EXPERIENTIA

Vol. 36 – Fasc. 2 Pag. 143–266 15.2.1980

GENERALIA

Sclerotization and coloration of the insect cuticle

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Summary. Evidence indicating linkage between the processes of cuticle hardening and coloration are reviewed. Data and observations for the mutants black and ebony in *Drosophila melanogaster* are used to construct a possible model for the relationship between these developmental events.

I. Introduction

The process of eukaryotic development must necessarily involve the coordination and articulation of diverse metabolic pathways. Much current research and models[1] have focused on transcriptional control and certainly an understanding of the mechanism(s) of this process would constitute the primary breakthrough in this field. However, even when achieved, a knowledge of transcriptional regulation will be insufficient to explain many of the complex metabolic relationships which must occur during development; the action and interaction of enzymes and their products are too far removed in time and space from primary gene action. A more comprehensive view of developmental regulation could be gained by a study of the final metabolic reactions involved in the interactions among developmental processes.

A promising area for such an investigation are the developmental events leading to the hardening (sclerotization) and coloration of the insect cuticle. Sclerotization occurs during the larval molts, and in holometabolic insects at puparium formation and adult eclosion. Hardening may or may not be accompanied by coloration, and studies in various insects[2–4] have revealed not only a lack of correlation between the degree of sclerotization and coloration, but that occasionally the dark coloration of some cuticles can be prevented without inhibiting normal hardening. Such observations have led to the suggestion that these processes are entirely separate. However when coloration occurs it is normally concomitant with sclerotization, and other experiments have demonstrated an intrinsic linkage between these events. It is the purpose of this review to summarize the most pertinent data on these processes. The information available indicates that these events are interrelated and underscores the complexity of the molecular processes involved in this interesting developmental interaction. Information has been drawn from studies of various insects but, because its genetics is best known, the major focus will be on *Drosophila melanogaster.* While much remains to be learned of the molecular details of these processes, it is possible at this time to present a tentative model for their relationship.

II. N-Acetyl dopamine as a sclerotizing agent

Early studies[5–8] established crosslinking of cuticular proteins as a mechanism for cuticular hardening, and in 1962, Karlson et al.[9] first identified N-acetyl dopamine (N-acetyl-3,4-dihydroxy-β-phenylethylamine) as a 'sclerotizing agent' responsible for this process in *Calliphora vicina (= C. erythrocephala).* N-acetyl dopamine has since been implicated as a sclerotizing agent in a number of insects[10–12], and its biosynthesis studied extensively in *Calliphora* and *Drosophila*[13–15]. In both organisms, synthesis proceeds from tyrosine via dopa (3,4-dihydroxyphenylalanine) and dopamine (3,4-dihydroxyphenylethylamine), and experiments in *Calliphora*[16,17] indicate that the messenger RNA for dopa decarboxylase (E.C.4.1.1.26), the enzyme responsible for the conversion of dopa to dopamine, is induced during puparium formation by the steroid molting hormone, ecdysone.

In the classical view of sclerotization[18], N-acetyl dopamine is oxidized by a cuticular phenol oxidase to N-acetyl dopamine quinone and crosslinking of the cuticular proteins occurs as the quinone molecules join spontaneously with their free amino groups forming protein linked catechol derivatives which, upon reoxidation by further quinone interaction, may link directly to other amino groups or with other molecules of N-acetyl dopamine quinone forming polymeres linking several distant amino acids. Implicit in this model is the possibility of concomitant tan coloration by accumulation of the benzoquinone formed by ring oxidation of N-acetyl dopamine, and studies of Drosophila mutations affecting dopa decarboxylase[19]

lend support to this mechanism. Null alleles of the structural locus have been isolated which reduce decarboxylase activity to 50% or less than wild type in heterozygotes. Although these mutations are recessive lethals, the enzyme is a homodimere[20] and some heterozygous combinations produce viable adults via intracistronic complementation. In some cases, complementation is incomplete and the flies die within 24 h, possibly by dessication through a poorly sclerotized cuticle. The linkage of coloration to sclerotization is clearly evident in these flies; those areas of the cuticle which normally become tan remain white, and the abdominal stripes fail to darken.

These experiments, as well as those of Bodnaryk (see below), demonstrate the involvement of the classical pathway to sclerotization in coloration, and a partial explanation for the discrepancy often observed between these processes has come from the experiments of Anderson[21] on the enzymatic action of the cuticular proteins toward N-acetyl dopamine. In addition to quinone formation catalyzed by ring oxidation, Anderson has found that the sclerotizing agent may instead be oxidized on the β-carbon yielding a colorless compound, and that the β-carbon activated molecule is also capable of crosslinking cuticular proteins[22]. Considerable variation in ring vs β-carbon oxidation was found in studies of isolated cuticular regions of various insects and, as expected, β-carbon oxidation was found to predominate in cuticular regions destined to sclerotize without tan coloration[23]. Ring and β-carbon activation appear to be the result of at least 2 separate enzymes[24], and cell autonomy of tan coloration may be explained by the type(s) of phenol oxidases active in various regions of the cuticle.

III. Possible roles of β-alanine: results from studies of Drosophila mutations

Since extensively hardened but uncolored cuticles may be formed by β-oxidation, it is hardly surprising to find a lack of correlation between sclerotization and coloration. However, since many cuticles, or parts thereof, are characterized by black pigmentation, it is obvious that something other than the brown quinone formed by ring oxidation of N-acetyl dopamine must be invoked to more fully explain coloration. Indeed studies of the 2 Drosophila mutations, black (2-48.5) and ebony (3-70.7) have indicated that N-acetyl dopamine is not the only compound involved in either process and emphasized the underlying molecular complexity of their relationship. These mutations are of special interest from the standpoint of developmental genetics as they show an intriguing temporal phenotypic dichotomy; the adults characterized by an intense black pigmentation extending over normally tan cuticular regions, and the pupa cases extremely light or white compared to the distinct tan coloration of wild type. Both mutations are characterized by the same molecular defect, a deficiency of the unusual amino acid, β-alanine, in the cells of the cuticle. Although ebony mutants synthesize β-alanine they are, in some way, defective in their ability to incorporate it into the cuticle[25,26]. Black mutations suffer a lesion in β-alanine synthesis, and wild type phenocopies can be produced by supplying the amino acid prior to the pupal[27] and adult[28] stages. Thus, in some way, a deficiency of cuticular β-alanine is involved in both the pupal and adult phenotypes, and a further understanding of the molecular mechanism(s) underlying these directly opposing phenes should contribute greatly to our understanding of the biochemistry of coloration.

Once again the connection between the 2 developmental events is evident in that β-alanine is involved not only in coloration but, at least to some extent, as a participant in sclerotization (see discussion in Chen[29]). Electron microscope studies[30] have shown that the pupal cuticles of both black and ebony are less compact than wild type, and β-alanine injection into early black pupae leads to normal compaction. Although the molecular mechanism of β-alanine involvement in sclerotization has yet to be defined, Jacobs has demonstrated in vitro binding of β-alanine to chitin accompanied by the formation of a tan color. This finding leads to a view of sclerotization as a 2-part process: the binding of cuticular proteins promoted by an oxidized form of N-acetyl dopamine and the crosslinking of chitin molecules by β-alanine. The latter process would promote tan coloration, while the former may or may not, as determined by the specificity of the cuticular proteins. Bodnaryk's experiments with the fleshfly, *Sarcophaga*[31–33] have revealed that the 2 mechanisms of sclerotization are interdependent and related to coloration. Precocious pupal hardening and coloration induced by molting hormone injection, presumably by induction of the classical pathway, was found to cause premature cuticular incorporation of β-alanine. Inhibition of normal pupation by injection of a dopa decarboxylase inhibitor was found to prevent β-alanine incorporation, and release of this block in the classical pathway by injection of either dopamine or N-acetyl dopamine stimulated β-alanine incorporation along with normal pupation. Interestingly, dopamine was found to be a much better antagonist to dopa decarboxylase inhibition than the sclerotizing agent itself, suggesting a link between the pathways subsequent to β-alanine and dopamine synthesis but not necessarily before synthesis of N-acetyl dopamine.

β-Alanine is a normal component of the sclerotized cuticles of many insects[29,34–39] and, in a number of species, coloration mutants have been associated with its absence. However, no simple correlation has been

established between deficiency and phenotypic manifestation in coloration; a situation analogous to the temporal dichotomy of black and ebony Drosophila. An understanding of the molecular basis of the phenotypes of these 2 mutants might therefore be extended to include situations which occur in other species. 2 hypotheses have been advanced to explain the Drosophila phenotypes. Jacobs[40] found that β-alanine inhibited phenylalanine degradation and suggested that the accumulation of unincorporated β-alanine in ebony adults promotes high levels of phenylalanine which might then be oxidized to black indole quinones. More recently Jacobs[30] has noted that the light puparium of both black and ebony can be explained if one assumes that the tan color of wild type at this stage is due to the reaction of cuticular β-alanine and chitin. This model, however, fails to account for the adult phenotype of black, unless some precursor prior to the metabolic lesion in β-alanine synthesis also inhibits phenylalanine degradation. A further problem with the explanation[30] is that it fails to incorporate the classical pathway, which the work of Bodnaryk and Wright clearly implicate as a major factor in both sclerotization and coloration. An alternative model based on the classical pathway has been presented by Hodgetts and Choi[28]. It had been determined previously[26] that although N-acetyl dopamine was the major compound detectable at puparium formation in wild type Drosophila, its immediate precursor, dopamine, was the major tyrosine metabolite accumulated at adult eclosion. On the basis of these measurements, as well as the intriguing fact that no mutation of *Drosophila melanogaster* produces a black pupal case while such mutations are found among other Diptera, Hodgetts and Choi suggested that the black coloration of ebony and black adults is due to polymerization of indole quinone molecules produced by oxidation of dopamine, a reaction largely prevented in wild type by crosslinking of the quinone molecules to β-alanine and the cuticular proteins. The authors note that this model is attractive in 2 respects: It can explain the dark puparium mutants found in other insects, by assuming that these species have high levels of dopamine at that time of development. It can also explain the black stripes which occur along the posterior margin of the tergites of wild type Drosophila adults. The cells which underlie these areas of the cuticle may like ebony, a mutant known to be cell autonomous[26], fail to incorporate β-alanine. However this model, as stated, fails entirely to account for the abnormally light pupa cases of either ebony or black.

IV. A tentative model on the basis of metabolic interactions

It is not surprising that the phenotypes of black and ebony are not fully explained by either model. The relationship between sclerotization and coloration as evidenced in these mutations may well indicate the complexity of developmental relationships in general, when viewed at the level of their final metabolic interactions. As yet, neither process is understood in detail and the nature of their relationship awaits further studies in both areas. Yet the literature on this subject is already voluminous and it seems possible, at the present time, to offer a tentative model explaining the phenotypes of wild, black and ebony Drosophila which may act as a guide for further research.

β-Alanine acts, as Jacobs suggests, by compacting chitin while forming a tan color, and the deficiency of this amino acid in the cuticles of black and ebony accounts for their light and poorly compacted pupal cases. However, since black and ebony nevertheless form viable puparia, the major sclerotizing event is the crosslinking of cuticular proteins by N-acetyl dopamine. This reaction makes little or no contribution to coloration because, at this stage of development, the sclerotizing agent is oxidized primarily on the β-carbon. Much the same process may occur during the larval molts: i.e. no chitin compaction and a lesser degree of sclerotization via β-carbon oxidation. In the adult, both mechanisms of sclerotization again occur, but the oxidation of N-acetyl dopamine is controlled by the cuticular proteins and is cell autonomous. Ring oxidation predominates in those regions of the cuticle which sclerotize with tan coloration, and β-carbon oxidation occurs in segments which eventually accumulate black pigment. The excess of dopamine found by Hodgetts and Knopka at this stage establishes the potential for melanization by oxidation and polymerization to indole-5,6-quinone. However, it has been demonstrated in vitro (Sherald, unpublished) that although dopamine oxidizes slowly to a black pigment, it rapidly forms a tan rather than black coloration when oxidized in the presence of β-alanine, and these observations may explain the adult phenotypes. Tan colored portions of the wild type cuticle result from ring oxidation of N-acetyl dopamine, and from the reaction of β-alanine with chitin and excess dopamine. The reaction with dopamine occurs quite readily and may explain the connection between the two pathways at this point as observed by Bodnaryk in Sarcophaga. 2 mechanisms, either singly or in combination may account for black regions of the wild type cuticle via polymerization of dopamine derived melanin. As suggested by Hodgetts and Choi, these areas may fail to incorporate β-alanine. It is also possible that accumulation of dopamine may be higher in cells where β-carbon oxidation of N-acetyl dopamine predominates, possibly via feedback inhibition. In either or both cases, the result would be an obvious accumulation of black pigment. In black and ebony, the absence or deficiency of β-alanine in the cuticles allows the gradual formation of indole-5,6-

quinone from dopamine, even in those regions of the cuticle where ring oxidation of the sclerotizing agent occurs. The resulting black pigment eventually obscures the tan color of N-acetyl dopamine quinone. These reactions are especially evident in weak alleles of ebony where normally tan areas of the cuticle clearly show both tan and black pigmentation.

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SPECIALIA

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Antidesmanol – a new pentacyclic triterpenoid from *Antidesma menasu* Miq. ex. Tul.[1]

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Summary. Antidesmanol (**1**), a new pentacyclic triterpenoid, has been isolated along with n-tritriacontane, friedelin, canophyllal and canophyllol from the aerial parts of *Antidesma menasu*. Based on chemical and spectroscopic evidence, its structure has been established as 3-keto-16α-hydroxyfriedelane. n-Tritriacontane and friedelin have shown antiinflammatory and diuretic activities respectively in experimental animals.

In a preliminary biological screening the petroleum ether extract of the aerial parts of *Antidesma menasu* Miq. ex. Tul. (Euphorbiaceae) showed antiinflammatory and diuretic activities. This prompted us to undertake its detailed chemical investigation. The column chromatography of this material on silica gel yielded n-tritriacontane (0.2% yield), friedelin (0.07%), canophyllal, canophyllol, and a new pentacyclic triterpene (0.044%) designated as antidesmanol (**1**). The present communication deals with the elucidation of the structure of **1** as 3-keto-16α-hydroxyfriedelane.

Antidesmanol (**1**), m.p. 277–280 °C ($CHCl_3$–MeOH); $C_{30}H_{50}O_2$; M^+ 442; $[\alpha]_D^{18°}$ −57° (c, 1, $CHCl_3$); ν_{max}^{KBr} 3400 (OH) and 1720 cm^{-1} (C=O) gave a benzoate (**2**), m.p. 90–91 °C; M^+ 546 $[\alpha]_D^{18°}$ −58.2° ($CHCl_3$). The chemical shift in PMR-spectrum (60 MHz; $CDCl_3$) of the hydroxymethine in antidesmanol acetate (**3**), m.p. 230–232 °C; $C_{32}H_{52}O_3$; M^+ 484; $[\alpha]_D^{18°}$ −38.5° ($CHCl_3$), revealed the secondary nature of the −OH function. The presence of signals for 7 tertiary (δ 0.7–1.18), 1 secondary (δ 1.30) methyl groups and 1 hydroxymethine (δ 3.65) in the PMR-spectrum of **1** coupled with a negative tetranitromethane test suggested it to be a derivative of friedelane group.

Careful examination of the various fragment ions[2–4] (m/e 193, 221, 273, 302, 357 and 371) present in its EI mass spectrum permitted the placement of the carbonyl and