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INVOLVEMENT OF REACTIVE OXYGEN SPECIES IN THE OXIDATION OF TYROSINE AND DOPA TO MELANIN AND IN SKIN TANNING

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The role of reactive oxygen ($^1O_2$ and $O_2^{\cdot-}$) in skin photosensitization and tanning reaction has been examined. Riboflavin (RF), hematoporphyrin (HP), 3-carbethoxypsoralen (3-CP), and 8-methoxypsoralen (8-MOP), upon photoexcitation under aerobic conditions, produced singlet O_2 ($^1O_2$). RF, 3-CP, and 8-MOP also produced superoxide anion ($O_2^{\cdot-}$). Reactive O_2 produced by photosensitized RF, 3-CP, and 8-MOP was found to oxidize tyrosine and dopa to dopachrome and subsequently their conversion to melanin. HP did not oxidize tyrosine to dopachrome, and 3-CP and RF revealed substantial oxidation of tyrosine. Dopa was oxidized to dopachrome and subsequently to melanin by all photosensitizers tested at a variable rate as follows: RF > 3-CP > HPD > 8-MOP. UVA alone and to a lesser extent UVB also produced $^1O_2$ which induced the oxidation of tyrosine and dopa to dopachrome and subsequently to melanin. The production of dopachrome was higher with dopa compared to tyrosine under all irradiation conditions. These observations appear to have relevance to the O_2-requiring immediate tanning reaction of the skin stimulated by solar radiation and in the induction of skin photosensitization.

Tyrosine and dopa can be oxidized enzymatically to form dopachrome, and dopachrome undergoes spontaneous polymerization to melanin (1,2). Melanin pigmentation of human skin, derived from tyrosine and catalyzed by tyrosinase, involves the formation, melanization, and transfer of pigment granules (melanosomes) by melanocytes to keratinocytes in the epidermis. Solar UVR profoundly stimulates melanin pigmentation (tanning) in human skin. In recent years, the use of UV-emitting (320 - 400 nm) high-intensity fluorescent lamps in the United States and Europe has increased significantly in stimulating the skin tanning response (4). Among the many reactions that occur in human skin

ABBREVIATIONS: 3-CP, 3-carbethoxypsoralen; dopa, 3,4-dihydroxyphenylalanine; DABCO, 1,4-diaza-[2,2,2]-bicyclo-octane; HP, hematoporphyrin hydrochloride; 8-MOP, 8-methoxypsoralen; $^1O_2$, singlet O_2; $O_2^{\cdot-}$, superoxide anion; NBT, nitro blue tetrazolium; RF, riboflavin; RNO, N,N-dimethyl-p-nitrosoaniline; GSH, glutathione; UVA, 320 - 400 nm radiation; UVB, 290 - 320 nm radiation; UVR, ultraviolet radiation (290 - 400 nm).

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when exposed to solar or artificially produced UVR, the primary events are observed in the form of immediate pigment darkening (an O_2-dependent photooxidation event), delayed sunburn (erythema), and tanning (neomelanogenesis) (3,4). Potent photosensitizers, such as 8-MOP and other psoralens which are used widely in the photochemotherapy of vitiligo and psoriasis, are known to stimulate melanin pigmentation in the skin of normal individuals, psoriatic patients, and in amelanotic skin areas of vitiligo patients after the oral or topical application of the drug and subsequent exposure of skin to UVA radiation (5,6). Although the cellular and morphological events associated with this increased skin pigmentation are fairly well understood (3,4,7), the role of reactive O_2 in melanin pigmentation has received limited attention. A wide variety of short-lived reactive O_2 species are known to be generated in skin photosensitization reactions in the presence of exogenous or endogenous photosensitizers (e.g., porphyrins and riboflavin) (5). Since psoralens are also known to generate $^1O_2$ and $O_2^{\cdot-}$ (5,8) and to oxidize dopa (9), it is likely the reactive O_2 produced by chemical agents or by UVR may have a biologic role in the UV-induced pigment darkening reaction and melanogenesis. We report the results of our tyrosine and dopa oxidation studies using known skin photosensitizers HP, 3-CP, and RF. The choice of HP and 3-CP was based upon their strong ability to generate $^1O_2$ under *in vitro* conditions (5,10), and RF was used because of its known $O_2^{\cdot-}$-producing ability (11). Tyrosine and dopa were also used as substrates for tyrosinase catalyzed enzymatic reaction in the formation of melanin (1,2).

Materials and Methods

L-Tyrosine, L-dopa, HP, and other chemical reagents, including mushroom tyrosinase used throughout this study, were purchased from Sigma Chemical Company, St. Louis, Missouri, USA. Crystalline 8-MOP was purchased from Elder Pharmaceuticals, Bryan, Ohio, U.S.A. 3-CP was a gift form Dr. June K. Dunnick, National Toxicology Program Center, Research Triangle Park, North Carolina, U.S.A. Photosensitization studies involving the production of $^1O_2$ and $O_2^{\cdot-}$ were carried out with UVA radiation. A horizontal planar array of four 4-foot long UVA-emitting fluorescent tubes (GTE Sylvania, Life Line) were used for irradiation (5). UVB radiation was obtained from two horizontally mounted, 4-foot long Westinghouse fluorescent sunlamps. The system at 8-cm distance produced UVA irradiance of 3.75 mW/cm^2. The UVB-emitting fluorescent sunlamp produced UVB irradiance of 0.75 mW/cm^2 at a 8-cm distance. UV radiometry was carried out with a radiometer model IL700 equipped with cosine-corrected UVA and UVB detectors, International Light Company, Newburyport, Massachusetts, USA. The production of $^1O_2$ was measured under aerobic conditions in an aqueous solution by the method proposed by Kraljic and Mohsni (12) involving the spectrophotometric measurement of a decrease in the

absorbance of N,N-dimethyl-p-nitrosoaniline at 440 nm and using histidine as a selective acceptor of $^1O_2$. The formation of $O_2^{\cdot-}$ was studied by monitoring the photosensitized reduction of nitro blue tetrazolium at 560 nm (13). The generation of $^1O_2$ and $O_2^{\cdot-}$ was further confirmed by using selective quenchers known for inhibiting the production of $^1O_2$ and $O_2^{\cdot-}$ and by carrying out quenching studies using sodium azide (10^{-6} - 10^{-2} M), DABCO (10^{-4} - 2.5×10^{-2} M), potassium sorbate (10^{-3} to 10^{-2} M), β-carotene (10^{-4}), mannitol (10^{-2} M), sodium benzoate (10^{-2} M), GSH (10^{-5} - 10^{-3}), and superoxide dismutase (100 - 250 units/ml). The generation of $^1O_2$ by RF, HP, 3-CP, and 8-MOP was additionally confirmed by carrying out the reaction in D_2O which enhances the lifetime of $^1O_2$. For experiments on the photosensitized production of $^1O_2$ and $O_2^{\cdot-}$ and on the oxidation studies of tyrosine (10^{-3} M) and dopa (10^{-3} M), the reaction mixtures (3.0 - 3.5 ml) were aerobically irradiated in stoppered-quartz cuvettes placed horizontally under UVA- or UVB-emitting light sources. The amount of $^1O_2$ or $O_2^{\cdot-}$ produced by UVA wavelengths present in the UVB-emitting fluorescent tubes was subtracted by carrying out the reaction with and without a mylar filter which transmitted wavelengths > 320 nm. The effects of N_2 and O_2 on these photoreactions were examined by irradiating the reaction mixture (10 ml) in 15 ml Pyrex tubes equipped with a gas purging system for O_2 or N_2. The N_2 or O_2 was bubbled through the solutions 15 mins before and throughout the course of irradiation. The photosensitized formation of dopachrome from tyrosine and dopa in the presence of various photosensitizers (2.66×10^{-5} M) was confirmed by the appearance of a characteristic pink-red color and by measuring spectrophotometrically the intensity of dopachrome color formed by recording an increase in the absorption values of two peaks at 305 and 475 nm. Tyrosine, dopa, RF, 3-CP, HP, 8-MOP, and the buffer system did not interfere with the spectral determination of dopachrome at 475 nm.

Results

Figure 1 shows the generation of $^1O_2$ by HP, 3-CP, RF, and 8-MOP at 2.66×10^{-5} M concentration of the photosensitizer at varying exposure doses of UVA. The photosensitized production of $^1O_2$ by 3-CP, psoralen, RF, HP, coproporphyrin, and uroporphyrin could be selectively quenched by NaN_3 (10^{-2} M). The extent of $^1O_2$ quenching induced by NaN_3 was 83, 93, 80, 86, 73, and 82 percent, respectively, for these photosensitizing compounds. Other well known $^1O_2$ quenchers such as DABCO, ethyl sorbate, potassium sorbate, and sorbyl alcohol produced 85 to 100% inhibition of $^1O_2$ as evidenced by examining the RNO bleaching reaction in the presence of 3-CP, psoralen, angelicin, and porphyrins (HP and uroporphyrin). β-carotene at 10^{-4} M inhibited 22 to 25% of $^1O_2$ production. The limited solubility of this agent appears to be a major factor in its limited ability of quenching $^1O_2$. In Figure 2, the production of $O_2^{\cdot-}$ by RF, 8-MOP, and 3-CP is shown. RF produced both $^1O_2$ and $O_2^{\cdot-}$, however, HP did not produce detectable levels of $O_2^{\cdot-}$. SOD (100 - 250 units per ml) caused 95 to 100% inhibition of NBT reduction by $O_2^{\cdot-}$. *In vitro* studies involving the formation of dopachrome from dopa (10^{-3} M) and tyrosine (10^{-3} M) with varying doses of UVA or UVB radiation also confirmed the formation of $^1O_2$ in the RNO bleaching reaction system; the photooxidation of Dopa

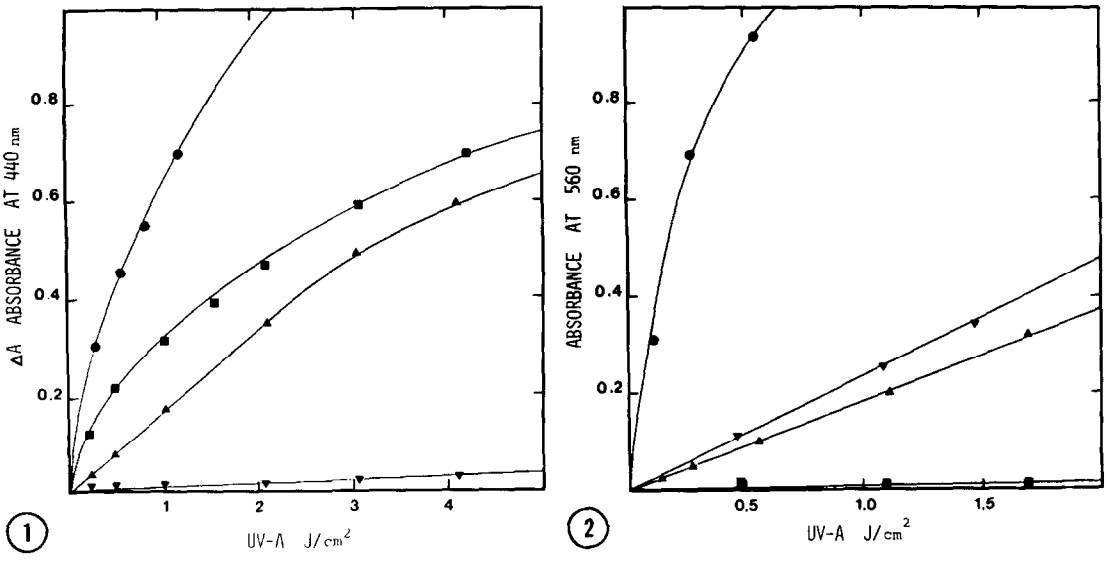
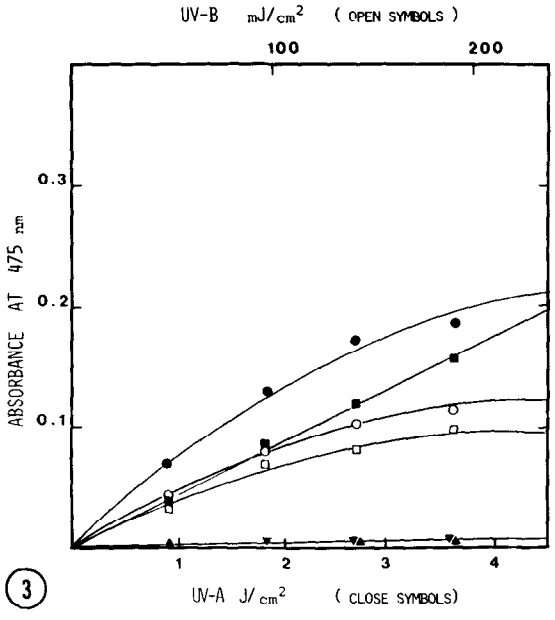


Figure 1. Production of $^1O_2$ by RF (●), HP (■), 3-CP (▲), and 8-MOP (▼) with varying doses of UVA. Concentration of all sensitizers used for $^1O_2$ production was at 2.66×10^{-5} M.

Figure 2. Production of $O_2^{\cdot-}$ by RF (●), 8-MOP (▼), and 3-CP (▲) with varying doses of UVA. HP (■) did not reveal $O_2^{\cdot-}$ production. Concentration of all sensitizers used was at 2.66×10^{-5} M.

or tyrosine to dopachrome could be quenched by NaN_3 (10^{-2} M) thus confirming the generation of $^1O_2$ by UVR. The effect of GSH (10^{-5} - 10^{-3} M) was also investigated for the production of $^1O_2$ and $O_2^{\cdot-}$. Unexpectedly, GSH reacted nonspecifically with RNO and NBT and its effect on the production of $^1O_2$ and $O_2^{\cdot-}$ could not be resolved.

HP and 3-CP, potent $^1O_2$-producing agents, were used as positive controls for investigating the effects of $^1O_2$ on the photooxidation reaction of tyrosine and Dopa. Tyrosine was oxidized to dopachrome by photosensitized RF, 8-MOP, and 3-CP when irradiation was carried out under UVA and UVB (Fig. 3). HP (2.66×10^{-6} to 2.66×10^{-5} M), a potent generator of $^1O_2$, did not induce oxidation of tyrosine to dopachrome or to melanin, indicating $^1O_2$ was not involved in the transformation of tyrosine to dopa. The formation of dopachrome from tyrosine by various test chemicals revealed the following order of their reactivity: RF > 3-CP >> 8-MOP. Dopa was oxidized to dopachrome by both $^1O_2$ and $O_2^{\cdot-}$ generating photosensitizers in the presence of UVA and UVB radiation at varying rates (Fig. 4) in the following order: RF > 3-CP > HP > 8-MOP. The comparison of results revealed a good correlation between the ability of the photosensitizer to produce active O_2 (Figs. 1 and 2) and their potential to oxidize tyrosine and dopa to dopachrome and



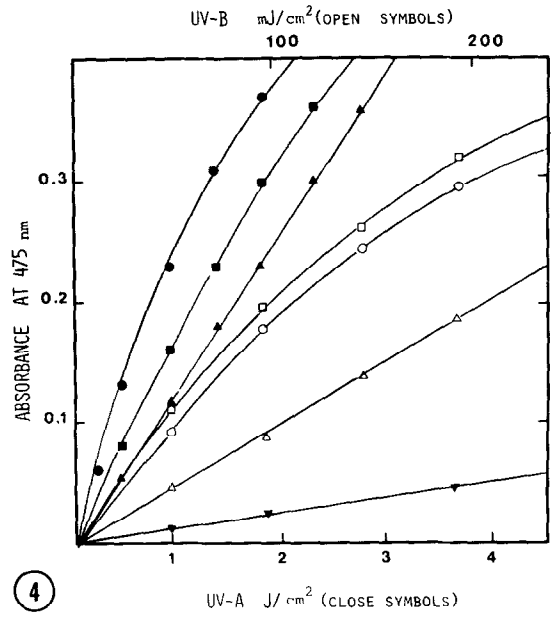


Figure 3. Production of dopachrome from tyrosine (10^{-3} M) by RF (●), 3-CP (■), HP (▲), and 8-MOP (▼) with varying doses of UVA (close symbols) and UVB (open symbols). Exposure dose for UVA is expressed in J/cm^2 and for UVB in mJ/cm^2. All sensitizers were used at 2.66 x 10^{-5} M.

Figure 4. Production of dopachrome from dopa (10^{-3} M) by RF (●), 3-CP (■), HP (▲), and 8-MOP (▼) with varying doses of UVA (close symbols) and UVB (open symbols). 8-MOP$_2$+ UVB produced no dopachrome. Exposure dose of UVA are in J/cm^2 and UVB in mJ/cm^2. All sensitizers were used at 2.66 x 10^{-5} M.

melanin. (Figs. 3 and 4). The yield of dopachrome was 3 to 5 fold higher with dopa and photosensitizers than with tyrosine and photosensitizers.

Studies with various quenchers revealed the involvement of both $^1O_2$ and $O_2^{\cdot-}$ in the oxidation of dopa to dopachrome. As indicated earlier, $O_2^{\cdot-}$ was primarily involved in the oxidation of tyrosine to dopachrome, whereas $^1O_2$ and $O_2^{\cdot-}$ were responsible for the oxidation of dopa and dopachrome. GSH and NaN_3 produced 100 and 90% inhibition of dopachrome formation, respectively. Potassium sorbate at 10^{-3} M and 10^{-2} M produced 40% and 95% quenching effect on the production of $^1O_2$. When RF, 3-CP, HP, or 8-MOP were used as sensitizers in the dopa to dopachrome reaction, GSH (10^{-3} M) induced 100% quenching, whereas the quenching effect of NaN_3 (10^{-2} M) and potassium sorbate ($^1O_2$) was decreased by approximately 20% indicating an involvement of other reactive O_2 species in this oxidation reaction. Mannitol (10^{-2} M) and sodium benzoate (10^{-2} M) did not show any quenching reactions involving tyrosine and dopa, thus ruling out the possibility of the involvement of hydroxy radicals ($^{\cdot}OH$) in the reaction. Unfortunately, DABCO, an

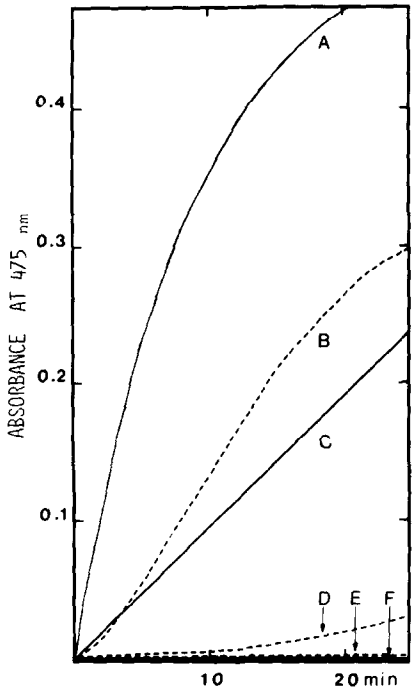


Figure 5. Enzymatic formation of dopachrome from tyrosine (10^{-3} M, dotted lines) and dopa (10^{-3} M, solid lines) catalysed by tyrosinase. A, dopa + tyrosinase (1.6 µg/ml); B, tyrosine + tyrosinase (6.6 µg/ml); C, dopa + tyrosinase + NaN_3 (10^{-2} M); D, tyrosine + tyrosinase + NaN_3 (10^{-2} M); E, tyrosine + tyrosinase + GSH (10^{-3} M); and F, dopa + tyrosinase + GSH (10^{-3} M).

effective quencher of $^1O_2$, was found to chemically react with dopa, and SOD, an effective quencher of $O_2^{\cdot-}$, interfered with the dopa oxidation studies; therefore, their effects could not be studied meaningfully. The involvement of molecular O_2 in the tyrosine-dopa oxidation reaction was also confirmed by carrying out studies in aerated and N_2-purged solutions. When N_2 was bubbled through the irradiation solution for 15 mins before and throughout the course of irradiation, the formation of dopachrome was not observed. When the photoreactions were carried out in D_2O instead of H_2O, an increase in the rate of $^1O_2$ production from 40 to 200% could be observed.

Figure 5 represents the enzymatic oxidation of tyrosine (10^{-3} M) and dopa (10^{-3} M) with tyrosinase (1.60 to 6.6 µg/ml) without the presence of light. The quenching effect of NaN_3 and GSH on the enzymatic formation of dopachrome are shown in Figure 5. The effect of NaN_3, GSH, and SOD on the tyrosinase-mediated oxidation of tyrosine and Dopa are shown in Table 1. The data on the quenching of

TABLE 1: QUENCHING OF ENZYMATICALLY AND NON-ENZYMATICALLY PRODUCED DOPACHROME

Quencher	Percent Quenching of Dopachrome Formation			
	Enzymatic		Non-Enzymatic	
	Dopa (10^{-3} M) + Tyrosinase (1.6 µg/ml)	Tyrosine (10^{-3} M) + Tyrosinase (6.6 µg/ml)	Dopa (10^{-3} M) + 3-CP (10^{-5} M)	Dopa (10^{-3}) + RF (10^{-5} M)
NaN_3, 1 x 10^{-2} M	60	95	80	85
GSH, 1 x 10^{5} M			5 - 10	
GSH, 1 x 10^{-4} M	50			
GSH, 2 x 10^{-4} M	80			
GSH, 1 x 10^{-3} M	100	100	95 - 100	100
mannitol, 10^{-2} M	0	0	0	5
SOD 100 to 250 units/ml	0	0	0	0
sodium benzoate, 10^{-2} M	0	0	0	0

nonenzymatic formation of dopachrome by NaN_3, GSH, SOD, mannitol, 6-carotene, and sodium benzoate are also shown in Table 1. The spectral properties of dopachrome produced enzymatically showed an absorbance maxima at 303 and 475 nm; these values were identical to dopachrome produced by photosensitized reaction and to the absorption values of dopachrome reported in literature (1). Tyrosinase catalyzed the production of dopachrome from dopa at a rate about 10 times faster than the rate of production of dopachrome by an equimolar concentration of tyrosine. Enzymatic reaction for dopachrome formation was quenched by specific $^1O_2$ and $O_2^{\cdot-}$ quenchers in a manner similar to that observed in photosensitized reactions (Table 1). GSH (10^{-3} M) induced 100% quenching of tyrosine plus tyrosinase reaction. NaN_3 (10^{-2}) produced 70 to 75% inhibition of dopachrome formation from dopa and tyrosinase. Mannitol (10^{-2} M), a known $^{\cdot}OH$ scavenger, did not show any effect in the reaction. The tyrosinase activity remained unchanged during exposure to UVA (0 - 10 J/cm^2).

Discussion

The purpose of this study was to consider the evidence implicating reactive O_2 species (e.g., $^1O_2$, $O_2^{\cdot-}$, and $^{\cdot}OH$) in clinically interesting reactions involving:

(a) photopolymerization of melanogenic precursors to yield melanin-like polymeric material which may be responsible for immediate pigment darkening and delayed tanning reaction commonly seen in individuals undergoing exposure to UVR; (b) skin photosensitization (erythema, edema, and post-inflammatory hyperpigmentation) induced by psoralens and porphyrins. Although porphyrins, psoralens, and several dyes have been reported to generate reactive O_2 species both under *in vitro* and *in vivo* conditions (5,8,9), the most difficult problem is the unequivocal assignment of the role of $^1O_2$ and $O_2^{\cdot-}$ in skin photosensitization and tanning reactions. The role of $^1O_2$ in psoralen-induced and porphyrin-induced skin photosensitization reactions has been recently reported by us (5,14) and involves membrane-lipid peroxidation and endothelial cell damage to evoke vasodilation (erythema and edema reaction). We will focus our comments on the role of reactive O_2 species in skin tanning responses induced by UVR.

Evidence has been presented in this study to indicate: (a) UVA as well as UVB radiation induce the production of $^1O_2$. (b) Skin photosensitizing agents such as psoralens, porphyrins, and RF produced $^1O_2$; and RF, 3-CP, and 8-MOP produced $O_2^{\cdot-}$. (c) Reactive $^1O_2$ produced by RF, 3-CP, and 8-MOP was found to oxidize tyrosine to dopachrome and subsequently its conversion to melanin. (d) Dopa, an intermediate in melanin formation, was found to be oxidized to dopachrome and subsequently to melanin at variable rates by $^1O_2$ and $O_2^{\cdot-}$ produced by 3-CP, RF, 8-MOP, and porphyrins. The production of dopachrome was higher with dopa compared to tyrosine. The rapid formation of dopachrome and melanin mediated by reactive O_2 produced by UVA, UVB, psoralens, HP, and RF indicates that such a mechanism may be operative in human skin subjected to UV exposure.

It is of interest to note that *in vivo* immediate tanning reaction is optimally induced by 315- to 400-nm radiation and is a strong O_2-dependent reaction. This reaction can be inhibited or appreciably decreased by reducing the O_2 tension of the tissue (skin) by temporarily blocking the cutaneous blood flow and causing ischemia and hypoxia (15,16). This suggests the production of $^1O_2$ by UVA under anoxic or hypoxic conditions is inhibited, and there is no rapid formation of dopachrome and melanin. Furthermore, Rosen et. al. (17) have recently observed

that melanocytes in human skin prior to UVA exposure were tyrosine negative and weakly dopa positive, and soon after exposure, they became tyrosine positive and strongly dopa positive when skin biopsies were examined within 24 hrs. This suggests UVA radiation in vivo possibly increases the formation of dopa and dopachrome in epidermis. These observations are in agreement with our earlier findings reported in 1968 concerning the photoenhancement of electron spin resonance (ESR) signals from pigmented human skin, hair, and synthetic dopa-melanin preparations (18). Evidence was presented to indicate the immediate tanning of human skin represented a photooxidation reaction in which a reduced form of melanin (the monomer units of 3,4-dihydroxyindole) is oxidized through the generation of semiquinone-like free radicals in the melanin polymer.

Our findings strongly favor the role of $^1O_2$ and $O_2^{\cdot-}$ in photopolymerization of melanogenic precursors in the form of immediate and delayed tanning reactions stimulated by UVR and mediated through the formation of dopa and dopachrome. Reactive O_2 species also play an important role in cutaneous photosensitivity reactions induced by porphyrins and psoralens.

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