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Autorizzazione del Tribunale di Napoli n. 3684 dell’11/12/87.
Pulsed irradiation studies of unstable intermediates of melanin pigment formation

The fast reaction techniques of flash photolysis and pulse radiolysis are known mainly for their enormous contributions to knowledge of free radical and excited state chemistry, and, in particular, the involvement of such species in the chemistry of biology and medicine (1). There is however a whole "spectrum" of unstable intermediates including, for example, orthoquinones, quinone-imines and quinone methides which are neither free radicals nor excited states, yet are too short-lived to be studied by normal methods, but are amenable to study by these pulsed radiation techniques. A variety of such transient intermediates are thought to be involved in the complex processes of melanin formation, and this article attempts to call attention to the largely unexplored potential of pulsed radiation techniques to elucidate these processes.

A common mode of decay of semiquinones is disproportion to give quinones and dihydroxybenzenes. Several unstable orthoquinones, for example, dopaquinone (I) (2-4) and the false melanin precursor anisyl-3,4-quinone (II) (5), can be prepared almost instantaneously by one-electron oxidation of the corresponding dihydroxybenzenes with sub-microsecond pulses of radiation. For dopaquinone, under the conditions chosen (3), formation of dopaquinone from stable dopa was complete within 5 ms of the pulse, and its decay, eventually to form dopachrome, followed over tens of ms to seconds, depending upon the pH. For anisyl-3,4-quinone, formation of the orthoquinone was complete within 20 ms of pulse irradiation (Aqueous solutions of the latter cannot be made by simple dissolution of solid quinone without significant polymerisation occurring during the process of dissolution). In the formation of both orthoquinones, one-electron oxidation can be performed either photochemically, via photoionization and/or photohomolysis, or radiation chemically, for example, via N_3^- radical oxidation, the corresponding semiquinone being formed initially which disproportionates to form the orthoquinone (and reform the parent dihydroxybenzene).

The corresponding orthoquinone (III) of 5-S-cysteinyldopa, which can only be similarly formed radiation chemically (3,4), decays by a different and competing cyclisation path to that pertaining to dopaquinone, the cysteinylnyl side chain amino substituent
attacking the carbonyl of the phenyl ring with loss of water to produce an unstable quinone-imine (IV), postulated by Prota et al (6). This quinone-imine then rearranges by a hydrogen shift to a more stable benzothiazine isomer.

Another important quinone which has long been postulated to be crucial to melanin pigment formation is 5,6 indolequinone (V). One-electron oxidation of 5,6 dihydroxyindole is thought to lead to the corresponding oxygen-centred semiquinone radical which probably disproportionates to 5,6 indolequinone and reform 5,6 dihydroxy-indole (7). The 5,6 indolequinone appears to be unstable, decaying by 1rst order kinetics into a substance, or substances, possessing considerable absorption around 540 nm where melanochrome has a maximum (8,9). One of these substances could be the rearrangement product (VI), reminiscent of dopachrome but with added conjugation, which may be a seed of the polymerisation which ultimately leads to melanin. Positive assignments of the above unstable species are difficult however to establish unequivocally, and it could be that the semiquinone from 5,6 dihydroxyindole, in part at least, decays by demerisation, the dimers initially formed, possibly (VII) and (VIII) for instance, being unstable and rearranging by unimolecular processes into more stable dimers of the type that have been isolated by Corradini et al (10) and d'Ischia and Prota (11).

Decarboxylations are also involved in melanin pigment formation and pulsed radiation techniques may help in elucidating such processes. For instance, tyrosinase catalyses the oxidative decarboxylation of 3,4-dihydroxymandelic acid leading to 3,4-dihydroxybenzaldehyde (12) although 4-hydroxymandelic acid and 3-methoxy-4-hydroxymandelic acid are inert. One-electron oxidation of 3,4-dihydroxymandelic acid via pulse radiolysis (13) led to the corresponding semiquinone which decayed by disproportionation into a species which subsequently decayed by 1rst order kinetics (k=2 s⁻¹) into a stable product with an absorption spectrum matching that of 3,4-dihydroxybenzaldehyde. Since one-electron oxidation of 4-hydroxymandelic acid did not appear to lead to 4-hydroxybenzaldehyde, it is suggested that the intermediate resulting from the second order decay of semioxidised 3,4-dihydroxymandelic acid is the orthoquinone (IX) rather than the corresponding quinone methide (X) previously postulated (12), the equivalent of which should also have been formed from 4-hydroxymandelic acid.

It is hoped that the above gives some idea of the scope of pulsed radiation techniques for studies of unstable melanin precur-
sors. Since the equipment in particular the sources of pulsed ionizing radiation for carrying out such experiments are expensive and comparatively rare, if any reader wishes to consider jointly pursing other ideas for investigating unstable intermediates relat-
ed to melanogenesis please get in touch.

Acknowledgements

I am grateful to J.M. Bruce, J.N. Chacon, M.R. Chedekel, C. Lambert, P.A. Riley, T. Sarna, A. Thompson and T.G. Truscott for
collaboration, G. Prota for comments, and the Cancer Research Campaign and Medical Research Council for support.

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    isolation of oligomers from 5,6-dihydroxy-1-methylindole.

(11) d'Ischia M, Prota G
    Photooxidation of 5,6-dihydroxy-1-methyl-indole. Tetrahedron
(12) Sugumaran M
Tyrosinase catalyses an unusual oxidative decarboxylation of

(13) Bouheroum M, Bruce JM, Land EJ
To be published
Improving the melanin formation assay for mammalian tyrosinase

Because of the multi-functionality of tyrosinase (EC 1.14.18.1) in the melanin biosynthetic pathway, there are several methods to measure its enzymatic activity. These methods differ in the substrate used, the step of the pathway measured, and the analytical technique used to follow the tyrosinase-catalyzed reaction. It is difficult to establish which method is the best one to evaluate tyrosinase activity. However, in samples where melanogenesis capability is the desired parameter to be determined, the melanin formation assay is surely the more suitable one. This assay was introduced by Chen and Chavin (1965) and further studied by Hearing and Ekel (1976) and ourselves (1988). Basically, the assay consists in the determination of the insoluble $^{14}$C-labelled eumelanin formed from L-(U-$^{14}$C)-Tyrosine.

The major problem of this method is the low percentage of L-Tyrosine hydroxylated by tyrosinase that is incorporated into the eumelanin polymer. Most of the substrate, once oxidized in the tyrosine-catalyzed reactions, remains as soluble intermediates of the pathway, and these intermediates are washed before their incorporation into the polymer. Comparison between tyrosine hydroxylase and melanin formation assays, shows that the percentage of L-tyrosine hydroxylated which actually is incorporated into the polymer ranges from less than 10% in 1 hour of assay, to 60% in assays performed for long times of incubation, 8 hours.

In our laboratory we have tested some modifications of the melanin formation assay in order to improve it by increasing the percentage of tyrosine hydroxylated which reaches the polymer using assays of 1 hour incubation time. These modifications enhance the radioactivity incorporated up to 7 times and they could be very useful when samples showing low tyrosinase activity are being dealt with.

Taking into account the methodology described by Hearing and Ekel (1976) or ourselves (Jara et al, 1988), there are two easy ways to improve the sensitivity of the method:

1. once the time of incubation of the sample with the substrate has elapsed, the reaction is stopped by addition of NaOH up to a 60 mM concentration. Then the reaction mixture is left in such a basic medium for 5 minutes, to allow radioactive intermediates to polymerize rapidly into eumelanin, since the rates of the chemical steps in the Raper-Mason pathway increase at high pH. Higher concentrations of NaOH must not be used, since some degree of solubilization of the polymer occurs. After this treatment, samples are applied to Whatman 3MM filter paper discs and washed in 0.1 M HCl and other solvents according to the published method (Hearing and Ekel, 1976).

2. Bearing in mind that divalent metal cations also increase the rate of the chemical steps in the Raper-Mason pathway, we tried the
addition to the reaction media of 1 mM of several cations. Ni²⁺ proved to be the most efficient one, increasing the radioactivity incorporated to the insoluble polymer up to 7 times in some samples. This effect and the one produced by NaOH were not additive, as it can be seen in the Table, mean and S.D. of five experiments performed using purified tyrosinase from Harding-Passey melanosomes, and 1 hour incubation time.

**Effect of 1 mM Ni²⁺ or basic treatment on melanin formation**

<table>
<thead>
<tr>
<th>Assay number</th>
<th>Ni²⁺</th>
<th>NaOH</th>
<th>net cpm/h</th>
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<tr>
<td>1</td>
<td>No</td>
<td>No</td>
<td>7802 ± 266</td>
</tr>
<tr>
<td>2</td>
<td>No</td>
<td>Yes</td>
<td>23427 ± 1046</td>
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<tr>
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<td>Yes</td>
<td>Yes</td>
<td>50091 ± 2198</td>
</tr>
<tr>
<td>4</td>
<td>Yes</td>
<td>No</td>
<td>50150 ± 1746</td>
</tr>
</tbody>
</table>

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**COMMENTARY**

**Color and paramagnetism of eumelanins**

The most familiar characteristic of eumelanins is their black color and it has been well accepted that this is due to the extended conjugation of the electronic network throughout the biopolymer. This argument has been used to explain the paramagnetism, blackness, and intractability of the material. In our studies we have begun to reexamine the nature of the mobility of the electrons within eumelanin polymers with specific attention paid to their possible origins.
From the original studies by Nicolaus (1) and Swan (2) came the proposal that eumelanin is a highly crosslinked heteropolymer of 5,6-dihydroxyindole, DHI, 5,6-dihydroxyindole-2-carboxylic acid, DHICA, and DOPA covalently bound in an irregular fashion. However, recent studies by Prota (3) and by Ito (4) have shown that both DHI and DHICA are generated from DOPA in substantial quantities. In the presence of metal-ions both compounds further react to give covalently bound oligomers. It is unreasonable to assume then that the formation of eumelanins from these precursor materials should be formed in a haphazard fashion, especially under biological conditions. Jimbow's SEM study on melanosome formation shows clearly the highly ordered structure of the eumelanin melanosome (5). As melanosome buildup is complete, the spaces fill between the initially formed strands making the total structure appear to have a highly compacted form. The harsh chemical treatments typically utilized in the removal of natural eumelanins from their native surroundings could severely alter the original chemical and physical nature of the melanosome such that subsequent analyses yield imprecise information.

We have found the oxidation of DHI gives a highly structured material that consists of oligomers with a molecular weight of about 3,000 Daltons (6). This oligomer has approximately 1.5 water molecules bound to each unit of DHI. ESCA studies revealed that 20~25% of the DHI unites are in an oxidized state similar to that of adrenochrome (7). X-Ray diffraction studies material further shows repeat spacings at 15 and 3.4 Å, which leads to a component cell length of 5 DHI molecules attached linearly across the benzene portion. Carbon 13 NMR studies using specifically enriched ($^{13}$C$_{2}$ and $^{13}$C$_{3}$) DHI further indicated that there is no change in the substitution at the pyrrole portion of the compounds (8). This suggests a structure for the oligomer of a two stranded helix of 5 DHI units in various stages of oxidati aligned in either a head to head or head to tail configuration held via hydrogen bonding from the water molecules. This structure places the hydrophilic sections of the molecules exposed to the surrounding environment. Intersetingly we also have found that as the melanochrome forms from DHI there is an EPR signal quite similar to that of the intact eumelanin (Figure). Moreover these initial eumelanin oligomers have a paramagnetic characteristic of 1 spin per 6~8 units. With a carbonyl content of ca. 20% for these oligomers this value is much lower than what would be expected for a semi-quinone free radical. In solution the oligomers are dark brown, but upon acidification they precipitate out as black particles. One possibility for this color change is that in the soluble form the oligomers both absorb light and are large enough to scatter it. Upon precipitation they aggregate to larger particles which further scatter light and appear black. If the perceived color of the eumelanins were due solely to light absorption, the solution and solids would appear more similar. Additionally this would require more of an extended conjugation of the -electron system of the DHI units than is possible for the molecular dimensions we find for this material. But the low value for the spin density certainly argues against solely the involvement of semi-quinone free radicals because they would not be delocalized throughout the oligomer. Rather the
paramagnetism may be due to the establishment of charge transfer complexes across the helix between the oxidized and standard state of the DHIs. Studies by Kuroda et al (8) for merocyanine dye films show the presence of a simple free radical with a g-value close to that of a free electron. They argue that this is due to charge transfer through the stacked dye molecules. A similar phenomenon may be operative in DHI eumelanin. We are therefore continuing to further unravel the nature of the paramagnetism of eumelanins with specific attention to the possibility that the signals originate from an intrinsic conduction network inherent in the polymeric chain.

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Figure: ESR and UV-visible spectrum of DHI in reaction with CuSO₄ at 1, 3, 15 minutes after mixing.
PIGMENT CELL RESEARCH BULLETIN

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- REVIEW  O
- LETTER TO THE EDITOR  O

TOPIC COVERED: .................................................................

NUMBER OF PAGES: ....... (not exceeding 6 pages in double spacing, including references)

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Laboratory of Oncology and Experimental Surgery
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Rue Héger-Bordet 1
1000 Bruxelles
1. MSH, OTHER HORMONES, DIFFERENTIATION

- Hruby VJ, Hadley ME, Dorr R, Levine N
Abstract: A method for stimulating melanin production in a mammal comprises topical administration of alpha-MSH and/or analogs. (Nle4,D-Phe7)-alpha-MSH was dissolved in PEG (25% PEG 400, 74% PEG 3350 by wt.) at 10^{-9}M and the ointment was applied topically to the skin of plucked mice. Microscopic examination revealed eumelanin within hair bulbs by 24 h following application of the analog. Proliferation of melanocytes was not restricted to the hair bulbs of the treated site but was observed microscopically in hair bulbs taken from untreated areas of the animal where hair growth was in progress.

- Osman AM, Amer TM
Abstract: The sensitivity of 2 established human melanoma cell lines to L-dopa (one of the intermediates during melanin synthesis) was investigated. The M-5A cell line showed 49 and 61% inhibition of cell proliferation after 1 h treatment with L-dopa at doses of 0.125 and 0.5 mM, respectively. Twenty-four hour treatment at a dose of 0.25 mM showed 96 and 49% inhibition in M-5A and SK-Mel-25 cells, respectively. Melanin content was increased with L-dopa treatment and there was a relation between the induction of melanin synthesis and the inhibition of cell proliferation.

- Pelletier G, Guy J, Desy L, Li S, Eberle AN, Vaudry H
Abstract: Melanin-concentrating hormone (MCH)-containing neurons have recently been localized in the dorsolateral region of the rat hypothalamus, an area where the second alpha-MSH system is found which contains only alpha-MSH and none of the pro-opiomelanocortin (POMC)-related peptides. In order to study the morphological relationships between the MCH and alpha-MSH neuronal systems, we have studied the immunocytochemical localization of both MCH and alpha-MSH in the rat hypothalamus. The same study was also performed in the human hypothalamus where there is only one alpha-MSH system.
which contains alpha-MSH as well as the other POMC-related peptides (first alpha-MSH system). In the rat dorsolateral hypothalamus, we could demonstrate that most neuronal cell bodies stained for MCH also contained immunoreactive alpha-MSH. In the human hypothalamus, neuronal cell bodies stained for MCH were observed only in the periventricular area whereas cell bodies containing alpha-MSH were exclusively located in the infundibular (arcuate) nucleus. In the rat, immunoelectron microscopy showed labelling for MCH in the dense core vesicles of positive neurons and double-staining techniques clearly demonstrated that both immunoreactive MCH and alpha-MSH could be consistently detected in the same dense core vesicles. These ultrastructural studies then suggest that these two peptides should be released simultaneously from neurons located in the rat dorsolateral hypothalamus.

- Powell KA, Baker BI


Abstract: Using immunocytochemical methods at the electron microscope level, immunoreactivity for both melanin-concentrating hormone (MCH) and alpha-melanocyte-stimulating hormone (alpha-MSH) has been demonstrated in the carp neurohypophysis. A double-labelling technique, using colloidal gold probes of different sizes showed that immunoreactivity to both molecules coexists within the same neurosecretory granules in some neurones, while in other neurones the granules exhibit only MCH-like immunoreactivity. These observations suggest that the two immunoreactivities are attributable to separate molecules; if they are derived from the same precursor molecule, then this must be cleaved differently in the two sets of neurones. The absence of adrenocorticotropic hormone (ACTH)-like immunostaining in any neurosecretory granule might suggest the alpha-MSH-like molecule is not derived from the conventional pro-opiomelanocortin precursor.

2. MORPHOLOGY OF PIGMENT CELLS AND PIGMENTARY DISORDERS

- Aliev GA, Rachkovskii ML, Krymova MA


Abstract: The suppressing effect on melanogenesis of genes determining high wool productivity in domestic sheep has been discussed for the first time. Morpho-physiological mechanism of suppression of the activity of pigment cells function in lambs of Tajik breed was shown: melanocytes lose their activity during some weeks-months after birth and migrate from the zone of usual localization on follicle papilla into forming hair. The result of genes-modifiers' action (the period of postembryonic development, when white wool starts growing) is determined by their interaction with alleles of genes A and E: the lower intensity of melanogenesis, the earlier and more effective the inhibitory effect of genes-
modifiers. Some factors which suppress pigmentation are characterized: these are an increase in SH groups content, deficiency of tyrosine, rise in growth rate and reduction of the diameter of hairs, intensification of antagonism between melanocytes and keratinocytes. The problem is also interesting in evolutionary aspect: since suppression of melanogenesis in the process of creating wooly sheep illustrates possible mechanisms of inhibition of one function, because of hyperfunction of other, such negative correlations could play significant role in evolution of organisms.

- Goldstein J, Hecht SD

- Green RM, Su WP

- Holick MF, Smith E, Pincus S

- Tal H, Landsberg J, Kozlovsky A

3. MELANIN CHEMISTRY, BIOLOGY, HISTOCHEMISTRY AND OTHER PIGMENTS

- Aver'yanov AA, Lapikova VP, Petelina GG, Dzhavakhiya VG, Umnov AM, Stekol'shchikov MV
Abstract: The damage of the spores of the fungus Pyricularia oryzae was studied in model systems generating superoxide (xanthine oxidase-xanthine and riboflavin-methionine-light) and OH (H2O2-Fe2+) radicals. The fungus mutant devoid of the melanin pigment was damaged several-fold higher than the normally melanized strain of the wild type. The mutant with a defect pigment occupied a medium position. Melanin isolated from the wild type strain and added to the spores afforded strong protection in all cases studied. Melanin inhibits both the reduction of Nitro Blue Tetrazolium by superoxide radicals and the oxidation of alpha-keto-gamma-methylthiobutyric acid by OH radicals. The catalase activity of melanin was also detected, but it was too low to afford protection. The protective effect manifests itself in scavenging of O radicals and (or) inhibition of the Haber-Weiss reaction. Presumably, the participation of melanins in the mechanism of fungal resistance involves the suppression of the cytotoxicity of reactive O species.

- Bathory G, Szuts T, Magyar K
Abstract: The in vivo melanin binding of (14C)selegiline was
studied in mice. Extensive accumulation was observed in the pigmented mouse eye, while in the albino animal uptake was low in the corresponding tissues. In vitro investigations demonstrated that the amphetamine derivatives tested can be taken up by melamins. Scatchard analysis of selegiline binding to dopamine melanin (structurally similar to neuromelanin) and beef eye melanin showed that >1 class of binding sites may be involved. The total binding capacity of the beef eye melanin was higher than that of the dopamine melanin. Selegiline inhibited the binding of the neurotoxic metabolite of MPTP to dopamine melanin. Selegiline may accumulate in pigmented nerve cells. Melanin affinity may contribute to the use of this compound for the treatment of Parkinson's disease or may play a role in its protective effect against MPTP neurotoxicity.

- Corradini MG, Napolitano A, Prota G
Abstract: Enzymatic oxidation, or autoxidation at pH 9, of the title compound (I) led to a mixture of fluorescent products the major of which was 5,6,5',6'-tetraacetoxy-1,1'-dimethyl-2,4'-biindolyl (II). In the presence of metal cations, e.g. Ni2+, autoxidation of I, at pH 7.5, gave besides II 5,6,5',6'-tetraacetoxy-1,1'-dimethyl-2,2'-biindolyl and 5,6-diacetoxy-1-methyl-2,4-di-(5',6'-diacetoxy-1'-methyl-2'-indolyl)indole. The unexpected oxidative coupling of I at the 2- and 4-position rather than the 3- and 7-position, as previously believed, is discussed in relation to the current views on the structure and biosynthesis of eumelans.

- Eastman CL, Young JS, Fechter LD

- Ferroni EN, de Lauro C
Alpha-MSH (melanocyte-stimulating hormone) and MCH (melanin concentrating hormone) actions in Bufo ictericus ictericus melanophores. Comp Biochem Physiol (A) 15-20, 1987.

- Ito S, Imai Y, Jimbow K, Fujita K
Abstract: Cysteine is known to be involved in pheomelanin synthesis. Available evidence, however, indicates that glutathione may also play an important role in melanogenesis. This in vitro study clarifies how cysteine and glutathione participate in melanogenesis. Melanins were prepared by tyrosinase oxidation of 3,4-dihydroxyphenylalanine (dopa) with various amounts of cysteine or glutathione, and were subjected to HCl hydrolysis. Synthetic melamins and the hydrolyzed melanins gave N/S molar ratios corresponding to those calculated from the ratio of dopa to cysteine or glutathione. On hydriodic acid hydrolysis, dopa plus cysteine-melamins and dopa plus glutathione-melamins gave aminohydroxyphenylalanine and cysteine, respectively, the yields of which were proportional to the sulfur content. The results indicate that
cysteine is integrated into benzothiazine units (phaeomelans) while glutathione is connected to dihydroxyindole units (eumelans) with the retention of glutathione moiety. Analysis of natural melanins, prepared by HCl hydrolysis of Sepia, B16, and Harding-Passey melanosomes, indicates that sulfur (0.4–1.4%) in these natural melanins may be derived artificially from the reaction of melanins with cysteine or cystine in the course of HCl hydrolysis.

- Jimbow K

- Kagedal B, Gawelin AL, Pettersson A

- McGovern J, Crocker J
The effect of melanin pigment removal on the peroxidase-antiperoxidase immunoperoxidase technic. Am J Clin Pathol 480-483, 1987. Abstract: With the increasing use of immunoperoxidase techniques, it may be difficult to differentiate between the dark staining of 3,3'-diaminobenzidine (DAB) compound reaction product and melanin pigment. The latter may be particularly observed in skin. Samples of both normal skin and melanotic malignant melanoma were treated for the removal of melanin by std. techniques both before and after a peroxidase-antiperoxidase (PAP) immunohistologic sequence. Many methods for the removal of melanin pigment resulted in diminished DAB staining intensity. Some also caused cellular disruption. However, the method of choice was found to be treatment with 0.25 g/dL potassium permanganate and 1 g/dL oxalic acid before the immunoperoxidase sequence.

- Miranda M, Bonfigli A, Zarivi O, Manilla A, Cimini AM, Arcadi A, Botti D

- Morishita F
Responses of the melanophores of the medaka, Oryzias latipes, to adrenergic drugs: evidence for involvement of alpha 2 adrenergic receptors mediating melanin aggregation. Comp Biochem Physiol (C) 69-74, 1987. Abstract: 1. Melanin-aggregation response of the medaka melanophores to a series of adrenergic drugs were examined. 2. Concentration-response curves for the drugs indicated that the melanin-aggregating effects of alpha 2 adrenergic agonists, naphazoline, tramazoline and clonidine, were more than 100-fold greater than that of alpha 1 agonists, phenylpropanolamine, phenylephrine, oxymetazoline and methoxamine. 3. The inhibitory effect of alpha 2 antagonist, yohimbine, on the cell responses to the agonists were also about 100-fold greater than that of alpha 1 antagonists, corynanthine and prazosin. 4. These results indicate that adrenergic receptors which mediate melanin-aggregation response of the cells are alpha 2 in nature.

**Abstract**: Skin-lightening lotions and ointments contain (1) an aqueous solution of kojic acid and/or its derivatives, and (2) an aqueous solution or suspension of vitamin E and/or its derivatives to inhibit melanin formation in the skin. Thus, a natural vitamin E 1.0, polyoxyethylene hydrogenated castor oil 4.0, polyethylene glycol 8.0, EtOH 16.0, and H₂O to 100% by wt. were mixed. This solution 5.0, polyoxyethylene hydrogenated castor oil 1.0, a perfume trace, EtOH 15.0, 4-hydroxybenzoate 0.1, citric acid 0.1, Na citrate 0.3, 1,3-butylene glycol 4.0, di-Na edetate 0.01, kojic acid 0.5, and H₂O to 100% by wt. were combined to give a skin lotion.

- Palumbo P, d'Ischia M, Prota G

- Rogers CB, Blum CA, Murphy BP

- Sakaguchi T, Nakajima A

**Abstract**: The U adsorbing abilities of various biopigments were investigated. Extremely high adsorption capacities for U were found in melanin and bioflavonols (quercetin and morin) having chelating positions with U. As a step toward improving the adsorption characteristics of the bioflavonols, quercetin and morin were immobilized on both Bemberg rayon fiber and polyaminostyrene, and the basic features of U adsorption by the immobilized bioflavonols were studied. The bioflavonols immobilized on Bemberg rayon fiber have a highly selective capacity to adsorb U. U recovery from seawater by the immobilized bioflavonols was markedly affected by the pH value of the seawater, and the uptake at pH 8, which is the pH value of natural seawater, was difficult. However, this adsorbent can accumulate large amounts of U from nonsaline water. Thus it can be used to remove and recover U from U refining waste water and other waste sources.

- Sato C, Ito S, Takeuchi T

**Abstract**: The pheomelanin content of TM10 cells increased when 0.2 mM L-DOPA was added to the culture medium, whereas the content of eumelanin was little changed. In contrast, 5-S-cysteinyldopa did not increase either type of melanin. L-DOPA increased the number of pheomelanosomes in the cytoplasm of the cells. L-DOPA phosphates also increased the pheomelanin content of the cells, with no effect on eumelanin. L-DOPA inhibited tyrosinase activity in the cells.
- Spiridonova NA, Kulieva AM, Kurbanov KhK
Abstract: Guinea pigs were given L-tyrosine or DL-methionine at 1 mmol the 1st day, at 2 mmol the 2nd day, and at 3 mmol the 3rd day orally; some animals of each group were exposed to a sunlight-heat stimulus (20 min sunlight at a temperature of 45-49 degree). L-Tyrosine and DL-methionine equally stimulated melanin formation by th skin associated with increased urinary excretion of p-hydroxy-phenylpyruvic acid and 5-S-cysteinyl-DOPA. Exposure to sunlight and heat also induced melaninogenesis and this process was accompanied by the increased formation of 5-S-cysteinyl-DOPA, the amount of which in urine is a good indicator of the extent of melanin formation.

- Ten LN, Stepanichenko NN, Mukhamedzhanov SZ, Aslanov KhA

- Vedralova E, Borovansky J, Duchon J
Abstract: Strategy of protein determination in melanin containing samples was analyzed, and the prerequisites under which the Lowry procedure could be used were established. (a) Complete solubilization of proteins from melanosomes was not reached until the treatment with 3% sodium dodecylsulfate at 100 degrees C was prolonged to 5 h. (b) It is necessary to correct the data obtained with Folin reaction to eliminate melanin interference. (c) The correction is based upon the determination of the extent of solubilized melanin and substraction of the corresponding Folin-Ciocalteu reaction values. (d) The extent of correction due to the melanin interference was found to be about 40% of the absorbance value. It was concluded that correction of the data obtained with Lowry assay is possible, but the procedure is complicated and time-consuming. However, a more simple modification has not been developed.

4. NEUROMELANINS
- Juurlink BH

5. PHOTOBIOLOGY AND PHOTOCHEMISTRY
- Friedmann PS, Gilchrest BA
Abstract: In humans the major stimulus for cutaneous pigmentation is ultraviolet radiation (UVR). Little is known about the mechan-
ism underlying this response, in part because of the complexity of interactions in whole epidermis. Using a recently developed culture system, human melanocytes were exposed daily to a physiologic range of UVR doses from a solar simulator. Responses were determined 24 hours after the last exposure. There was a dose-related increase in melanin content per cell and uptake of $^{14}$C-DOPA, accompanied by growth inhibition. Cells from donors of different racial origin gave proportionately similar increases in melanin, although there were approximately tenfold differences in basal values. Light and electron microscopy revealed UVR-stimulated increases in dendricty as well as melanosome number and degree of melanization, analogous to the well-recognized melanocyte changes following sun exposure of intact skin. Similar responses were seen with Cloudman S91 melanoma cells, although this murine cell line required lower UVR dosages and fewer exposures for maximal stimulation. These data establish that UVR is capable of directly stimulating melanogenesis. Because cyclic AMP elevation has been associated in some settings with increased pigment production by cultured melanocytes, preliminary experiments were conducted to see if the effects of UVR were mediated by cAMP. Both alpha-MSH and isobutylmethylxanthine (IBMX), as positive controls, caused a fourfold increase in cAMP level in human melanocytes and/or S91 cells, but following a dose of UVR sufficient to stimulate pigment production there was no change in cAMP level up to 4 hours after exposure. Thus it appears that the UVR-induced melanogenesis is mediated by cAMP-independent mechanisms.

- Gallas JM
Optical lens system incorporating melanin as an absorbing pigment for protection against electromagnetic radiation. 1987.

- Kollias N, Bager AH

Abstract: In this paper we propose that human melanin absorbs visible radiation through two distinct mechanisms: one that is in effect over the entire visible range and is linear in wavelength, and a second one that is evident at wavelengths in the range 400-500 nm and is exponential in frequency. These mechanisms are apparent in all human diffuse reflectance spectra that we have collected. We show that the absorber is the same in all human volunteer skin samples. By studying the diffuse reflection spectra of DOPA-melanin in solution and DOPA-melanin in powder form, we find that we can correlate the absorption mechanisms, one with melanin in solution (a low molecular weight form) and the other with melanin in powder (a high molecular weight form). Therefore, we propose that melanin exists in two distinct states. This interpretation for the diffuse reflection spectra obtained from delayed pigment (UVB-induced) and immediate pigment (UVA-induced). Delayed pigment appears as an increase of both forms of melanin (neomelanogenesis), whereas immediate pigment appears as an increase in the higher molecular weight form with a commensurate decrease in the lower molecular weight form: the two mechanisms change independently of each other. Finally, we show that we can distinguish spectroscopically between the delayed pigment and the immediate
pigment.

- Musk P, Parsons PG
Abstract: Solar irradiation of a panel of human cell lines revealed 3 phenomena relevant to understanding the biological role of melanos, i.e. a heavily melanized melanoma line (MM418) was considerably more resistant to solar killing compared with HeLa and amelanotic melanoma cells of similar size and DNA content. MM418 cells were also resistant to killing by artificial UVB and by H₂O₂ generated in situ with extracellular glucose oxidase, and no difference in survival between the cell lines was found using 254 nm UV or gamma-radiation. MM418 cells were resistant to sunlight when irradiated as attached monolayers but not when irradiated in suspension. Further studies showed that resistance to solar radiation in MM418 cells was not due to less DNA damage, as judged by inhibition of semiconservative DNA synthesis, or to enhanced constitutive or induced repair determined by reactivation of irradiated adenovirus. These results indicate that melanization protects human cells from solar UVB in vitro and that the mechanism is associated with protection from H₂O₂-type damage rather than direct shielding of DNA.

- Raaphorst GP, Azzam EI

- Ranadive NS, Menon IA
Abstract: A review with 154 references. Skin reactions to solar radiation in people with various ethnic origins are studied. The photobiological effects of the large amounts of reactive O species and free radicals produced from UV-irradiated-melanos are discussed. The role of mast cells in these reactions is described. A probable mechanism of photoinduced cutaneous inflammation is suggested.

6. MELANOMA

- Dreux C, Launay JM, Garnier JP

Abstract: The antitumor effect of cysteinylphenol (CP) and cysteaminylphenol (CAP) analogs in melanoma were studied. Both 4-S-
cysteinylnphenol (4-S-CP) (73243-10-4) and 4-S-cysteaminylphenol (4-S-CAP) (91281-34-4) increased the life span of B16 melanoma-bearing mice and inhibited the tumor growth while their 2-S-isomers and catechol derivatives did not. Also, 4-S-CP and 4-S-CAP were substrates of melanoma tyrosinase (9002-10-2) while their 2-S-isomers were not. 3H-labeled 4-S-CP was selectively accumulated in melanoma tissue. Apparently, 4-S-CP and 4-S-CAP are melanin precursors and can act as potent antimelanoma agents which are selectively incorporated into melanoma cells and destroy them through the presence of tyrosinase. The synthesis of the compounds is not described.

- Kanno J, Matsubara O, Kasuga T
Induction of melanogenesis in Schwann cell and perineural epithelium by 9,10-dimethyl-1,2-benzanthracene (DBMA) and 12-O-tetradecanoylphorbol 13-acetate (TPA) in BDF1 mice. Acta Pathol Jpn 1297-1304, 1987.
Abstract: Six-week-old female mice were treated with a single topical application of DMBA (I) followed by repeated application of TPA on the clipped dorsal skin. Several weeks after DMBA application, the intradermal melanocytes of perifollicular melanocytic network began to proliferate to form melanocytic tumors in all treated mice skin. Besides these changes, single membrane-bound melanosomes and premelanosomes were found in the cytoplasm of perineural epithelia and Schwann cells with mesaxons of the nerve bundles involved; the melanogenic activity of the Schwann cell and the perineural epithelium of the dermal peripheral nerve bundle is discussed in terms of a common feature of neuroectodermal cells. For diagram(s), see printed CA issue.

7. TYROSINASE AND OTHER ENZYMES
- Bomirski A, Wrzolkowa T, Arendarczyk M, Bomirska M, Kuklinska E, Slomiński A, Moellmann G
Abstract: A spontaneous, hypomelanotic variant (MI) of the highly melanotic transplantable hamster melanoma of Bomirski (Ma) is the subject of this report. Tyrosinase activity is 2-3 times higher, but melanin content significantly lower than in the parental Ma melanotic melanoma. Acid phosphatase activity is similar in both, but beta-glucuronidase and aryl-sulfatase A are 2-3 times higher in the hypomelanotic variant. Transplanted MI melanomas grow more slowly than the parental tumor, but metastasize with similar incidence and localization. Hypomelanotic variant melanoma cells, even those in grossly nonnecrotic parts of the transplants, show signs of low viability like swelling of the cytoplasm of cellular condensation, and disintegration. Autophagic vacuoles are numerous. They appear to be formed by enclosure of a portion of cytoplasm by cisternae of smooth endoplasmic reticulum or trans-Golgi network. These limiting cisternae contain tyrosinase as evidenced by deposition of electron dense reaction product on incubation with tyro-
sine or DOPA. Other sites of ultrastructural tyrosinase reaction are melanosomes and the smooth-surfaced cisternae and vesicles of the trans-Golgi network. We postulate the low cell viability, associated with autophagosome formation, is the cause for the growth retardation of the MI variant, and that the lower melanin content of these tyrosinase-rich cells is due to sequestration of a substantial portion of newly synthesized enzyme into autophagic vacuoles before it has the chance of being incorporated into melanosomes.

- Fuller BB, Iman DS, Lunsford JB

Abstract: Melanogenesis in mammalian pigment cells is regulated by changes in the activity of tyrosinase, the rate-limiting enzyme for melanin synthesis. Because recent evidence suggests that this enzyme may exist in pigment cells in both active and inactive stages, a competitive enzyme-linked immunoabsorbent assay (ELISA) was developed to compare tyrosinase levels in amelanotic and melanotic melanoma cell clones. The melanotic cell line used for this study, MEL-11A, had basal tyrosinase levels approximately 40 times that of the amelanotic cell line, AM-7. Both cell lines responded to melanocyte-stimulating hormone by demonstrating large increases in tyrosinase levels in these two clones, microtiter plates were coated with purified tyrosinase, and trypsinized cell extracts were tested for their ability to compete with bond tyrosinase for antibody binding. Although tyrosinase activity in the amelanotic clone was 1/40 that of the melanotic clone, immunoreactive tyrosinase levels in AM-7 cells were found to be approximately one-half that present in the melanotic clone. Additional evidence for the presence of an inactive (or at least, catalytically less active) enzyme in AM-7 cells was obtained from immunotitration analysis of tyrosinase in cell extracts from both cell lines. These results suggest that at least some amelanotic melanoma cells may contain significant levels of catalytically inactive tyrosinase molecules and that the level of pigmentation in mammalian melanocytes may be regulated by a tyrosinase activation process.

- Hearing VJ, Jimenez M

- Korytowski W, Sarna T, Kalyanaraman B, Sealy RC

Abstract: The oxidation of 4 catechol(amine)s by tyrosinase was studied by ESR and optical methods. Rates of O consumption and of dopaquinone and dopachrome formation during the oxidation of dopa were measured and compared with rates of dopasemiquinone production measured by spin-stabilization procedures. In the presence of spin-stabilizing metal ions, production of semiquinone was approxi-
mately quant. Time-dependent ESR spectra obtained from dopa and dopamine showed a slow regeneration of semiquinone, suggesting that a semiquinone precursor is slowly reformed. In contrast, time-dependent spectra for 4-methylcatechol and N-acetyldopamine showed decay of the primary semiquinone together with buildup of a secondary semiquinone apparently derived from the corresponding 6-hydroxy catechol(amine). Thus, catecholamines that give rise to a cyclizable quinone show a pattern of behavior that differs from those that produce a noncyclizable quinone. These results are discussed in terms of their possible significance to melanogenesis and the toxicity of catechol(amine)s, which has been attributed to production of semiquinones and(or) other O radicals.

Movaghar M, Hunt DM
Abstract: Tyrosinase activity at the time of phaeomelanin synthesis in neonatal mice is lower in agouti than in black skin and hair bulb tissue, and this depressed activity is associated with a reduction in the electrophoretically distinct de novo form of the enzyme. Direct chemical measurements of sulphhydryl compounds show elevated levels in agouti hair bulb tissue at this stage of development. The addition of exogenous copper to hair bulb extracts raises the activity of tyrosinase in agouti to approximately the black level but has no affect on black itself. These results are discussed in relation to the role of sulphhydryl compounds and copper availability in regulating tyrosinase activity and turnover.

Wood JM, Schallreuter KU
Abstract: The ESR spectrum of the reduced thioredoxin/tyrosinase inhibitor complex resembles that of the model biscysteiny1-Cu(II) complex (Hanaki; Yoko1 1986), It can be concluded that reduced thioredoxin reacts with the binuclear Cu(II) center of tyrosinase to reduce 1 Cu(II) atom and form a stable bisthiolate with the 2nd Cu(II) site. This result presents additional in vitro evidence in support of the NADPH/thioredoxin reductase/thioredoxin/tyrosinase feedback mechanism for the inhibition of melanin biosynthesis.
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THE NEXT ESPCR MEETING WILL BE HELD IN UPPSALA, SWEDEN, DURING THE PERIOD 18-21 JUNE, 1989. THE FIRST ANNOUNCEMENT WILL BE DELIVERED DURING SUMMER