

Minireview

Molecular mechanisms for mammalian melanogenesis

Comparison with insect cuticular sclerotization*

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Melanogenesis is an important biochemical process for the production of skin pigments which protect many animals from the damage of solar radiation. The abnormalities in melanogenesis are associated with albinism, vitiligo, as well as malignant melanoma in humans. In the lower forms of animals viz., insects, the exoskeleton is hardened to protect their soft bodies by a process called sclerotization, which is often accompanied by melanization. Recent advances in the biochemistry of sclerotization and melanization reveal remarkable similarity between these two processes. The seven stages of sclerotization are: (a) enzymatic oxidation of *N*-acetyldopamine, (b) Michael-1,4-addition reactions of *N*-acetyldopamine quinone methide to 1,2-dehydro-*N*-acetyldopamine, (c) tautomerization of quinone to quinone methide, (d) Michael-1,6-addition of quinone methides, (e) tautomerization of *N*-acetyldopamine quinone methide to 1,2-dehydro-*N*-acetyldopamine, (f) enzymatic oxidation of 1,2-dehydro-*N*-acetyldopamine, and (g) the reactions of resultant quinone compounds. Amazingly, striking similarities in the reaction sequences are found in the melanization process starting from dopa. These comparisons predict a central role for quinone methides as reactive intermediates during melanization. Accordingly, recent studies provide increasing evidence in favor of this proposition.

Melanogenesis; Cuticular sclerotization; Tyrosinase; Dopachrome conversion factor; Quinone isomerase; Quinone methide

1. INTRODUCTION

Melanin is the general term assigned to a group of phenolic pigments derived from tyrosine and related compounds by oxidative polymerization [1]. Melanins range in color from yellow to red-brown, as in the case of pheomelanins to the brown to black of eumelanins. These phenolic pigments are of widespread occurrence in the animal kingdom and are responsible for the different colors of hair and skin in animals. Apart from providing coloration, melanins play a crucial role of protecting the body from damaging solar radiation. Abnormalities in melanin biosynthesis result in pathological conditions such as albinism, vitiligo, and malignant melanoma.

Melanization is not unique to higher animals, as lower forms such as arthropods can also utilize this

biochemical machinery for exoskeletal pigmentation. Interestingly, arthropods employ the melanin biosynthetic system not only for coloration, but also for their own protection against invading microorganisms and other parasites. Foreign organisms too big to be phagocytosed are found encapsulated and melanized in arthropod blood [2,3]. Therefore, melanin production seems to be a key immune reaction in arthropods. Apart from skin pigmentation and insect immunity, there is a good deal of evidence to show that melanization reactions are also involved in wound healing reactions in insects [4].

The biochemistry of melanogenesis has been intensively investigated for over seven decades [5-10]. In spite of these persistent efforts, only a few basic aspects of the process have been elucidated, but most of the intricate and complex reactions leading to the synthesis of the melanin polymer, and the detailed chemical structure of melanin remain unsolved. The difficulties in solving the structure of melanin and the reactions leading to its production stem partly from the highly reactive intermediates formed during the process which elude conventional identification techniques but mostly from the structural complexity, insolubility and heterogeneity of melanin pigments. The two primary pigments found in higher animals are the eumelanin arising from oxidative polymerization of dopa, and the pheomelanin

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Abbreviations: NADA, *N*-acetyldopamine; Dehydro NADA, 1,2-dehydro-*N*-acetyldopamine; DHI, 5,6-dihydroxyindole; DHICA, 5,6-dihydroxyindole-2-carboxylic acid; DCF, dopachrome factor

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lanins formed by the oxidative polymerization of cysteinyl dopas, generated by the coupling of cysteine to dopaquinone. Eumelanin forms the primary focus of this article.

2. RAPER-MASON PATHWAY

The biosynthesis of eumelanin is achieved by a complex series of chemical and biological transformations starting from tyrosine and its hydroxylated product, dopa. Most of our present knowledge on the biosynthesis of melanin comes from the classical work of Raper and Mason, termed the Raper-Mason pathway. The early studies of Raper [5] and Mason [6,7] led to the establishment of the following preliminary sequence of reactions involved in eumelanin biosynthesis: (a) enzyme catalyzed hydroxylation of tyrosine to dopa; (b) biochemical oxidation of dopa to dopaquinone; (c) non-enzymatic conversion of dopaquinone to leucochrome; (d) oxidation of leucochrome to dopachrome; (e) aromatization of dopachrome to 5,6-dihydroxyindoles; and (f) oxidative polymerization of 5,6-dihydroxyindoles to generate ultimately eumelanin via melanochrome.

For convenience, the melanogenesis pathway can be split into two phases: Phase I comprises the reactions converting tyrosine to the indolequinones, and Phase II comprises the polymerization of indolequinones to me-

lanin via melanochrome. It has been well accepted that the melanin biosynthetic pathway employs a single enzyme viz., tyrosinase, which possesses both monophenol monooxygenase (E.C. 1.14.18.1. monophenol, dihydroxyphenyl alanine: oxygen oxidoreductase) and catechol oxidase (E.C. 1.10.3.1. *o*-diphenol: oxygen oxidoreductase) activities. The subsequent steps beyond dopaquinone were believed to occur non-enzymatically. However, in the last decade, increasing evidence indicates the presence of additional protein factor(s) controlling melanogenesis.

Studies by Pawelek and his associates [11-14] led to the discovery of a new protein factor controlling the biosynthesis of melanin. This factor, which acts on dopachrome, has been termed as DCF. Initially, it was thought that DCF from mammalian systems catalyzes the conversion of dopachrome to DHI [11-14]. Subsequently, however, it has been established that this enzyme catalyzes the conversion of dopachrome to DHICA and not to DHI [15]. This coupled with the fact that there is a large retention of the carboxyl group from dopa in the naturally occurring melanin [16] pigment indicates a central role for DHICA in the biogenesis of eumelanins. Progress on the purification and characterization of mammalian DCF was extremely slow, but we have successfully purified an insect DCF to near homogeneity and characterized some of its properties [17].

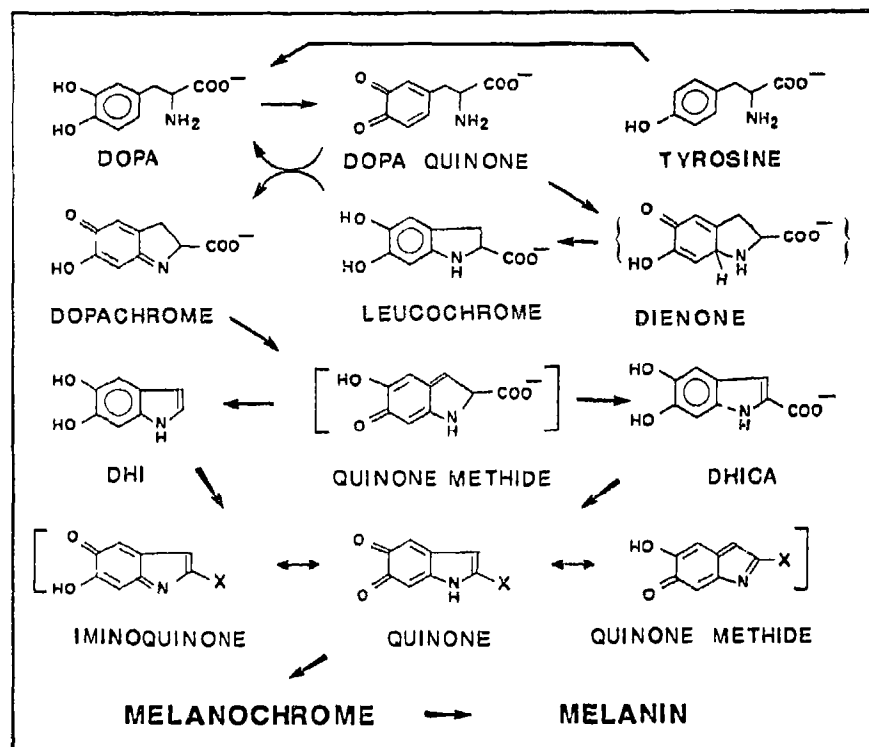


Fig. 1. Modified Raper-Mason pathway for melanogenesis. Tyrosinase catalyzes the hydroxylation of tyrosine to dopa and the oxidation of dopa to dopaquinone. Dopaquinone undergoes non-enzymatic intramolecular cyclization to form leucochrome via a dienone intermediate. Oxidation of leucochrome generates dopachrome which serves as the substrate for DCF. Mammalian DCF converts dopachrome to DHICA, while insect DCF converts dopachrome to DHI via the quinone methide intermediate. Oxidation of 5,6-dihydroxyindoles results in the production of tautomeric indole quinonoid species that polymerize to form melanin viz., melanochrome.

The DCF from *Manduca sexta* catalyzes the conversion of dopachrome to DHI.

Studies of *Manduca* DCF provided the first evidence for the occurrence of a new intermediate in the melanin biosynthetic pathway. Although it was established nearly ten years ago that oxidative cyclization of α -methyl-dopa ethyl ester generated a stable quinone methide by the pioneering studies of Castagnoli and his co-workers [18,19], it was not until two years ago that the facile conversion of α -methyl dopachrome methyl ester to the quinone methide was demonstrated [20]. Following these studies, not only did we predict a central role for quinone methides in melanin biosynthesis [21], but we also proved the hypothesis by establishing that during the enzymatic conversion of dopachrome, quinone methide is generated as an intermediate through mechanistic studies [17]. Our studies ultimately helped us to complete the Phase I reactions of melanogenesis.

Fig. 1 shows our modification of the Raper-Mason pathway for Phase I of eumelanogenesis. Interestingly, quinone methide seems to be a key reactive intermediate

not only during the dopachrome conversion step, but also during the subsequent reactions of indolequinone [22].

3. SCLEROTIZATION OF INSECT CUTICLE

As stated earlier, melanization is a widespread occurrence and arthropods seem to utilize this process not only for exoskeletal pigmentation, but also during immune response and wound healing. Unlike mammals, arthropods do not possess an internal skeletal system, but instead use their outer skin (cuticle or exoskeleton) for attachment of their muscles and organs. In addition, the hard cuticle also forms a formidable barrier for invading microorganisms and other parasites. The cuticle does not, however, allow continuous growth. Therefore, insects and other arthropods often shed their old cuticle and build a new larger one to allow for expansion. Freshly-made cuticle is soft and pale, but soon becomes hard and sometimes tanned by sclerotization reactions. During sclerotization, catecholamine derivatives such as NADA and *N*- β -alanyldopamine are acti-

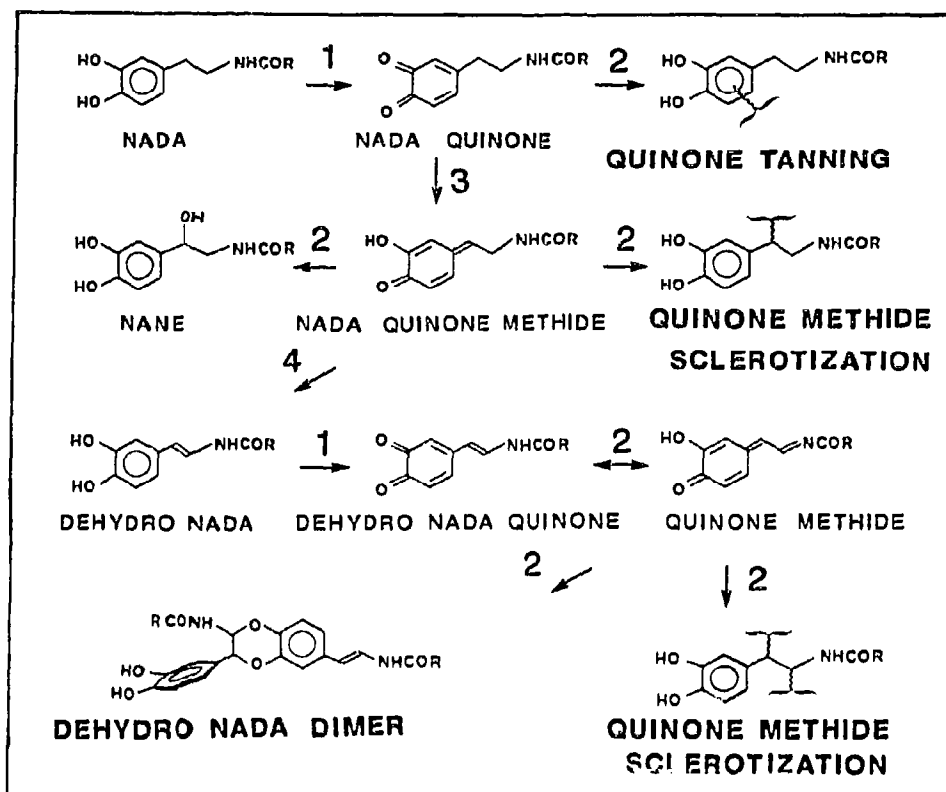


Fig. 2. Unified mechanism for insect cuticular sclerotization. Sclerotizing precursors such as NADA are oxidized by phenoloxidase to their corresponding quinones which participate in non-enzymatic reactions with cuticular nucleophiles, accounting for quinone tanning. Quinone isomerase produces quinone methides from 4-alkyl quinones and provide them for quinone methide sclerotization. For instance, the reaction of NADA quinone methide with (a) water results in the formation of *N*-acetylnorepinephrine (NANE); and (b) cuticular nucleophiles results in the generation of quinone methide adducts. Alternately, NADA quinone methide can also serve as the substrate for quinone methide isomerase and yield dehydro NADA. Phenoloxidase oxidizes this compound to its corresponding quinone which after isomerization to quinone methide imine forms both dimers and cuticle adducts. Originally, this process was termed as β -sclerotization, but because of the key role of quinone methides in this process, it has been renamed as quinone methide sclerotization. 1, phenoloxidase; 2, chemical reactions; 3, quinone isomerase; 4, quinone methide isomerase.

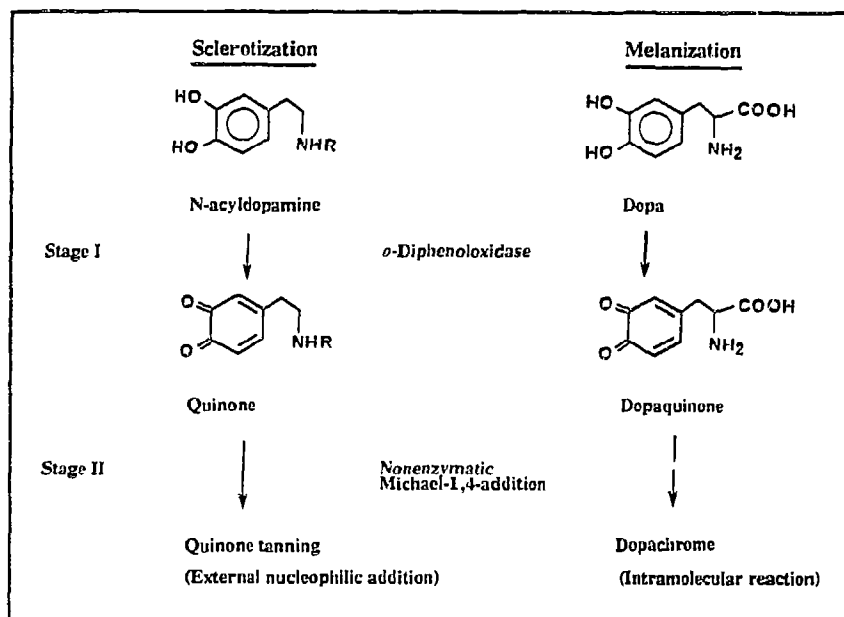


Fig. 3. Comparison of sclerotization and melanization. Stage I: oxidation of *o*-diphenols to *o*-quinones; stage II: non-enzymatic reactions of quinone.

vated by cuticular enzymes to reactive intermediates (sclerotizing agents) that crosslink cuticular structural components such as proteins and chitins to ultimately produce hardened cuticle [23–25]. Sclerotization and melanization occur both simultaneously and independently in the same cuticle. For the past five decades, numerous workers have devoted their attention to the study of sclerotization. As early as 1940, Pryor [26,27] identified quinones to be one type of sclerotizing agents. Subsequently, others confirmed this contention (see ref. [24] for review). According to the quinone tanning hy-

pothesis, catecholic substrate(s) are oxidized by cuticular phenoloxidase(s) (a form of tyrosinase) to the quinones. Quinones thus formed react with available nucleophiles on proteins and chitin to form protein-catechol and chitin-catechol adducts. Further oxidation and coupling to other structural components ensures the generation of protein–protein, protein–chitin, as well as chitin–chitin crosslinks necessary for strengthening the cuticle.

A different type of sclerotization was discovered by Andersen and his associates [28,29]. Andersen's school

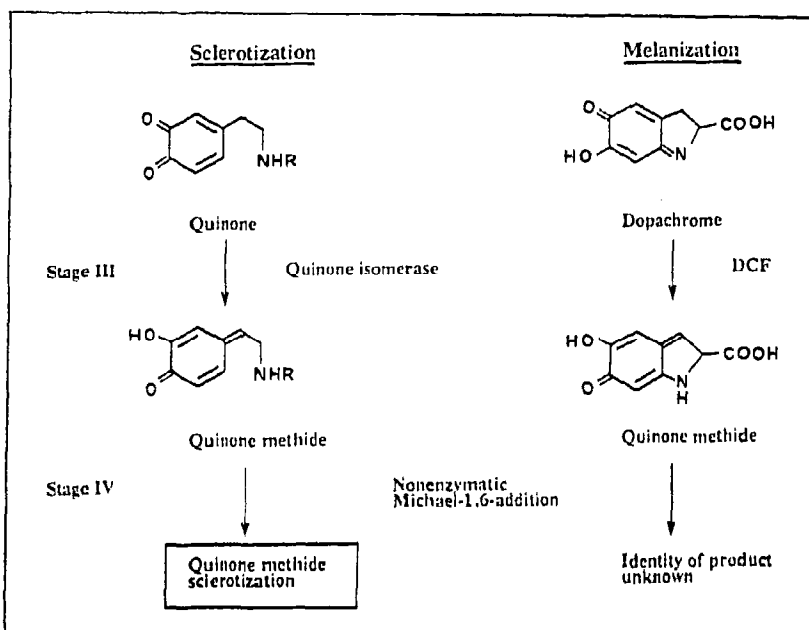


Fig. 4. Comparison of sclerotization and melanization. Stage III: conversion of quinonoid compounds to quinone methide; stage IV: reactions of quinone methide.

found that one of the primary cuticular tanning precursors, NADA, is amazingly desaturated at the side chain. The resultant dehydro NADA is oxidized by phenoloxidase and utilized for crosslinking purposes. He termed this process β -sclerotization and suggested the presence of a specific NADA desaturase which introduces a double bond in the side-chain of NADA as a key enzyme of sclerotization in insects. Independently, a third type of sclerotization mechanism was discovered in this laboratory [30,31] whereby catecholamine derivatives are activated by cuticular enzymes to form reactive 2-hydroxy-quinone methides (tautomers of 4-alkyl-quinones), which could also crosslink cuticular components. We argued that β -sclerotization is a side reaction of quinone methide chemistry and proposed quinone methide as the sclerotizing agent involved in β -sclerotization [23–25]. However, the enzymes participating in either of these processes remained elusive until three years ago.

Our successful solubilization and characterization of enzymes of quinone methide sclerotization [32–34] led to the finding that all three sclerotization mechanisms are unified by three enzymes. Thus, phenoloxidase generates and provides 4-alkylquinones for the quinone isomerase. Quinone isomerase produces quinone methides necessary for quinone methide sclerotization. The third enzyme viz., quinone methide tautomerase converts NADA quinone methide to dehydro NADA. Phenoloxidase then acts on dehydro NADA and the resultant quinonoid species serves as a crosslinking agent for β -sclerotization. Since quinone methides are involved in the latter process also, it has been classified as quinone methide sclerotization only. The combined mechanisms are illustrated in Fig. 2.

4. COMPARISON OF SCLEROTIZATION AND MELANIZATION

Both sclerotization and melanization are different processes. Therefore, only similarities are given below. For the purpose of effective comparison of these two key processes, cuticular sclerotization can be divided into seven stages. The first stage in cuticular sclerotization is the phenoloxidase catalyzed oxidation of the sclerotization precursors viz., such as NADA to its *o*-quinone. The parallel reaction in melanogenesis is the tyrosinase-catalyzed conversion of dopa to dopaquinone (Fig. 3). At stage II of sclerotization, the enzymatically generated quinones undergo non-enzymatic Michael-1,4-addition reactions. In insect cuticle, this process leads to quinone tanning. In the case of melanogenesis, the predominant reaction of initially-formed dopaquinone is the intramolecular Michael-1,4-addition reaction. This generates a transient dienone, which undergoes rapid dienone phenol rearrangement to produce the leucochrome (Fig. 1). Dopaquinone can also react with cysteine to form cysteinyl-dopa, which serves as the precursors of pheomelanin [9,35].

Stage III in cuticular sclerotization is the enzyme-catalyzed isomerization of NADA quinone to the quinone methide tautomer (Fig. 4). Recently, we have purified quinone tautomerase to apparent homogeneity from the hemolymph of *S. bullata* [36]. This enzyme, which exhibits a wide substrate specificity, can attack a number of 4-alkyl quinones and isomerize them to quinone methide. A parallel reaction in melanogenesis is the isomerization of dopachrome to quinone methide catalyzed by dopachrome conversion factor [17].

Quinone methides are highly reactive compounds and

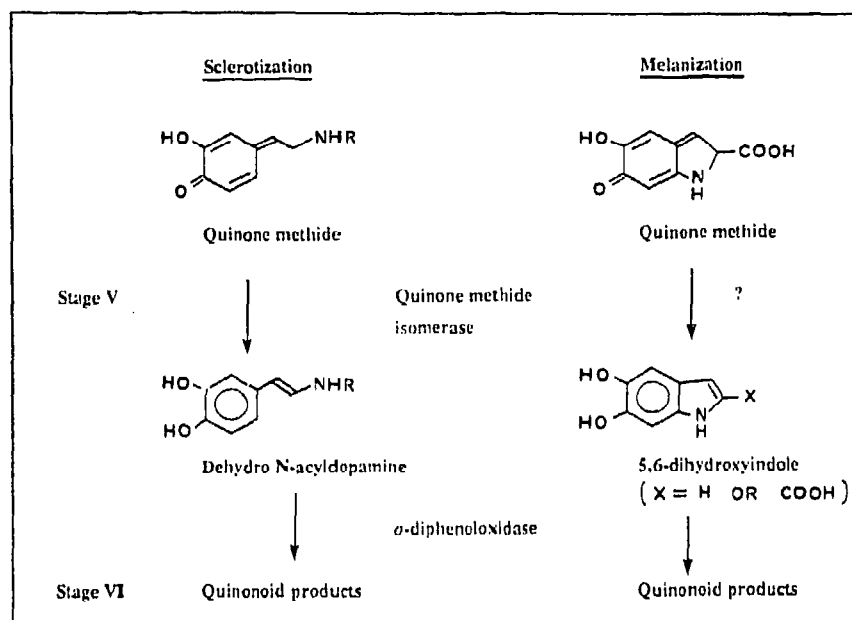


Fig. 5. Comparison of sclerotization and melanization. Stage VI: introduction of double bond in the side chain; stage VI: enzyme catalyzed generation of quinonoid species.

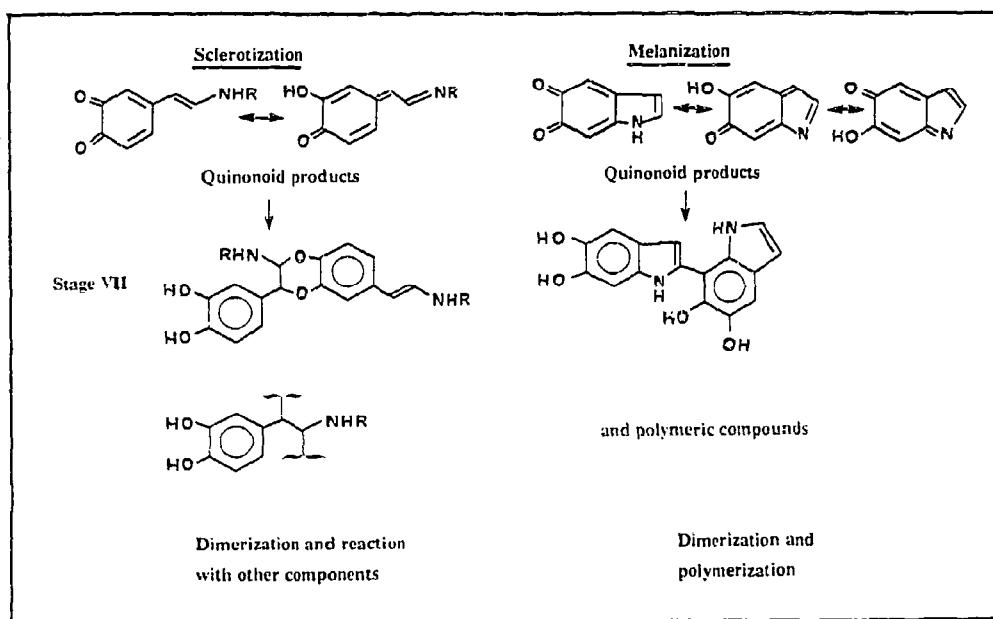


Fig. 6. Comparison of sclerotization and melanization. Stage VII: reactions of quinonoid species.

usually undergo non-enzymatic rapid Michael-1,6-addition reactions [23–25]. Since this regenerates the aromatic ring, it is a highly favored reaction. A number of nucleophiles such as OH, *o*-alkyl, *o*-aryl, SH, COOH, NH₂, and imidazole can add on to quinone methides. These reactions lead to the quinone methide sclerotization proposed by this laboratory [23–25] and constitutes stage IV. In the case of melanization, the quinone methide formed by tautomerization of dopachrome can undergo a similar reaction (Fig. 4), but proof for the existence of such a pathway must come from product analysis.

Apart from participating in quinone methide sclerotization, quinone methides can also undergo prototropic rearrangement to generate a side chain desaturated product (stage V). This reaction was first demonstrated in chemical models by our group with dihydro caffeic acid derivatives [37,38]. Both the methyl amide and methyl ester of this compound upon oxidation generated the quinone which rapidly isomerized non-enzymatically to the corresponding quinone methides. The quinone methides thus formed exhibited the internal rearrangement reaction by prototropic transformation to generate caffeic acid derivatives [37,38]. The electron withdrawing carbonyl group and the quinone methide nucleus seems to make one of the methylene protons acidic and hence easily removable. This coupled with the aromatizing tendency of the quinone methide nucleus leads to the non-enzymatic production of side chain denatured compounds. Similar conversion of *N*-acetyldopa quinone methide ethyl ester to *N*-acetyldehydrodopa ethyl ester has been reported recently [39,40]. However, in the case of NADA quinone methide, we could not observe the non-enzymatic tauto-

merization at all, but a new enzyme catalyzing the conversion of dehydro NADA to NADA quinone methide has been identified in this laboratory [34].

The comparable stage at melanogenesis is the production of DHI and DHICA from the quinone methide tautomer of dopachrome (Fig. 5). Although this reaction remarkably resembles the conversion of NADA quinone methide to dehydro NADA, it is not clear at present whether a separate enzyme similar to NADA quinone methide tautomerase is necessary to catalyze the parallel reaction in melanogenesis. First of all, the driving force for the reaction can simply come from the indolization reaction. Secondly, the high rate of non-enzymatic as well as metal ion catalyzed conversion of dopachrome to dihydroxyindoles [41,42] make it unlikely that a second enzyme participates in this conversion. Finally, DCF itself might carry out both iminochrome tautomerization (stage III) and indolization (stage V) reactions. However, further studies on DCF are essential to confirm this contention.

The next stage (VI) is the enzymatic oxidation of side chain desaturated compounds (Fig. 5). In the case of sclerotization, *o*-diphenoloxidase further oxidizes dehydro NADA to the quinonoid derivatives [43]. Similarly, in melanogenesis, 5,6-dihydroxyindoles are further oxidized to quinonoid products [22].

At stage VII, simple quinonoid compounds generated by enzymatic oxidation of catecholamine derivatives seem to undergo dimerization, polymerization and crosslinking reactions (Fig. 6). Determination of the structure of the dimeric product(s) formed, greatly facilitated the establishment of initial reactions [43]. The exact stereochemical nature of the dimer(s) is yet to be elucidated, but HPLC analysis reveals that the dehydro

NADA dimers consist of at least three different, yet stereochemically related benzodioxan adducts. Quinonoid derivatives of dehydro NADA not only form adducts with dehydro NADA, but also with a variety of other compounds such as NADA, catechol, *o*-aminophenol, etc [43]. In cuticle, its reaction seems to result in the initial crosslinking of cuticular components with the regeneration of catecholic groups (Fig. 2). However, further reactions of dehydro NADA quinone and quinone methide need to be delineated. Much less is known at the parallel stage in melanogenesis. Recently, Protá and his associates established the formation of simple dimers of 5,6-dihydroxyindoles [44]. Beyond this stage the sequence of reactions is speculative in nature and needs to be studied in detail.

5. CONCLUSIONS

From the above comparison, it is clear that both sclerotization and melanization share several common (bio)chemical transformations. These comparisons predict that quinone methides could be a key reactive intermediate even in melanogenesis. Recently, studies from our group [17,20,21] and British workers [22] strongly support this contention.

I hope the above comparison will help investigators in both fields to study the detailed biochemistry with a new perspective. In my mind such a study will no doubt lead to the development of new control measures not only for insect pests, but also for pathological conditions arising from the abnormalities in melanogenesis such as malignant melanoma.

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REFERENCES

- [1] Nicolaus, R.A. (1968) *Melanins*. Herman, Paris.
- [2] Johansson, M.W. and Soderhall, K. (1989) *Parasitology Today* 5, 171-176.
- [3] Sugumaran, M. (1990) *UCLA Symposium on Molecular Cell Biol. New Series* 121, 47-62.
- [4] Lai-Fook, J. (1966) *J. Invest. Physiol.* 12, 195-226.
- [5] Raper, H.S. (1928) *Physiol. Rev.* 8, 245-248.
- [6] Mason, H.S. (1948) *J. Biol. Chem.* 172, 83-99.
- [7] Mason, H.S. (1955) *Adv. Enzymol.* 16, 105-184.
- [8] Swan, G.A. (1974) *Fortschr. Chem. Org. Naturst.* 31, 521-582.
- [9] Protá, G. (1988) *Med. Res. Rev.* 8, 525-556.
- [10] Crippa, R., Horak, V., Protá, G., Svoronos, P. and Wolfram, L. (1989) *The Alkaloid* 36, 253-323.
- [11] Korner, A.M. and Pawelek, J. (1980) *J. Invest. Dermatol.* 75, 192-195.
- [12] Pawelek, J., Korner, A., Bergstrom, A. and Bologna, J. (1980) *Nature* 286, 617-619.
- [13] Korner, A. and Pawelek, J. (1982) *Science* 217, 1163-1165.
- [14] Murray, M., Pawelek, J.M. and Lamoreux, M.L. (1983) *Dev. Biol.* 100, 120-126.
- [15] Korner, A.M. and Getting, P. (1985) *J. Invest. Dermatol.* 85, 229-231.
- [16] Ito, S. (1986) *Biochim. Biophys. Acta* 883, 155-161.
- [17] Sugumaran, M. and Semensi, V. (1991) *J. Biol. Chem.* 266, 6073-6078.
- [18] Musson, D.G., Karashima, D., Rubiero, H., Melmon, K.L., Cheng, A. and Castagnoli Jr., N. (1980) *J. Med. Chem.* 23, 1318-1323.
- [19] Cheng, A.C., Shulgin, A.T. and Castagnoli Jr., N. (1982) *J. Org. Chem.* 47, 5258-5262.
- [20] Sugumaran, M., Dali, H. and Semensi, V. (1990) *Bioorg. Chem.* 18, 144-153.
- [21] Sugumaran, M. (1990) in: *Biological Oxidation Systems* (Reddy, C.C., Hamilton, G.A. and Madyastha, K.M., eds.) Vol. I, pp. 347-363. Academic Press, N.Y.
- [22] Lambert, C., Chacon, J.N., Chedekel, M.R., Land, E.J., Riley, P.A., Thompson, A. and Truscott, T.G. (1989) *Biochim. Biophys. Acta* 993, 12-20.
- [23] Sugumaran, M. (1987) *Bioorg. Chem.* 15, 194-211.
- [24] Sugumaran, M. (1988) *Adv. Insect Physiol.* 21, 179-231.
- [25] Sugumaran, M. (1991) in: *The Physiology of Insect Epidermis* (Binnington, K. and Retnakaran, A., eds), pp. 143-170. Inkata Press, Victoria, Australia.
- [26] Pryor, M.G.M. (1940) *Proc. R. Soc. London. Ser. B.* 128, 378-393.
- [27] Pryor, M.G.M. (1940) *Proc. R. Soc. London. Ser. B.* 128, 393-407.
- [28] Andersen, S.O. and Roepstorff, P. (1982) *Insect Biochem.* 12, 269-276.
- [29] Andersen, S.O. (1985) in: *Comprehensive Insect Physiology, Biochemistry, and Pharmacology* (Kurkut, G.A. and Gilbert, L.I., eds), Vol. 3, pp. 59-74. Pergamon, New York.
- [30] Sugumaran, M. and Lipke, H. (1983) *FEBS Lett.* 155, 65-68.
- [31] Lipke, H., Sugumaran, M. and Henzel, W. (1989) *Adv. Insect Physiol.* 17, 1-84.
- [32] Saul, S.J. and Sugumaran, M. (1989) *FEBS Lett.* 251, 69-73.
- [33] Saul, S.J. and Sugumaran, M. (1990) *Arch. Insect Biochem. Physiol.* 15, 237-254.
- [34] Saul, S.J. and Sugumaran, M. (1989) *FEBS Lett.* 255, 340-344.
- [35] Deibel, R.B. and Chedekel, M.R. (1982) *J. Am. Chem. Soc.* 104, 7306-7309.
- [36] Saul, S.J. and Sugumaran, M. (1990) *J. Biol. Chem.* 265, 16992-16999.
- [37] Sugumaran, M., Dali, H., Kundzicz, H. and Semensi, V. (1989) *Bioorg. Chem.* 17, 443-453.
- [38] Sugumaran, M., Semensi, V., Dali, H. and Saul, S. (1989) *FEBS Lett.* 255, 345-349.
- [39] Rzepecki, L.M., Nagafuchi, T. and Waite, H.J. (1991) *Arch. Biochem. Biophys.* 285, 17-26.
- [40] Rzepecki, L.M. and Waite, H.J. (1991) *Arch. Biochem. Biophys.* 285, 27-36.
- [41] Palumbo, A., d'Iochia, M., Misuraca, G. and Proto, G. (1987) *Biochim. Biophys. Acta* 925, 203-209.
- [42] Leonard, L.J., Townsend, D. and King, R.A. (1988) *Biochemistry* 27, 6156-6159.
- [43] Sugumaran, M., Schinkmann, K. and Dali, M. (1990) *Arch. Insect Biochem. Physiol.* 14, 93-109.
- [44] D'Isochia, M., Napolitano, A., Tsiakas, K. and Protá, G. (1990) *Tetrahedron* 46, 5789-5796.