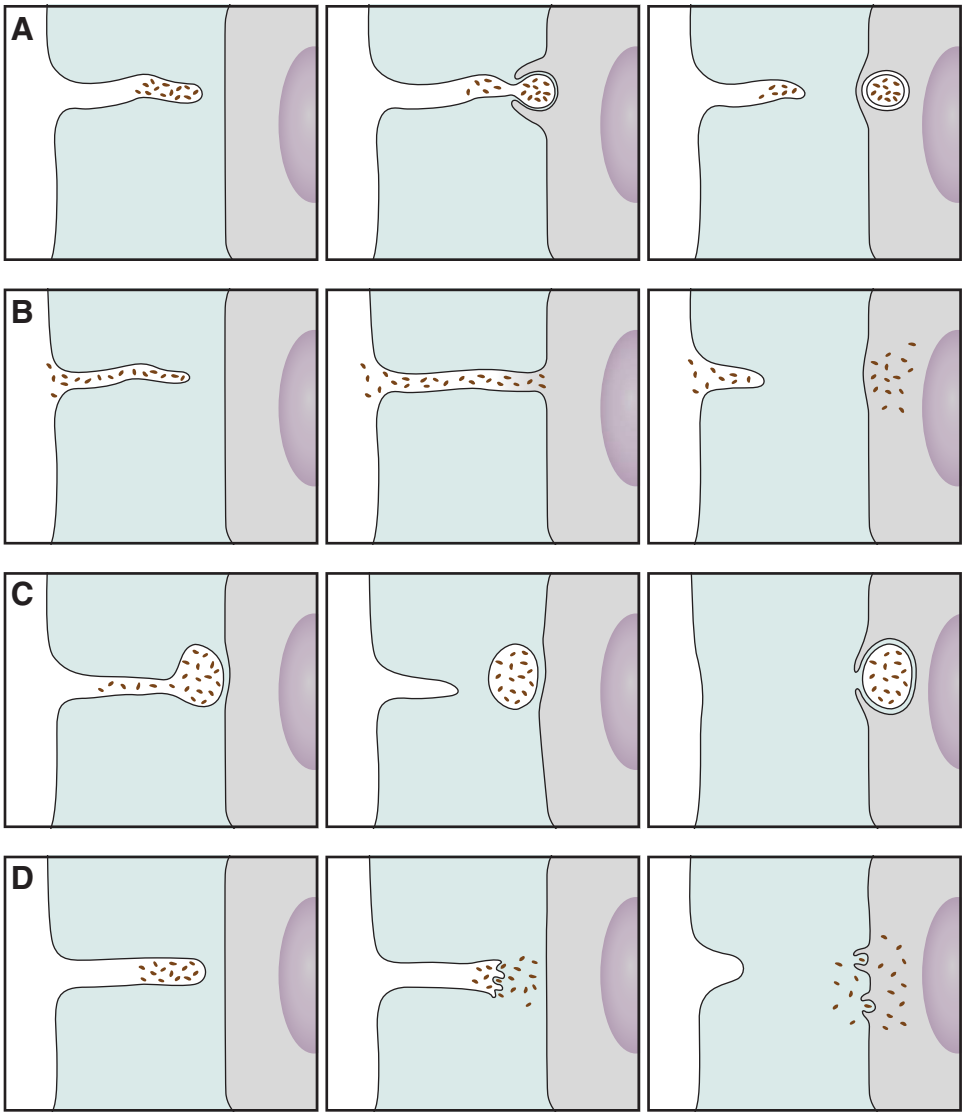

FIGURE 4. Models of transfer of melanosomes between melanophores and keratinocytes based on the mechanisms proposed by Van de Bossche et al. (146). *A*: the cytophagocytosis model. *B*: the fusion model. *C*: the model of vesicle transfer. *D*: the exocytosis model. In all cartoons, melanophores are shown on the left in white and keratinocytes are shown on the right, shaded in gray.

tron and immunoelectron microscopy, including evidence of uptake and arrangement of membrane-free synthetic melanosomes (59), of human skin cells support this hypothesis (29, 60, 142).

C. Other Interactions With Melanocytes

Melanocytes in the vertebrate integument often form interactions with the epidermal keratinizing cells. In animals including crustaceans, cephalopods, fish, amphibians, and reptiles, melanophores are present in both the dermis and epidermis.

In reptiles, epidermal melanophores are scattered in the stratum basale in varying concentrations. Melanophores donate melanosomes to keratinocytes forming what is known as the epidermal melanin unit (12). The distribution patterns of epidermal melanophores vary with coloration patterns and are species-specific.

Dermal melanocytes often form a single layer and do not transfer melanosomes to keratinocytes; however, in ectothermic vertebrates, dendritic processes can project through pigment cells like iridophores and xanthophores (74) constituting the dermal pigmentary effector system (56). The rapid mobility of dermal melanophores is under hormonal and neural control (i.e., physiological color change). This mobility leads to rapid changes in color that can be used for signaling or photoprotection.

During physiological color change, melanosomes move synchronously along microtubules within melanophores making the whole animal appear darker, lighter, or speckled depending on the dispersion or aggregation of melanosomes (2).

In crocodilians, the color of the skin results from different combinations in the distribution of pigment cells. For example, dark spots derive from the accumulation of melano-

phores in the epidermis; the brown or gray background in the skin is the result of the homogeneous presence of melanophores along the basal part of the epidermis and/or the stratum corneum. Sometimes, melanophores can also be present in the superficial dermis.

D. Post-Transfer Changes in Melanosome Morphology

In general, melanosome morphology remains consistent between the melanocyte and keratinocyte. However, birds have unique melanosome shapes, including hollow spheres, hollow rods, and hollow platelets (39) found nowhere else in metazoans. How flattening occurs is as yet unclear, but evidence suggests that some melanosomes become hollow after their deposition in the developing feather cells (e.g., in *Meleagris gallopavo*, Ref. 129; **FIGURE 5**). The solid cores of melanosomes in the melanocyte gradually disintegrate and become hollow in the barbule plate cell, perhaps through loss of less-stable pheomelanin in the new chemical milieu (129). Similar pheomelanin-eumelanin core-shell arrangements in iridal melanosomes have been identified using various techniques including photoemission electron microscopy (130). Why the pheomelanin cores are stable in eyes but may not be in feathers is unclear, and using these techniques to verify the core-shell chemistry of developing hollow melanosomes would be a good first step towards clarification.

V. RELATIONSHIP BETWEEN MELANOSOME SHAPE AND CHEMISTRY/COLOR

Because most melanin-based colors are produced by a mixture of pheo- and eumelanin in varying concentration (94), color cannot be diagnosed solely on the presence of a single melanosome type. That is, the binary distinction between "eumelanosomes" and "pheomelanosomes" does not capture the true diversity of both melanosome shape and chemistry found in nature. In birds, crown clade avian melanin-based brown colors range from the bright rufous colors of American Robin (*Turdus migratorius*) breasts to the dark cryptic browns of many seabirds that are nearly indistinguishable from black (94; **FIGURE 6**). The biochemistry, function, and evolution of these different shades vary tremendously, and thus it is critical to distinguish between them. Bright oranges, for example, are thought to be produced by nearly pure phaeomelanin that forms uniformly spherical melanosomes, while darker browns frequently have larger percentages of eumelanin that form both rod-shaped and spherical melanosomes (76, 77). Gray colors are even more variable both in color and in morphology and arrangement of melanosomes (77). While the majority of melanosomes producing grays are intermediate in size and shape between browns and blacks, they can approach those of pure phaeomelanosomes and exceed those of eumelanosomes producing the darkest black (77).

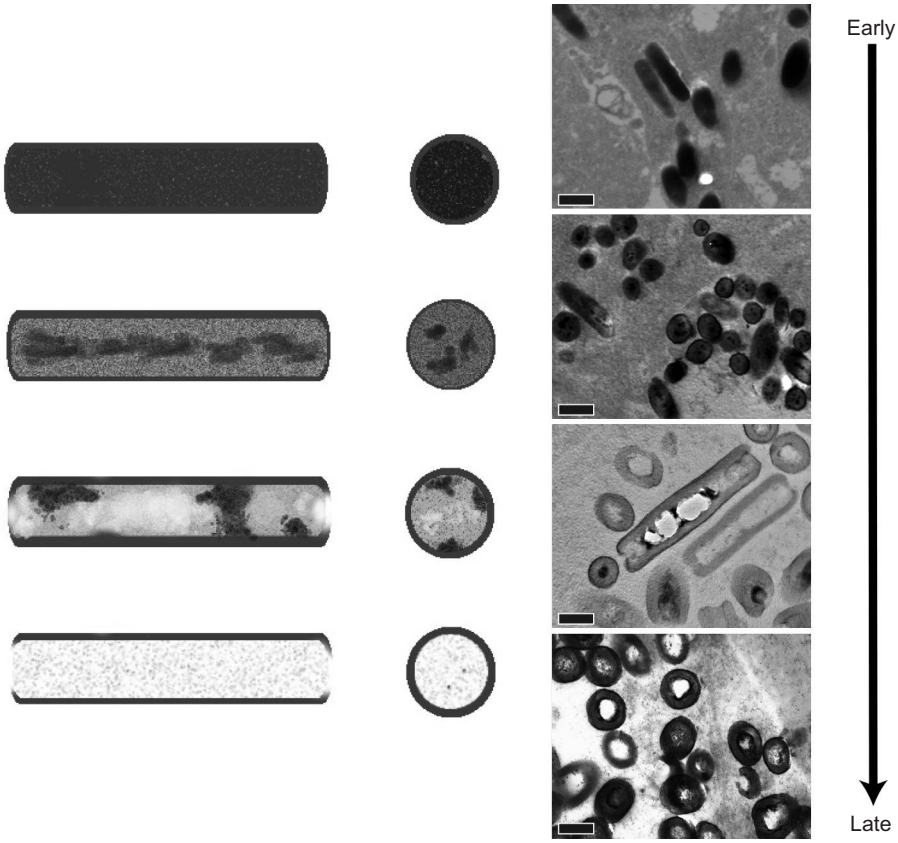

FIGURE 5. Changes in melanosome morphology in iridescent feathers of *Meleagris gallopavo*. A successive degradation of the pheomelanin core of melanosomes takes place during the formation of feathers. [From Shawkey et al. (129).]

FIGURE 6. Examples of melanin-based color diversity, ranging from light brown to iridescent purple in extant birds.

Intriguingly, the relationship between color and melanosome morphology appears to exist only in birds and mammals (78). These two groups exhibit both rod-shaped and spherical melanosomes that correspond roughly with browner and blacker colors. In contrast, all sampled squamates and turtles showed an invariant oblong melanosome morphology despite variation in color comparable to that in birds and mammals. Moreover, in birds and mammals, it has been demonstrated that most melanosomes contain mixtures of eu- and pheomelanin (61), whereas in fish and molluscs (*Sepia oficinallis*) melanosomes contain pure eumelanin (61).

Thus variable melanosome morphology may be a derived feature of birds and mammals that could correlate with changes in the broader melanocortin system or with the evolution of protein fibrils and associated PMEL genes (see below).

This variation in melanosome morphology is also almost certainly associated with variation in chemistry. However, nearly our entire understanding of this relationship is based on studies of mammal (mostly human) hair (130) with only a few studies on which to base inferences in birds. For example, zebra finch cheek feathers have nearly 100% pheomelanin (94) contained in irregular spherical melanosomes (167), while red-winged blackbird feathers contain nearly 100% eumelanin in rod-shaped melanosomes (76). While these data suggest a similar chemistry-to-morphology relationship to that seen in mammal hairs, these represent only the extreme ends of distribution. For example, mallards have a high percentage of eumelanin in their feathers (94), but very high aspect ratio melanosomes. Thus this relationship is likely to be complex and needs further investigation through combined analysis of melanosome chemistry and morphology.

VI. MELANOSOMES AND STRUCTURAL COLOR

Colors can be produced by either selective absorption of certain wavelengths of light by pigments or by light scattering by tissues arranged at the nanometer scale (called structural colors) (115). Pigments produce a limited palette relative to structural colors in part because their reflected color

is based solely on pigment biochemistry. Melanin is the most widespread color-producing pigment in extant birds and creates a broad range of black, brown, and gray colors (82; **FIGURE 6**), and when arranged into discrete nanostructures, melanin is a critical component of some of the brightest iridescent colors found in nature (115). Colors produced by nanostructural feather constituents, termed structural colors, can be further subdivided into two categories: iridescent and non-iridescent (115). Broadly defined, iridescent structural colors change in appearance with angle of observation or illumination, while non-iridescent colors generally remain similar in appearance regardless of angle of observation (105). Integumentary melanins are indirectly responsible for non-iridescent colors, such as blue feathers where they provide a dark background that enhances saturation of the color produced by keratin and air matrices in the feather barb (127). However, iridescent structural colors are generally produced directly by melanosomes in feather barbules (115). These colors are created by laminar or crystalline arrays of melanin granules embedded in keratin (39); light is scattered constructively by laminar or crystal-like arrays consisting of alternating layers of materials with different refractive indexes, namely, keratin, melanin, and sometimes air. Although all iridescent plumage colors are produced by the same underlying processes, considerable variation exists in the structure and arrangement of the alternating layers of keratin and melanin and, consequently, in the appearance of these different colors **(FIGURE 6)**.

Melanin granules can be rod- or disk-shaped, solid or hollow, and can be arranged in single or multiple layers (39, 88). Furthermore, recent work has elucidated a fundamental dichotomy in the types of iridescent colors. "Thick films" have a single layer of melanin granules below a single superficial keratin layer (13, 37, 115). Brink and van der Berg (13) showed that the coppery-purple iridescence in the dark plumage of the hadeda ibis, *Bosthrychia hagedash*, is produced primarily by coherent scattering from an unusually thick (~0.8 μm) and uniform keratin cortex. The single, underlying layer of elliptical melanin platelets apparently serves mainly to define the thickness of the superficial keratin layer (13). In contrast, "thin films" are composed of multiple layers of melanin granules and keratin (39, 115, 169). The brilliantly colored iridescent gorgets of many species of hummingbirds (Family Trochilidae), for example, are produced by coherent light scattering from multiple, alternating layers of keratin and air-filled, disk-shaped melanin granules (51, 72). Melanosomes may also be arranged two dimensionally as, for example, in the hexagonal close-packed configuration that confers diverse iridescent colors to dabbling duck wing patches (41).

Slight variations in melanosome diameter and spacing can cause dramatic changes in color, but these colors are limited relative to what is theoretically possible, likely due to energetic constraints during development. Hollow melanosomes introduce an additional low refractive index material (air) into hexagonal nanostructures and thereby produce brighter colors (42). Such melanosomes have convergently evolved numerous times in some lineages with bright colors (e.g., hummingbirds, African starlings) and have recently been shown to accelerate both the rate of color evolution and speciation in clades that possess them (88). The high RI of melanin enables a strong contrast with the lower-RI keratin (~1.56) surrounding it, producing frequently vivid colors and iridescence. The high optical absorbance of melanin enhances the saturation of these colors (162), while also reducing their overall brightness. Hollow melanosomes allow particularly high RI contrasts due to the extremely low RI of air (42).

How melanosomes are arranged in precise patterns during feather growth is also largely unstudied, but initial data suggest that it is not actively guided by the cell (87). Instead, the physical and chemical interactions between melanosomes and polymerizing keratin appear to passively produce the nanoscale patterns producing color. Variation in the relative concentration of melanosomes and keratin in the developing barbule may lead to variation in patterning. Similar processes produce even more complex patterns in synthetic materials (168), so this hypothesis is reasonable. Indeed, the evolutionary lability and widespread presence of iridescence among birds suggests that it is relatively simple to evolve, perhaps because of this developmental simplicity. Patterning that required, for example, active guidance of melanosomes via cytoskeletal elements would likely be more difficult to evolve.

This phylogenetic lability suggests that melanosome-based colors may play critical roles in the diversification of birds. In African starlings, for example, the evolution of hollow and flattened melanosomes was likely a key innovation that dramatically increased their rates of color evolution and, speciation (88). However, how these novel forms have impacted broad avian evolution has never been examined.

VII. EVOLUTION OF MELANIN AND MELANOSOMES

Melanins are a very diverse group of pigments, present in organisms from all taxa across the tree of life. This ubiquity **(FIGURE 1)** and the diverse functions of these pigments across taxa suggest that the ability to produce melanin is of great evolutionary importance (134). However, little work has been done to address either the pattern or process of the evolution of melanin and melanosomes. Our **FIGURE 1** is, as far as we know, the first attempt to provide a broad overview of the pattern of chemical and morphological evolution. This figure suggests that diffuse eumelanin is likely melanin's ancestral form, but higher resolution sampling is needed to state this definitively and, more broadly, to un-

derstand melanin's significance to biotic evolution. Some fundamental questions include: What selection pressures drove the evolution and maintenance of melanin? What major shifts have occurred in its chemistry and morphology? In particular, what has propelled the evolution of melanosomes? How did these changes affect evolutionary patterns? These are fundamental questions that we have barely begun to address.

The evolution of pheomelanin is particularly intriguing. Despite its association with higher levels of skin cancer in humans (101), likely due to its weaker absorbance of UV light than eumelanin, it is found in at least two major lineages (Fungi and in animals: birds, mammals, squamates, insects). One potential explanation for this conundrum is that it may serve some function that counterbalances its potentially negative effects. It is worth noting that an association between skin cancer and pheomelanin has only been demonstrated in humans and may thus not be relevant to other animals whose skin is more completely covered by integumentary structures like hair or feathers. This should be investigated more thoroughly before assuming a trade-off between the negative and positive effects of pheomelanin. Alternatively, at least in animals pheomelanin could have enabled the production of more diverse integumentary colors. These could have allowed for production of more effective camouflage or diverse signals that could be favored by sexual selection. If this were true, we predict an expanded colorspace occupied by clades that produce pheomelanin than those that do not. This expansion should have initiated at around the time of the origin of pheomelanin in each clade.

The maintenance and evolution of eumelanin has received more attention.

It has been hypothesized that, since early in animal evolution, the presence of melanin as a dark pigment shielding photoreceptor cells had the function of reducing backscattered light (71) as well as providing protection from indirect light and removing free radicals (15). For example, the melanin contained in the eyes of the cnidarians *Tripedalia cystophora* and *Cladonema radiatum* (155) shields photoreceptor cells and performs the same function as in vertebrates eyes, suggesting an ancestral condition. Similarly, close communication between melanin-containing cells and photoreceptors is observed throughout the evolution of animal eyes, suggesting that photoreception and photoprotection functions are coupled.

Melanin's diverse protective roles may help explain its conservation and ubiquity. Melanin protects fungi from the presence of toxins and allows habituation to stressful environments that include high insolation, low temperature, elevated reactive oxygen species, and increased radioactivity (4, 50). In many pathogens and plants, melanin is also related to wound healing, stress, and immune responses (89).

External melanin deposition might associate with a mechanical protection function in many organisms. Protective melanin deposits are often observed after cellular disruption, i.e., due to tissue injury bringing the tyrosinase enzyme into contact with their phenolic substrates. For example, allomelanins (catechol-melanins) present in the outer cells of some plant seeds or in senescent leaves (103), or produced by plants on tissues after injury (143), help resist mechanical damage. In invertebrates, melanin is an essential response against pathogens (34) and the combined presence of proteins and melanins may also be involved in the hardening process of the outer skin of insects and arthropods (116).

What drove the evolution of melanosomes themselves? In vertebrates, melanin packaging is associated with the production of amyloid fibrils within melanosomes (see sect. IV). Amyloid fibrils template and direct melanin production to encapsulate foreign intruders, avoiding the biosynthesis of diffuse melanin (34), which could be harmful to surrounding cells due to the production of toxic melanin intermediates. For bacteria that produce melanin extracellularly, prevention of cytotoxic melanin intermediates is probably not imperative, as melanin tends to form a layer in the cell wall external to the cell membrane (109). The coupled production of melanin and amyloid fibrils seems to be evolutionarily conserved and tightly associated with the stress response; amyloidogenesis and melanogenesis are the result of integrated immune and endocrine pathways (106). Both in invertebrates (52) and vertebrates (140), the amyloid fibril formation is accompanied and even inducible by melanocortins ACTH and α-MSH.

Given the ubiquity and multi-functionality of melanin, and that melanism and immunity share the melanin-producing pathway (122), we can hypothesize that melanin packets or melanosomes could be present in organisms since the origin of the immune system. However, our assessment of when melanosomes first appeared is limited to their availability in the fossil record. Currently the oldest known fossil bearing melanosomes, the *Tullimonstrum* bilaterian, only dates back to the Carboniferous (307 Ma; Ref. 26). However, older specimens have not yet been sampled and may reveal an earlier origin of melanosomes.

VIII. MELANOSOMES IN THE FOSSIL RECORD

Almost a decade ago, evidence of melanosomes preserved in the fossil record was discovered (148, 150). This discovery was a reanalysis of earlier findings by Davis and Briggs (33) who had described what they thought were feather-degrading bacteria in fossil plumage. Noticing that melanosomes were preserved in ink sacs of 60-myo squid, Vinther et al.

(148) reasoned that they may be preserved in similarly-aged fossil feathers, and with collaborators examined a feather with dark and white stripes. Scanning electron microscopy revealed the presence of micron-sized, rod-shaped structures in the dark, but not light stripes. While this morphology is superficially consistent with both bacteria and melanosomes, this distribution was much better explained by the latter. This same group then showed that the organized arrays of melanosomes that make iridescent structural colors could also be identified in fossil feathers (149). Finally, two independent research groups nearly simultaneously published papers showing that the shape of these melanosomes could be used to predict colors of both non-avialan dinosaurs and basal birds (early parts of Avialae; Refs. 25, 76, 77, 167). These discoveries showed, first, that the colors of fossil organisms could be accurately reconstructed, offering unprecedented insights into their functions and evolution. Second, that melanosome morphology shows an unanticipated diversity that contrasts with the previously assumed bimodal distribution (e.g., Ref. 167). Finally, they had directly demonstrated that melanin was present at least ~250 million years ago (26). Indeed, the mixed rod and sphere morphology in the eyes of *Tullimonstrum* are so similar to that found in modern vertebrates that it was used to categorize it as a vertebrate after years of ambiguous phylogenetic classification.

The deposition of melanin in melanosomes provides the opportunity for detection through morphological analysis, typically involving scanning electron microscopy (SEM). Typically, small pieces of the fossil are excised using a bevel tool, mounted on SEM stubs using carbon tape, sputter-coated and viewed on an SEM. Although samples are taken destructively, they are small enough that no damage on the fossil is visible to the naked eye. In one case, an SE with an unusually large chamber was used to hold the entire isolated Archaeopteryx feather so that no destructive sampling was needed (19). In either protocol, fossilized melanosomes are identified through scanning at low magnification and then imaged at higher magnification to obtain morphological details. Lack of melanosomes in a sample is also recorded, along with images of the matrix (77).

Thus far, primarily SEM has been used to characterize melanosomes in fossils, but other techniques may prove useful. Atomic force microscopy (AFM) is a well-established technique that uses mechanical force rather than light or electrons to visualize the surface of materials and objects (48). It provides a level of detail on surface texture greater than any other technique, visualizing and quantifying their roughness and features to the resolution of less than a nanometer. AFM is also ideally suited to paleontological work because, unlike SEM, it can accommodate larger samples and is done without coating, eliminating any need for destructive sample preparation. It is also useful because evidence indicates that different melanosomes types have different surface textures. Both eu- and phaeomelanosomes are composed of smaller melanin subunits, but eumelanosomes are relatively smooth while phaeomelanosomes are rough and globular (86). Thus, when fossil melanosomes are exceptionally well preserved, we may be able to identify types associated with different colors by their surface features. Both eumelanosomes isolated from a wild turkey *Melleagris gallopavo* and fossilized melanosomes from the Messel shale show the small subunits characteristic of eumelanosomes **(FIGURE 7)**, indicating that this technique can be successfully applied.

Several exciting new papers have detailed methodology that may at some point allow identification of melanin-based colors through chemistry, either alone or in conjunction with established morphological methods. Measuring fossilized melanin through trace chemical signatures may allow color reconstruction as well as study of the evolution of color and of the pigments themselves at unprecedented levels of detail. Wogelius et al. (157) and Barden et al. (3) describe a nondestructive technique that could be added to the reconstruction toolbox: detection of trace metals associated with eumelanin pigments. They clearly show that copper associated with feathers of several ancient bird species is of biological origin, and likely from feathers. However, other work has shown that this technique is prone to false positives (151).

Other methods have similarly shown strong evidence for the presence of preserved chemical eumelanin in association with fossil melanosomes. Lindgren et al. (83) showed that time of flight secondary ion mass spectrometry (ToF-SIMS) and IR absorbance patterns of a fossil fish eye from the early Eocene were similar to a eumelanin standard. Glass et al. (49) and Simpson et al. (132) used additional direct chemical techniques and again showed strong evidence for preservation of eumelanin in Jurassic fossils. While these data further strengthen the melanosome hypothesis, they again do not enable assignment of colors because they do not demonstrate presence of phaeomelanin. Simpson et al. (132) applied pump-probe microscopic imaging, a method that can differentiate pheo- from eumelanin, to a fossil squid ink sac, which is known to contain only eumelanin. While serving as a good negative control, application to another fossil with mixed melanosome composition (e.g., *Anchiornis*) or one with putative pheomelanosomes (*Sinosauropteryx*) will be an excellent next step. Some fossil material has been tentatively described on pheomelanin based on chemical analyses (27), but the difficulty in characterizing pheomelanin in even extant animals makes caution warranted. If pheomelanin can be detected in fossils, the rush will then be on to identify how color relates to relative pheomelanin/eumelanin composition so that assignments based on morphology can be verified through chemistry and vice versa.

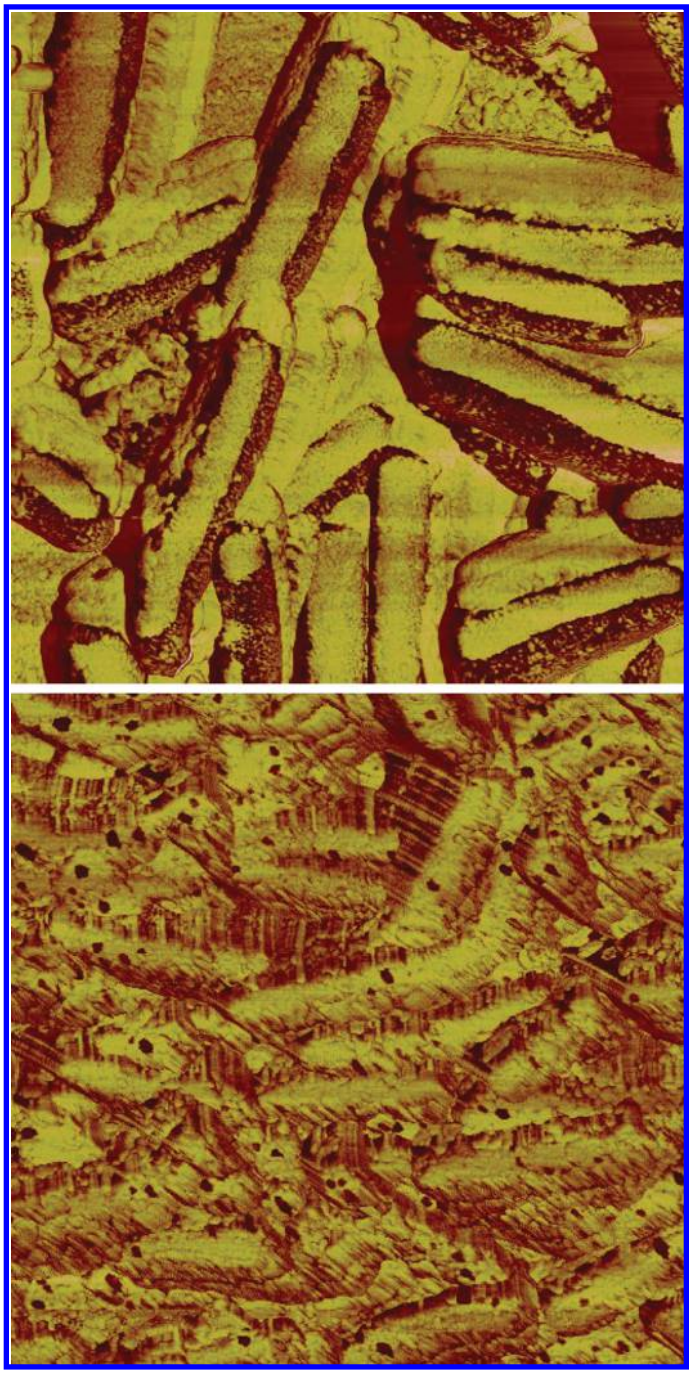

FIGURE 7. Atomic Force Microscope images showing melanosomes extracted from extant wild turkey feathers (*top*) and from fossilized melanosomes from a bird from the Messel shale (*bottom*).

An alternative or complementary approach will be to build a database of melanin chemistry similar to that built using morphology. While morphology can be used to predict color and may play some role in color production through scattering, its chemistry likely has the most significant effect on its color. Thus identifying patterns in melanin composition of differently colored feathers and comparing it with that of fossilized melanin could give great insights into the relationship between melanin chemistry and color. Avian melanosomes have an unparalleled diversity of form that almost certainly mirrors a diversity in chemistry. However, these two features remain virtually unstudied, even though they are critical to understanding their role in producing colors and are also involved in numerous physiological pathways (95). While most melanin has been replaced during diagenesis, recent results from several independent groups (49, 83, 157) suggest that sufficient quantities for analysis may be preserved.

Because they are based on organized arrays of melanosomes, detection of iridescent structural colors may be easier than distinguishing between black, brown and gray. Indeed, two papers have presented evidence that they were present in both fossil birds and feathered theropods. Vinther et al. (149) examined a fossil feather with visible purple coloration and found organized arrays of melanosomes highly similar to those of extant birds with iridescent plumage. Subsequently, Vitek et al. (151) used focused ion beam (FIB) milling to examine this same fossil in cross section, revealing its organization into a single thick layer of oriented melanosomes even more clearly. The pristine preservation of this fossil is not typical, particularly in older fossils, so alternative methods of detection are needed. Li et al. (77) expanded their CDA sampling and found that melanosomes from iridescent feathers were characterized by a distinctively thin (low aspect ratio) morphology. They found most melanosomes from a well-preserved Microraptor specimen had similar morphology and were therefore predicted as iridescent. Another theropod appeared to have flattened melanosomes most similar to those in hummingbirds (58). Thus, even in the absence of pristine preservation of melanosome organization, iridescence may be detectable in the fossil record through melanosome morphology. However, a change in melanosome length and width (but not aspect ratio) with high temperature and pressure suggests that melanosomes shrink somewhat fossilization (90). We thus have to statistically account for this difference in analyses likely through comparison of melanosomes versus their impressions and from our experimental observations (27, 58, 77). If melanosomes forming iridescent nanostructures have distinct chemical signatures, then chemical techniques could be used to detect them. However, this does not appear to be true (Refs. 81 and 163 both showed that melanosomes from iridescent feathers were not highly chemically distinctive from other melanosomes) and even if it is possible, visual analysis will be needed to determine the morphology and arrangement of melanosomes.

However, caution is warranted when classifying colors based on morphology. To determine the level of difference in melanosome size between the two preservation types, melanosomes preserved both in three dimensions and as imprints from several fossil feather deposits should be compared (78). The size, shape, and chemical composition of melanosomes before and after diagenesis can be analyzed as in References 27 and 90. These experiments examined the

expected composition of fossil organic substances (53, 54) under high pressure and temperature conditions (27, 65, 90). The product was studied under SEM for its morphological preservation in comparison to its original condition and analyzed using Fourier transform infrared spectrometry to detect the levels of functional groups, such as OH- and COOH-. The chemical structure has also been studied with PY-GCMS (pyrolysis–gas chromatography mass spectrometry), ToF SIMS (time of flight secondary ion mass spectrometry), and VUV-LDMS for its molecular preservation. Chemical changes may also have to be taken into account when diagnosing melanin chemistry of fossils (27). These experiments will give us a better understanding of the fossilization of melanin and how is it altered chemically and morphologically (91). This will obviously be critical when using chemistry and/or morphology to diagnose color. Since both morphology and chemistry change to varying degrees during diagensis, there is no reason to favor or disregard evidence from one or the other, so long as these changes are taken into account during analysis. Indeed, a combined approach would be ideal, but is probably not needed for every analysis (150). Despite the vigorous debates over methodologies and inferences, the study of fossil melanin has already provided great insights into the evolution of melanin-based colors and melanosomes. For example, their colorful melanin-based patterns suggest that very early feathers were used for sexual display even before flight (76, 77, 167). Even more sophisticated analyses will only continue to provide greater insights in the future.

IX. CONCLUSIONS AND FUTURE DIRECTIONS

We have here broadly summarized and synthesized the available data on melanins and melanosomes in a holistic manner, enabling us to produce some new hypotheses and focal areas for future research. While the basic structure of eumelanin is fairly well understood, whether it is a heterogeneous polymer that is chemically cross-linked or a group of oligomers assembled through physical interactions is not. The structures and assembly of natural pheomelanin and combined pheo/eumelanin are even less well understood. Characterizing protein content and metal coordination chemistry of natural melanins will also be critical to obtaining a fuller understanding of these polymers and their properties. Moreover, how does its hierarchical chemical structure affect its functions, e.g., what structural differences explain the differences in absorbance between pheo- and eumelanin? What chemical aspects contribute to the high fossilization potential of melanosomes? The connection between chemistry and the development and morphology of melanosomes should also be elucidated. Why do melanosomes with mixed pheo/eumelanin content have shapes distinct to pure pheo- and eumelanosomes? Moreover, why does this pattern seem to only hold true in birds and mammals; is it caused by differences in the Pmel genes or an aspect of the melanocortin system between them and, e.g., squamates? Does melanosome shape affect organization of melanosomes into organized nanostructures after deposition into feather keratinocytes? More thorough sampling of diverse organisms is needed to enable further insight into the evolutionary history of melanin and melanosomes. How common is melanin in invertebrates, for example? And when present, is it contained in melanosomes or diffusely deposited? Why is diffuse melanin so seemingly rare? What insights can the fossil record give us into the evolution of melanosomes? Despite a long history that has provided tremendous insight, the study of melanin and melanosomes is ripe for future work at both the proximate and ultimate level.

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Address for reprint requests and other correspondence: L. D'Alba or M. Shawkey, Evolution and Optics of Nanostructures Group, Dept. of Biology, Univ. of Ghent, 9000 Ghent, Belgium (e-mail: liliana.dalba@ugent.be, matthew.shawkey@ugent.be).

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