Sclerotization of mosquito cuticle[1]

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Summary. The mode of sclerotization of *Aedes aegypti* pupal and adult cuticle was examined by employing biochemical and radioactive techniques. During larval-pupal metamorphosis, tyrosine is converted to tanning precursors and is incorporated into aryl-amino adducts and β-crosslinks. The major hydrolysis product of β-crosslinks in pupal cases is identified to be arterenone. Examination of tanning modes in five different mosquito species shows that the ratio of quinone to β-sclerotization not only differs within the life stages of the insects, but also differs between species.

Key words. Mosquito cuticle; quinone tanning; β-sclerotization; arterenone; *Aedes aegypti.*

During larval-pupal transformation as well as adult development, stabilization of insect cuticle in general is achieved by insertion of aromatic bridges between structural protomers and chitin backbone[2]. Two models viz., covalent crosslinking and noncovalent hydrophobic interaction have been proposed to account for the hardening and tanning of arthropod cuticle[3–6]. The covalent crosslinking hypothesis invokes the reactions of quinones and related reactive intermediates with cuticular components yielding aryl-protein adducts as well as aryl-protein crosslinks[3–5]. The noncovalent crosslinking hypothesis calls for hydrophobic interaction of cuticular components with polymers derived from aromatic compounds[6,7].

Recently we provided evidence for the presence of crosslinks in sarcophagid pupal cuticle by employing radioactive tracer technique[8–10]. Since cuticle formation, and in particular sclerotization of exoskeleton, is a unique process of insects and other arthropods, a clear understanding of the molecular mechanisms of sclerotization could lead to the development of more specific insecticides.

Mosquitoes are the vectors for malaria, filariasis, yellow fever, and other dangerous diseases afflicting man. In the past, control of these noxious insects was achieved by organochlorine insecticides such as DDT. The development of resistance to such compounds by mosquitoes and the persistence of these compounds in the environmnent have created the need for new vector control measures. With this view in mind, we studied the mode of tanning of mosquito cuticle and report some of our results in this communication.

Materials and methods. Aedes aegypti eggs were generously donated by Dr Andrew Spielman of the Harvard University, School of Public Health, Boston, MA, USA. They were hatched in a beaker of water under vacuum for 20 min. The larvae were raised in plastic tanks and fed daily with Tetra Min aquarium food. The temperature of the growth chamber was kept at 30 °C with relative humidity of 60%. Under these conditions pupation starts in about 6 days.

Radioactive tyrosine (2 µCi) was administered to mosquito larvae as outlined by Zomer and Lipke[11]. Isolation of catecholamine derivatives from cuticular hydrolyzates was achieved by the method of Murdock and Omar[12]. Aryl amino acid adducts were analyzed in cuticular hydrolyzates as described by Sugumaran and Lipke[8]. Quinone to β-sclerotization ratio was determined by the published procedure[13,14]. Scintillation counting was carried out after mixing aliquots (1 ml) of radioactive samples with 5 ml of aquasol (New England Nuclear Co., Boston, MA, USA) in a Packard Model 3420 liquid scintillation counter. Higher performance liquid chromatography of phenols from mosquitoes was carried out using a high pressure pump (Altex,

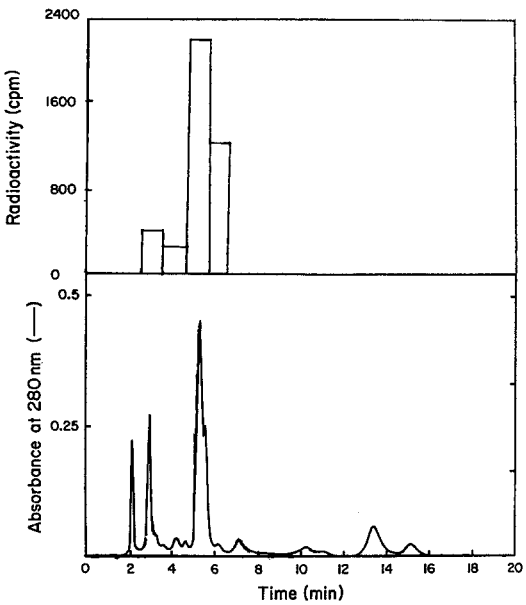

Figure 1. HPLC separation of radioactive phenols from mosquito larvae. U-[^{14}C]-tyrosine [2 µCi] was administered to late fourth instar mosquito larvae as outlined by Zomer and Lipke[11]. Prior to pupation the larvae were homogenized in 80% methanol and centrifuged. The clear supernatant was concentrated and subjected to HPLC analysis as outlined in Materials and Methods. The major radioactive peak was identified to be tyrosine by HPLC, spectral and amino acid analyses.

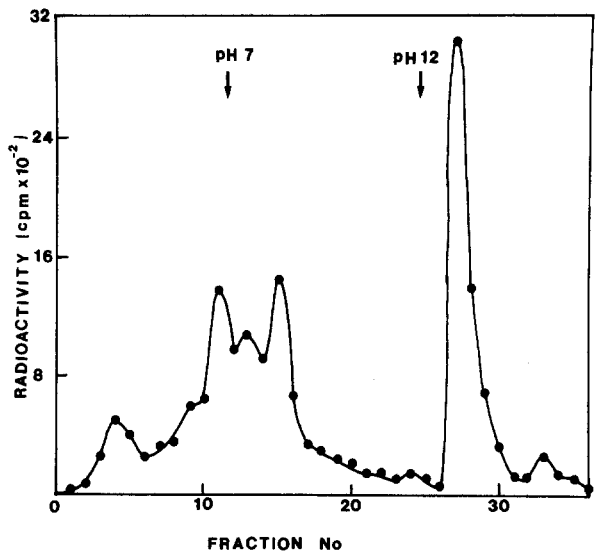

Figure 2. Dowex 50 column chromatography of U-[^{14}C]-tyrosine labeled cuticle hydrolyzate. 5 mg of U-[^{14}C]-tyrosine labeled cuticle (sp. act. 2930 cpm/mg) was hydrolyzed and chromatographed on Dowex 50-H$^+$ column using step gradients, as outlined by Sugumaran and Lipke[8]. The broad peak (pooled fractions) at the pH 7 fraction, which corresponds to unmodified tyrosine, carries approximately 38% of the total radioactivity loaded on the column. The basic aryl-amino acid adducts appear at pH 12 fraction and carry 36% of the radioactivity.

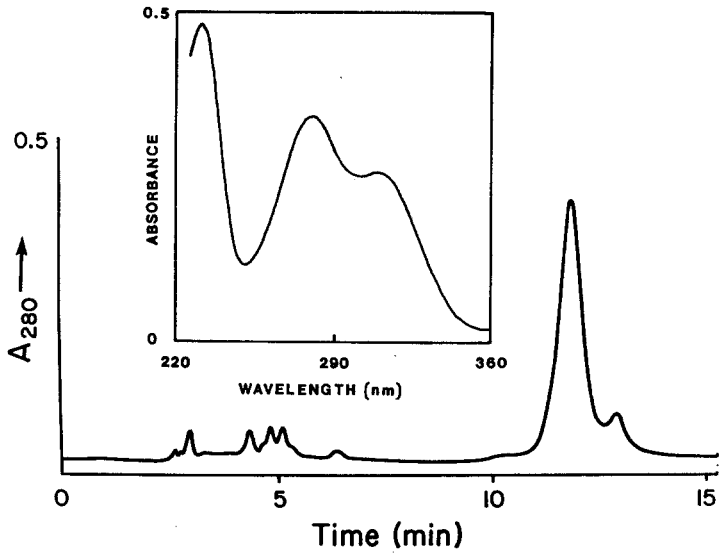

Figure 3. HPLC of catechol fraction isolated from cuticular hydrolyzates. Clean pupal cuticle (25 mg) was hydrolyzed with 0.2 ml of 6 N HCl and the catechols released from β-crosslinks were adsorbed onto alumina as outlined by Murdock and Omar[12]. After elution with 1.0 M acetic acid, they were subjected to HPLC analysis. The major peak corresponds to arterenone, the shoulder corresponds to dopamine.
Inset: UV absorption spectrum of the major peak in 0.2 M acetic acid.

Model 100A), a 50-µl loop injector (Altex, Model 210), a variable wavelength UV detector set at 280 nm with a flow cell (Hitachi spectrophotometer, Model 100-30) and a recorder-integrator (Altex, Model C-R1A). Reversed-phase chromatography was carried out using a 5-µ Ultrasphere ODS column (25 cm × 4.6 mm I. D., Altex). Isocratic elution with methanol: water 1:4, v/v, containing 0.05 M acetic acid and 0.2 mM sodium octylsulfonate was used at a flow rate of 1 ml/min at 2200 psi to achieve separation.

Results and discussion. It is now well established that tyrosine forms the major precursor for most of the sclerotizing agents in insect cuticle[15]. Hence U-[^{14}C]-tyrosine was administered to late fourth instar mosquito larvae and its fate analyzed. Nearly 50% of the tyrosine supplied was incorporated into the larvae within 24 h. Figure 1 illustrates the chromatographic separation of radioactive phenols extracted from mosquito larvae. The major radioactive peak corresponded to unmodified tyrosine. In order to confirm this possibility, the methanol extract was fractionated on a Bio Gel P-2 column, and the major radioactive peak was isolated, concentrated and rechromatographed on HPLC column. This resulted in the identification of a single UV positive and radioactive peak. The position and radioactivity were unaffected by acid hydrolysis. Upon cochromatography with tyrosine, this peak chromatographed as a single symmetrical peak. By comparison of elution times in HPLC as well as Biogel P-2 column, cochromatography with authentic sample and amino acid analysis, it was concluded that this radioactive peak was due to tyrosine. The minor component had a retention time similar to that of dopa but due to limited quantity of the sample further analysis was not carried out. In several insects, tyrosine is stored in the hemolymph in protected forms such as tyrosine-O-phosphate[16], β-alanyltyrosine[17], and tyrosine-O-glucoside[18]. However, in *Aedes aegypti*, it seems to be stored as free tyrosine only.

The mode of tanning of mosquito pupae was then examined after administering radioactive tyrosine to fourth instar larvae by procedures developed in this laboratory[8,14]. Pupal cuticle labelled with U-[^{14}C]-tyrosine was hydrolyzed and the hydrolyzate chromatographed on Dowex 50 chromatography. From figure 2 it can be seen that 38% of the total counts of radioactivity incorporated into the cuticle emerged as tyrosine while 36% was incorporated into basic aryl-amino acid adducts. Recovery of significant amounts of aryl-amino acid adducts clearly rules out the hydrophobic hypothesis[6,7] and supports the covalent crosslinking model as the major mechanism for stabilization of mosquito cuticle.

Even among covalent crosslinks, 2 types exist viz., the quinone type aryl-amino acid adducts and β-crosslinks[5,8]. Hence, the ratio of these two crosslinkings in mosquito cuticle was checked using dihydroxyboryl cellulose chromatography[14]. The results show that *Aedes aegypti* pupal cases are tanned 40% by quinone tanning and 60% by β-sclerotization (table).

There is no study in the literature to show whether the ratio of quinone to β-sclerotization in any cuticle is constant within a species or not. Therefore, we examined the tanning modes of 5 different mosquito pupae and adults. Examination of the results in the table reveal that considerable variations in the ratio of quinone to β-sclerotization are observed between the various mosquito species tested. In general, mosquitoes seem to prefer β-sclerotization over quinone tanning for stabilization of their exoskeleton with the exception of adult *Anopheles albimanus*. It is also interesting to note that pupae and adults use these two modes of tanning differentially to stabilize their cuticle. Thus the ratio of quinone to β-sclerotization is different not only between different life stages but also between the same stages in different species.

Quinone: β-sclerotization tanning modes in Culicine pupae and adults

Species	Stage Pupae			Adults		
	Quinone	B-	Q:B	Quinone	B-	Q:B
Anopheles albimanus	14	86	0.16	67	33	2.0
Anopheles quadrimaculatus	42	58	0.72	33	67	0.49
Anopheles gambiae	24	76	0.32	29	71	0.41
Culex quinquafasciatus	27	73	0:37	28	72	0:39
Aedes aegypti	40	60	0.67	41	59	0.69

The ratio of quinone to β-sclerotization was determined in the cuticle after hydrolysis as outlined by Sugumaran and Lipke[14]. Values in percent of summed crosslinks. Average of 2 determinations.

Since β-sclerotization is associated with the presence of covalently bound catechols, the cuticular hydrolyzates were examined for the presence of catecholamine derivatives. Quantitative and specific isolation of catecholamine derivatives from cuticular hydrolyzate was achieved by alumina adsorption technique[12]. HPLC analysis of catechols thus isolated from cuticular hydrolysates revealed the presence of a major component eluting at about 12 min and a minor component eluting at about 13 min as a shoulder to the major peak (fig. 3). Comparison of retention time with related catecholamine derivatives led to the tentative characterization of the major peak to be arterenone and the minor to be dopamine. Cochromatography of the isolated peaks with authentic compound further confirmed this contention. The positive response of the major compound to Arnow's test for o-dihydroxyphenols[19], 2,4-dinitrophenyl-hydrazine and fluorodinitrobenzene indicated the presence of catecholic, carbonyl and amine groups on the molecule, respectively. UV absorption spectrum of the major peak in 0.2 M acetic acid (fig. 3, inset) corresponded well with that of the authentic compound, confirming its identity as arterenone. Similar studies led to the characterization of the minor peak to be dopamine.

Andersen and Roepstroff[20] have identified as many as eleven catechols from cuticular hydrolyzates. However, these authors failed to take precautions to avoid the decomposition of catechol during hydrolysis. For instance, hydrolysis was carried out in an oxygen rich atmosphere which causes decomposition of catechols[21]. Since our procedure involves hydrolysis in vacuum and protection of catechols with alumina during isolation, artificial product formation is highly minimized. This accounts for the isolation of a single ketocatechol. i.e. arterenone, from cuticle. The mechanism by which β-sclerotization is initiated was not clarified until recently. Andersen's group isolated 1,2-dehydro-N-acetyldopamine from β-sclerotized cuticle and suggested it to be the reagent initiating β-sclerotization[22]. As the condition used to extract this compound from cuticle certainly is drastic, involving hot alkali treatment, it is questionable whether this compound is present freely in the cuticle. Moreover, generation of colorless cuticle by the quinone of this compound as suggested by Andersen[22] is also doubtful. Alkyl substituted quinones with conjugated double bonds absorb more strongly in the visible region as compared to unconjugated quinones. As a result, the quinone from 1,2-dehydro-N-acetyldopamine is expected to give dark colored cuticle rather than light colored cuticle. On the other hand, quinone methide intermediate not only accounts for the presence of catechols in cuticle, but also for the formation of colorless cuticle[2,23]. Isolation and characterization of arterenone as a single ketocatechol from mosquito cuticle is also consistent with quinone methide formation and is inconsistent with the involvement of 1,2-dehydro-N-acetyldopamine in β-sclerotization. Recently, we synthesized 1,2-dehydro-N-acetyldopamine and tested for its participation in sclerotization of sarcophagid cuticle. Studies with radioactive trapping experiments reveal that this compound is not involved in the tanning of sarcophagid cuticle and that quinone methides are the reactive species for tanning[24].

Further studies on the characterization of aryl-amino acid adducts and the nature of enzyme system(s) involved in sclerotization reaction(s) are in progress in our laboratory.

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Ornithine decarboxylase, S-adenosyl-L-methionine decarboxylase and arginine decarboxylase from *Mycobacterium bovis* (BCG)

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Summary. Ornithine decarboxylase (ODC), S-adenosyl-L-methionine decarboxylase (AMDC) and arginine decarboxylase (ADC) activities were detected for the first time in extracts of *Mycobacterium bovis* (BCG). All the decarboxylases differed from corresponding known bacterial decarboxylases in that: a) ODC did not require GTP for activity; b) ODC was not inhibited by any known inhibitor of bacterial ODCs; c) AMDC and ADC did not require Mg^{2+}-ion for activity and were not markedly inhibited by any known inhibitor of the decarboxylases of other bacteria.

Key words. Polyamine biosynthesis; *Mycobacterium bovis* (BCG); inhibition of polyamine biosynthesis.

Numerous studies have shown that polyamines (putrescine and spermidine) are necessary for the growth of bacteria[1,2]. We have shown earlier that ethambutol, which is an effective antituberculosis drug[3], specifically inhibited spermidine synthesis in *M. bovis*, but not in non-mycobacteria[4,5]. To study the early steps of polyamine biosynthesis in *Mycobacteria* we measured the activity in *M. bovis* of the different decarboxylases needed for the biosynthesis of putrescine and tested the effect of several