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Pheomelanogenesis in the Mouse: A Review of Its Genetic, Developmental and Morphological Features. W.C. Quevedo, Jr., R.D. Fleischmann, T.J. Holstein. Brown Univ., Providence, RI

Pheomelanin is synthesized within melanocytes of hair follicles in mice of appropriate Agouti (a)- or Extension (e)- locus genic constitution. Alleles at the a-locus program pheomelanogenesis by altering the internal environment of hair follicles whereas alleles at the e locus do so by direct action through the genome of the melanocyte. For example, although transplanted recessive yellow (e/e) melanoblasts synthesize pheomelanin within "eumelanic (a/a, B/B, E/E) hair follicles," dominant (A/y/-) lethal yellow melanocytes produce eumelanin. Injections of melanocyte-stimulating hormone induce pheomelanocytic melanocytes of A/y/- mice, but not those of e/e mice, to switch to eumelanin synthesis. Although it is generally maintained that pheomelanin synthesis is restricted to follicular melanocytes in mice, similarities between the ultrastructure of epidermal and follicular melanocytes in recessive yellow mice question this interpretation. A follicular melanocyte in agouti (A/A) hair follicles synthesizes both pheomelanosomes and eumelanosomes. Apparently, both types of melanosomes derive from a common multivesicular body-like structure which contains filaments as well as microvesicles. Filaments become the major component of the internal matrix in eumelanosomes whereas microvesicles predominate in pheomelanosomes. Microvesicles may serve to transport tyrosinase from the GERL/GOLGI complex where it has undergone glycosylation and, possibly, to isolate tyrosinase from inhibitory melanogenic substrates such as cysteine within the melanosome. Compared to eumelanocytes, tyrosinase activity is reduced in pheomelanocytes and its electrophoretic profile altered. At present little is known about how genes trigger pheomelanin synthesis and the extent to which the type of melanin synthesized influences melanosome morphology. The literature available suggests a tentative model for discussion.

Hormone Receptors and Vertebrate Pigment Cells. Mac E. Hadley, Christopher B. Heward, Victor J. Hruby, Tomi K. Sawyer, Young C.S. Yang. The Univ. of Arizona, Tucson, AZ

Melanocyte stimulating hormone (α-MSH, α-melanotropin) stimulates melanin synthesis within vertebrate epidermal melanocytes and melanoma cells and melanosome dispersion (translocation) within dermal melanophores. α-MSH mediates its action through pigment cell membrane receptors. α-MSH and related analogs have been prepared by organic synthetic methods. Structure-function studies of these α-MSH analogs have provided data on the
comparative aspects of melanocyte-melanophore receptors. Melanotropin analogs have been synthesized for use as radioligands for radioreceptor assays utilizing melanoma membranes. There is a calcium requirement for signal transduction between the melanotropin receptor and adenylyl cyclase. This is an MSH receptor-specific cation requirement since prostaglandin or beta adrenergic receptor stimulation of adenylyl cyclase is without such a requirement. Nucleotide, nucleoside, and metal ion modulation of MSH stimulated melanoma adenylyl cyclase activation has been characterized. MSH elevates cyclic AMP within melanoma cells and this results in tyrosinase activation and melanin production. Both of these processes are dependent upon transcriptional and translational events related to protein synthesis. a-MSH regulates melanosome dispersion within melanophores through cyclic AMP production. A model for melanosome translocation is provided wherein cyclic AMP mediates its effect through removal of cytosolic Ca2+ with resulting activation or deactivation of melanofilaments. Thus, Ca2+ plays a pivotal bifunctional and compartmentalized role in melanosome movements as regulated by MSH and other melanotropic agents.

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**Oxidation of Dopaquinone Addition Products.** H. Rorsman, C. Hansson, E. Rosengren, G. Agrup. University of Lund, Lund, Sweden

Dopaquinone is formed by the action of tyrosinase in the melanocytes. This compound is a most reactive intermediate which has not been isolated as such. Quinones are prone to react with nucleophilic compounds. Thiols are the most reactive nucleophiles in the cell. Cysteinyl-dopa and glutathionedopa are products formed by nucleophilic addition to dopaquinone.

Oxidation of tyrosine or dopa by tyrosinase in the absence of thiols leads to the formation of 5-OH-dopa, a compound previously unknown in melanogenesis. 5-OH-Dopa is produced by nucleophilic addition of water to dopaquinone. Water is a weak nucleophile but present in enormous amounts. 5-OH-Dopa, first detected as an unknown compound on high-pressure liquid chromatography (HPLC) of the dopa-tyrosinase incubate, was identified by comparison with the authentic compound in several chromatographic systems and by mass spectrometry. 5-OH-Dopa is a substrate for tyrosinase and the reaction can be monitored by HPLC by disappearance of 5-OH-Dopa.

Presence of thiols will limit or prevent the formation of 5-OH-Dopa. Instead thioethers of dopa are formed by nucleophilic addition of the SH-compounds. The presence of cysteine and glutathione in the cells favours this type of reaction. Under in vivo conditions the oxidation of cysteinyl-dopas is therefore of great interest in melanogenesis. 5-S-Cysteinyl-dopa is a substrate for tyrosinase. The reaction leads to the formation of 5-S-cysteinyl-dopaquinone which compound may react with a second nucleophile.

Oxidation of 5-S-cysteinyl-dopa by mushroom tyrosinase was studied by measuring 5-S-cysteinyl-dopa consumption by HPLC. A self-catalyzed oxidation induced by enzymatically formed 5-S-dopaquinone also takes place. The enzymatic oxidation of 5-S-cysteinyl-dopa is markedly accelerated in the presence of dopa. This can be explained by dopaquinone oxidation of 5-S-cysteinyl-dopa, which has a lower oxidation potential than dopa.

**Ion Exchange Studies in Melanins.** T. Sarna, W. Korytowski, M. Pasenkiewicz-Gierula. Institute of Molecular Biology, Jagiellonian University, Kraków, Poland

Natural and synthetic melanins were studied for their ion exchange properties. Electron spin resonance and Mössbauer spectroscopies were employed to characterize the spatial distribution, the molecular nature, and the geometry of the melanin metal ion complexes. The multivalent metal ion binding capacity of melanin was estimated by atomic absorption. The number and affinity of melanin binding sites to selected metal ions were determined by equilibrium binding studies.

The results show that depending on pH DOPA-originated melanins can form with cupric ions at least four complexes with the involvement of carboxyl, amine, and phenolic hydroxyl ligands. The ferric ions, on the other hand, appear to show more specificity in binding to melanin, forming predominantly one type of complexes. The formation of chelate complexes between melanin ortho-semiquinones and zinc (II) ions was studied in detail as a function of pH, metal concentration, temperature, and incubation time.

It can be concluded that melanin has a variety of metal ion binding sites randomly distributed
Malignant Melanoma—Abnormal Proteins Synthesized in Murine and Normal Tissues. Vincent J. Hearing, Amy E. Newburger, Thomas M. Ekel, Paul M. Montague. National Institutes of Health, Bethesda, MD

The presence of proteins unique to malignant tissues has been described in a variety of different tumor systems. An earlier study from our laboratory demonstrated the presence of abnormal proteins in human malignant melanoma; many of these proteins were electrophoretically identical to unique proteins described in the B-16 murine melanoma, both with regard to their molecular size and their charge [Arch Dermatol 113:19]. Some of these murine proteins have been more fully characterized and have been reported elsewhere [Cancer Biochem Biophys 4:59]. We have now studied melanoma tumor proteins from 11 different patients and have isolated several proteins from excised tissues by gel chromatography and by preparative gel electrophoresis, and subsequently characterized them by amino acid analysis. The data show that the amino acid contents of the proteins from all the human melanomas more closely resemble the corresponding proteins from the B-16 mouse melanoma, than they do those from the normal melanocyte. We have shown that the abnormal proteins in the mouse melanoma seem to result from the deletion of segments of primary structure from proteins normally synthesized in normal melanocytes. Our results suggest that the mechanism(s) responsible for the production of unique proteins in the mouse melanoma may be similar to those functional in the synthesis of unique proteins in the human melanoma. We have recently published data which suggest that these tumor-specific proteins may be detectable in the serologic fluids of melanoma patients [J Nati Cancer Inst 64:29]. Work is under way in our laboratory, not only to more completely elucidate the mechanism of formation of abnormal proteins in these neoplastic cells, but also to determine their possible immunologic import in the detection and/or control of human malignant melanoma.

Iodination of Melanotropin: Production of Biologically Active $^{125}$I-$\beta$MSH. Drew T. Lambert, Janos M. Varga. Yale Univ. School of Medicine, New Haven, CT

It has recently been reported [Heward et al: Biochem Biophys Res Comm 88:266–273, 1979; Heward et al: Hoppe-Seyler's Z Physiol Chem 360:1851–1859, 1979] that iodination of melanotropin (a- and $\beta$-MSH) by chloramine T results in extensive modifications of the peptides with concomitant loss of biological activity, leading these authors to suggest that "chloramine T-induced iodination of a- or $\beta$-melanotropin, using radioactive iodine, will not provide a labeled hormone derivative which is biologically active and suitable for radio-receptor assay." In contrast to the above studies, which employed a 10–100 fold molar excess of chloramine T over MSH, we have found that milder oxidation conditions followed by reduction with dithiothreitol (0.75M in borate-buffered saline, 37°C for 18–24 hours) result in a completely active labelled hormone, as measured by stimulation of tyrosinase in Cloudman S91 melanoma cells. We have investigated two milder procedures for iodinating $\beta$-MSH. The first was to simply use brief (15 sec) oxidation with levels of chloramine T equimolar to $\beta$-MSH and Na$^{125}$I. The second procedure employed a water-insoluble analog of chloramine T: IodoGen (1,3,4,6-Tetrachloro-3a, 6a-dihydropyralcoluril) at a 5:1 molar ratio (IodoGen: $\beta$-MSH). In both cases, biological activity was substantially reduced, but could be recovered by dithiothreitol treatment. These milder conditions do not label all the $\beta$-MSH molecules in the reaction mixture, but the iodinated material can be easily separated from native peptide by ion exchange chromatography, using DEAE Sephacel 25 and an ammonium acetate gradient (5→200 mM, pH 6.8). Supported by grants CA 26081 and 5 KO4 AI 00154 from USPHS.

Is Dynein Involved in Melanosome Movement? Masataka Obika, Sumiko Negishi, Kazuo Ogawa. Keio University, Yokohama, and National Center for Biological Sciences, Okazaki, Japan

Teleost fish melanophores respond to hormonal and nervous stimuli with rapid centrifugal or centripetal translocation of melanosomes and other intracellular vesicles. Dendritic processes of
these cells possess well-defined arrays of microtubules and centripetal transport of pigment granules is markedly impaired by treatments with antimitotic reagents. Total disruption of microtubules produces an extreme delay in pigment aggregation. The presence of actin microfilaments is observed only in limited species, and cytochalasin B has little or no effect on pigment translocation itself, although it induces a prominent change in cell shape. In *Oryzias* melanophores, it partially inhibits centrifugal, but not centripetal, movement of melanosomes when applied together with colchicine. These and other circumstantial evidences indicate that the rapid centripetal transport of pigment granules is dependent on microtubule system though microtubule-independent mechanism may also be involved in granular transport in general. It remains uncertain, however, if microtubules actually provide motive force for the movement.

Recently, we found that dynein isolated from trout spermatozoa is immunologically similar to that from sea urchin sperm axonemes. We therefore attempted to localize dynein in rainbow trout melanophores using ferritin-labeled antidynein antibody raised against sea urchin dynein 1.

**Do the Melanins from Blue and Brown Human Eyes Differ?** I.A. Menon, S. Persad, H.F. Haberman. Univ. of Toronto C.J. Kurian. Alpha Laboratories, Inc. P.K. Basu. Univ. of Toronto, Canada

Differences among the physical and chemical properties of melanins of diverse colors have been reported.

This investigation compares the properties of melanins isolated from blue and brown human eyes.

Human eye bank eyes were divided into two groups on the basis of the color of the iris: viz., blue and brown. The uveal tissues were pooled and homogenized with water. The supernatants after removal of debris were mixed with equal volumes of 12N HCl. The mixture was refluxed for 48 hrs. The protein-free melanin was washed with water and dried.

The melanin from blue eyes had a bluish tinge and that from brown eyes was brown. There was no significant difference between the UV-vis spectra of the two melanins. The *ir* spectra showed a characteristic major band in the region of 1,100 cm⁻¹ which was absent in dopa melanin. Red and black hair melanin showed a negligible absorption in this region. Two sharp bands were observed at about 2850 and 2950 cm⁻¹ which were also present in red hair melanin. Both blue and brown eye melanins showed the same composition of C, H, N and S. Protoporphyrin, chlorpromazine, and paraquat formed complexes with these melanins. Both melanins catalyzed a few oxidation-reduction reactions. No significant difference in their catalytic activity in the coupled oxidation of NADH and reduction of ferricyanide was observed. However, the brown melanin-protoporphyrin complex showed decreased activity in the oxidation-reduction reaction as compared with blue melanin complex.

These studies show that although there are some differences in the physical and chemical properties of these melanins, they are similar in most respects.

**Melanin-Water-Ultrasound Interactions: Switching and Memory Effects and Their Relation to Cytotoxicity.** R. Kono, H. Yoshizaki, T. Yamaoka. Defense Academy, Japan Y. Miyake, Y. Izumi. Hokkaido Univ., Sapporo, Japan J.E. McGinnness, M.D. Anderson Hospital, Houston, TX

A certain amorphous polymer, melanin, which is contained in a human tumor is capable of absorbing ultrasound in the 1 MHz range and converting this energy into a form which kills the tumor cell. A significant sound absorption is found in diethylamine (DEA) melanins for a hydration of 20 wt percent around 1 MHz between 40° and 60°C. By this experimental evidence it has been proposed that the absorption of energy from the ultrasound (phonon) is the initial step of the cytotoxic events and that the melanosome converts the phonon energy into cytotoxic products. A detailed survey on the absorption mechanism undoubtedly yields a useful means of treating this particular tumor.

A large absorption is found in the hydrate with different pH values ranging from 2 to 12. A melanin modified by drug and dopa melanin suspended in water also have significant absorption. The magnitude of absorption depends on the pH value, water content, and degree of order associated with spatial molecular structure essentially defined in stacking of indole monomer unit. The absorption is assigned to a stochastic resonance characterized in radiationless transition rate S₂₋₂.
Synthetic melanins and melanin in melanosomes exhibit the unusual features of an amorphous semiconductor switch together with memory effect. Both of these effects can be interpreted in terms of the three energy states. If the applied frequency of ultrasonic wave matches to $S_{32}/12$, the switching happens to cause electric breakdown with powerful phonon radiation; this is hypersound at a Debye frequency which kills the cancer.

**Electret State and Hydrated Structure of Melanin.** M. Bridelli, R. Capelletti, P.R. Crippa. Institute of Physics, University of Parma, Italy

All known solid state properties of melanins (as threshold switching, absorption and dispersion of sound waves, electrical and photo-conductivity and charge and/or polarization storage) strongly depend on their water content. Therefore, a tentative explication of the biological role of melanins in their natural state by means of physical models requires the proper consideration of the hydration degree in order to better reproduce the physiological conditions. On the other hand, due to the polyelectrolyte character and the complex structure of the melanin random polymer, a functional relationship with the hydration structure may be reasonably inferred.

We performed experiments with the TSDC method (Thermally Stimulated Depolarization Currents) on bovine eyes and L-dopa synthetic melanin in order to determine the state of the water bound to the macromolecule. Our results can be summarized as follows:

- Natural melanins in the pigment epithelium-choroid complex are natural electrets at physiological temperature;
- Measurements on synthetic melanin confirm this behavior and reveal a clean signal due to the temperature-induced release of water;
- The calculations of the reorientation energy for water dipoles from TSDC peaks suggest the presence of both an "easy water" and an ice-like "hard water";
- On these bases we may hypothesize a model of hydration structure for melanin suitable for the interpretation of the solid state physical properties.

**Electronic Excited Molecules in the Formation and Degradation of Melanins.** D. Sławinska, J. Sławinski. Institute of Physics and Chemistry Univ. of Agriculture, Poznan, Poland

Generation of electronically excited molecules, part of which deactivate by the light emission (chemiluminescence CL) in model systems relevant to the melanogenesis and melanin oxidation, has been established. Light emission was observed when melanins' precursors: L-DOPA, adrenaline, adrenolutin, and adrenochrome in aqueous solutions were undergoing oxidative polymerization *in vitro* in three classes of model reactions: 1. photooxidation with UV or visible light, 2. autooxidation (O$_2$, pH > 7.5), and 3. peroxidation with hydrogen peroxide in the presence of catalysts (peroxidase, cytochrome c, hemin, ferricyanide, Mn (II)). In the same reactions synthetic melanins also exhibit CL.

The spectral distribution and the total quantum yield of CL (in some cases also the quantum yield of excitation) were measured. On the basis of CL kinetics and spectra, absorption and fluorescence spectra, thermochemical calculations as well as literature data, it has been shown that the elementary exergonic reaction of chemiexcitation, common for majority of the processes, is the oxidative opening of the six-membered ring of indoloquinone. In the case of adrenaline and its analogues, the transfer of excitation energy from primary products to strongly fluorescing hydroxyindoles, e.g., adrenolutin, occurs (sensitized CL). There are also some data indicating the formation of excited singlet molecular oxygen and its CL.

Physiological implications of the generation of excited molecules and the so-called "photochemistry without light" for melanin biosynthesis and degradation are discussed.

The work was performed under project PAN 03.10.

**Quantitative Approaches to Melanin Structure.** Messod Benathan, Hugo Wyler. Institut de Chimie organique, Université de Lausanne, Switzerland

There is a lack of reliable criteria for the characterisation and comparison of melanins. In order to arrive at a meaningful stoichiometry one ought to combine the analytical data in such a way that gravimetric uncertainty which is mainly due to sample conditioning cancels out. Prolonged hydrolytic treatment of melanosomes as usually practised is shown to be unneece-
sary. An interesting relationship was found to be the ratio of the two moderate and weakly acidic functions present in both natural and synthetic melanins. This is measured by a special titration procedure in presence of p-hydroxybenzoic acid. The ratio in natural melanins (~1.1), i.e., hydrolysed melanosomes of sepiia ink or bovine iris, differs from that found with autoxidative (~1.7) or enzymatically (~0.5) prepared dopa-melanins.

The main defect in melanin chemistry is the fact that so far no oligomeric fragment has been seized on degradation which might have served as structural evidence. The tiny amounts of pyrrolic and indoic acids found have never given satisfaction. In studying the stoechiometry of oxidative degradation of melanins using periodate and permanganate, a rational relationship is found which is not in contradiction with a polyindolic texture. About 25 percent of the originally contained carbon is recovered in form of oxalic and oxamic acids (4:1) and less than 1 percent in form of a mixture of pyrrolic acids. A close analogy is noticeable between dopa-melanin and melanin from sepiia ink melanosomes.

**Binding of Protoporphyrin to Melanin and Oxidation-Reduction Properties of Melanin-Protoporphyrin Complex.** H.F. Haberman, S. Persad, I.A. Menon. Clinical Sciences Division, University of Toronto, Toronto, Ontario, Canada

Several drugs have been reported to be found to melanin *in vivo* and *in vitro*. Some of these drugs are photosensitizers. An important role of melanin in skin is considered to be protection against ultraviolet radiation. Melanin catalyzes several oxidation-reduction reactions. It has been suggested that these oxidation-reduction properties of melanin may have a role in the protective action of melanin. This report deals with the binding of a biological photosensitizer, protoporphyrin, to melanin and the effects of the binding of protoporphyrin on the oxidation-reduction properties of melanin.

Melanin was isolated from human black hair. To study the binding of protoporphyrin to melanin, suspensions of melanin were incubated with varying concentrations of porphyrin. The melanin was separated by centrifugation and then washed several times with the same buffer. The amount of porphyrin in the supernatant and washings were determined by fluorescence. The porphyrin bound to melanin was extracted with 3 N HCl, and the fluorescence of the extract was determined. The activity of the melanin-porphyrin complex to catalyze the coupled oxidation of NADH and reduction of ferricyanide was determined.

The results showed that protoporphyrin was bound to the melanin under these conditions. Calculations based upon the Langmuir’s Isotherm showed that 2.5 μg of the porphyrin was bound per mg of melanin. The melanin-porphyrin complex had less reaction activity in catalyzing the oxidation reduction. When 100 μg/ml melanin was incubated with 0.1 and 2.0 μg/ml porphyrin, the oxidation of NADH was decreased by 31 percent and 53 percent respectively.

These results indicated that the binding of natural photosensitizers such as porphyrins, as well as other metabolites or drugs, to melanin *in vivo* might affect the activity of these compounds as well as the protective action of melanin against ultraviolet radiation.

**Biochemical Activities of Melanin: Inactivation of Enzymes.** Atsushi Oikawa, Michie Nakayasu. Research Inst. TB and Cancer, Tohoku Univ., Sendai, and Natl. Cancer Center Res. Inst., Tokyo, Japan

Melanin synthesized *in vitro* inactivates various enzymes, including muscle lactate dehydrogenases of skeletal and heart muscles, pancreatic ribonuclease, and deoxyribonuclease I. However, heart lactate dehydrogenase, Lactobacillus leichmannii D-lactate dehydrogenase and Bacillus subtilis α-amulase are highly resistant to melanin.

The enzyme-inactivating activity of melanin decreases slightly on its aging, but is not due to “nascent melanin,” since aged preparations and those prepared from melanoma tissue by refluxing in 6N hydrochloric acid still retain activity.

The inactivation of enzymes by melanin seems to be due to irreversible, rather unspecific binding of melanin with the enzyme protein, because (1) bovine serum albumin prevents this inactivation, but does not reactivate already inactivated enzymes, and (2) an enzyme which was susceptible to melanin was co-precipitated with it on high-speed centrifugation, while one that was resistant was not.

The susceptibilities of enzymes to melanin was not related to their enzymological, chemical, or physico-chemical properties so far documented, or to the source of these enzymes.
The Reactivity of Melanin with Oxygen. W. Korytowski, T. Sarna. Institute of Molecular Biology, Jagiellonian University, Kraków, Poland

Under certain conditions melanin exhibits substantial chemical reactivity. Of particular interest may be its ability to interact with molecular oxygen. The effect of melanin on oxygen consumption was studied as a function of pH, illumination by visible and UV light, temperature, the presence of proteins, and the type of natural and synthetic melanins.

Each factor affected the rate of oxygen consumption in a complex way indicating the role of phenolic subunits of the melanin polymer. The data also indicated a correlation between the rate of oxygen consumption and the concentration of the induced free radicals in melanin. Spectral characteristics of the photo-dependent melanin paramagnetism and oxygen consumption revealed significant similarities with the quantum yield rapidly increasing in the UV region. These results strongly suggest the involvement of the extrinsic free radicals of melanin in the interaction with oxygen.

The protein component of the natural pigment decreases the reactivity of the polymer with oxygen. However, the rate of oxygen consumption, in the presence of natural melanin, greatly increases after illumination by UV or visible light and/or after raising the pH.

The role of reactive, intermediate products of the interaction of melanin with oxygen (such as superoxide radical, hydrogen peroxide, and singlet oxygen states) is also briefly discussed.

Donor-Acceptor Properties of Melanins in the Light of New Electrochemical and Electron Spin Resonance (ESR) Data. K. Reszka, Z. Matuszak, J. Dobrucki, S. Lukiewicz. Jagiellonian University, Kraków, Poland

The simultaneous electrochemical-electron spin resonance (SEESR) method is a technique which makes it possible to reduce or oxidize electrochemically the investigated sample with simultaneous examination of its free radical content.

A recent application of this methodical approach to the study of redox and paramagnetic properties of natural and synthetic melanins and melanoproteids has brought direct evidence that melanins are electrochemically active and that their electroreduction and electrooxidation proceed via two single-electron steps.

The present paper summarizes further investigations along this line which have shown that electrochemical treatment affects not only "quantitative" but also "qualitative" parameters of the ESR spectrum of melanins.

The observed changes in the g-value and shape of the ESR signals suggest that the electrochemically induced paramagnetic centres are different from the native ones.

The reversible decrease and increase in the ESR signal amplitude occurring during electroreduction and electrooxidation indicate that all the types of examined melanins are able to accumulate or release electrons, revealing in some cases a huge donor-acceptor capacity.

The possible role of these findings in biological regulative processes is discussed.

Molecular and Cellular Basis of Different Radiosensitivity in Pigmented and Non-Pigmented Hamster Melanoma Cells. S. Łukiewicz, B. Pilas, J. Nowicka, K. Cieszka, R. Gurbiel. Jagiellonian University, Kraków, Poland

Experimental data are presented which indicate that the content of melanin in plant and animal cells can in some cases be correlated with their radiation response: the greater the amount of pigment, the higher the level of radioreistance. Similar relations were shown to be valid for two lines of Bomirski hamster melanoma differing in their pigmentation, the melanotic form being twice as resistant to X-rays as its amelanotic counterpart.

Using electron spin resonance spectroscopy, the work analyses some of the factors responsible for this difference in radiosensitivity, for example: (a) scavenging activity of melanins, (b) interactions of melanin with oxygen.

At the cellular level an important feature characterizing melanotic cells was found: this was a very high rate of oxygen consumption, roughly twice exceeding that of amelanotic ones. This property could be associated with at least two additional oxygen utilizing processes, absent in non-pigmented cells. As a result, amelanotic cells have less ability to counteract efficiently the local accumulation of oxygen, whereas pigmented cells must naturally tend toward creating an atmosphere of oxygen deficiency in their interior and close surroundings. This seems to be another, previously unknown, factor, apart from the scavenging capacity of melanins, which
might account for the difference in response to low LET radiations of pigmented and non-pigmented melanoma. Such a difference is not observable in the case of fast neutrons.

Laboratory Tests and Clinical Trials on Chemical Radiosensitization of Pigmented Cells (Including Hamster and Human Melanoma). S. Łukiewicz, H. Mach, E. Link, Z. Maciejewski, B. Pilas, J. Nowicka. Jagiellonian University, Kraków, and Medical Academy, Kraków, Poland

Attempts were made to verify experimentally the efficacy of “radio-chelation therapy,” using non-living model systems and pigmented plant, animal, or human cells (including melanoma). This approach is based on the assumption that melanins are engaged in the activity of cellular regulatory mechanisms, comprising a system of “endogenous radioprotectors,” and that chemical interference with the functions of this system may produce a decrease in a usually high radioresistance.

“Radio-chelation therapy” simply means a combined action of radiations and chelating agents as radiosensitizers. Among the latter compounds a number of drugs are known which inhibit or at least reduce melanogenesis, most probably by binding \( \text{Cu}^2+ \) ions.

Several plant and animal organisms, if grown in the presence of chelators, may develop as entirely non-pigmented forms. This is followed by a drastic radiosensitization. The “depigmentation” of melanotic tumours in situ by a similar treatment can, however, hardly be achieved. The paper describes the results of investigations indicating that some of the tested chelating drugs can sensitize pigmented tumours to low LET radiations in both animal systems (hamster melanoma) and humans (choroidal melanoma). It also demonstrates, on the basis of the electron spin resonance data, that the sensitizing action of chelators may be due to the depression of oxygen consumption of melanotic cells brought about by these compounds.

New Radioactive Drugs Selectively Accumulating in Melanoma Cells. Ewa Link, Stanisław Łukiewicz. Jagiellonian University, Kraków, Poland

The possibility of utilizing the selective sorption of radioactive compounds on melanins for therapeutical purposes was reexamined in spite of rather discouraging conclusions from earlier trials of other authors with the use of \( ^{35}\text{S}\)-chlorpromazine.

The rationale for applying this kind of treatment is based on the assumption that endoirradiation of pigmented tumors with \( \alpha \) - or \( \beta \)-rays emitted from the incorporated radionuclide can be intense enough to produce effects of therapeutical value.

In order to make such effects as strong as possible, a number of phenothiazine derivatives less toxic than chlorpromazine were selected and tested for their strength of binding to melanin.

It was found that both the isotopically labelled compounds chosen in this way and their inactive counterparts accumulate in pigmented melanoma cells in substantial amounts. The biological action of the incorporated \( ^{35}\text{S} \) was then evaluated \textit{in vitro} and \textit{in vivo} by watching the number of cells and kinetics of tumor growth.

Brief, repeated, \textit{in vitro} incubations of melanoma cells in \( ^{35}\text{S}\)-carrier containing medium brought about the suppression of mitotic activity within 150 hr, whereas the same concentration of an inactive carrier did not affect normal proliferation.

If the cells were in contact for 3 hr with \( ^{35}\text{S}\)-carrier in non-toxic concentration before reimplantation into the skin, a clear-cut correlation between \( ^{35}\text{S} \) radioactivity and the capacity of growth initiation could be found.

The growth of melanoma in situ could also be delayed by injecting the \( ^{35}\text{S}\)-carrier to tumor-bearing animals.

Role of Melanosomes in the Binding Mechanism of Chlorpromazine (CPZ) to Melanoma Cells. Takafumi Nakanishi, Masamitsu Ichihashi, Yutaka Mishima. Kobe University School of Medicine, Kobe, Japan

In 1965, Ilois reported that intraperitoneally administered CPZ to melanoma-bearing mice accumulated in the eye and melanoma. He postulated that CPZ combined with melanosomes to form a charge-transfer complex, and selectively incorporated into melanoma cells. However, direct analysis of the binding mechanism of CPZ to melanoma cells with respect to their melanogenic activity or melanosome content has not been studied.
Selective Thermal Neutron Capture Therapy of Malignant Melanoma Using Its Specific Melanogenic Activity. Yutaka Mishima, Masamitsu Ichihashi, Takaumi Nakanishi, Masayuki Tsuji. Kobe University School of Medicine, Kobe, Japan

Using accentuated melanogenesis in malignant melanoma, we have been establishing selective thermal neutron capture therapy. We have synthesized 7 melanoma-seeking 10B-compounds. Among them 10B2,-chlorpromazine (10B2,-CPZ) and 10B1,-para-boronophenylanine (10B1,-BPA) have been found to be highly effective in killing melanoma cells by 10B(n,a)7Li reaction. Our previous results lead us to clarify the further three lines of research.

I. Establishment of in vitro radiobiological analysis of the specific killing effect of various 10B-compounds: 10B2,-CPZ and 10B1,-BPA preincubation have shown marked enhanced killing effect on melanoma cells with D0 values of 0.9 × 1017 n/cm2 and 0.9–1.2 × 1017 n/cm2, respectively. D0 of thermal neutron alone is 2.8 × 1017 n/cm2. 10B2,-CPZ and 10B1,-BPA can concentrate 10B into melanoma cells as much as 4.5 and 11 times the 10B concentration in the medium.

II. In vivo evaluation of melanoma therapeutic effect: Greene's melanoma-bearing hamsters treated with 10B-compounds and neutron irradiation exhibit almost complete inhibition of tumor growth for 16–18 days, while neutron alone gives only an average of 6 days. These melanomas show only 4 days' growth suppression even after 5,000 rad of X-ray.

III. Affinity of 10B-compounds to melanoma cells—Quantitative analysis: Using a new chemical assay (Kakihana et al., 1980), it is found that 28 hours after 10B1,-BPA·HCl ip-injection into melanoma-bearing hamsters, the melanoma-to-blood ratio of 10B becomes 11.5 and the melanoma-to-liver ratio is 15. This affinity is approximately parallel to the melanogenic activity of the tumor.

Further an LD50 of 10B1,-BPA·HCl obtained by iv-injection into mice is 840 mg/Kg, indicating a low acute toxicity. We have also carried out preclinical therapeutic experiments using spontaneously occurring malignant melanoma in Duroc pig skin.

Radiosensitivity of Hamster Melanoma to Fast Neutrons. K. Cieszka, B. Łazarska, R. Gurbieł, E. Tyralaska, S. Łukiewicz. Jagiellonian University, Kraków, and Institute of Nuclear Physics, Kraków, Poland

The radiosensitivity of pigmented and non-pigmented cells to fast neutrons was compared under in vitro and in vivo conditions using Bomirski hamster melanoma.

The cells were irradiated either immediately before or on the next day after reimplantation into the skin. In both cases a total dose of 2, 3, or 4, 5 Gy was delivered at a single exposure. A volume of 0.2 ml of cell suspension in MEM Eagle's solution per sample was placed in sealed cylindrical plastic tubes for irradiation. During in situ exposures the dose was distributed over the whole body of the animals.

In a separate series of experiments a fractionation into two equal subdoses, given with a one-week interval, was additionally adopted.

The growth curves of the tumours indicate that the applied doses were high enough to bring about measurable changes in tumour mass, did not kill the animals before 30 days, and inhibited the development of both pigmented and non-pigmented melanoma to a similar extent. It should be pointed out here that these two lines of hamster melanoma distinctly differ in their response to low LET radiations, as previously shown in this laboratory. The results of present...
fast neutron experiments seem to suggest that the above-mentioned difference in radiosensitivity of melanotic and amelanotic cells may be determined by a greater contribution of oxygen effect to radiation damage of non-pigmented cells.

Microheterogeneity of Tyrosinase. Kazuhiro Miyazaki, Noriko Ohtaki. Tokyo Medical and Dental University, Tokyo, Japan

Multiple forms of tyrosinase have been reported in mammalian melanomas since 1950s. We have reported that the difference in isoelectric points of both T1 and T2 tyrosinases are mainly ascribable to their different contents of carbohydrate residues, especially sialic acid groups. At the same time, we strongly suggested that T1 tyrosinase was acidic glycoprotein containing sialic acid groups that could be removed by neuraminidase, and that it also contained presumably neutral sugars which were not attacked by neuraminidase. While our purified T1 tyrosinase sample shows only a single activity, the affinity chromatography ConA-Sepharose have fractionated the absorbed and unabsorbed groups.

The present report is some further characterization of the carbohydrate content, especially neutral sugars, of mouse tyrosinase as glycoprotein.

Inactivation of Tyrosinase by Dopa. Y. Tomita, A. Hariu, C. Mizuno, M. Seiji. Tohoku University School of Medicine, Sendai, Japan

Tyrosinase in a melanosome is known to be inactivated during melanin formation in vivo, and a similar inactivation was observed in vitro when melanosomes isolated from Harding Passey mouse melanoma were incubated with dopa. Tyrosinase, whether particle-bound or in soluble form, was inactivated during the dopa-tyrosinase reaction and the reduction rate of its activity was proportional to the reaction time. When ascorbic acid was added to the reaction system, in which dopaquinone, an oxidation product of dopa which is immediately converted back to dopa by ascorbic acid thus preventing melanin formation, tyrosinase inactivation also occurred. When "C-dopa or "C-ascorbic acid were added to the reaction mixture, these radioactive substances were not recovered from the inactivated enzyme protein fraction after incubation. In addition this inactivation of tyrosinase by dopa was not inhibited by any of: 1,4-diazabicyclo[2.2.2]octane, scavenger for singlet oxygen; D-mannitol, that for hydroxyl radical; superoxide dismutase, that for superoxide anion; and catalase, cleaving enzyme for hydrogen peroxide. Thus the inactivation of tyrosinase appears to be due to neither these radicals, nor reaction products from dopa or ascorbic acid, but to changes in the enzyme itself.

Phaeomelanogenesis in the Absence of Tyrosinase. Shosuke Ito, Keisuke Fujita. Institute for Comprehensive Medical Science, School of Medicine, Fujita-Gakuen University, Toyoake, Aichi, Japan

The first step in the biosynthesis of phaeomelanin is the tyrosinase-catalyzed formation of cysteinyl dopas from dopa and cysteine or glutathione. Oxidation of dopa in the presence of cysteine with mushroom tyrosinase produces 5-S-cysteinyl dopa (I), 2-S-cysteinyl dopa (2), and 2,5-S,S-dicysteinyl dopa (3) in 15:3:1 ratio, which is consistent with the biosynthesis in vivo. We have studied whether the formation of cysteinyl dopas can be promoted by peroxidase-H2O2, superoxide radical, and oxygen in the presence of metal ion.

By peroxidase-H2O2. Incubation of dopa (10 mM) and cysteine (20 mM) at pH 6.8 with peroxidase-H2O2 (10 mM) afforded I, 2, and 3 in yields of 13, 4, and 6 percent, respectively, with a 60 percent recovery of dopa. Oxidation of I under similar conditions produced 3 in 20 percent yield with a 47 percent recovery of I. Additional products in these reactions were the two isomeric dihydrobenzothiazine derivatives of 3 and 2,5,6,S,S,S-tricysteinyl dopa.

By superoxide radical. Formation of cysteinyl dopas was also mediated by superoxide radical generated by the action of xanthine oxidase on hypoxanthine at pH 7.4. 20 µM of I and traces of 2 and 3 were produced from dopa (500 μM) and cysteine (1,000 μM) in the first 5 min of the reaction with superoxide radical (initial rate: 21 µM/min). The similar reaction of I yielded 3 at an initial rate of 2.2 µM/5 min.

By oxygen in the presence of metal ion. Formation of cysteinyl dopas by autoxidation was negligible in the absence of metal ion. However, the conjugation reaction was effectively catalyzed by Fe2+ and Fe3+. Air-oxidation of dopa (500 μM) and cysteine (1,000 μM) at pH 7.4, catalyzed by Fe2 or Fe3 (50 μM) in the presence of EDTA (500 μM), afforded 35-40 μM of
Chemical Induction of Pheomelanogenesis in Vivo. M. Nishimura, S. Hoshino, G.A. Gellin, K. Fukuyama, W.L. Epstein. Univ. of California, San Francisco, CA

Previously we reported (Clin Res 28:569A, 1980) that an increase of pheomelanosomes and a decrease of eumelanosomes occur in tissue-cultured human melanoma cells after treatment with 4-tertiary butyl catechol (TBC), an antioxidant which causes depigmentation in man. In order to further elucidate the chemically induced pheomelanogenesis we conducted in vivo studies.

USCD strain hairless mice which have naturally pigmented ears and a white flank were used. Pigmentation was induced on flank skin by UVB irradiation twice a week for one month. 1 M TBC dissolved in DMSO-acetone was topically applied on the flank and ear 3 times at 48 hr intervals. Control mice were treated with DMSO-acetone.

Biopsies taken from skin sites 24 hr after the last treatment were processed for electron microscopy. Melanocytes were photographed at 60,000 X and melanosomes in 10 melanocytes/mouse for each experimental group were classified as eu- or pheo-melanosomes or undetermined, according to the description by Jimbow et al. (J Invest Dermatol 73:278, 1979) and the number of melanosomes compared statistically. Stage I melanosomes were excluded.

| Sites               | melanosomes counted | Eumelanosome (%) | Pheomelanosome (%) | Undetermined (%) |
|---------------------|---------------------|------------------|--------------------|-----------------|
| Ear (control)       | 110                 | 74.55 ± 10.05    | 11.75 ± 1.45       | 13.70 ± 8.60    |
| Ear (TBC treated)   | 98                  | 34.70 ± 3.20     | 35.75 ± 0.15       | 29.55 ± 3.35    |
| Flank (control)     | 176                 | 83.60 ± 0.30     | 53.35 ± 1.75       | 11.05 ± 1.45    |
| Flank (TBC treated) | 172                 | 41.70 ± 6.40     | 31.30 ± 2.50       | 27.00 ± 3.90    |

The data showed that TBC application induces an increase in pheomelanosomes in both naturally pigmented and UVB-induced pigmented skin (p < 0.02).

The findings confirm the in vitro study and suggest that the two types of genetically determined melanogenesis can be modified chemically.

Dynamics of 5-S-Cysteinyldopa Production in Cultured Melanoma Cells. Manoj Mojamdar, Masamitsu Ichihashi, Yutaka Mishima. Kobe University School of Medicine, Kobe, Japan

The role of 5-S-cysteinyldopa, a sulphur-containing amino acid believed to be produced only in the melanocytes, has been studied in cultured melanotic melanoma cells in normal and tyrosine, cysteine and glutathione free media. 5-S-cysteinyldopa has been found to be greater in the exponential phase as compared to plateau phase. Greene's melanotic melanoma cells showed a dopa concentration dependent 5-S-cysteinyldopa production. This dopa dependent 5-S-cysteinyldopa production has been found to be inhibited by low temperature and sodium diethylthiocarbamate, a tyrosinase inhibitor. In the presence of dopa and cysteine, large amounts of 5-S-cysteinyldopa have been found also in cell-free media. However, in the presence of glutathione and dopa only cell-cultured media have shown large amounts of 5-S-cysteinyldopa in contrast to none in cell-free media. The dopa and glutathione dependent 5-S-cysteinyldopa production in cells has been found to be inhibited by iodoacetamide, a specific inhibitor of r-glutamyl transferase. Further selective incorporation of radioactive dopa and glutathione into 5-S-cysteinyldopa and melanin gives an understanding of the dynamics of 5-S-cysteinyldopa in different types of melanogenesis.

Methionine and Pigmentation. Ch. Aubert, F. Rougé, J-R. Galindo, C. Hansson, H. Rorsman, E. Rosengren. I.N.S.E.R.M., U. 119, Marseille, France; University of Lund, Lund, Sweden

Pigmented and non-pigmented melanocytes obtained by culture of primary and metastatic malignant melanomas were studied with different amounts of methionine in the culture medium.

The dedifferentiation to fibroblast-like cells, the differentiation to melanocytes, and the establishment of permanent cell lines were studied by electron microscopy and by determination of the melanocyte metabolite 5-S-cysteinyldopa (5-S-CD).
The dedifferentiation during the first subcultures was less pronounced in a medium containing less methionine. Redifferentiation to a melanocyte line was more easily obtained in methionine-poor medium. The amount of 5-S-CD secreted by the melanocytes was less in methionine-poor medium.

The higher amount of methionine in tissue culture medium facilitates dedifferentiation and prevents redifferentiation to melanocytes. The higher amount of 5-S-cysteinylidopa in melanocytes cultured in medium rich in methionine may reflect the differentiation of the melanocytes but may also be due to a more direct interference with melanogenesis.

**Pheomelanin in Albino Negroses.** R. Aquaron, F. Rougé, Ch. Aubert. University of Marseille, I.N.S.E.R.M., Marseille, France

Human oculocutaneous albinism is an hereditary disease frequent in the black subjects living in the Cameroons. It occurred in a pathological pathway in biosynthesis of melamins.

The urinary 5-S-cysteinylidopa (5-S-CD) excretion in the black and albino (20) children and adult Cameroons subjects was studied. Cutaneous fragments and hair bulb were processed for electron microscopy.

The albino children had much higher amounts of 5-S-CD (5,800 nmol/ml ± 2000) than the black children (160 nmol/ml ± 84) (S > 0.001). The albino adults had higher amounts (4,420 nmol/ml ± 980) than the black adults (1,070 nmol/ml ± 200) (S > 0.01).

However, one population of black subjects had high amounts of 5-S-CD.

Two families presenting both black and albino subjects were studied for 5-S-CD excretion. Ultrastructural examination showed modified melanosomes decreased in number in albino Negroses.

Further observations are necessary to study the existence of black heterozygotes with a high 5-S-CD amount and the mechanism(s) of 5-S-CD excretion in the black oculocutaneous albinism.

**Structural Analysis of Phaeomelanin from Yellow Mice.** Sumu Matsueda, Chikako Nebuya, Takuji Takeuchi. Hirosaki University, Hirosaki, and Tohoku University, Sendai, Japan

Phaeomelanin was isolated from the hairs of genotypically yellow mice (C57BL/6J-A') and analyzed. We confirmed that the yellow pigment was derived from cysteinylidopa as shown in other organisms (Prota, 1972). Phaeomelanin was extracted by saturating the hairs with 0.1N NaOH followed by hydrolysis with boiling HCl. A lower molecular weight fraction (Y1, Ikejima and Takeuchi, 1978) was isolated by gelfiltration with 0.1N NH4OH on Sephadex G15 and G25, respectively. The spectral data on Y1 were as follows:

\[ \text{IR}_{\text{cm}^{-1}}: 1680 \text{ and } 1660 \text{ (o-quinonoid); } 840 \text{ (2, 6-position 2H); } 700 \text{ (pc-s).} \]

\[ ^{1}H-\text{NMR (D}_{2}O, \text{ ppm}): 2.50 \text{ (S-CH}_{3}); 3.24 \text{ (Ph-CH}_{2}); 3.93 \text{ (}-\text{CH(NH}_{2})_{2}-\text{COOH); } 8.08 \text{ and } 8.98 \text{ (o-quinonoid 2, 6-position 2H).} \]

\[ \text{MS:m/e Amine fragment cource: } 240 \text{ (M}^{+}\text{-CH(NH}_{2})_{2}\text{COOH); } 207 \text{ (240-}\text{NH}_{2}\text{OH). Carboxylic acid cource: } 253 \text{ (M}^{+}\text{-NH}_{2}\text{COOH); } 165 \text{ (253-CH}_{2}\text{CH(NH}_{2})_{2}\text{-COOH); } 152 \text{ (165-CH). Ringelose cource: } 241 \text{ (M}^{+}\text{-CO}_{2}\text{NH}_{2}\text{CH); } 225 \text{ (241-NH}_{2}); 153 \text{ (225-CH}_{2}\text{CHCOOH); } 211 \text{ (225-CH}_{2}). \]

These results indicate that Y1 is 5-S-cysteinylidopaquinone, and that mouse phaeomelanin contains cysteinylidopa derivative.

**Biological Significance of Kynurenine in Rat Hair.** Hitoshi Takahashi, Yoshiko Fukushima. Toxicology Institute, Kumamoto University Medical School, Kumamoto, Japan

In 1957 a large amount of kynurenine (KYN) was found as a fluorescent substance in albino rat hair by Rebell. The significance of this KYN was one time thought to be an interruption of its hydroxylation to ommochrome pigment which might be related in some way to phaeomelanin melanogenesis. When L-KYN was added in vitro to the system of L-dopa and mammalian tyrosinase (Harding-Passey mouse melanoma) or polyphenol oxidase (mushroom), KYN was less recovered from the incubation mixture as the melanogenesis proceeded. In vivo, however, the concentration of KYN in hair was almost the same between Wistar albino and DA agouti rats. In the HO (hooded) rats there was no difference in KYN concentration between pigmented and non-pigmented hairs, indicating that KYN excretion and melanogenesis might be processes
Correlation Between Glutathione Levels and Types of Pigmentation in Guinea Pig Skin. J.P. Benedetto, J.P. Ortonne, C. Voulot, G. Prota. FRA INSERM N° 11 and INSA—Lyon, France; University of Naples, Italy

In recent years, it has been clear that in addition to tyrosinase, glutathione reductase could be involved in the regulation and control of melanogenic activity of melanocytes. Indirect evidence came from the discovery of the phaeomelanin pathway as well as from more recent studies showing the occurrence of cysteinyldopa derived units in brown and even black eumelanin type pigments from mammals.

The role of glutathione reductase in mammalian pigmentation has been investigated. We report biochemical studies on the correlation between glutathione levels and different types of pigmentation (white, black, brown, and yellow) in guinea pig skin. Reduced (GSH) and oxidized (GSSG) glutathione levels were measured by a specific fluorometric method.

The lowest levels of reduced glutathione (GSH) were found to be associated with eumelanin type pigments whereas the highest levels were associated with phaeomelanin type pigments. White skin which contains no active melanocytes gave intermediate GSH levels.

Analysis of the ratio GSH/GSSG and GSH levels, reflecting mostly the glutathione reductase activity, suggests that the level of the enzyme in the environment of melanocytes could influence strongly their melanogenic activity.

Some New Aspects of Melanogenesis in Cephalopods. G. Prota, J.P. Ortonne, C. Voulot, C. Khatchadourian, G. Nardi, A. Palumbo. University and Stazione Zoologica, Naples, Italy; FRA INSERM N° 11 and INSA, Lyon, France

Since the work of Szabo et al., the ink-gland of cephalopods has been recognized as the most perfect model for the study of melanogenesis. However, very little further attention has been devoted to this organ.

In connection with a study on the structure and biosynthesis of adrenochrome, a most unusual type of phaeomelanin pigment found in octopus species, we have reexamined some aspects of melanogenesis in the ink gland of cephalopods.

Biochemical evidence is presented that, in addition to melanin, the ejected ink of the cephalopods, Octopus, Sepia, and Loligo, contains substantial amounts of free tyrosinas in a relatively pure form. Despite differences in molecular size and electrophoretic mobilities, the kinetic properties of the cephalopod enzymes paralleled those of tyrosinas from other sources.

The occurrence of free tyrosinas in the ink coupled with a reexamination of the morphological events accompanying melanogenesis throw new lights on the biological and physiological significance of the cephalopod ink.

Post Tyrosinase Regulation of Melanogenesis in Extracts of Newborn and Fetal Mouse Skins. M. Murray, J. Pawelek, A. Kroner. Yale Univ. School of Medicine, New Haven, CT

Efforts in our group have recently been directed toward characterizing three factors that appear to regulate melanogenesis at points distal to tyrosinase activity in Cloudman melanoma cells (Korner and Pawelek, J Invest Derm; Pawelek et al., Nature; 1980, in press). Dopachrome conversion factor (DCF) catalyzes the conversion of dopachrome to 5,6-dihydroxyindole-2-carboxylic acid. 5,6-dihydroxyindole conversion factor (ICF) catalyzes the conversion of 5,6-
Electron microscopy studies reveal dihydroxyindole into melanin. 5,6-dihydroxyindole blocking factor restricts melanogenesis at 5,6-dihydroxyindole and protects cells from the cytotoxicity of melanin precursors. This report describes the presence of similar activities in extracts of fetal and newborn mouse skins. Skins were removed from the animals, homogenized in buffered Triton X-100 (0.5 percent), and centrifuged (30,000g, 15'). Activities were found in the supernatant fractions. We have assayed these fractions from different genotypes and found the three factors as well as measurable tyrosinase activity. To date our most detailed study has been with c-locus albino skins, the results of which are as follows. As expected, the albino skins contained negligible tyrosinase activity (measured by the Pomerantz assay). Activities similar to DCF, ICF, and blocking factor were present, however, and their levels changed dramatically during development. Extracts of fetal skins contained ICF (i.e., they readily formed melanin from 5,6-dihydroxyindole) and some DCF. By 24 hours post-partum, ICF had disappeared while DCF and blocking factor began to increase. This increase in DCF and blocking factor plateaued after 4–5 days. Thus, (1) extracts of albino mouse skins contain melanogenic factors similar to those observed in melanoma cells; (2) the factors seem to be regulated during development; and (3) c-locus albino skins do not form pigment from dopa or tyrosine (presumably from a lack of tyrosinase activity), but can, at least in fetuses and newborns, form pigment from 5,6-dihydroxyindole or dopachrome. We recognize that while these findings are of considerable interest, we cannot assign a role for the factors in melanogenesis until their structures and exact functions have been elucidated.

Post Tyrosinase Regulation of Melanogenesis and Precursor Cytotoxicity in Cultured Melanoma Cells. A. Körner, J. Pawelek, A. Bergstrom. Yale Univ. School of Medicine, New Haven, CT

It has generally been assumed that the only regulated steps in the melanin biosynthetic pathway are those governed by tyrosinase since in a test tube melanin can be generated from dopa quinone through a series of spontaneous reactions. However, our studies have revealed that steps distal to tyrosinase activity also appear to be under regulatory control (Körner and Pawelek: J Invest Derm; Pawelek et al: Nature, in press, 1980). In this report we describe three new factors that appear to control melanin synthesis. We have observed these factors in three separate melanoma lines as well as mushrooms, and have partially purified the factors from Cloudman melanoma tumors. The first, dopachrome conversion factor (DCF), catalyzes the conversion of dopachrome to 5,6-dihydroxyindole-2-carboxylic acid. The second, 5,6-dihydroxyindole conversion factor (ICF), catalyzes the conversion of 5,6-dihydroxyindole into melanin and is active in heavily pigmented cells or cells exposed to MSH. The third, 5,6-dihydroxyindole blocking factor, restricts melanogenesis at 5,6-dihydroxyindole, protects cells from the cytotoxic effects of melanin precursors, and is present in amelanotic cells but removed when the cells are exposed to MSH. The two conversion factors seem to exist in a complex with tyrosinase since they each co-chromatograph with tyrosinase during early purification procedures. They are isolated by Triton X-100 extraction of melanosomes. The relevance of the three factors to melanogenesis remains to be demonstrated. We have, however, recently observed similar activities in homogenates of fetal and newborn mouse skins. In addition to implications for the control of hair and skin pigmentation, the findings could be of relevance to the establishment of a rational therapy for human melanoma.

Electron Microscopic Study on the Origin of Melanosomes, the Nature of Their Matrix, and the Differentiation of Their Limiting Membrane. P. Stanka. Ruhr-Universität Bochum, Fed. Rep. Germany

In the retinal pigment epithelium of chick embryos it is shown by electron microscopy that mostly a continuous cisternal complex is transformed simultaneously into premelanosomes. These early premelanosomes grow and develop without a continuity with the granular endoplasmic reticulum. Therefore, it can be concluded that the main constituents of the matrix are non-proteinous. Carbohydrates are demonstrated in the premelanosome matrix by electron microscopic cytochemistry.

Comparative measurements of cytomembranes reveal significant changes in the thickness of the melanosome limiting membrane during melanogenesis. It is suggested that the variations in thickness, as an expression of structural changes, indicate functional differentiations of this membrane. However, the thickness variations seem to be independent of tyrosinase activity, as is shown in newborn rats of different coat color.
Ultrastructural Comparison of Giant Pigment Granules (Large Melanosome Complexes) with Macromelanosomes in Various Cutaneous Pigmented Lesions. T. Horikoshi, K. Jimbow, S. Sugiyama. Sapporo Med. Coll., Sapporo, Japan

How the macromelanosomes (macroMSs) are formed is still unknown. To elucidate whether macroMSs are formed simply through an autophagic degradation of normal melanosomes (MSs) or through complex processes involving the synthesis of abnormal MSs which eventually become an extraordinarily large-sized MS, we compared fine structure of giant pigment granules present in pigmented nevus, seborrhic keratosis, and malignant melanoma with that of macroMSs found in café-au-lait macules, lentigo simplex, and lentigo maligna. Light microscopically a large MS complex, which is formed simply through an auto- or hetero-phagocytosis of MSs, was aggregated with fine brownish grains of MSs, while macroMS was darker, more homogenous, and often surrounded by a halo. Electron microscopically macroMS was characterized by the presence of (a) vesiculo-globular bodies (VGBs) in the cortical zone and (b) electron-lucent globular structures embedded in the dense, amorphous matrix. In contrast, large MS complex did not reveal any of these subunits, but did a clear space below the outer membrane that is seen in ordinal auto- or hetero-phagosomes. In addition, melanocyte (MC) forming macroMSs possessed infrequently large MS complexes and developmental stages of macroMSs. These macroMSs often revealed degraded MSs in the central matrix and also VGBs accumulated in the cortical zone. Occasionally macroMSs were found in the tip of dendrite of MC and encircled by numerous 10 nm filaments, suggesting transfer of macroMSs to keratinocyte (KC). Thus macroMS appears to be formed by complex processes involving (a) synthesis of abnormal MSs easily degraded, (b) aggregation and degradation of these abnormal MSs in the central matrix through an autophagic process, and (c) accumulation of VGBs in the cortical zone, which is a differential point of macroMSs from a regular MS complex. MacroMS is, then, transferred by motive force of 10 nm filaments to KC.

Characterization of Structural Proteins for Morphological Differentiation of Melanosomes. K. Jimbow, M. Jimbow, H. Kanoh, M. Kiyota. Sapporo Med. Coll., Sapporo, Japan

Melanosomes were isolated from B16 and Harding Passey (HP) mouse melanomas, which produce the ellipsoidal forms with lamellar matrix and spherical forms with granular materials inside, respectively. From these morphologically different melanosomes, structural proteins were solubilized to characterize how the biochemical properties are related to the morphogenesis of melanosomes. B16 and HP melanosomes revealed distinct morphological changes after treatment with dissociating agents, i.e., BRIJ-35 and guanidine HCl (GH). The BRIJ treatment affected only to the outer surface of melanosomes, revealing also some decrease in electron density of cortex in both B16 and HP. Subsequent treatment with GH, however, revealed a decrease in density and dissociation of inner matrix in B16 whereas it showed a marked dissociation to an extent that inner matrix was completely fragmented in HP. Solubilized proteins were fractionated, under SDS electrophoresis, into 11 to 12 species with five major common subunits, one of which (mol wt: 36 x 10^3 daltons) was quite different in percent distribution between B16 and HP. Mol wt and number of proteins ranging from 75 x 10^3 to 94 x 10^3 daltons were also different between B16 and HP. Densitometric tracing indicated that the surface of B16 melanosomes is composed of four main components with mol wt of 45, 73, 84, and 86 x 10^3 daltons whereas that of HP is three main components with mol wt of 45, 73, and 75 x 10^3. Their inner matrix appeared to be composed of two main common subunits with mol wt of 50 and 55 x 10^3 daltons and one with mol wt of 36 x 10^3 daltons present only in B16. Thus, the 36 x 10^3-dalton protein and several other unique subunit-proteins are the major biochemical properties responsible for the morphological differentiation of melanosomes in B16 and HP melanomas.

SDS Soluble but Triton X-100 Insoluble Normal and Malignant Murine Melanosomal Proteins. Jesse M. Nicholson. However Univ., Washington, D.C., Vincent J. Hearing. NIH, Bethesda, MD

Our laboratory has been concerned with the elucidation of the mechanism(s) by which unique, or altered, melanosomal proteins from normal and malignant melanocytes may arise. Recently, we discovered that purified melanin granules which have been extensively extracted with Triton X-100 (until no further proteins are solubilized), can then be extracted with a
mixture of 1 percent sodium dodecyl sulfate (SDS) and 0.5 percent 2-mercaptoethanol (2-MSH) at room temperature overnight. Utilizing polyacrylamide gel electrophoresis, we have been able to resolve and determine the molecular weights of thirty-five SDS-soluble proteins from purified melanin granules isolated from normal (C57B1) epidermal melanocytes, and of thirty-four proteins from purified melanosomes isolated from malignant (B-16) melanoma melanocytes. The proteins present in the SDS/2-MSH extracts seem to be quite different from those extracted with Triton X-100; however, just as has been shown for the Triton X-100 extracts, these SDS extracts contain several proteins which are unique to either the C57 or B-16 tissues, as well as many other proteins which are common to both types of melanocytes.

Electron microscopic examination of C57 and B-16 melanin granules before and after Triton X-100 extraction reveal sharp contrasts with those obtained after the SDS/2-MSH treatment. The electron micrographs indicate that Triton X-100 only solubilizes the membrane proteins; whereas the SDS is able to solubilize proteins within the internal matrix of the granules. This indicates that the internal matrix of the melanosomes consists of multiple protein species, as opposed to a single protein (although more work is necessary to demonstrate this more definitively).

Finally, preliminary results from gel electrophoresis on SDS/2-MSH extracts of purified melanin granules from human malignant melanoma, isolated and solubilized as described above, indicate that the protein banding patterns resemble those obtained from the B-16 preparation very closely.

The Localization of Tyrosinase in Mouse Hair-Bulb Melanocyte. Hiroaki Yamamoto, Takuji Takeuchi. Tohoku University, Sendai, Japan

An attempt was made to localize tyrosinase antigen by using anti-tyrosinase antibody. Tyrosinase was purified from B16 mouse melanoma and was demonstrated to oxidize tyrosine. Antiserum against the enzyme protein was prepared. Horse radish peroxidase (HRPO) was conjugated with Fab' which was prepared from the antiserum and normal serum. By using the conjugates, immunoelectron microscopic localization of tyrosinase was studied in hair-bulb melanocyte of black (C57BL/6J, a/a) mice.

DOPA-reaction product and tyrosinase antigen were found on the same organelles, namely on premelanosomes, melanosomes, GERL and Golgi vesicles. This result seems to suggest that the conventional cytochemistry using DOPA as the substrate of tyrosinase is appropriate to localize tyrosinase. We also suggest that tyrosinase is present in a form of granule-like structure inside GERL cisterna being associated with its membrane. The result with black mice was compared with that of yellow mice (C57BL/6J, A²/a).

Photoaffinity Labelling of MSH Receptors of Xenopus Melanophores. P.N.E. de Graan, A.N. Eberle, F.C.G. van de Veerdonk. Zoological Laboratory, State University of Utrecht, Utrecht, The Netherlands; Institute of Molecular Biology and Biophysics, Swiss Federal Institute of Technology (ETH), Zürich, Switzerland

Four new aMSH derivatives carrying photolabile groups at positions 1, 2, 9, and 13 have been prepared by classical peptide synthesis: (a) 4'-azidophenacylserinel-αMSH, (b) 4'-azidophenylalanine²-αMSH, (c) 2'[2'-nitro-4'-azidophenylsulfenyl]-tryptophan⁶-αMSH and (d) 4'-azidophenylalanine¹⁵-αMSH. In a new in vitro melanophore bioassay, all these substances were full agonists of αMSH with relative biological activities of 50 percent (a), 3 percent (b), 30 percent (c), and 65 percent (d). The kinetic patterns during incubation and wash-out phases of the assay were identical to the one of αMSH when the experiments were performed under normal lamplight. However, UV-irradiation of the photolabile substances at 338 nm during the incubation phase caused an "irreversible" pigment dispersion; repetitive washings during four hours did not reaggregate the melanosomes. The duration of the dispersion depends on the temperature during the wash-out phase as well as on the concentration of the photolabile MSH derivative used during photolysis. Control experiments proved that (i) the cells remained intact and completely responsive to αMSH after UV-irradiation, (ii) neither αMSH without a photolabile group nor non-MSH peptides containing a photolabile group were effective in producing an irreversible dispersion, (iii) synthetic aMSH present in a large excess could prevent the effect of the photolabile derivatives, and (iv) the presence of a scavenger-like 4-aminobenzoic acid did not diminish the long-lasting stimulation. Thus, it is concluded that
labeling of the αMSH receptors is specific and that hereby formed covalent MSH-receptor complexes generate a continuous signal resulting in the observed irreversible pigment dispersion. This new system permits a more detailed study of the mechanism of αMSH action, e.g., receptor localization and turnover, the role of calcium and of cyclic AMP.

Toward the Isolation of Mutant Melanoma Cells with Impaired Receptors for MSH.

J.R. Emanuel, J. Pawelek, J. Varga, A.B. Lerner. Yale Univ. School of Medicine, New Haven, CT

The structure and function of MSH receptors are of central importance to the regulation of melanoma cells by MSH. On Cloudman cells the receptors bind MSH in the G2 phase of the cell cycle and become localized in discrete areas overlying the Golgi apparatus. The MSH-receptor complex is then internalized. In the process, adenylate cyclase is activated and a variety of cellular events ensue, including increases in pigmentation and changes in rates of proliferation and cellular morphology. In order to better understand the role of the receptor in these processes we have designed a procedure for isolating mutant cell lines having impaired receptor function. The parental line we have chosen for the studies is unique in that at low temperature (33°C) it displays an absolute requirement for elevated cAMP in order to proliferate. It is thus dependent upon a culture medium supplemented with agents which raise cAMP levels such as MSH or methylisobutylxanthine (MIX, a phosphodiesterase inhibitor). Our selection procedure is as follows. Cells are exposed to the mutantagen, ethylmethanesulphonate, and then cultured at 33°C in the presence of MSH and colchicine. Mutant cells lacking MSH receptors do not divide. Cells with functional MSH receptors proliferate, arrest in mitosis, and are discarded. The remaining cells are then incubated in culture medium containing MIX to stimulate their growth. After three cycles of selection, cells are plated in medium containing both MSH and MIX. With both of these agents present cells which still retain functional receptors become highly pigmented and cease dividing. We have isolated several clones of cells by this procedure and are in the process of testing them for MSH receptors. Results indicate that many of the cells are stimulated to grow by MIX but to a much lesser extent by MSH. It appears that the basic cAMP system is intact, but that MSH cannot activate it. In testing these clones for such impairments, we hope to find a spectrum of mutants with altered receptor functions.

Cyclic Nucleotides in Fish Melanoma Cells: Radioimmunological Determination.

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In amphibian melanophores where pigment dispersion is induced by melanophore-stimulating hormone (MSH), concomitant increase of cyclic AMP level in the integument was observed. Both cyclic AMP and its dibutylryl derivative mimic the effect of MSH in a variety of lower vertebrates, indicating that hormonal stimulation results in pigment dispersal through the increase of intracellular level of the nucleotide. In cells whose responses are controlled by adrenergic nerves, it is generally assumed that stimulation of beta adrenoceptors results in the elevation of intracellular level of cyclic AMP whereas that on alpha receptors induces cellular responses through the decrease in cyclic AMP. Pharmacological and physiological studies on fish chromatophores appear to be in agreement with this assumption, although cyclic GMP is also effective in producing pigment dispersal in some instances.

Since the intracellular level of cyclic nucleotides has not yet been estimated in pigment cells, radioimmunoassay of cyclic AMP and cyclic GMP was carried out using epinephrine and theophylline-stimulated melanoma cells of platyfish-swordtail hybrids.

The Effect of Melanotrophic Peptide Hormones on the Adenylate Cyclase Activity of B16 Mouse Melanoma.

S.S. Bleehen, S.K. Johnson, S. Mac Neil, B.L. Brown, S. Tomlinson. Royal Hallamshire Hospital and University of Sheffield, Sheffield, England

The effect of melanotrophic peptide hormones has been studied on the adenylate cyclase activity of whole cell lysates of pigmented cell lines of B16 mouse melanoma. We have found
Cyclic AMP-Dependent Protein Kinase Activity in Cloudman Mouse Melanoma Cell Cultures. Jean B. Burnett. Michigan State Univ., East Lansing, MI, and The Univ. of Arizona, Tucson, AZ. David E. Birch, Bryan B. Fuller, Mac E. Hadley. The Univ. of Arizona, Tucson, AZ

Cloudman mouse melanoma cells in culture respond to melanocyte-stimulating hormone (MSH) with an increase in tyrosinase activity. MSH has been shown to elicit an increase in melanoma cell membrane adenylate cyclase activity [1] and to increase cyclic AMP levels in the cell cytosol. A tenfold increase in cyclic AMP concentration over that in control cell cultures was observed within 20 min following exposure of the cells to \( \alpha \)-MSH (10^{-7}M). Within 90 min, the level of cyclic AMP fell to approximately twice that of the controls and remained stable for over nine hours. The presumed mediator of all cyclic AMP action within the cell is cyclic AMP-dependent protein kinase. A time-dependent stimulation of cyclic AMP-dependent protein kinase by \( \alpha \)-MSH has been observed. Maximal cyclic AMP-dependent protein kinase activity occurs 30 min following incubation with 10^{-7}M \( \alpha \)-MSH and declines in about 60 min to levels which remain 1.5 to 2 times above control values. An increase in kinase activity can be detected with as little as 10^{-8}M \( \alpha \)-MSH. Both type I and type II cyclic AMP-dependent protein kinase isozymes are present in three distinct mouse melanoma cell lines which differ in their degree of basal tyrosinase activity. The ratios of type I and type II kinases have been determined. Taking into consideration several parameters, the effect of \( \alpha \)-MSH on these kinases and their respective ratios is being determined.

[1] Kreiner P, et al: Yale J Biol Med 64:583-591, 1973

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Synthesis and Activity of [Nle^4, D-Phe^7]-\( \alpha \)-Melanocyte Stimulating Hormone: A Highly Potent Melanotropin Analog with Prolonged Activity. Jean B. Burnett. Mich. State Univ., E. Lansing, MI, and The Univ. of Arizona, Tucson, AZ; Mac E. Hadley, Christopher B. Heward, Tomi K. Sawyer, Pauline J. Sanfilipo, Victor J. Hruby, Michael H. Engle, Brent R. Larsen. The Univ. of Arizona, Tucson, AZ

Melanocyte stimulating hormones (\( \alpha \)- and \( \beta \)-MSH) darken frog skins by stimulating melanosome movements within melanophores. Heat-alkali treatment of these melanotropins results in an extraordinarily prolonged biological activity of the peptides. It has been suggested that such treatment leads to racemization (conversion of \( L \) to \( D \) enantiomers) of amino acids within the peptides. Quantitative gas chromatographic analysis of heat-alkali treated \( \alpha \)-MSH revealed racemization of a number of the amino acids, particularly at 4 (methionine) and 7 (phenylalanine) positions. Synthesis of [Nle^4]-\( \alpha \)-MSH yielded an analog that reversibly darkens frog skins but exhibits prolonged activity after heat-alkali treatment. Synthesis of the [Nle^4, D-Phe^7] analog of \( \alpha \)-MSH resulted in a peptide with prolonged biological activity, apparently identical to that of heat-alkali treated \( \alpha \)-MSH or [Nle^4]-\( \alpha \)-MSH. This [Nle^4, D-Phe^7]-\( \alpha \)-MSH analog appears to be totally resistant to enzymatic degradation by serum enzymes. This peptide exhibits dramatically increased biological activity as determined by (1) frog skin bioassay, (2) activation of mouse melanoma cell adenylate cyclase and cyclic AMP production, and (3) stimulation of melanoma tyrosinase activity. This [Nle^4, D-Phe^7] analog of \( \alpha \)-MSH is the most potent melanotropin so far synthesized. Frog skins darkened by the peptide either in vitro or in vivo remain maximally darkened for days. This darkening can be temporally reversed by
melatonin indicating the physiological nature of the response. Either the hormone is irreversibly bound to the MSH receptor, or else it irreversibly activates the transduction signal between the receptor and adenylate cyclase. Supported by NSF grant PCM77-07031 and USPHS grants AM 17428, AM 21085, and CA 20547.

Preparation of a Radiolabeled Melanotropin Suitable for Use as a Tracer in a Radioreceptor Assay: [125I-NLE4]a-Melanotropin. Christopher B. Heward, Mac E. Hadley, Tomi K. Sawyer, Victor J. Hruby, Michael H. Engle, Brent R. Larsen. The Univ. of Arizona, Tucson, AZ

Iodinated derivatives of alpha-melanotropin (a-MSH) and 4-norleucine-alpha-melanotropin (NLE4)a-MSH were prepared using a variety of labeling techniques for use in a radioreceptor assay on S91 mouse melanoma cells. Under iodination conditions commonly used for other peptides, a substantial loss of biological activity of the native hormone was observed. This loss of hormonal activity was primarily a consequence of oxidation of methionine and occurred regardless of the oxidant used (Chloramine-T, Lactoperoxidase-hydrogen peroxide, or Iodogen). Under similar iodination conditions using[NLE4]a-MSH, satisfactory incorporation of 125I into the peptide was accomplished without significant loss of biological activity. Data is presented suggesting that [NLE4]a-MSH is far superior to a-MSH for the preparation of a radioactive melanotropin for radioreceptor assay. Although some success was achieved with this peptide using all three iodination methods, the simplest and most consistent methodology involved the use of Iodogen followed by purification of the labeled product using high performance liquid chromatography (HPLC). The suitability of [125I-NLE4]a-Melanotropin for studying the MSH receptor is demonstrated by preliminary data from the radioreceptor assay. Supported by NSF grant PCM77-07031 and USPHS grants AM 17420, AM21085, and CA 20547.

The Clonal Basis of Mouse Pigmentary Development. Beatrice Mintz. Inst. for Cancer Research, Fox Chase Cancer Center, Philadelphia, PA

The origins and gene control of pigmentary development in mice have been retrospectively analyzed by constructing allogenic animals in which cells of two different genotypes co-exist throughout ontogeny and serve as markers. Coat patterns in such individuals have furnished valid models of development in single-genotype mice. The entire coat pigmentary system is determined early in embryonic life. From the neural crest, 17 pairs of cells are set aside as clonal initiators of melanoblasts which proliferate and migrate laterally, forming wide transverse bands. From the somites, approximately 85 pairs of cells become the precursors of hair-follicle dermal cells and form narrow transverse clones. These archetypal or undisturbed clonal patterns can be modified in reproducible ways by many genes with specific effects on cell proliferation, migration, or viability. Some "pigment" genes are expressed only in melanoblasts; or only in hair follicles with secondary effects on melanoblasts, thereby generating subclonal differences. However, many pigment genes are apparently expressed both in melanoblasts and in hair follicles, with different products. Phenotypic heterogeneity can also occur, despite genetic identity, among melanoblast clones or among hair-follicle clones, in many genotypes even when the relevant loci are homozygous. Thus, at the genetic and the cellular levels, various mechanisms act to provide pigmentary cell sub-types that offer the possibility of clonal selection and greater adaptability during development. From the evolutionary point of view, there is the advantage of increased cellular heterogeneity without a greatly expanded genome. All pigment loci may be complex assemblages of genetic units with flexible transcriptional possibilities.

Gene Expression in Melanosome Formation. Takuji Takeuchi. Tohoku University, Kawauchi, Sendai, Japan

Melanosome, a specialized organelle of the melanocyte, has been shown to be under gene control. Several genetic variations in melanosome morphology have been reported in various organisms. The presence of these genetic variations indicates that some gene products seem to be directly involved in melanosome architecture. On the other hand, there are cases where melanosome morphology is modified by a cytoplasmic factor which seems to be the direct or indirect product of a gene. For example, during agouti pattern formation in mouse hair, the form of melanosomes is modified from eumelanosome to phaeomelanosome and vice versa by
the factor controlled by \( A \) allele at the agouti locus. Similar changes in melanosome formation have been experimentally reproduced by the use of UV light, MSH, and such reagent as cAMP. In the melanocytes of genotypically yellow mouse skin cultured in the medium containing MSH, melanosomes of various intermediate types were observed. The observation on the induced change in melanosome formation might provide a clue to the mechanism of the organelle formation. In addition, the melanosome formation seems to be regulated in an ON-and-OFF manner during the differentiation of the melanocyte. The presence of factors involved in the regulation of this type has been demonstrated by hybridizing mouse melanoma cells with chick embryonic cells from various organs.

**Gene Expression and Pattern Development.** Thomas C. Mayer. Rider College, Lawrenceville, NJ

Pattern development in vertebrates continues to be one of the most fascinating topics involving interactions between cells of diverse embryological origin. Although the early neural crest-skin transplantation studies on amphibian development revealed that either the melanoblast or skin can play a determining role in pattern development, the precise ways these genes act remain unknown. Alterations in melanoblast migration, differentiation, proliferation, and survival are commonly suggested mechanisms.

The complex pattern of pigment distribution in the plumage of birds has been examined by the use of melanoblast transplantation techniques between various breeds and species. In all cases the pattern of pigment distribution within the feather corresponds to the genotype of the donor melanoblasts that are incorporated into the feather epidermis. It appears that a common stem melanoblast produces either red or black pigment depending on the tract specificity of the feather (wing, breast, or tail) and, when formed, the two melanocyte types are fixed in expression. Various types of barred patterns (wide or narrow stripes) are also melanoblast specific, and may be related to the cyclical production of an inhibitor and different levels of melanoblast sensitivity.

Mammalian hair and coat patterns have been studied extensively in the mouse because of the variety of mutants and availability of genetically uniform inbred strains. White spotting genes restrict pigment occurrence in the coat and, depending on the gene, produce small white spots, piebald type patterns, or totally white animals with pigmented eyes. Three hypotheses are commonly used to explain pattern development: (1) number of primordial melanoblasts migrating from the neural crest; (2) organization of viable and inviable primordial melanoblasts in the neural crest; (3) interactions between melanoblasts and tissue environments. Hair patterns (agouti) are determined by the hair follicle environment, and the phaeomelanic (yellow) and eumelanic (black) melanocytes are freely interconvertible.

**Regulation of Transdifferentiation by Microenvironmental Factors in Vertebrate Pigmented Epithelial Cells Cultured in Vitro.** Goro Eguchi. Institute of Molecular Biology, Nagoya University, Nagoya, Japan

Capacities of the pigmented epithelial cell (PEC) of the eyes for transdifferentiating into lens cells were widely demonstrated in adult newts, avian embryos, and even in human fetuses by means of *in vitro* cell culture techniques. In particular, the pluripotency of clonal progenies of newt PECs has been clearly shown by observations that lens cells, neuronal cells, and pigmented cells differentiated simultaneously in some of identical clonal colonies derived from single PECs. The following evidences, which are highly relevant to understanding of regulation of transdifferentiation in PECs, have been so far accumulated through cell culture analyses. (1) When dissociated PECs from chick embryos were cultured on the collagen substratum, their differentiation as pigment cells was stably maintained, and their transdifferentiation into lens cells was completely suppressed. (2) Chick embryonic PECs easily transdifferentiated into lens cells in the presence of 0.5–1.0 mM 1-phenyl-2-thiourea, and this promotive effect of phenylthiourea was markedly amplified by testicular hyaluronidase. (3) Newt PECs in clonal colonies stably maintained their differentiation as pigment cells, when they closely communicated with each other. However, in clonal colonies, in which PECs communicated less, they rapidly lost their specificity as pigment cells and easily switched their differentiation into lens cells. These facts are invaluable for elucidating the control mechanisms of transdifferentiation of PECs *in vitro* and *in vivo*. 
Non-Random Distribution of Amelanotic Melanocytes in the Retina of Chinchilla-Mottled Mice and Its Significance. M.S. Deol, Gillian M. Truslove. University College, London, UK

The albino locus "allele" chinchilla-mottled (cm) in the mouse gives rise to a coat that appears to be similar to that of albino-pigmented allophenic mice. For this reason it is thought that the developmental mechanism underlying mottling is essentially the same in both types of animal. However, an examination of the pigmented epithelium of the retina showed that chinchilla-mottled mice are fundamentally different from allophenic mice. The distribution of melanotic and amelanotic melanocytes is random in allophenic mice, whereas in chinchilla-mottled mice it follows a clear pattern. In these animals the vast majority of amelanotic melanocytes are concentrated in the close vicinity of the optic nerve, and they are all virtually confined to the proximo-ventral quarter of the eye-cup. The significance of these findings in relation to the nature of this "gene," its mode of action, and the role of the tissue environment in its expression will be discussed, and the effects of other mottling genes will be considered in the light of these findings.

Age-Specific Incidence, Differentiation, and Variation in Hereditary Melanomas in Xiphophorus Fish Hybrids. Kenjiro Ozato, Yuko Wakamatsu. Yoshida College, Kyoto University, Kyoto, Japan

Hereditary melanomas developing in the Xiphophorus fish hybrids carrying Sp gene were classified in two types, the adult and the fry melanomas, based on the age of the onset. The adult melanomas occurred in a wide range of adult ages in F1 and backcross generation (F1 x sword-tail). The cumulative incidence of this type of melanomas increased in proportion to the third power of the age of the hybrid fishes. By contrast, the fry melanomas occurred in a narrow range of early ages mainly in backcross generation.

These two types of melanomas were different in the degree of differentiation. In general, the adult melanomas were in less differentiated states in morphology, pigmentation, and response to epinephrine of melanoma cells in primary culture, and in ultrastructure, than the fry melanomas.

The adult melanomas, however, showed great variation in these phenotypic traits among different tumors. Stability of these phenotypic traits of the adult melanomas was followed in the same tumor with the course of tumor growth. The cell size and cell shape were relatively stable, while the pigmentation and response to epinephrine were variable. On the other hand, variation of the fry melanomas was rather small.

From all these facts, it is suggested that different genetic mechanisms are involved in formation of these two types of melanomas. The adult melanomas may arise from somatic mutations in addition to genetic changes in germ cells by hybridization, whereas the fry melanomas may arise as a direct result of hybridization.

Biochemical Analysis of Interrupted Melanogenesis in the Cultured Melanoma Cells Induced by Inhibited Carbohydrate Metabolism Using Glucosamine and Tunicamycine. Genji Imokawa, Yutaka Mishima, Gakuzo Tamura. Kobe University School of Medicine, Kobe, and University of Tokyo, Japan

The addition of glucosamine (Gln, 1 mg/ml) or tunicamycine (TM, 0.4 μg/ml) to the highly pigmented B-16 melanoma cells in vitro has induced their marked pigment loss with moderate decrease in growth curve. This inhibitory effect disappears about 10 cultured days after removal of Gln or TM from culture media. It is seen that the addition of Gln or TM primarily causes specific suppression of mannosc uptake without an apparent effect on the synthesis of DNA, RNA, and protein. Further, the cellular uptake of dopa, glutathione, and Cystein is not substantially reduced. In order to clarify this interrupted melanogenic mechanism, biochemical analysis including tyrosinase assay has been carried out in comparison with tyrosinase activity present in melanosomes as revealed by dopa reaction. Biochemical assay of Gln-treated cells has revealed no substantial decrease in the tyrosinase activity of 12,000 g supernatant fraction and of the small granule fraction, but decrease in the large granule fraction. Electrophoresis of tyrosinase in the 30,000 g supernatant fraction demonstrates an increase in T1 form, while disappearance or decrease in membrane bound tyrosinase (T13) is seen in the sedimental fraction in accordance with the finding of dopa reaction that the amelanotic cells contain a markedly
Increased number of tyrosinase-negative premelanosomes with the ratio of 49 percent in Gln-treated cells and 70 percent in TM-treated cells compared to 12 percent in the control. This ratio is also found to be parallel with that in altered melanosomes. It is thus suggested that the inhibition of mannose uptake in melanoma cells could induce decreased tyrosinase activity in melanosomes and their subsequent interrupted melanization.

**Overexpression of Tyrosinase in Hybrids Derived from Mouse Melanoma and Spleen Cells.** R. Halaban, L.Q. Uribe, G.E. Moellmann, J.M. Eisenstadt, A.B. Lerner. Yale Univ. School of Medicine, New Haven, CT

The effect of allelic substitution in the c locus on the overexpression of tyrosinase in hybrid cells was investigated. Mice that carry the extreme dilution (c5) or the himalayan (c1) or the albino (c) mutations as well as C57BL/6 which have the wild-type (C) tyrosinase were used as the source for spleen cells. The spleen cells were fused to Cloudman melanomas (PCHPRT-) and approximately 30 hybrid clones, with wide ranges of tyrosinase activity, were isolated from each fusion.

| Subclones of PCHPRT- | Ranges of Tyrosinase activity | % of clones with overexpression |
|----------------------|------------------------------|---------------------------------|
| Hybrid cells         | cpn/min/mg protein           |                                 |
|                      | -MSH | +MSH |                                 |
| PCHPRT × C57BL/6     | 50—2,847 | 218—6,287 | 57.6                          |
| PCHPRT × albino      | 391—17,810 | 1,740—41,430 | 57.6                         |
| PCHPRT × himalayan   | 117—13,000 | 1,203—27,190 | 18.5                         |
| PCHPRT × extreme dilution | 161—10,256 | 1,098—19,608 | 42.9                         |

Karyotypic analyses confirmed the hybrid nature of the clones and revealed no correlation between the mean number of chromosomes and activity of the enzyme. Rather, the upper limits of the ranges of tyrosinase activity in the different classes of hybrid were a reflection of the "c" locus present in the parental animals, from which the spleen cells were derived. In supermelanotic hybrids derived from melanoma and normal diploid cells (Halaban et al: Somatic Cell Genetics 6:29, 1980) the diploid parental cells might have included normal melanocytes. The findings reported here indicate that the overexpression of tyrosinase activity in hybrid cells is a frequent event that is probably independent of the presence of melanocytes in the tissue from which the diploid cells were derived. The spleen of the C57BL/6 parent was examined by light and electron microscopy, and no melanocytes were identified. The hypothesis that the overexpression is due to activation of the silent tyrosinase gene in a previously non-expressing cell will be examined. NC15R01CA04679; NIHGM21873; and ACSB3C3M.

**Reconstructed Cells Show Melanogenesis Is under Nuclear Control.** John A. Brumbaugh, Kathy P. Fuza, Dan O. Ullmann. Univ. of Nebraska, Lincoln, NE

Reconstruction fusions between mouse fibroblast (A9) nuclei (minicells) and pigmented mouse melanoma (B-16 subclone) cytoplasts (cytoplasts) and reciprocal combinations were produced. Flasks of subconfluent cells were filled with cytochalasin B medium (10 μg/ml) and centrifuged. Nuclear donor cells were labeled with 1 μ beads (90 percent > 20 beads/cell). Minicell pelets were collected after centrifugation and plated out for two hours to reduce whole cell contaminants. Meanwhile, the cytoplasm-bearing flasks were rinsed and allowed to regain normal morphology. Enculeation of > 90 percent of the cells was usually accomplished. Fusion of components was attained using inactivated Sendai virus and ca. 5 × 10⁵ minicells/enculeated flask. In all cases, reconstructed cells were identified by their paucity of beads since minicells averaged < 1 bead/minicell. In early experiments the nuclear donor cells were prelabeled with ³H-thymidine, thus reconstructions were verified autoradiographically 24 hours post-fusion. Frequently, selective techniques were used to select against melanoma cells and for A9 nuclei through 6-thioguanine medium (A9's are HGPRT- and derived from a pigmented mouse) or against A9 cells and for melanoma nuclei through HAT medium. In other cases reconstructed cells were picked on the basis of morphology.

Clones from reconstructed cells were analyzed statistically as to frequency, electron cytochemically for DOPA oxidase activity, and chromosomally as to nuclear donor (A9 or B-16). In all cases, clones exhibited the characteristics of the nuclear donor, i.e., A9 nuclei in melanoma.
cytoplasmic produced DOPA-negative fibroblasts, while B-16 nuclei in fibroblast cytoplasmic produced DOPA-positive melanomas. Therefore melanogenesis is primarily under nuclear control.

**Filopodium and Dendrite Formation with Associated Pigment, Carotenoid Droplets, Movement.** Szecheng J. Lo, T.T. Tchen, John D. Taylor. Wayne State Univ., Detroit, MI

Treatment of cultured goldfish xanthophores by hormone (ACTH) or c-AMP induces dispersion of carotenoid droplets from a large aggregate within the perinuclear region into dispersed droplets throughout the cell with the exception of the lamellae. This phase is completed in less than 15 min and is followed by a second phase in which carotenoid droplets disperse into newly formed filopodia which eventually become new dendrites. The second phase is shown to proceed as follows. First, the edge of the cytoplasmic lamellae takes on a scalloped contour with numerous protrusions. These presumably serve as nucleation centers where short microfilament bundles are assembled. Later, these microfilament bundles elongate, often resulting in an extension of the protrusions to become filopodia while the other end of the microfilaments penetrates into the thicker portion of the existing dendrite which now houses the carotenoid droplets. Carotenoid droplets migrate along the microfilament bundles, or cytoplasmic channels associated with them, into the newly formed filopodia. Some filopodia are not stable and retract back into the lamellae while others become broader, thicker and laden with carotenoid droplets. These are recognized by light microscopy as pigmented dendrites. The microfilaments are labile to cytochalasin B treatment, thus preventing filopodium formation, and have been shown to be actin filaments by their size, the size of their subunits, and decoration by heavy meromyosin. The elongation of the actin bundles is not inhibited by puromycin and appears to be achieved by recruitment of short actin strands found in random orientation in adjacent areas.

**MSH Effects on RNA Polymerase Activities in Mouse Melanoma Cell Cultures.** Bryan B. Fuller, Thomas J. Lindell. The Univ. of Arizona, Tucson, AZ

Previous studies have revealed that transcriptional requirements exist for the MSH-induced differentiation response (increased tyrosinase activity) in Cloudman S91 mouse melanoma cell cultures. To continue the investigation of the possible role of transcription in mediating the action of MSH, we have measured the activities of RNA polymerases I, II, and III in nuclei prepared from melanoma cells exposed to MSH (2 x 10^-4M) for various times. Within five minutes of hormone treatment, a marked decrease in the activity of both polymerases I and II was observed. This decrease was transient and by 60 minutes RNA polymerase I activity had returned to control values while the activity of polymerase II (the enzyme associated with mRNA transcription) increased to more than twice control values. RNA polymerase III activity was stimulated at 10 minutes after exposure of cells to MSH but by one hour had decreased below control levels. Since MSH is thought to exert its cellular effects through the second messenger, cyclic AMP, the effects of either dibutyryl cyclic AMP (10^-4M) or the phosphodiesterase inhibitor, theophylline (1 mM), on the activity of each of the three RNA polymerases over the time were also determined.

The activities of melanoma cell RNA polymerases were found to be density dependent in that the basal activity of all three polymerases increased as cell density increased. The relative effect of MSH on the activities of RNA polymerases I, II, and III, however, remained the same regardless of cell density.

**Interrelationship of Nervous Control among Chromatophores of the Medaka.** Kiyotugu S. Iwata, Takushi Takahashi, Yukiko Yamazaki. Okayama University, Tsushima, Okayama, Japan

We have accumulated experimental evidences that the fish melanophores are under the control of the sympathetic post-ganglionic nerve fibers, but nervous control of leucophores and xanthophores has been only poorly studied. Interrelation of nervous control among these three kinds of chromatophores in the scale of the medaka was investigated in the experiments on the pulsations induced by Ba ion, since Ba-pulsations in melanophores have been shown to
originated from rhythmic activity of the controlling nerve. The results obtained are summarized as follows: (1) Leucophores and xanthophores, just like melanophores, can be initiated to pulsate by treatment with isotonic BaCl₂ for several minutes. (2) Ba-pulsations of melanophores and xanthophores are blocked by dibenamine, a potent α-adrenoceptor blocking agent, while those of leucophores are blocked by propranolol, a β-adrenoceptor blocking agent. (3) Pulsations of neighboring chromatophores of all kinds are well synchronized with one another and the pulsations of melanophores hold the same phase relation as those of xanthophores, and hold the reverse phase relation as those of leucophores.

These results can be most adequately interpreted by assuming that chromatophores of all kinds are innervated by the same adrenergic nerve fibers, at the excitatory phases, initiating pigment aggregation in melanophores and xanthophores via α-adrenoceptors, while on the other hand initiating pigment dispersion in leucophores via β-adrenoceptors.

Reflecting Platelet Formation in Iridophores. Yoshihisa Kamishima. Okayama Univ., Tsushima-naka, Okayama, Japan

Iridophores are typical structural chromatophores which produce colors from colorless organelles, such as reflecting platelets, by their optically precise arrangement so as to split and reflect the incident light. Iridophores are found throughout the whole animal kingdom, and are especially abundant in those with thin and transparent body walls in the lower vertebrates and mollusks. In these animals, iridophores are not only observed in the integument, but also densely distributed in the internal tissues, such as the peritoneum, the pericardium, and the perineural membrane. These cells exhibit the most prominent and diverse coloration in the animal world, ranging from milky white of the abdomen in many animals to brilliant metallic blue or purple of sea shells and tropical fishes.

In spite of such special contribution to animal coloration, however, no detailed works have ever appeared on the process of organellogenesis in these cells. By observing the platelet formation electron microscopically in iridoblasts of frogs, fishes, cephalopods and clams, the following process is generalized. (1) The platelet envelope is derived from the RER, and initially a double-membraned saccule. (2) Cytoplasmic and nuclear components, such as ribosomes, are directly taken up into the saccular lumen. (3) Golgi vesicles incorporate to the saccule during the earlier phase of the development. (4) Material deposition in the envelope differs in animals. In vertebrates, it crystallizes in a concentric pattern along the envelope margin, while it accumulates lengthwise to the platelet in molluscan iridophores. (5) A cytochemical work demonstrates strong nucleate activity on the cell membrane of fish iridoblasts. (6) Biological significance of the iridophore will be discussed in relation to its development and distribution.

Tyrosinase and Acid Phosphatase Pathways in Regenerating Feather Melanocytes of the Fowl. Roger R. Bowers, Douglas W. Chun. California State Univ., Los Angeles, CA

Growth of regenerating feathers in the fowl involves the enlargement and further addition of keratinocytes and melanocytes by mitosis to the proximal end of the feather. This mechanism permits the study of temporal as well as linear cellular differentiation of feather melanocytes. Melanocytes were treated to demonstrate tyrosinase (DOPA reaction) and acid phosphatase (GOMORI reaction) and thin cross sections (designated as level 0, level 500, level 1000, etc.) were made every 500 μm along the length of the specimen up to the 5000 μm level distal to the beginning of the barb ridges.

From level 0 to level 250, tyrosinase activity was found primarily in the Golgi apparatus, with some reaction product observed in Golgi associated smooth endoplasmic reticulum (GERL) and in a few coated vesicles (CV). Little or no tyrosinase activity was observed in the Golgi apparatus and GERL from level 250 to level 2500 but was found in numerous coated vesicles. From level 2500 to level 5000, no tyrosinase activity was observed in the melanocyte cytoplasm other than that assumed to be located on the stage IV melanosome. Thus a temporal pathway of tyrosinase activity was observed, originating in the Golgi apparatus, transporting through the GERL to the CV, and depositing on the premelanosome.

Only scant acid phosphatase activity was observed in these melanocytes until level 4000 whereby reaction product was obvious in the Golgi apparatus, smooth endoplasmic reticulum,
and lysosomes. Larger amounts of this enzyme activity were observed in the adjacent pulp epithelial cells from level 2500 to level 5000. Portions of melanocyte cytoplasm and large aggregates of melanosomes were observed also in these cells. These latter results support earlier observations which suggested that pulp epithelial cells play a major role in the phagocytosis of degenerating feather melanocytes.

**Biology of the Retinal Pigment Epithelium.** Yin-Lok Lai, Raymond Lug, Seiji Hayasaka. Yale Univ. School of Medicine, New Haven, CT; Tohoku University Medical School, Sendai, Japan

Maintenance of the subretinal space is dependent upon fully developed apical microvilli of the retinal pigment epithelium (RPE). Volume of subretinal fluid, and photoreceptor outer segments. Loss of contact between the apical microvilli and outer segments, as may occur with presence of subretinal macrophages, results in loss of apical microvilli. Pathological changes in the RPE, interfering with the development of normal apical microvilli, interrupt both the removal of photoreceptor outer segments and the biochemical interactions between the neuroretina and the choroid, resulting in a reduction of the subretinal space.

Age-related reduction in number of photoreceptor cells and shortening of photoreceptor inner and outer segments in primate retinas relate to the loss of apical microvilli in the adjacent RPE.

RPE cells are capable of becoming epithelial macrophages, migrating from their single layer adjacent to Bruch's membrane to become free cells in the subretinal space. Migration is preceded by a proliferation of apical microvilli, projecting over neighboring cells in the direction of movement. Migration is most frequently observed in the peripheral retina, and rarely seen elsewhere, except in response to a lesion. Once detached from the RPE layer, the epithelial macrophages lose their typical surface microvilli. In the aging primate retina, RPE cells have been observed moving underneath the RPE, between the RPE basal surface and the Bruch's membrane.

Low intensity continuous light exposure damaged the RPE before the photoreceptor cells. This was characterized by an increase in the electron density of the cytoplasmic matrix, modifications of the endoplasmic reticulum, changes in the number and distribution of lysosomes, and changes in the number and size of apical microvilli and their population of inclusions. Continuous light exposure caused an increase of lysosomal enzymes in the retinal pigment epithelium.

**Intraepidermal Nerve in Guinea Pig: Its Relation to Melanocyte.** Motoyuki Mihara. Tottori Univ. School of Medicine, Yonago, Japan Ken Hashimoto. Wright State Univ. School of Medicine, Dayton, OH Masanobu Kumakiri. Hokkaido Univ. School of Medicine, Sapporo, Japan

Intraepidermal nerves were studied in spotted (black and white) guinea pigs in order to examine the distribution density of them. Specimens were obtained from white, black, and gray areas. Twenty blocks were made from each area and sections were examined with the electron microscope. Intraepidermal nerves were found in the interfollicular epidermis of all three areas. The distribution density of them for the black areas was about twice as that for the white areas. The frequency of them in the gray areas was about medium between those for the black areas and for the white areas. There was no difference for the ultrastructure among the three areas. Intraepidermal nerves contacted directly with the melanocytes in the interspaces between the basal lamina of the epidermis and the cytomembrane of melanocyte or with the dendrite of melanocyte in the intercellular spaces between basal keratinocytes. In most cases, their axons were ensheathed by the Schwann cells. In some cases, a part of axon expanded like a balloon about 200–300 nm in diameter. The ballooning structure contained several vacuoles about 40–60 nm in diameter. These vacuoles seemed to be secreted into extracellular spaces. In such cases, the accumulation of dense materials was observed along the opposed membrane of vacuole and the cytomembrane of melanocyte. In another case, the cytomembranes of axons and those of melanocytes seemed to form synapse-like structures. Intradermal injection of 5-hydroxydopamine or 6-hydroxydopamine did not alter their axonal structures. It could not be confirmed that intraepidermal nerves belonged to sensory nerves or autonomic nerves.
Physiological and Electronmicroscopical Studies on Melanophores from Xenopus Laevis Tadpoles in Primary Culture. R. Seldenrijk, W. Berendsen, F.C.G. van de Veerdonk. State University of Utrecht, Utrecht, The Netherlands

Melanophores are isolated from the ventral tail-fin of *Xenopus laevis* tadpoles by digestion with acetyltrypsin and collagenase. After isolation the cells are spherical and covered with microvilli. After adhesion to the substratum, the melanophores start the development of protrusions: filopodia and afterwards cell processes, which often show branchings. Transmission electronmicroscopic comparison of primary cultured melanophores and integumental ones does not reveal striking differences: the cells contain similar organelles, located in the same ways. Scanning electronmicroscopy (SEM) reveals that in the aggregated state cell body is spheroidal, due to the aggregation of numerous melanosomes. Dispersing stimuli initiate a spreading of these melanosomes into the processes of the cell with a concomitant flattening of the cell body. From time to time the cell shape changes, caused by retraction and new development of processes. Often the processes of adjacent melanophores are situated in a straight line and contact each other. In two contacting processes nor border between the cells can be observed in SEM. These close contacts are formed by both collinear and branched-off processes.

Fully developed melanophores in primary culture respond to α-MSH and to cyclic AMP by dispersion of the melanosomes. Upon washing out these agents, the pigment granules regagrate. In darkness about 40 percent of the cells show pigment dispersion, whereas reillumination or addition of melatonin results in a rapid aggregation. Both the MSH-induced dispersion and the melatonin-induced aggregation are Ca²⁺-dependent. Both morphological and physiological criteria demonstrate the cultured cells to retain their specialized function.

Long-Term Cultivation of Amphibian Melanophores. Hiroshi Kondo, Hiroyuki Ide. Tokyo Metropolitan Institute of Gerontology, Tokyo, and Biological Institute, Tohoku University, Sendai, Japan

Melanophores were isolated from the tail skin of bullfrog tadpoles (*Rana catesbeiana*) and cultivated with 1:2 or 1:4 split ratio for two or three years. Melanophores at early passages proliferated rapidly. With the progress of population doublings (PD), cell number per dish declined gradually and cell size increased. After cell growth became low rate or ceased, cells initiated to proliferate again and after that cells continued to proliferate rapidly. This result was also revealed from the observation of clonal growth. Most of melanophores at early passages (PD 10) proliferated rapidly. With the progress of passages (PD 26), the number of rapidly growing cells decreased and the number of slowly growing cells and non-dividing cells increased. At PD 56, rapidly growing cells disappeared, the number of slowly growing cells decreased, and the number of non-dividing cells increased. However, at PD 70 most of cells proliferated rapidly. Chromosomal analysis showed that in one cell line chromosomal number changed from diploid (2n = 26) to hyperploid (2n = 30) and in other cell lines it did not change. These results show that phase II phenomenon (cellular aging in vitro) was observed and after phase III neoplastic transformation occurred.

Melanin granules were observed not only in cells at primary culture but in large cells at phase III, and were also observed in transformed cells. α-MSH induced melanin dispersion with cells at early and advanced passages. With the progress of passages, cells with dispersed melanin increased gradually.

Co-Cultivation of Amphibian Melanophores with Epidermal Cells and Fibroblasts. Hiroyuki Ide. Biological Institute, Tohoku University, Sendai, Japan

Bullfrog melanophores distribute in various regions of the skin, that is, epidermal layer, dermal layer, and subdermal layer, and the shape of the melanophores differs with these regions. Dermal and subdermal melanophores are dendritic and thick, but epidermal melanophores of adults are thin and dendritic and those of tadpoles are spindle-shaped.

To elucidate the role of surrounding tissues on the morphogenesis of melanophores, tadpole melanophores were co-cultivated with epidermal cells and fibroblasts. As the fibroblast, FT cell line derived from bullfrog tongue was used.

Melanophores inoculated onto the sheet of epidermal cells of bullfrog tadpoles showed elongated and spindle-shaped morphology, which resembles that of epidermal melanophores of tadpole. Electronmicroscopic observation of the cross section of the culture revealed the
presence of the melanophore between the epidermal sheet and plastic dish. Melanophores co-cultivated with epidermal cells isolated from adult tree frog showed thin and dendritic morphology. Melanophores located between the epidermal cells and dish.

On the other hand, the melanophores intermingled with FT cells and cultured or inoculated onto plastic dish directly showed thick and dendritic shape of dermal melanophores. In the former case, melanophores located on and in the multiple layer of the FT cells. These results suggest that epidermal cells or their products control the shape of melanophores in vitro and in vivo.

Mutual Repulsion between Epidermal Melanocytes. I. Rosdahl, S. Lindström. University of Göteborg, Sweden

The distribution of epidermal melanocytes has been studied in split-skin preparations from the ear of C57BI mice. Closely related melanocytes were rare, despite the fact that the epidermal melanocyte population is continuously renewed by mitoses. Thus, newly formed melanocytes seem to migrate apart during interphase. Using Poisson statistics it could be demonstrated that the melanocyte population was more evenly dispersed than would be expected by a random distribution process. This suggests that the melanocyte migration is controlled by some repelling influence between the cells. It is possible that this interaction is mediated via dendritic contacts or close apposition of dendritic processes, as the size of dendritic domains of individual melanocytes varied inversely with the local population density and there was minimal overlapping of the domains.

A mutual repulsion between epidermal melanocytes seems functional, since it would ascertain an even distribution of pigment in the epidermis.

Growth and Differentiation of Cultured Pigment Cells in Chemically-Defined, Serum-Free Media and the Effects of NGF. Raul I. Garcia, Luke Whitesell, George Szabo. Harvard School of Dental Medicine and Harvard Univ., Boston and Cambridge, MA

Chick embryo retinal pigment epithelium (CERPE) cell cultures have been used to study relationships of growth and differentiation in normal, non-neoplastic melanin-producing cells. The CERPE culture system provides easily isolated and maintained isotopic pigment cell cultures. CERPE from 8-day White Leghorn embryos was cultured on plastic Falcon dishes at 37°C in 5 percent CO₂ in air for periods of up to three weeks in a modified Ham's F12 supplemented with calcium, glucose, bicarbonate, and Gentamycin, with/without chick embryo extract and fetal bovine serum (FBS). After two weeks growth in serum-supplemented medium, cultures reach confluency and organize into a monolayer but are still unable to fully express their differentiated as far as melanin production is concerned. We have, for the first time, maintained cultures of normal pigment cells in completely chemically defined medium. However, not only did CERPE cells survive, the absence of serum resulted in hyperpigmentation of cultures. Evidence was gained suggesting the presence of a specific melanin synthesis inhibiting factor in FBS. Serum withdrawal from confluent cultures resulted in a burst of melanin synthesis producing a visible hyperpigmentation within 24 hr. The variable mitogenic effect of FBS in relation to its inhibitory effect on melanogenesis was also studied in conjunction with and in comparison to embryo extract-supplemented cultures. The possible role of Nerve Growth Factor (NGF) on pigment cells was studied. Unlike the effects of NGF on neural crest-derived melanocytes, NGF was found to be not essential for CERPE cell survival, growth, nor pigmentation, in neither early sparse cultures nor late confluent cultures, over a wide range of nanogram concentrations used. This was true in both defined and serum-supplemented media cultures. This absence of NGF effects points to important differences between neural crest-derived melanocytes and the neuroectoderm-derived CERPE in regards to differentiative processes and regulatory mechanisms. (Supported by USPHS Grant AM-20669 from the NIAMDD.)

Use of Density Gradient Centrifugation to Produce Pure Melanocyte Cultures. L.M. Wilkins, S. Wachs, G. Szabo. Harvard School of Dental Medicine, Boston, MA

Previous attempts at establishing pure melanocyte cultures have relied on modifying culture media, often detrimentally, to obtain a differential survival rate favoring melanocytes over
keratinocytes. We have developed a unique method which exploits inherent differences in cell densities between melanocytes (M) and keratinocytes (K) in order to separate these cell types. Epidermal sheets from human and guinea pig skin were obtained by skin-splitting with 0.25 percent trypsin treatment overnight at 4°C, and cells were dispersed by incubation in 0.02 percent EDTA at 37°C for 10 min. Gradients of 60 percent (human) or 70 percent (guinea pig) Percoll (Pharmacia) were formed by centrifugation at 20,000 RPM for 20 min in an SS34 rotor. Cells were layered onto these gradients and centrifuged at 6,000 RPM for 40 min at 4°C. Twenty 0.5 ml fractions were plated into plastic petri plates and incubated for 3-4 days in Minimal Essential Medium + 10 percent fetal calf serum (guinea pig) or Basal Medium, Eagle + 0.5 percent dialyzed fetal calf serum (human). Cultures were then fixed and cell types enumerated.

In both Negroid and Caucasoid human neonatal foreskin, the highest number of melanocytes and keratinocytes were found in the same fractions. Also, the percentage (M/M + K) of black skin melanocytes was found to be higher (40.5 percent) than that of white skin melanocytes (30.8 percent) indicating a higher plating efficiency in our fraction cultures, since it is known that their numbers are equal in vivo. In contrast, Percoll gradients of black guinea pig skin produced separate peaks for melanocytes and keratinocytes. Almost no keratinocytes were found in cultures of the four highest density fractions, which provided melanocyte cultures of >95 percent purity. In summary, the separation of melanocytes from keratinocytes by Percoll gradient centrifugation was only possible for guinea pig epidermis, because the density of human melanocytes and keratinocytes is too similar to be differentiated by this technique.

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**Influence on the Elongation and Elaboration of Dendrites of Guinea Pig Melanocytes *in Vitro***. S.N. Klaus, S. Bencharit, M. Bernhardt. West Haven VA Medical Center and Yale Univ. School of Medicine, New Haven, CT

Monolayer cell cultures of guinea pig epidermis were grown in Cruickshank chambers in Eagle's minimal essential media supplemented with 10 percent fetal calf serum. The formation and elongation of melanocyte dendritic processes were studied. Changes in cell outlines were recorded by serial still and time lapse cine microscopy. Selected cells were examined by transmission and scanning electron microscopy. Measurements of the number and complexity of the dendrites and of the rates at which they elongated were made from camera lucida drawings and film tracings.

Two patterns of dendrite growth were identified: (1) elongation proceeded directly from the tip of a small dendrite “bud” that emerged from the melanocyte perikaryon; (2) elongation occurred by the movement of a pigment cell away from a spot where the distal end of a dendrite had become fixed to either the glass substrate or to an adjacent cell.

Contact between melanocytes and keratinocytes stimulated both patterns of dendrite elongation. Scanning electron micrographs demonstrated microspikes extending from the dendritic processes. Transmission electron micrographs showed both microfilaments and microtubules in the growing portion of the dendrites. Generally, dendrites grew over the free surface of the keratinocytes, away from the glass substrate.

Nerve growth factor (10-100 nanograms/ml) had no effect on dendrite elongation. Dibutyryl cyclic AMP (5 x 10^{-3}M) encouraged dendrite growth and elaboration, even without keratinocyte contact. Colchicine (1 x 10^{-3}M) caused shortening of pre-existing dendrites but did not inhibit new dendrite formation.

**The Establishment of Various Types of Pigmented Cell Lines from Harding-Passey Mouse Melanoma**. Hideaki Takematsu, Takeo Maruyama, Seiki Kataoka, Makoto Seiji. Tohoku Univ. School of Medicine, Sendai, Japan

Harding-Passey mouse melanoma has been extensively used for the biochemical and morphological studies as a model of melanin-forming cells. It is necessary to establish pigmented cell lines from Harding-Passey mouse melanoma. We report the establishment of various types of pigmented cell lines. The melanoma was excised from the Swiss mice 21 days after transplantation. The tumor was cut into small pieces and treated with a Hanks solution (containing 0.5 percent trypsin). After rinsing the cells in growth medium (70 percent Ham's F12 + 30 percent fetal calf serum), the cell suspensions were passed through a 50 μm pore filter.
and were dispersed in the growth medium. All cells were grown in the growth medium routinely in Falcon dishes (#3001) in a humidified atmosphere of 5 percent CO₂ and 95 percent air at 37.5° C. During the culture, the pigmented cells increased gradually in number and they formed colony-like aggregates which rested in fibroblasts on the bottom of the chamber. Using a micromanipulator attached to phase contrast microscope most of the fibroblasts were removed from the dishes. The pigmented cells were transferred to dishes by micromanipulator and cultured in the growth medium. The same procedure replaced three times in culture. Then the cells proliferated in our culture system. These cells were transplantable to Swiss mice, and typical melanotic tumors were produced after the injections of suspensions of these cells. The other cell lines could be established from the tumors produced by the inoculated culture cells in the same procedures as described above. The cells of established cell lines and those of tumors produced by the injections of cell suspensions were observed under light and electron microscopy. The cells retained the characteristics of melanocytes. The pattern of tyrosinase activity, karyotype, and sensitivities to growth factors will also be discussed.

Establishment of Continuously Melanin-Forming Human Melanoma Cell Line and Its Biological Properties. T. Kasuga, I. Nojiri, O. Matsubara, S. Seki, T. Furuse. Tokyo Med. & Dent. Univ., Tokyo, and Natl. Inst. Radiol. Sci., Chiba, Japan

So far, no human melanoma cell lines which produce melanin pigments over the 100th passage have been reported. The cell line already established(HMV-I) derived from primary pigmented vaginal melanoma of 65-year-old female turned into amelanotic since the 90th passage. Therefore, the second attempt was made to establish a human melanoma cell line with melanin production, by using a stock melanoma cell with melanin-forming ability. The stock cells had been frozen at the 27th passage of the same cell line(HMV-I) with liquid nitrogen for five years. The cells were cultivated in Falcon plastic dishes with 0.5 percent soft agar layer (Agar Noble) and growth medium at 37° C in 5 percent CO₂ gas incubator, performed up to the 100th passage. The growth medium used consisted of F10 medium, 20 percent fetal bovine serum and 10 percent human serum. After that, the cell line was maintained with the growth medium with 15 percent fetal bovine serum without soft agar layer in the culture flasks with rubber caps. Passage level of the cell line at time of the present study was the 236th for six years(HMV-II).

The continuous cell line with melanin production(HMV-II) consisted mostly of spindle and dendritic stellate cells with light brownish cytoplasm and moderate-sized nuclei containing nucleoli. Strongly melanin-pigmented cells scattered among the monolayered cells positive for DOPA reaction and melanosomes were found electronmicroscopically. Cell clots of the cell line collected by the centrifuge revealed brown to brownish black in color.

The two human melanoma cell lines, HMV-I and HMV-II, had the difference of the biological properties; these include the melanin synthesis, cell morphology, growth properties, and ultraviolet sensitivities, in spite of no difference between these cell lines in regard to chromosomal modes and X-ray sensitivities. Details of the investigation will be presented.

Establishment of a Human Melanoma Tumor Stem Cell Assay in Soft Agar. F.L. Meyskens, Jr., M.H. Henley, S.E. Salmon. Cancer Center Division, Univ. of Arizona, Tucson, AZ

We have developed a soft agar bioassay for human melanoma tumor stem cells (TSC). Melanoma colony growth was observed in 70 percent of 54 samples with a mean cloning efficiency of 0.01 percent. Biopsy site did not appear to affect cloning efficiency. Two distinct types of clusters/colonies were identified: collections of 15 to 100 light, large cells (LL) and aggregates of 15 to 100 small, dark cells (SD). Cells in the colonies have premelanosomes and melanosomes and demonstrate abnormal karyology. TSC was reduced when the initial cell preparation was depleted of host phagocytic macrophages with carbonyl iron (8 of 8 cases) and restored with adherent cell repletion (3 of 3 cases). Both proliferation and differentiation in the two colony variants of TSC were modulated by neurohormones, including nerve growth factor, melatonin, MSH, FSH, and LH.

The assay has also been used to study chemotherapeutic effects in vitro. Evaluation of 215 in vitro responses to 21 different drugs showed (1) that TSC were resistant to drugs with which the patient had never been treated, and (2) in only 26 of 215 in vitro drug exposures was a greater
Melanogenesis of Melanoma Cells Cultured in a Chemically Defined Medium. Kunio Iwata, Naomichi Inui. The Japan Tobacco and Salt Public Corporation, Hatano, Kanagawa, Japan

Although a number of reports have been published on melanogenesis in melanoma cells, most in vitro studies have been performed with media containing serum. Since serum contains many factors with unknown biological effects, the results of experiments using media containing serum may not afford the exact features of melanoma cells in relation to melanogenesis.

We, therefore, established melanoma cell lines cultured in a serum-free chemically defined medium. These cells have been cultured for over 70 passage generations (Proc Japan Acad 56B:146–151, 1980).

This report deals with the stimulation of melanogenesis on mouse B16 melanoma cells cultured in a serum-free medium by treatment with various substances, such as fetal bovine serum (FBS), dimethylsulfoxide (DMSO), and theophylline. Each substance has shown different manner of effects on cell growth, melanin synthesis, and tyrosinase activity of cells. FBS and DMSO markedly induced tyrosinase activity and/or melanin synthesis of cells without inhibition of cell growth. Theophylline treatment, on the other hand, gave rise to an increase in the melanin content and tyrosinase activity of cells accompanied with inhibition of cell growth. The relationship among cell growth, melanin synthesis, and tyrosinase activity will be discussed.

BrdU Induced Alteration of Gangliosides in B16 Mouse Melanoma Cells. Yoshito Kinoshita, Takuji Takeuchi. Tohoku University, Sendai, Japan

Gangliosides, sialic acid containing glycolipids, of cultured B16 mouse melanoma cells were characterized. Effect of BrdU on their composition and content was also investigated.

Ganglioside fraction was prepared from crude chloroform/methanol lipid extract of cell pellet, employing Folch partition and lyophilization. This fraction was analyzed by thin layer chromatography on silica gel. Only two resorsinol positive (i.e., ganglioside) bands were detected. Cellular ganglioside content was determined as sialic acid content of the ganglioside fraction. Relative amount of two ganglioside bands was estimated by densitometry. Chemical identification of both gangliosides was performed by gas-liquid-chromatography-mass-spectrometry.

Of two ganglioside bands, the major was N-acetyleneuraminyl-hematoside (NANA-GM3) and the minor was N-glycolyneuraminyl-hematoside (NGNA-GM3). In control melanotic cells, NGNA-GM3 accounts for about 10 percent of the total gangliosides. When cells were treated with BrdU, where the cells became amelanotic and less transformed, the ratio of NGNA-GM3 increased up to over 20 percent. Cellular content of total ganglioside did not change significantly and no other additional ganglioside band appeared. Comparison between the melanotic and amelanotic cell lines as to the ratio of NGNA-GM3 in total gangliosides revealed that the ratio in the amelanotic cell lines was higher than that in the melanotic ones.

Cell Type Conversion in a Mouse Melanoma Cell Clone. Seiji Sato, Takuji Takeuchi. Tohoku University, Sendai, Japan

A melanoma cell clone was isolated from cultured B16 mouse melanoma cells. This clone, conv, which is characterized by rounded spindle-shaped cell morphology, is not highly melanotic under the normal culture condition but has high tyrosinase (DOPA oxidase) activity. When the cells were seeded on a plastic dish to form colonies, two kinds of cell types appeared. One was cytochemically DOPA-positive spindle-shaped cells (S type) with the same phenotypes as those of the parental cells. The other was DOPA-negative fibroblast-like cells (F type) that
possessed no melanosomes. The conversion from S type to F type has been reproducible even after quite a number of recloning.

The cell type conversion was shown to be dependent on the following two factors. The first is the concentration of calf serum. As long as S type cells were seeded on a plastic dish to form colonies, most of the colonies exhibited F types in the medium with a concentration of calf serum, 10 to 20 percent. However, they remained S types in the medium with a low concentration of calf serum (2 percent). The second is the substratum of culture dish. When the colony formation of S type cells was performed on a glass dish with a concentration of calf serum, 10 to 20 percent, the conversion rate was low. Cloned F type cells reverted in a low frequency to S type cells after several cell passages, indicating that the cell type conversion was reversible.

Tyrosinase Activity of Cultured Mouse Melanoma Cells under Different Culture Conditions. Hisaaki Saeki, Atsushi Oikawa. Res. Inst. for TB and Cancer, Tohoku Univ., Sendai, Japan

The tyrosinase activity of cell-free extracts (TyH) of cultured B-16 melanoma cells changes in a bell-shaped pattern during culture with a peak at the late exponential phase of growth in Eagle's MEM, while in Leibovitz's L-15 medium it does not drop but continues to keep a high level. This high activity is due to the presence of galactose instead of glucose in L-15, because the same is true in a modified MEM in which glucose is replaced by galactose (Gal-MEM). This difference in tyrosinase activity was analyzed as a shift of balance between synthesis and degradation of the enzyme, assuming that TyH reflected the amount of tyrosinase. Experiments were conducted with stationary phase cultures in the presence and absence of cycloheximide (1 μg/ml).

In the stationary phase, (1) the melanoma cells did not synthesize tyrosinase in MEM, but in Gal-MEM they continued to synthesize tyrosinase. (2) In a pH range between 6.3 and 7.2, the rate of the enzyme synthesis was higher at higher pH in Gal-MEM. (3) The degradation rate did not depend on sugars and was lower at higher pH, the half life at pH 6.3 was about one-third of that at pH 7.2, where it was about 1.8 days. (4) Degradation of tyrosinase at low pH was reduced to that at neutral pH by NH₄Cl (10 mM) or leupeptin (100 μg/ml), indicating that the lysosomal proteases were responsible for the degradation of tyrosinase. (5) The effect of sugars or medium pH on the amount of the enzyme was reversible. (6) Tyrosinase activity expressed in culture (TyC) usually changed similarly to TyH by sugars or medium pH.

The bell-shaped pattern of tyrosinase activity of cells cultured in Eagle's MEM is explained by these facts.

Selective Aberration and Pigment Loss in Melanogenic Subcellular Compartments of the Cultured Melanoma Cells Induced by Inhibited Carbohydrate Metabolism Using Glucosamine and Tunicamycine. Yutaka Mishima, Genji Imokawa, Gakuzo Tamura. Kobe University School of Medicine, Kobe, and University of Tokyo, Japan

Since we reported much higher tyrosinase activity in the GERL and coated vesicle fraction than that in premelanosome fraction at the last Conference (1977), we have further investigated the specific function of melanogenic subcellular compartments including GERL, coated vesicles, and premelanosomes by their dynamics following the selective inhibition of the glycoprotein synthesis. This is found here to interrupt melanization of melanoma cells in vitro. We have found that the addition of glucosamine (Gln, 1 mg/ml) or tunicamycine (TM, 0.4 μg/ml), a specific inhibitor of lipid-carrier dependent glycosylation of protein, to cultured B-16 melanoma cells produces a marked loss of pigmentation. It has been found by electron microscopy that Gln- and TM-induced amelanotic cells are now forming uniquely altered melanosomes containing no or little melanin, although their population is not substantially reduced. Among melanogenic compartments, selective aberration of melanosomes is seen as deformity, bulging and segregation of their interior membrane, as well as the intramelanosomal formation of irregularly concentric lamellar structure. No apparent structural deformity of Golgi apparatus, GERL, and coated vesicles has been observed. Quantitative analysis of altered and non-altered melanosomes has revealed that the ratio of altered premelanosome to
Effect of Theophylline on the Growth and Expression of Different Surface Antigens of Cultured Human Melanoma Cells. S.K. Liao, P.C. Kwong, P.B. Dent. McMaster University and the Ontario Cancer Treatment and Research Foundation, Hamilton Clinic, Hamilton, Ontario, Canada

Previous studies with mouse melanoma have shown that inhibition of cyclic nucleotide phosphodiesterase by agents, such as theophylline (TH), impairs growth potential, promotes maturation, and decreases tumorigenicity of cultured melanoma cells. We now report that TH may have similar effects on human melanoma cells. Human melanoma cells (CaCL 73-36 line) cultured in medium containing TH, at concentrations of 0.1, 0.5, 1 and 2 mM reduced plating efficiency by 16, 35, 64, and 99 percent of that in control cultures. Treatment of melanoma cells with 1 mM TH resulted in approximately 30 percent reduction in saturation density as compared to the control. 1 mM of TH was chosen for further investigation of its effect on binding of cells to antibodies against melanoma-associated antigens (MAA) and β2 microglobulin (β2m) which are known to be present on these cells. The cells cultured in the presence and absence of TH for 16, 64, 96, and 168 hr were used for quantitative absorption with a monkey anti-MAA (Liao et al: Cancer Res 39, 1979) and a rabbit anti-β2m antisera, followed by the measurement of residual antibody reactivity by mixed hemadsorption assays. TH treated cells showed a maximum fourfold increase in MAA and a fivefold increase in β2m expression at 96 hr, at which time the cultures were approaching the stationary phase of growth. Untreated cells did not change in their ability to absorb antibody during log and stationary phases of growth. To rule out the possibility that TH nonspecifically induces neoantigens or promotes expression of hidden antigens, we showed that the Daudi lymphoma cells lacking MAA and β2m grown in medium with and without TH failed to remove antibody reactivity. The data demonstrate that TH can enhance the expression of both MAA and β2m surface antigens and has antiproliferative activity on human melanoma cells. It remains to be shown whether TH enhances antigen expression in an uncloned population of melanoma cells by inhibiting cell growth or by selective elimination of weakly antigenic subpopulations.

Insulin and the Proliferation of Cloudman Melanoma Cells. R. Kahn, M. Murray, J. Pawelek. Yale Univ. School of Medicine, New Haven, CT

Insulin is a potent, reversible inhibitor of proliferation in Cloudman S91 mouse melanoma cells. The inhibition seems to be unique to the Cloudman line since five other cell lines, including the mouse B16 and hamster Greene melanomas, were unaffected by insulin under the same culture conditions. Variants of Cloudman S91 cells were isolated which differed from wild-type (WT) cells in their response to insulin. Most of these variants were resistant to insulin (INS<sup>res</sup>) and had the same generation time independent of the presence of the hormone. One line (INS<sup>dep</sup>) was found to require insulin for growth. This line was about 15 times more sensitive to the proliferative effects of insulin than the WT. Revertants of the INS<sup>dep</sup> line were selected for their ability to proliferate in the absence of insulin. Five out of five such revertants were insulin resistant, suggesting that the INS<sup>dep</sup> line arose as a result of at least two separate mutations. Both WT and INS<sup>dep</sup> cells showed enhanced uptake of <sup>14</sup>C-o-aminoisobutyric acid (AIB) when exposed to insulin. Dose-response curves of the stimulation of AIB uptake in WT and INS<sup>dep</sup> cells were superimposable. Stimulation of AIB uptake and stimulation of proliferation by insulin were not under coordinate control since AIB uptake was increased equally in the wild-type cells when proliferation was inhibited and in INS<sup>dep</sup> cells when proliferation was enhanced. Binding of <sup>125</sup>I-insulin was used to demonstrate the presence of specific, high affinity insulin receptors on the cells. INS<sup>res</sup> variants generally had fewer receptors than WT, but in no case did the magnitude of this effect appear to be sufficient to explain the insensitivity to insulin. The INS<sup>dep</sup> variant showed a greater than twofold increase in the number of insulin receptors per cell, compared to WT. Revertants of the INS<sup>dep</sup> line had the same number of receptors as WT. The specificity for both binding and for the effects on proliferation were the same in WT.
and INS<sub>dep</sub> cells. Since the effects of insulin on proliferation were opposite in the two lines, we propose at least two distinct sites of insulin action on the cells. Further isolation and analyses of Cloudman lines with unusual responses to insulin should be useful for understanding the molecular basis of action of this hormone.

**Fatty Acids and Melanogenesis.** S. Shono, K. Toda. Tokyo Teishin Hospital, Tokyo, Japan

The supplementation of selective nutrients in promotion of the physiological growth of melanocytes and in the formation, melanization, and degradation of melanosomes in melanogenesis is becoming increasingly important. This study was designed to examine the role of two fatty acids, palmitic acid and linoleic acid, in melanogenesis. C-57BL/6 mouse epidermal cells were cultured for 20 days in McCoy's 5a medium supplemented with 1 percent fetal calf serum. The tyrosinase activity of these organ cultured cells with and without the supplementation of 0.04 percent palmitic acid and 0.04 percent linoleic acid were examined by a colorimetric method. Tyrosinase was found to increase with the addition of palmitic acid, whereas the addition of linoleic acid to the medium markedly decreased tyrosinase activity. Direct effects of these two fatty acids on tyrosinase activity and on the synthesis of melanosomes were also evaluated in B-16 mouse melanoma cells in a logarithmic growth phase cultured in Ham's F-12 medium which was supplemented with fatty acid-free 1 percent bovine serum albumin. Tyrosinase activity of melanoma cells grown from 1, 2, 3, 4, 5, 7, and 9 days was determined by incubation of cells in buffered Dopa solutions. Tyrosinase activity was found to increase by 40 percent after five days with 0.04 percent supplementation of palmitic acid; however, when cells were grown with the addition of 0.04 percent linoleic acid a 40 percent decrease in tyrosinase activity was observed. There was no difference in the growth rate of these cultured cells grown with or without the addition of fatty acids. Likewise, the production of melanosomes in these melanoma cells was found to be increased with palmitic acid and decreased with linoleic acid. These studies suggest that tyrosinase activity and the formation of melanosomes is markedly affected either by the metabolism of fatty acids or by the enzymes involved in the metabolism of fatty acids. The activity of prostaglandins and cyclic nucleotides can also be influenced by palmitic acid and linoleic acid; and these, in turn, may also influence the melanogenic activity of these cells.

**Extinction of Cytochemical Dopa Oxidase Activity in Mouse Melanoma—Chick Embryo Fibroblast Heterokaryons: A Single Cell Analysis.** Marlene S. Schwartz, John A. Brumbaugh. Univ. of Nebraska, Lincoln NE

Cells from a pigmented subclone of B-16 mouse melanoma were treated for three hours with 10 μg/ml of mitomycin C and fused with 3H-thymidine-labeled primary chick embryo fibroblasts, which were wild-type with respect to eumelanin production. Visually, the melanoma cell would contribute an unlabeled nucleus (incapable of division) and pigment granules to the fusion product, while the fibroblast would contribute a labeled nucleus.

Fifty hours post-fusion the cells were fixed for transmission electron microscopy and incubated in 5 mM DOPA to test for DOPA oxidase activity. The epon embedded cells were emulsion coated for light microscope autoradiography so that heterokaryons could be recognized. Electron microscopic examination of the identified heterokaryons showed a significant reduction of DOPA oxidase activity compared with control cells ($\chi^2 = .005 < P < .01$).

The population of heterokaryons examined was heterogeneous with respect to DOPA reaction product and other cytoplasmic components. A few of the binucleates showed no evidence of extinction in that they contained DOPA-positive golgi stacks, premelanosomes, and melanosomes. Other heterokaryons, however, seemed to be autophagocytizing their melanosomes. In the extreme cases, fused cells showed no DOPA reaction product but had an abundance of vesicles, golgi stacks, and/or swollen endoplasmic reticulum.

**Activation and Suppression of Melanogenesis in Hybrid Cells between Mouse Melanoma Cells and Chick Embryonic Cells.** Sei-ichi Ishiguro, Takuji Takeuchi. Tohoku University, Sendai, Japan

Sendai virus-induced fusion of 6-thioguanine-resistant mouse melanoma cells (TG14) with various types of chick embryonic tissue cells resulted in the formation of hybrid cells. Each type of hybrid clones showed characteristic tyrosinase activity in relation to melanin production. An
extensive production of melanin was observed in the hybrid cells between TG14 cells (not highly pigmented) and retinal pigment cells. The melanin content of the hybrid cells was much higher than the original melanoma cells, whereas the tyrosinase activity was the same level as the original cells. Electrophoretic analysis showed that banding patterns of tyrosinase were not chick type but mouse melanoma type. Numerous stage III and IV melanosomes of mouse melanoma type were observed in the pigmented hybrid clones. It seems likely that chick embryonic pigment cells provide a factor which accelerates melanogenesis without the activation of tyrosinase. On the other hand, hybrid cells between mouse melanoma cells and chick embryonic liver cells exhibited low melanogenic activities.

Mouse Model of Tyrosinase Positive Type Oculocutaneous Albinism: Tyrosinase Activity and Ultrastructure of Melanocytes. Yoshiaki Hori, Masako Mizoguchi, Atsushi Kukita. University of Tokyo, Tokyo, Japan

Human tyrosinase negative type oculocutaneous albinism exhibits no tyrosinase activity of melanocytes in vivo as well as in vitro. In contrast, tyrosinase positive type has positive tyrosinase activity of melanocytes in vitro and partial pigment formation in melanosomes in vivo.

An albino mouse has no tyrosinase activity and no melanin formation in melanocytes. However, there is a tyrosinase positive type albino mouse which has white hair and pink eyes and is called as a "chinchilla" mouse. The two types of albino mice cannot be distinguished phenotypically. Although this relationship is similar to tyrosinase negative type and tyrosinase positive type of human oculocutaneous albinism, the chinchilla mouse does not increase its pigmentation with advancing age.

Genotypes of chinchilla mouse are a/a, b/b, cch/cch, d/d and p/p.

Melanosomes of hair bulb melanocytes and retinal pigment epithelial cells of the chinchilla mouse were observed under an electron microscope and tyrosinase activity of these cells were examined. When a hair bulb was incubated in L-tyrosine or L-dopa solution, pigmentation occurred in the hair bulb. Most of the melanosomes in hair bulb melanocytes and retinal pigment epithelial cells were in Stages II or III. After incubating in L-tyrosine or L-dopa solution, melanosomes increased melanization. Reaction products were recognized in the melanosomes, GERL and some vesicles near the Golgi complexes.

In albino mice (A. Jackson and DDY), however, hair bulb melanocytes showed no pigmentation after incubating in L-tyrosine or L-dopa solution and all melanosomes in melanocytes were in Stages I or II.

Ultrastructure, Histochemical and Biochemical Studies of the Melanin Metabolism in Pallid Mouse. M. Ito, K. Hashimoto. Veterans Administration Medical Center, Dayton, OH  D. Organisciak. Wright State Univ., Dayton, OH

The pallid mouse (C57/6J-Pa/Pa), which is considered to possess genetically abnormal Mn metabolism and high lysosomal activities, has a peculiar pale coat color and dark red eyes. Although the coat color is determined by genetic factors, it is still unknown what components of the melanin metabolism are important in the determination of coat color. To solve this question, the eyes and the hair follicles of pallid mice and those of black mice (C57/6J-+/Pa) were examined ultrastructurally, histochemically, and biochemically.

In pallid mice, the cells in the pigment epithelium of retina had a small amount of melanin granules. In the choroid, abundant immature melanin granules were observed. They often aggregated and constituted membrane-bound large granules, which might be melanolysosomes because some of them were acid phosphatase-positive. Biochemically, paranitrophenolphosphate hydrolysis technique showed the acid phosphatase activity of pallid eye was higher (13.06 ± 0.70 n moles/min/mg. protein) than that of black eye (12.07± 0.24) (0.01 < p < 0.025). The melanosomes in the melanocytes and the keratocytes of hair follicles of pallid mice were more immature and smaller than the those of black mice, and some of them showed acid phosphatase reactions.

Dopa reactions at light and ultrastructural levels indicated that Dopa oxidase activity was present in the melanocytes of both eye and hair of the pallid mice as well as those of the black mice. Dopa reaction seemed to be equal or slightly higher in pallid mice.

From these results it was concluded that the pallid color is not related to low Dopa oxidase activity but by immaturity of melanosomes and high activities of lysosomal enzymes; melanoly-
ososomes seemed to digest some of these immature melanosomes and therefore contribute to the light color of pallid mice.

Interestingly, we found that some of the melanocytes in the eyes and the hair follicles of pallid mice produced macromelanosomes which were similar to those found in café-an-lait spot and multiple lentigines syndrome.

**Premature Loss of Melanocytes from Hair Follicles of Light ($B^b$) and Silver (si) Mice.** W.C. Quevedo, Jr., R.D. Fleischmann, J. Dyckman. Brown Univ., Providence, RI

In mice, follicular melanocytes normally synthesize melanosomes from early in the hair growth cycle (Anagen III) to the end of Anagen VI (ca. day 17), a time when active proliferation of the hair matrix also terminates. In light ($a/a$, $B^b$ / $B^b$) mice, however, melanocytes disappear prematurely from the hair bulbs, often by the ninth day. The result is that hairs are produced which are pigmented at their tips but devoid of pigment over variable lengths of their bases. Light microscopy reveals that $B^b$ melanocytes are uprooted and incorporated either in toto or as large fragments into the developing hair. Prior to uprooting, the melanocytes usually become enlarged when dense aggregates of melanosomes of varying size accumulate within their perikarya. The dendrites of these melanocytes are poorly developed and their nuclei are frequently pycnotic.

Electron microscopy indicates that $B^b$ melanocytes synthesize abnormal melanosomes and isolate them within autophagosomes, producing some of the pigment clumps seen at the light microscope level. In addition, large masses of melanosomes and other organelles are found within secondary lysosomes of adjacent keratinocytes. Although observations on silver ($a/a$, si/si) mice have been restricted to light microscopy, the pattern of melanocyte performance appears to be comparable to that found in $B^b$ mice. In both genotypes, the atypical behavior of melanocytes is limited to the hair follicles, for epidermal melanocytes of UVL-irradiated trunk and tail skin are not obviously abnormal in structure or function.

**Differentiation of Melanocyte in Culture in the Black Eyed White Mouse.** Kazuo Ito, Takuji Takeuchi. Tohoku University, Sendai, Japan

$m^b$ is an allele at $m$ (microphthalmia) locus. When homozygous it usually eliminates all the pigment in the coat but not eyes, which remain black. Thus homozygous $m^b$/$m^b$ mice totally lack melanocytes in the skin.

In order to analyze the mechanism of gene action of $m^b$, we performed a series of organ and cell culture experiments. For the organ culture, the membrane origami method was used. When a piece of dorsal skin from wild-type mice was organ-cultured, differentiation of DOPA-positive melanocytes was observed. These cells displayed the characteristic response to MSH and theophylline treatments. On the other hand, in the explants of the mutant skin, no DOPA-positive cell was found. MSH and theophylline were ineffective.

When newborn wild-type skin was cell-cultured, numerous DOPA-positive cells appeared. The DOPA-positive cells significantly increased in number of MSH and theophylline treatments. In the cell culture from the newborn mutant skin, occurrence of the DOPA-positive cells was also observed, although the number of the cells was less than that of wild type. The occurrence of the DOPA-positive cells was also enhanced by MSH and theophylline treatments. These results suggest that $m^b$/$m^b$ neonatal skin contains melanoblasts, and that the site of primary gene action is in skin environment, not in the melanoblast.

**An Experimental Analysis of Agouti Gene Function in Vitro.** Hidetoshi B. Tamate, Takuji Takeuchi. Tohoku University, Sendai, Japan

In the house mouse, the agouti gene is known to control the type of melanin formed in hair-bulb melanocytes. Animals homozygous for recessive $a$ gene produce dark melanin (eumelanin), while $A^t$/a mice produce yellow melanin (phaeomelanin). Several reports have shown that melanocytes are primarily capable of producing both types of melanin. However, little is known about the control mechanism that determines the type of melanin produced. In order to find the primary action of the agouti gene, an attempt was made to induce eumelanin formation in genotypically yellow skin.

Skin explants from newborn mice with genotype $A^t$/$a$ were wrapped in membrane filters,
and cultured in Ham's F-12 medium supplemented with 20 percent fetal calf serum. The explants in control culture continued phaeomelanin synthesis throughout a 10-day culture period, whereas eumelanin was produced in the explants cultured in the medium containing α-MSH. This change in melanin synthesis was observed within 12 hours after the addition of α-MSH. The explants continued eumelanin synthesis for over 10 days after removal of α-MSH from the medium. Eumelanin synthesis was also induced by the addition of cAMP and theophylline to the culture medium.

Ultrastructural and biochemical changes during the “yellow to black” conversion were investigated.

Changes in Melanogenesis from Eumelanin to Phaeomelanin. K. Okazaki, S. Akiu, S. Ota, F. Morikawa, V. Mizuhira, M. Seiji. Shiseido Co., Ltd., Tokyo, Tokyo Medical and Dental University, Tokyo, and Tohoku University, Sendai, Japan

Ultrastructural and biochemical characteristics of melanosome and melanin were examined in hair of C3H/He-strain mice. The agouti band is formed by the changes in pigments from black to yellow then yellow to black in the hair follicular melanocytes. Various stages of the hair follicle, at black stage, at yellow stage, and at color-shifting stage, were examined by an electron microscope.

Ultrastructurally, typical eumelanosomes, and phaeomelanosomes were observed in the melanocytes at the black stage and yellow stage of the hair follicles, respectively. At a particular stage of anagen through which hair bulb changes its color from black to yellow, specific ultrastructure of melanosome having the characteristics of both eumelanosome and phaeomelanosome were found in the melanocyte as a single melanosome; lattice-like fine structure and granular melanin depositions.

Biochemically, trichochrome F and 5, S-cysteinyldopa were detected by spectrophotometry, fluorometry, and mass-fragment-graphy in the extracts of the agouti hairs.

The ultrastructural and biochemical findings will be discussed in relation to the mechanisms of hair color shift in the agouti hairs, with special reference to the changes in morphogenesis and melanogenesis of the hair follicular melanocytes.

Cytogenetics of Heritable and Induced Melanoma in Xiphophorus. A. Anders, G. Kollinger, K. Chatterjee. Institute of Genetics, Justus Liebig University, Giessen, Federal Republic of Germany

Melanoma develops in some Xiphophorus hybrids “spontaneously” (heritable), in others following the exposure to carcinogens (induced). Histological, ultrastructural, and chromosome analyses reveal significant differences between these two types of neoplasia. The heritable melanoma is a compact mass of tissue mainly formed of incompletely differentiated pigment cells. A number of cells undergo a process of nuclear fragmentation. Electron microscopic study shows frequent occurrence of nuclear pockets and projections in the highly melanized melanocytes. The majority of the cells have a diploid karyotype with no side line. The induced melanoma differs from the heritable melanoma in having comparatively less amount of pigment cells and an enhancement of that of the connective tissue. No nuclear fragmentation has been encountered. At the ultrastructural level the melanocytes are less melanized and have rounded nuclei without any pockets and projections. Various types of structural aberrations in the chromosomes belonging to the categories of chromatid and isochromatid gaps and breaks are present. They seem to be randomly distributed in the karyotype. In the chromosomes, however, they have a nonrandom distribution usually occurring in the distal half. We conclude that those abnormalities present in the induced melanomas but absent in the heritable ones and vice versa are without direct causal relevance to neoplasia, but are essentially what may be called epiphenomena and the real genetic change underlying neoplasia is a subchromosomal event.

Implying that the discrimination between the heritable and the induced melanoma in Xiphophorus might also be valid for humans, it could be of prognostic value, because the heritable melanoma as proved in Xiphophorus is much more deleterious than the induced melanoma.

Experimentally Induced Promotion of Differentiation of Melanoma Cells in Xiphophorus. M. Schartl, A. Schartl, A. Anders. Institute of Genetics, Justus Liebig University, Giessen, Federal Republic of Germany

In Xiphophorus the stage of differentiation of the melanoma cells determines the malignancy
of melanoma. The objective of the experiments was to find out, whether modulation of cell differentiation by (a) diffusible factors from non-tumorous cells, and (b) treatment with differentiation-promoting substances leads to an alteration of malignancy.

(a) Tissue of extremely malignant melanoma has been grafted to 2-3 day-old non-tumorous embryos. 5-6 days after transplantation, increased differentiation of melanoma stem cells to terminally differentiated transformed melanophores is observed and subsequently the cells are removed by macrophages. These processes lead to benignisation of the grafted melanoma. Some of them grow throughout the whole life of the host as extreme benign melanoma; in some cases the melanoma cells even regain the ability for normal regulation of growth. Other melanomas show total regression.

(b) Treatment of neonate and adult melanoma-bearing fish with both cyclic AMP and testosterone shows opposite effects. In the melanoma of neonates, where most pigment cells are in an early stage of differentiation and not yet competent for neoplastic transformation, the cells become competent via differentiation. This process results in an increase of growth and malignancy of the treated melanoma. On the contrary, in the melanoma of adults, where most pigment cells are in an advanced stage of differentiation and already neoplastically transformed, the cells become terminally differentiated by the substances applied, and are removed by macrophages. These processes result in benignisation of the melanoma leading to a partial regression.

Characterization of Established Cell Lines of Hereditary Melanomas in Xiphophorus Fish Hybrids. Yuko Wakamatsu, Kenjiro Ozato, Atsushi Oikawa, Masataka Obika. Yoshida College, Kyoto University, Kyoto, Research Institute for Tuberculosis and Cancer, Tohoku University, Sendai, and Keio University, Yokohama, Japan

Cell lines of hereditary melanomas developing in interspecific hybrids of the Xiphophorus fish were established for making a cytotogenetical study of this melanoma system. Melanotic and amelanotic melanomas were obtained from melanotic F₁ hybrids between the spotted platyfish and the green or albino swordtails. The tissues were dissociated and cultured in Eagle's minimum essential medium supplemented with 10 percent fetal calf serum. These cell lines have been subcultured for more than two years since their initial isolation.

The cells of these lines show dark and granulous cytoplasm under a phase contrast microscope, but no apparent melanosome is observed. The cells are mono- or poly-nucleated. Giant lobulated nuclei are often seen in large epithelial cells. The cells show no contact inhibition and grow in an overlapping manner to form many clumps. Adhesion of these cells and cell clumps to the substratum is so weak that they can be dislodged by gentle pipetting. The cultures extensively acidify the medium.

The cells show various degrees of Dopa-reaction. The most intensive reaction is observed in small round cells. The proportions of this type of cell vary among the cell lines. Various degrees of tyrosinase activity are detected among cell lines. The cell lines are heteroploid in chromosome number. Ultrastructure of the cell lines is studied in comparison with that of the melanoma tissues in vivo.

Ultrastructure and Physiology of Shrimp Integumental Chromatophores. P.Y. Noël, C. Chassard-Bouchaud, M. Hubert. Univ. Paris-VI, France

Caridean shrimps have at least four types of integumental chromatophores which accumulate specifically either black (omomochrome), white (isoxanthopterine and riboflavine), yellow, red or diversely colored (carotenoid) pigments. These cells often assemble to make colored multicellular units (chromatosomes).

Ultrastructural studies reveal that, unlike many poikilothermic vertebrates, there is only one type of chromatogranule in the chromatophore. One exception is known with some Palaeoninae where the astaxanthin can be found either as a lipocarotenoid droplet without bounding membrane (red pigment) and as a carotenoprotein crystal (blue pigment). Only one nucleus is found in each cell; the other usual organelles in caridean chromatophores are mitochondria and microtubules associated with endoplasmic reticulum that often presents a scalariform arrangement as in nervous fibers. Golgi apparatus is never found in normal shrimps; it appears only after removal of neuroendocrine complex of the eyestalk. Centriole and centrosome are absent.

In most of the shrimps whose chromat physiology is known, chromatophores are purely hormonally controled, as in other Crustacea, by polypeptidic hormones issued from the central
nervous system. In addition, in Processidae, direct photosensitivity of erythrophores occurs, as well as a nervous control through peripheric nerves, since unilateral eyestalk ablation gives unilateral color changes, as in some fish and reptilians. The chromatophores of several species of caridean shrimps do, or do not, present in vitro responses to chromactivating hormones, and/or to neuromediators (serotonin, adrenaline...). This indicates the presence of differences in the control of pigment granule migration through membrane receptors.

Action of Melanophore-Stimulating Hormone on Dermal and Epidermal Melanophores of the Siluroid, *Parasilurus Asotus.* Ryozo Fujii, Yoko Miyashita. Sapporo Medical College, Sapporo, Japan

For a more precise understanding of the mechanisms regulating chromatophores, it is necessary to develop better methods for assessing the motile responses of these cells. An attempt was first made to improve the photometric technique, in which a silicon photocell is employed to transduce the light transmitted through an isolated skin preparation into electric current. By making use of a high input-impedance, low bias-current operational amplifier, a current-to-voltage converter with an excellent linearity was built. Thus, the transmittance could be recorded exactly on an electronic chart recorder.

In *Parasilurus asotus*, both dermal and epidermal melanophores were found to be motile. Generally, their responses proceeded in the same direction. Thus, the photoelectric method was justifiably applied in most experiments. However, the time course of the response of the epidermal melanophores was generally slower than that of the dermal cells, so that it was therefore sometimes desirable to estimate the progress of the responses of these two kinds of cells separately. For this purpose, we employed conventional photomicrography.

Alpha-MSH was extremely effective in dispersing melanosomes in both dermal and epidermal melanophores, in which the pigment had previously been aggregated by the action of melatonin. The threshold concentration of the hormone was estimated to be less than $10^{-10}$ M. Ca ions were required for the MSH action. Ba$^{2+}$ and Sr$^{2+}$ replaced the Ca$^{2+}$, while Mg$^{2+}$ was only slightly effective. Ions of transition elements, including Mn, Co, Zn, and Cd, and of monovalent alkaline metals, i.e., Li, K, Rb, and Cs, did not replace the Ca$^{2+}$. It was also found that even Na ions were unnecessary for the MSH action. Pharmacological analyses suggested that cAMP is the intracellular mediator of the hormonal effect.

Purification of Goldfish Xanthophores and Their Response to Hormone or Cyclic AMP. Szecheng J. Lo, Dale G. Kern, Tom J. Lynch, T.T. Tchen, John D. Taylor. Wayne State Univ., Detroit, MI

In order to study biochemically the hormonal effects on pigment cells, particularly pigment organelle translocation, we have undertaken to purify goldfish xanthophores and to define the cell biology of these cells as a prelude to biochemical studies. We shall describe a simple procedure by which one can isolate, in one day, milligram quantities of hormone-responsive xanthophores of over 90 percent in purity. We shall also present evidence for the following conclusions: (1) ACTH, but not c-AMP or theophylline, induces plasma membrane internalization with disappearance of ACTH-receptors from the cell surface. All three induce carotenoid droplet dispersion. (2) In the absence of ACTH, the pigment aggregates slowly but is accelerated by treatment with epinephrine which also induces plasma membrane internalization. However, ACTH receptors are still present on the cell surface, suggesting specificity of the plasma membrane internalization process induced by epinephrine versus ACTH. (3) Pigment aggregation proceeds through an initial phase of "clumping" which requires F-actin and a later phase of formation of a single larger aggregate which requires microtubules. (4) ACTH stimulates biochemical processes including protein synthesis.

Comparative Pharmacology of Receptors for Neurotransmitter and Hormones of Fish Chromatophores. Tetsuro Iga. Shimane University, Matsue, Japan

The neurotransmitter and hormone receptors mediating the movement of pigment granules within chromatophores, especially melanophores and leucophores, of fishes and their pharmacological features were studied, using isolated scale preparations of freshwater teleosts, *Zacco temmincki* and *Oryzias latipes.* In a physiological solution, the melanophores keep a state of melanosome dispersion, while the leucophores assume a state of leucosome aggregation. Nerve
stimulation and catecholamines induced melanosome aggregation and leucosome dispersion. These responses were induced through alpha adrenoceptors in the melanophores and beta adrenoceptors in the leucophores. In the melanophores, the order of potency of catecholamines was norepinephrine (NE) > epinephrine (E) > epinine (Ep) > dopamine (D) > isoproterenol (ISO), whereas in the leucophores, the order was ISO > NE > E > D. The existence of beta adrenoceptors in the melanophores, which mediate melanosome dispersion, and of alpha adrenoceptors in the leucophores, which mediate leucosome aggregation, was recognized. In the melanophores, the order of potency of catecholamines was ISO > E > NE > Ep > D, whereas in the leucophores, the order was E > NE > ISO. Melatonin was effective in causing melanosome aggregation. MSH acted to disperse the pigment within the leucophores and the melanophores. It was argued that the alpha adrenoceptors and the MSH receptors in both melanophores and leucophores possess sulphydryl groups on or near their active sites.

**Cell Cycle Controls of Melanoma Cells Grown in Serum-Containing and Serum and Hormone-Free Media.** J.M. Varga, D. Burkholder, G.E. Moellmann, D.T. Lambert. Yale Univ. School of Medicine, New Haven, CT

Cloudman S91 melanoma cells have been subcultured and transferred more than twenty times in a medium containing only essential nutrients, no serum, and no hormones. Although these cells failed to respond to MSH by induction of tyrosinase and melanin synthesis, morphologically they appeared to be melanoma cells. The cell cycle of these cells was analyzed by quantitative flow cytometry. We found that cells grown in the presence of serum have a "slow-down" in early S-phase. When they are cultured in the hormone-free medium, they grow faster than in the presence of serum and the cells pass through S-phase without a slow-down. When the cells are cultured in the hormone-free medium in the presence of dibutyryl cAMP or Prostaglandin E1 or Cytochalasin B, they show an early S-phase block which appears at the same relative position of DNA synthesis as the "slow-down" in the presence of serum. Cell cycle dependence of MSH receptors was studied by two-parameter flow cytometry of cells stained with propidium iodide (DNA) and a biologically active FITC-MSH conjugate. We have found that cells grown in the presence of serum express receptors for MSH predominantly in the G2 phase. Supported by grants #CA 26081 and 5 KO4 Al00154 from USPHS.

**Mechanism of Action of Melanotropin-Daunomycin.** J.M. Varga, G. Wiesehahn, N. Asato, J.E. Hearst. Yale Univ. School of Medicine, New Haven, CT, and U.C. Berkeley, CA

We have previously demonstrated that a melanotropin-daunomycin conjugate has receptor-mediated cytotoxicity in cultured murine melanoma cells (Varga et al: Nature 267:56, 1977). Preliminary experiments had shown that internalization of melanotropin-daunomycin was necessary for toxicity of the conjugate. Therefore, it was important to see the effects of transglutaminase inhibitors which are known to inhibit clustering and internalization of polypeptide hormones (Davies et al: Nature, 283:162, 1980). We have found that ammonium acetate, methylamine, and chloroquin decreased the cytotoxicity of melanotropin-daunomycin significantly, showing that endocytosis is indeed essential for the toxic action of the conjugate. We have also investigated the interaction between purified DNA and melanotropin-daunomycin by spectrophotometry and by an agarose gel assay that detects unwinding of DNA due to ligand binding. We found an association constant of 4.8 × 10⁶ L/M and unwinding angle of 10 ± 1.5° for daunomycin. With the melanotropin-daunomycin conjugate, however, there was no detectable binding and the association constant was less than 4.8 × 10³ L/M. We conclude that the cytotoxicity of melanotropin-daunomycin is not due to direct binding of the conjugate to DNA. We assume that the conjugate is metabolically activated after it is internalized, freeing the daunomycin in a form that can bind to DNA and thereby interfere with transcription and replication. Supported by grants #CA 26081 and 5 KO4 Al 00154 from USPHS.

**Ultrastructural Variation in Skin Color and Comparison of Sun-burning Capacity in Light- and Dark-Skinned Solomon Islanders.** Paul L. Garcia, Robert E. Richardson, Ruth E. Mitchell, George Szabo. Harvard School of Dental Medicine, Harvard Univ., Boston, MA

Ultrastructural differences exist between Solomon Islander populations in the mode of
melanosome packaging found in keratinocytes, and these correlate with reported linguistic, anthropometric, and serologic variation in the Solomons. Our findings represent the first clear-cut genetic difference found between Solomon Islanders and suggest that considerable genetic diversity may have existed in the original founding populations. We have used skin biopsy specimens and reflectance data obtained by the Harvard Solomon Islands Expeditions from the lighter skinned Kwaoi of Malaita Is. and the darker Nasioi of Bougainville Is. in a natural experiment on the effects of skin color on suntanning capacity.

Natural selection is believed to have played a role in development of human skin color variation, finding a correlation with dark skin and high UV intensities related to latitude. However, equatorial and higher latitudes differ not only in average UV intensities but also in seasonal variations that are quite dramatic at higher latitudes. It has been suggested that persons with lighter skin may be able to respond more effectively to changes in UV intensities than those with darker skin. We have tested this hypothesis using UV-exposed forearm skin and unexposed buttock skin from different Solomon Islander groups. We have found: (1) a direct correlation between skin reflectance data and histologic melanin content, (2) that both Kwaoi and Nasioi have received equivalent exposure to UV in forearm skin, and (3) that contrary to the hypothesis, both the light- and dark-skinned groups have produced equal absolute amounts of melanin per equal dose of UV exposure. The analytic methods developed in this study are of use in the examining of skin color variation of other human populations. Additional skin reflectance data from the Solomon Islands project may be used to shed light on the origins of skin color variation in human populations. (Supported by USPHS Grant AM-20669 and by the Milton Fund of Harvard University)

Psoralen Plasma Levels and Erythema during Photochemotherapy. D.M. Carter, D.P. Goldstein, B. Ljunggren, J.S. Burkholder. Yale Univ., New Haven, CT

The treatment of choice for restoring cutaneous pigmentation in vitiligo is photochemotherapy with psoralsen and UV-A. Oral 8-methoxypsoralen (8-MOP) and 4′, 8-trimethylpsoralen (TMP) are effective whether administered separately or together. Pharmacological packaging of capsules, food intake, and duration of therapy affect psoralen plasma levels which determine therapeutic response. We have developed a method for measuring 8-MOP or TMP at sensitivities of 10 ng/ml.

Plasma levels of 8-methoxypsoralen (8-MOP) and photosensitivity to UV-A were measured 1–8 hr after an oral dose (0.5–0.8 mg/kg) of 8-MOP in 19 human volunteers undergoing photochemotherapy with 8-MOP and UV-A (PUVA). Five small patches of skin on the buttocks were irradiated (365 nm, 2–28J) with fluorescent lamps (Sylvania FR72T12) at each of four times, 2–6 hr after ingesting 8-MOP. Minimal erythema doses (MED) were scored 48 hr later for each irradiation time. After benzene extraction of plasma, 8-MOP levels were determined by high pressure liquid chromatography (HPLC) in a silica particle column, eluted with methylenechloride: acetonitrile (95:5).

We observed similar patterns of 8-MOP plasma levels in repeat studies of fasting subjects. Patterns varied, however, when subjects' diets and environmental conditions were not controlled. No erythema was observed when 8-MOP plasma levels were less then 30 ng/ml. Varying degrees of erythema were observed in subjects who received radiation at times when their plasma levels of 8-MOP were between 48 and 782 ng/ml. Peak plasma levels of 8-MOP were reached 1–6 hr after ingestion of drug. Minimal MEDs were reached 2–6 hr after ingestion of drug. In most patients, peak plasma level and minimal MED were recorded at the same time.

All subjects received approximately the same psoralen dose (mg/kg), but peak plasma concentrations of 8-MOP varied widely from person to person and there was no correlation between dose and level. For each subject, however, there was a constant and predictable response at each time point, there being a direct correlation between plasma levels and MEDs. The more pigmented the skin the greater the MED, but pigment alone did not explain patient variability.

These data emphasize the importance of having quantitative information on all aspects of photochemotherapy: UV dose, drug levels, and patient factors.

The Effect of Colchicine and Cytochalasin B on the Migration of Pigment Granules in the Xanthophores of the Prawn, Macrobrachium Kistnensis. R. Nagabhushanam. Marathwada University, Aurangabad, India

The xanthophores of the prawn, Macrobrachium kistnensis, are controlled by pigment-
dispersing and pigment-concentrating hormones. The effects of colchicine and cytochalasin B on the migration of pigment granules in xanthophores were investigated. Colchicine in a concentration of 25 mM inhibited responses to both the yellow pigment-concentrating and dispersing hormones by the xanthophores. Cytochalasin B, in a concentration of 10 μg/ml, also inhibited both centrifugal and centripetal migrations of the pigment granules in the xanthophores. The results are discussed in relation to previous published data on the mechanism of action of these drugs on integumentary chromatophores.

**Energy Requirements for Pigment Migration in Teleost Melanophores.** A.M. Castrucci. Instituto Biociências, Univ. São Paulo, São Paulo, Brasil

The effects of metabolic inhibitors on melanosome dispersion and aggregation of *Bathygobius saporator* were studied, as the literature about the subject is rare and controverted. The following specific blockers were used at 1 mM; 0.1 mM; 0.001 mM; 0.00001 mM:2-deoxy-D-glucose, sodium fluoride, sodium malonate, sodium cyanide, sodium azide, and 2,4 dinitrophenol.

Complete and irreversible inhibition of dispersion was obtained with 1 mM deoxyglucose, 1 mM dinitrophenol, and 1 mM and 0.1 mM ouabain. Deoxyglucose and ouabain induced *per se* melanosome aggregation. Inhibition of dispersion in 50 percent of the cells was induced by 1 mM fluoride, 1 mM malonate, 1 mM azide, and 1 mM cyanide, the latter producing an irreversible effect. Only 1 mM dinitrophenol irreversibly inhibited both aggregation and dispersion.

In view of these results, one might admit: 1. Dispersion is the active phase of pigment movement or, at least, where energy is most needed. 2. Glycolysis seems to be essential for energy production, since deoxyglucose greatly interfered in the process of migration more than other drugs, indicating that glucose is the major source of energy. 3. The energy provided by glycolysis accounts only for part of energy required; the aerobically produced ATP is also essential, for the uncoupling promoted by dinitrophenol completely inhibited centrifugal and centrifugal melanosome movements. 4. The partial inhibition caused by malonate can be explained because it still permitted some aerobic formation of ATP, in addition to those provided by glycolysis. 5. Sodium concentration is closely related to granule displacement, since extracellular excess induced dispersion, while intracellular excess induced aggregation (as shown by ouabain blocking of sodium pump).

**Evidences of Double Innervation on Teleost Melanophores.** M.A. Visconti, A.M. de L. Castrucci. Instituto Biociências, Univ. São Paulo, São Paulo, Brasil

Pharmacological studies were performed in melanophores of *Bathygobius saporator* (Gobiidae, Teleostei), employing the following drugs 0.01 mM:norepinephrine, epinephrine, isoproterenol, acetylcholine, carbachol, pilocarpine, acetyl-beta-methylcholine, benzoylcholine, atropine, neostigmine, phenolamine, propranolol.

Norepinephrine and epinephrine promoted maximal aggregation (in terms of potency:time for norepinephrine aggregation<time for epinephrine aggregation) while isoproterenol was ineffective. Phenolamine inhibited the aggregating action of both catecholamines, while propranolol was ineffective. The time for dispersion in the different drugs was compared with the time for dispersion in physiological solution. After epinephrine aggregation the relative potencies were: pilocarpine>acetylcholine>nicotine>acetyl-beta-methylcholine>benzoylcholine. After norepinephrine aggregation, nicotine>acetyl-beta-methylcholine>benzoylcholine. Isoproterenol was ineffective. After catecholamine aggregation both atropine and neostigmine induced *per se* melanosome dispersion.

The experiments performed suggest: 1. Pigment aggregation is probably mediated by postsynaptic alpha adrenergceptors; beta adrenergceptors seem to be absent. 2. Cholinergic fibers could take part in melanosome dispersion; the cholinceptors appear to be undifferentiated. 3. The dispersion induced by atropine could be due to the agonistic effect, also observed in classical pharmacological preparations, when used in low doses. 4. The dispersion elicited by neostigmine could be explained because it protected some endogenous choline ester from cholinesterase action.

**Implication of Actin Filaments in Pigment Aggregation of Swordtail Erythrophores.** Toyoko Akiyama, Jiro Matsumoto. Keio University, Yokohama, Japan

The distribution of actin filaments in swordtail erythrophores was examined at varying stages
of their pigment aggregation by means of light and electron microscopic immunocytochemistry. For this purpose, the antibody was prepared against carp skeletal muscle actins and assayed on monolayer-cultured erythropoies. Indirect immunofluorescence assay with use of FITC labelling revealed that actin filaments in these cells appeared in two forms; one occurred as thin bundles stretching out from the cell center to the cell peripheries in an astral arrangement, without showing any marked changes in their distribution upon pigment aggregation, while another appeared as a ring of thick bundles, first along the distal margins of the cells at the onset of pigment translocation and then in the regions progressively closer to the cell center, circumscribing aggregating pigment masses.

Electron microscopy combined with peroxidase or ferritin immunoassay clearly indicated that these erythropoies contained an abundance of 6–7 nm microfilaments which were conjugated with the anti-actin antibody. These actin microfilaments frequently occurred in a form of irregularly twisted masses which were loosely associated with each other by linearly stretched similar filaments. Such an organization of actin microfilaments became distinct with the progress of pigment aggregation, particularly in the peripheral area of aggregating pigment masses. The intracytoplasmic localization of actin filaments in the cells under pigment aggregation as revealed by immunoelectron microscopy was in good accordance with the observations provided by immunofluorescence. From these findings, actin filaments were presumed to be involved in pigment aggregation not merely as static cytoskeletons for the maintenance of stellate cell shape but also as contractile elements yielding a motive force.

**Induction of Melanosome Migration in Cultured Mouse Melanoma Cells.** Yoh-Ichi Koyama, Takuji Takeuchi. Tohoku University, Sendai, Japan

Mouse B16 melanoma cells, subline NC, in culture were treated with 2 μg/ml cytochalasin B(CB). Melanosomes localized in dendrites as well as those in the peripheral cell body formed aggregates, which then showed centripetal migration. After 24 hr of treatment, melanosomes were found as large aggregates near the nucleus. However, CB showed no effect on the distribution of mitochondria and endoplasmic reticulum. When cells were released from the 24-hr-treatment with CB, each melanosome began centrifugal migration. In this process, melanosomes were situated in the periphery, and were in close association with microtubules. Side arm-like structures were observed between microtubules and melanosomes, mitochondria, or endoplasmic reticulum. On the other hand, when subline N, in which melanosomes tend to stay around the nucleus, was treated with 1 percent N, N-dimethyl formamide (DMF), melanosomes were found to localize in the cell periphery after a few days of treatment. In the cytoplasm, numerous microtubules and network-like structures were found. These results seem to indicate the presence of a mechanism(s) specific to melanosome migration in the melanoma cells, and possible participation of microtubules in the melanosome migration.

**Structure and Regulation of the Messenger RNA Coding for the Corticotropin-β-Lipotropin Precursor.** Shosaku Numa. Kyoto University Faculty of Medicine, Kyoto, Japan

Messenger RNA from the pituitary was translated in a cell-free protein-synthesizing system to yield a large precursor that was found to contain not only corticotropin (ACTH) but also β-lipotropin (β-LPH) including β-melanotropin (β-MSH) and β-endorphin. The mRNA coding for the ACTH-β-LPH precursor was shown to represent a major mRNA species in the intermediate lobe of the pituitary, its translation product amounting to almost one-third of the products encoded by total translatable mRNA. The mRNA was purified to homogeneity from bovine pituitary neurointermediate lobes. Double-stranded DNA complementary to the purified mRNA was cloned, and the nucleotide sequence of the cloned cDNA was determined. The amino acid sequence deduced indicates that the cryptic portion of the precursor protein contains a third MSH sequence, named γ-MSH. The first 26 amino acid residues starting with the initiative methionine, which include a large number of hydrophobic amino acids, represent a signal peptide characteristic of secretory proteins. Thus, the ACTH-β-LPH precursor molecule is composed of three repetitive units, each containing an MSH sequence, plus a signal peptide. Each of the repetitive units, as well as the MSH sequences themselves, is separated by a pair of basic amino acids. This structure of the common precursor protein implies that its component peptides are formed by proteolytic processing. Furthermore, the presence of an unusually large number of repeated nucleotide sequences within the ACTH-β-LPH precursor mRNA suggests
that the structural gene for the precursor protein has evolved by a series of genetic duplications.

The cellular level of the ACTH-\(\beta\)-LPH mRNA is depressed by glucocorticoids. The effect of various steroids on the mRNA level correlates well with the binding specificity of the glucocorticoid receptor. This suggests that glucocorticoids regulate the expression of the ACTH-\(\beta\)-LPH precursor gene via the glucocorticoid receptor.

**Chemical Leukoderma.** Patrick A. Riley. University College School of Medicine, London, U.K.

Depigmentation of the skin caused by environmental chemicals has been recognised for many years and a large number of substances exhibiting this activity are now known. Leucoderma is invariably associated with a reduction in the population density of epidermal melanocytes in the affected zones, and is apparently the result of selective necrosis of pigment cells.

The recognition, some ten years ago, that certain phenolic materials with a depigmenting action are able to serve as substrates for tyrosinase has led to studies which go some way towards the elucidation of the melanocytotoxic action that is responsible for their depigmenting action.

Selective toxicity to melanocytes is also manifested by some agents that are not analogues of tyrosine and which do not take part directly in the reactions of the melanogenic pathway.

Whether they are indirectly implicated with melanogenesis is conjectural.

**Genetic Leukoderma.** Thomas B. Fitzpatrick, David B. Mosher, Syozo Sato. Harvard Medical School, Massachusetts General Hospital, Boston, MA

Hereditary, congenital or acquired leukodermas may conceptually arise from aberrations of the complex biologic process known as melanogenesis. Macroscopic melanin pigmentation, normal or abnormal, is the end result of embryologic, anatomical, biochemical, and biological events.

Hereditary leukoderma in man illustrates some of the specific genetic defects in the morphologic and metabolic pathway of epidermal melanin formation.

Steps 1 and 2 — Migration and differentiation of melanoblasts is probably the basis for piebaldism. In the belted mouse, the receptor spotted area prevents survival or differentiation of melanoblasts. In the \(W^v\) mutant mouse the neural crest appears to be the site of the defect; the melanoblasts never migrate to the dermo-epidermal junction.

Step 3 — Formation of melanosomes is defective in the hypomelanotic macule that occur in patients with tuberous sclerosis.

Step 4 — Melanization of melanosomes is abnormal in oculocutaneous albinism in which melanosomes are present in melanocytes but have reduced or absent melanin deposition. The \(c\) locus parallels in humans the tyrosine-negative oculocutaneous albinism.

Step 5 — Transfer defects are illustrated by a recently described syndrome of pigment dilution and an associated severe immunodeficiency syndrome. The pigment dilution occurs because of failure of melanin transfer. The animal (mouse) model is the dilute (\(d/d\)) and the leaden (\(ln/ln\)) in which the melanocytes are structurally defective and have "stubby" dendrites.

Step 6 — Degradation of melanosomes within melanocytes or keratinocytes is not yet illustrated by a human pigment defect.

**Vitiligo-Like Leukoderma Produced by Substituted Phenols.** S.S. Bleehen. Royal Hallamshire Hospital and University of Sheffield, Sheffield, U.K.

Light and electron microscopic studies have been carried out on multiple biopsies from four patients with progressive vitiligo-like leukodermas due to contact with two substituted phenols — the monobenzyl ether of hydroquinone (MBEHQ) and \(p\)-tertiary butylanil (\(pTB\)). Three of the patients had been treated with skin bleaching creams containing MBEHQ and one patient was exposed to \(pTB\) at work.

In the amelanotic areas of skin there was an almost complete lack of melanin and melanocytes. In all four patients there was a marked reduction in the number of dopa-positive melanocytes in the depigmented areas of skin with only a few melanocytes remaining, whereas in the adjacent pigmented skin the population density of melanocytes was normal. The few remaining melanocytes were either highly dendritic or had lost all their dendrites and were only weakly dopa-positive. Ultrastructural studies showed an almost complete absence of melano-
cytes and melanosomes in the vitiliginous areas of skin. The residual melanocytes contained only a few small granular melanosomes. The melanocytes in the basal layer of the epidermis appeared to be replaced by Langerhan's cells.

The histopathological findings in these patients with vitiligo-like leukoderma induced by contact with MBEHQ and pTBP appeared to be identical to what has been found in true vitiligo.

Malignant Melanoma in Oculocutaneous Albinism. A. Makita, K. Tajima, H. Miyasato, S. Ikeda. Saitama Medical School, Moroyama, Iruma-gun, Saitama, Japan

Malignant melanoma in oculocutaneous albinism (OCA) is so rare that only ten cases have been reported. A case of "amelanotic" malignant melanoma in tyrosinase-positive OCA is presented.

A number of moles are seen on the almost whole body, and a part of them are light-brown. Histologically, the nevus cells of the dermo-epidermal junction and the upper dermis show positive DOPA reaction. A brown colored tumor on the lt-leg is malignant melanoma of stage Ia, pT2pNOMO, and in the perifocal area of the tumor pagetoid pattern is observed. With electron microscopy, the malignant melanoma cells have a number of atypical melanosomes in cytoplasm and most of them are stage III or IV.

So far, malignant melanoma in OCA has been described to be "amelanotic" owing to the deficiency or the defect of tyrosinase, but in the tyrosinase-positive OCA, malignant melanoma as well as mole never restrained to synthesize melanin. In the case that many moles appear in OCA, no descriptions have been made about their malignancy, that is, the possibility of changing into malignant melanoma.

Prognostic Features in Melanoma. Vincent J. McGovern, Helen M. Shaw, Gerald W. Milton, George A. Farago. Royal Prince Alfred Hospital, Camperdown, New South Wales, Australia

The computerized records of 861 patients with clinical stage 1 cutaneous melanoma were reviewed in order to determine the relative prognostic value of their histological features. It was found that the difference in survival between nodular and superficial spreading melanoma was due to the greater proportion of thick lesions in the former and that for similar depth of invasion they had similar survival rates. Melanoma arising in Hutchinson's melanotic freckle behaved differently from the other forms and had a much better prognosis for every level of thickness.

Thick tumours generally had more mitoses, less pigment, and fewer lymphocytes at the invading front than thin lesions but these features derived their significance only from their correlation with thickness. A greater proportion of thin lesions exhibited partial regression, some of which were found subsequently to have metastasized, but otherwise, there was no prognostic significance in the presence of regression.

Women had better survival rates than men for every level of melanoma thickness. Furthermore they had more lesions of the extremities where melanomas are thinner than those of the axial regions. Our conclusion is that the only independent histological variable of prognostic significance for nodular and superficial spreading melanoma is depth of invasion.

Levodopa and Dopamine as Therapy for Malignant Melanoma in Man. Michael M. Wick. Sidney Farber Cancer Inst., Harvard Medical School, Boston, MA

Malignant melanoma cells contain tyrosinase, which catalyzes the conversion of levodopa and dopamine to the pigment melanin. This biochemical feature provides a rationale for the design of chemotherapeutic agents. We have shown that levodopa and dopamine are selectively incorporated by pigment-producing cells in vitro and in vivo and that levodopa methyl ester and dopamine cause a significant prolongation in survival of B-16 melanoma-bearing mice following treatment. A principal biochemical effect of levodopa and dopamine on human melanoma cells was selective inhibition of thymidine incorporation into DNA. We are now evaluating the clinical value of this approach and have examined the effect of dopamine and levodopa upon the labelling index of metastatic human melanoma since biochemical inhibition is a necessary pre-condition for biologic response.

Nine patients with advanced metastatic melanoma have been treated. Four patients have
received levodopa (sinemet) and five patients have received dopamine. The maximum daily dose of levodopa has been 16 gm/day while dopamine has been given by a five-day infusion at 25
μg/kg/min without irreversible toxicity. Each of the five patients treated with dopamine had a
significant inhibition of incorporation of radioactively labelled thymidine into tumor tissue.
Percent labelling indices of the five tumor samples prior to treatment were 3.0, 2.0, 1.0, 3.0, and
2.0. Following treatment with dopamine, repeat biopsies gave the following results: 0.2, 0.2, 0.1,
0.2, and 0.2, respectively. One clinical response was seen in a patient treated with dopamine who
experienced a resolution of a hepatic nodule.

Dopamine and levodopa appear to have potential as antitumor agents for human malignant
melanoma. The recent development of a more effective non-neurotoxic analog such as 3,4-
dihydroxybenzylamine and N-acetyldopamine suggest that novel and effective agents for this
disease may be available at last.

Cloudman S-91 Melanoma Colony Assay in the Lungs of Mice and the Profiles of
the Biochemical Tumor Markers, Tyrosinase and Polyamines. K. Nishioka, H. Takami,
R.D. Noyes, G.F. Babcock. The Univ. of Texas System Cancer Center, M.D.
Anderson Hospital and Tumor Institute, Houston, TX.

Melanoma lung colonies were established by injecting cell-cultured Cloudman S-91 mouse
melanoma cells into the tail vein of DBA/2 mice. Each week thereafter, blood was collected
from at least six mice which were then sacrificed and the number of melanoma colonies was
determined macroscopically. In melanoma-injected mice, no tumor colonies were usually seen
in the lungs during the first three weeks post-inoculation. In a typical experiment using 1 × 10^5
melanoma cells, the number of colonies detected was as follows: 15.3 ± 9.3 (mean ± S.D.) at
four weeks post-inoculation, 116.3 ± 28.8 at five weeks and 200—400 at six weeks. After seven
weeks, confluent colonies were seen in the lungs. Melanoma colonies were found only in the
lungs.

This in vivo system once established was used to study several biochemical tumor markers.
The activity of tyrosinase was measured in serum of melanoma-bearing mice using tritiated
tyrosine. The enzyme activity rose as tumor burden increased with detectable activity being
found before the melanoma colonies became visible. The polyamines including putrescine,
spermidine, and spermine, which are all regulators of cell proliferation were measured in
erthrocytes using Durrum D-500 high pressure amino acid analyzer. While putrescine
increased as early as one week after inoculation and reached maximum levels at four weeks,
spermidine and spermine levels significantly increased at four weeks and continued to increase
markedly during the entire observation period.

Thus, the melanoma lung colony assay coupled with the monitoring of biochemical markers
appears to be a very useful system to examine antimelanoma activities of various agents.
Supported by the Grant No. 983 from the Kelsey and Leary Foundation, the Virginia P.
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Cell Surfaces and Arrest Properties of Metastatic B16 Melanoma. G.L. Nicolson.
Univ. of Texas System Cancer Center, M.D. Anderson Hospital and Tumor
Institute, Houston, TX

B16 melanoma sublines have been selected which metastasize preferentially to lung (Fidler:
Nature NB 242:148, 1973), ovary (Brunson, Nicolson: J Supramol Struct 11:517, 1979), liver
(Tao et al: Int J Cancer 23:854, 1979) or brain (Brunson et al: Nature 272:543, 1979). Many of
these show differences in exposed cell surface glycoproteins (Brunson et al: Nature 272:543,
1978; Nicolson: Biol. Markers in Neoplasia. Edited by R.W. Rudder. New York, North-
Holland, 1978, p 227) which may be important in cellular recognition. B16 sublines spontaneously
shed intact PM vesicles, and these can fuse with PM of cells from homologous and
heterologous cells (Poste, Nicolson: PNAS 77:399, 1980). Fusion of vesicles from highly
metastatic B-16-F10 that localizes in lung with cells from poorly metastatic B16-F1 significantly
increases the ability of F1 cells to implant and form lung metastases. Vesicle-modified F1 cells
revert to their original properties following removal of F10 components from the PM. Vesicles
from other B16 sublines that are poorly metastatic to lung (B16-F1, F1L^T^-EM,-F10L^T^-EM,-B1) do
not modify the metastatic properties of B16-F1 cells (Poste, Nicolson: PNAS 77:399, 1980).
Interaction of B16 cells with the endothelium was examined in vitro using vascular endothelial
cell monolayers which produce a basolateral extracellular matrix similar to basement mem-
brane (Birdwell et al: PNAS 75:3273, 1978). B16 cells and certain normal cells such as leukocytes bind to the cell monolayers stimulating rupture of endothelial cell interactions, retraction and exposure of underlying matrix (Kramer, Nicolson: PNAS 76:5704, 1979). B16 cells migrate and adhere to the exposed matrix and eventually induce its enzymatic destruction (Kramer, Nicolson: PNAS 76:5704, 1979). The extracellular matrix is a much better adhesive substrate compared to endothelial cell surfaces possibly due to the presence of fibronectin in the matrix (Birdwell et al: PNAS 75:3273, 1978), and the net movement of B16 cells to the basement membrane matrix may be due to this adhesive difference. (Supported by USPHS grants R01-CA15122 & R01-CA28260 & ACS grant CD-71C.)

Interactions of Environment, Genotype, and Behavior in the Etiology of Human Malignant Melanoma. John A.H. Lee. School of Public Health, Univ. of Washington, Seattle, WA

The incidence and mortality from malignant melanoma are increasing rapidly in at least Europe and North America. The eye melanomas are not changing perceptibly. The incidence and mortality of malignant melanoma increase with nearness of residence to the Equator of white populations, where, as in the U.S. or Australia, the populations have been well mixed by migration. The reverse gradient is found in Europe, with very high rates among the Scandinavians. Melanomas select the anatomic sites that are exposed—giving for ears male:female ratios of 6:1, and for legs 0.3:1.

Melanoma incidence and mortality is related to socioeconomic status in at least Britain and the northern U.S., being highest in the most prosperous groups. No excess incidence or mortality was found in these areas among outdoor workers. There is thus great difficulty at the present time in accepting a simple dose-response relationship between exposure and incidence.

These problems are of great practical importance because of the lethality of the melanomas and the expected intensification of the UV flux consequent on the buildup of atmospheric halocarbons.

HLA-DR Dependent Autologous T-Cell Proliferation Induced by Cultured Primary Melanoma. D. Guerry, M.A. Alexander, J.P. Fuhrer, M.F. Herlyn, K.F. Mitchell. Cancer Center, Univ. of Pennsylvania and The Wistar Institute, Philadelphia, PA

The histology of primary melanoma is typified by infiltrating lymphocytes, cells usually absent in metastatic melanoma. We assessed the proliferative response of blood lymphocytes to cultured autologous melanoma to explore for a functional correlate of these histopathologic findings. Melanoma cell lines were established from eight patients: four lines were derived from primary lesions, each expressing HLA-DR determinants; four lines were established from metastases, 2 DR(+) and 2 DR(-). Mitomycin-C treated melanoma cells (stimulators) were co-cultured for four days with an equal number of autologous mononuclear cells or purified T lymphocytes (responders), and the incorporation of 3H-thymidine measured. A stimulation index (SI) was calculated by dividing the cpm incorporated by co-cultured responders by that of responders cultured alone. Cell lines derived from each primary melanoma strongly stimulated autologous lymphocytes (mean SI = 23.1, range 8.7–50.0). With one line so tested, xenogeneic anti-DR serum abolished this response in autologous T cells. In contrast, no metastatic line was stimulatory to autologous lymphocytes (mean SI = 1.3, range 0.1–1.8) whether cultured in autologous, allogeneic, or fetal bovine serum. Tumor cell lines stimulated normal, allogeneic lymphocytes according to the DR status of the melanoma lines: DR(+) stimulated and DR(−) did not. Similarly, lymphocytes from all eight patients were stimulated by allogeneic, DR(+) melanoma cells and lymphoblasts. These data suggest that a fundamental difference in primary and metastatic melanoma may lie in the capacity to stimulate proliferation of autologous lymphocytes. Further, the expression of DR determinants by the malignant cell may be necessary but not sufficient to provoke this response.

Acral Melanoma in Japan. Masaaki Takahashi, Makoto Seiji. Tohoku Rosai Hospital, Sendai, and Tohoku University School of Medicine, Sendai, Japan

Among cutaneous malignant melanomas, acral melanoma, which usually occurs on the hands and feet, has been designated by Mihm et al. in 1979, based upon specific clinical and histological characteristics. Racial differences are quite pronounced as to incidence and
predilection sites of melanomas. According to Japanese statistics (Kawamura et al. in the Annual Meeting of J.D.A., 1978), there is a relatively high incidence of melanomas occurring on the plantar surface of the foot among Japanese (32.1 percent among 333 cases of cutaneous melanomas). It is very characteristic as compared with a high incidence occurring on the trunk among Caucasians. Most of the melanomas occurring on the plantar region have specific clinical and histologic characteristics as follows: This type of melanoma is characterized by a biphasic growth pattern, i.e., growth within the epidermis, radial growth; and deep invasion into the dermis, vertical growth. Distinctive clinical and histological features are exhibited in their macular component where there are large atypical melanocytes with large, often bizarre nuclei and nucleoli, and cytoplasm filled with melanin granules. Two striking features stand out in this type of melanoma. One, a relatively short period of radial growth, aids in distinguishing the lesion from lentigo maligna melanoma (LMM) where the radial growth phase may be present for decades. The other is that this melanoma appears to be biologically much more aggressive than LMM, since a five-year survival rate is only 25.6 percent. In this study, we are going to summarize the clinical and histologic characteristics and statistics of Japanese acral melanomas which occurred on soles, palms, and in subungual regions.

**Histopathology of Melanoma: Unusual Types and Risk Factors.** Martin C. Mihm, Jr. Harvard Medical School, Boston, MA

The biphasic growth pattern of primary human malignant melanoma will be discussed especially as it affects acral and mucosal sites. Various aspects of the vertical growth phase of primary malignant melanoma will be explained with emphasis on determination of risk for metastases and death. These aspects will include: thickness, level of invasion, inflammation, regression, and lymphatic spread. A series of patients will be reviewed that have been analyzed by the method of the Cox regression analysis to determine the significance of each of these factors. Thickness of the primary tumor in this study has been shown to be the factor most useful in prognostication.

**Relevance of Xiphophorus Melanoma for Understanding Neoplasia.** F. Anders. Institute of Genetics, Justus Liebig University, Giessen, Federal Republic of Germany

Four types of etiology of melanoma which might be relevant to neoplasia in general were elucidated. In all the cases the tumors are mediated by a “tumor gene” (Tu) which might be related to an endogenous virus. Type 1 can be induced easily in those genotypes, the Tu of which is derepressed but cannot become active because pigment cell differentiation is delayed in a non-competent stage. Promoters may push the non-competent cells to the competent stage. The resulting melanoma originates multicellularly, grows by both transformation and proliferation, and is non-hereditary. Type 2 can be induced easily in genotypes where Tu is repressed by only one regulating gene (R); typically this R is Tu-linked. Carcinogens can induce mutations in this R in a competent cell that subsequently becomes transformed. The origin of the developing melanoma is unicellular. If, however, the mutated cell is not yet competent, it remains normal. After having reached competence, its descendants will be transformed. The melanoma originates multicellularly, although it can be traced back to a single mutation. Melanomas of this type grow only by proliferation, and are non-hereditary. Type 3 develops following germ line mutation of the R in the same genotypes that may produce the Type 2 melanomas. Transformation occurs in the progeny as soon as the cells become competent. Such melanomas are of multicellular origin, grow by both transformation and proliferation, and are heritable. Type 4 develops spontaneously following the replacement of R-carrying chromosomes with R-lacking ones by crossings. It is of multicellular origin and grows by both transformation and proliferation as the pigment cells become competent by differentiation.

**The Unique Properties of Cultured Fish Erythrophoroma and Irido-Melanophoroma Cells: Pluripotency for the Expression of Pigmentary Phenotypes.** Jiro Matsumoto, Takatoshi Ishikawa, Prince Masahito, Shozo Takayama. Cancer Institute, Tokyo, and Keio University, Yokohama, Japan

The objective of the present study is to clarify the properties of cultured neoplastic pigment cells derived from erythrophoromas and irido-melanophoromas occurring in fish, with particu-
lar reference to their differentiation of pigmented phenotypes. Thus far, we have established four permanent cell lines and their derived several clones from spontaneous erythrophoromas in goldfish and two uncloned cell lines from spontaneous irido-melanophoromas in sciænid fish (*Nibea mitsukurii*). The goldfish erythrophoroma cells *in vitro* were competent for an autonomous synthesis of pteridines and pterinosomes, the phenotypic markers of erythrophores and xanthophores, whereas the sciænid fish irido-melanophoroma cells are so in forming reflecting platelet-like organelles and melanosomes, the phenotypic markers of iridophores and melanophores. During successive cultivation of the erythrophoroma cells, even after cloning, either DOPA oxidase-positive or melanin-laden cells appeared in their population, more drastically upon treatment with dimethylsulfoxide. The sciænid fish irido-melanophoroma cells frequently formed red pigments (presumably drosopertins) and pterinosomes. Electron microscopy disclosed an occurrence of phenotypic mosaicism in heterotopically pigmented foci. Experiments to induce differentiation in the erythrophoroma and irido-melanophoroma cells indicated that their growing abilities are lost or markedly diminished with the progress of pigmentation. All these findings indicate that the fish erythrophoromas and irido-melanophoromas are composed of "chromatoblast"-like cells which are fundamentally pluripotent in manifestation of pigmented phenotypes.

**Iridosarcoma in a Pine Snake, *Pituophis Melanoleucus Melanoleucus*.** Elliott R. Jacobson, William O. Iverson, Wayne Ferris, Joseph T. Bagnara. College of Veterinary Medicine, Univ. of Florida, Gainesville, FL; Univ. of Arizona, Tucson, AZ

An adult northern pine snake was submitted with ulcerative irregularly thickened ventral post-gular scales. All scales involved were white. The snake was anesthetized with a halothane/O₂ mixture and several involved scales were biopsied. Histologic examination of skin specimens revealed a darkly pigmented dermis. The cells contained small to large amounts of rectangular, yellow-brown granules of variable size that were strongly anisotropic. A few similarly pigmented cells were scattered in the epidermis. Electron microscopic examination revealed the presence of cells containing organelles fitting the classic descriptions of iridophore reflecting platelets. Some were long and rectangular, while the profiles of others were almost square. In none of the cells did there seem to be any specific orientation of reflecting platelets. In some cells, only a single very long reflecting platelet was observed. Sections stained with lead citrate displayed the usual empty reflecting platelet profiles, but in unstained sections, the shattered purine contents of reflecting platelets was retained. Often, in cells containing mature reflecting platelets, many small vesicles were also observed. It is possible that these represent a form of pre-reflecting platelet. While cells containing reflecting platelets were more numerous in the dermis, such cells were also observed in the epidermis. It appears that we are dealing with an iridophore tumor that should be properly designated an iridosarcoma.

**Specificity of Melanoma-Directed Cellular Immunity in Man.** A.J. Cochran. UCLA School of Medicine, Los Angeles, CA R.M. Mackie, L.J.A. Morrison, A.M. Jackson, G. Todd. Western Infirmary, Glasgow, Scotland

We have previously reported the selective reactivity of melanoma patients' leukocytes with intact and fractionated melanoma cells using both the direct capillary leukocyte migration inhibition assay and a two-stage lymphokine generation technique. Fractionation of melanoma-derived materials on Sephadex G150 and on DEAE cellulose indicates a molecular weight of between 41,000 and 95,000 for the active components. Similar to some degree cross-reactive molecules are present on melanomas from different individuals. Subgroups of auto-immunogenic melanoma-associated antigens are suggested by the patterns of reaction of melanoma patients with panels of tissue from different melanomas. Melanoma patients' leukocytes are also selectively inhibited by materials from first trimester fetuses and from neocytes and hyperplastic and dysplastic melanocytes. The active molecules may thus include oncofetal and differentiation type antigens. In a study of *in vitro* tuberculoprotein sensitization in cancer patients and normal individuals we found no evidence of cross-reactivity between melanoma-associated antigens and mycobacterial antigens (BCG). Melanoma patients, especially those with relatively bulky tumor detectable on clinical examination, were less frequently reactive with BCG than were normal individuals residing in the Glasgow area. There is no evidence that we are detecting transplantation or blood group antigens.
Degradation of Soluble Melanoma Associated Antigens (MAA). J-C Bystryn, A.M. Boctor. New York Univ. School of Medicine, New York, NY

Soluble tumor antigens are believed to have a major impact on host immune defence mechanisms. Consequently, the factors which influence their accumulation in body fluids may influence tumor growth. In prior studies we have shown that one such factor is the ability of viable melanoma cells to rapidly shed tumor antigens (MAA) expressed on their surface. The following experiments were conducted to study the susceptibility to degradation of cell-surface MAA following their release by melanoma cells.

Surface macromolecules on melanoma cells in culture were radioiodinated by the lactoperoxidase technique, and the cells incubated in fresh medium. The medium, containing shed macromolecules, was collected after 3 hr. The susceptibility of shed material to degradation was studied by incubating aliquots of the medium with replicate confluent plates of unlabelled cells. Aliquots of medium were collected at the onset of incubation and at intervals thereafter and assayed for MAA (by immunoprecipitation with specific antibodies) and for labelled macromolecules (by precipitation with trichloroacetic acid). Degradation was calculated from the loss in CPM in the test sample in comparison to that in control aliquots of medium incubated without cells. It was found that surface MAA and unrelated macromolecules shed by melanoma cells could be degraded by these cells and by normal human cells. However, there were marked differences in the rates of degradation. Shed MAA were degraded much more slowly by melanoma cells than by normal allogeneic macrophages or fibroblasts (0.6 percent vs 19.5 percent vs 51.1 percent/10^6 cells/24 hr, respectively). This was not due to the inability of melanoma cells to catabolize material, since unrelated shed macromolecules were degraded at similar rates by melanoma and allogeneic fibroblasts (4.5 ± 1.0 percent SE vs 8.1 ± 2.1 percent/10^6 cells/24 hr, respectively).

These findings indicate that melanoma cells have a selective defect in their ability to degrade some MAA. This may favor the local accumulation of MAA around tumors and consequently increase their ability to escape immune destruction.

Relationship of Melanoma Associated Antigens to Histocompatibility Antigen and β-2 Microglobulin in Material Spontaneously Shed by Cultured Human Melanoma Cells. M. Khosravi, S.K. Liao, P.B. Dent. McMaster University and The Ontario Cancer Foundation, Hamilton Clinic, Hamilton, Ontario, Canada

Tumor associated antigens are thought to share certain characteristics of histocompatibility antigens (HA) in that they may be associated with β2 microglobulin (β2m) in the cell membrane and they may crossreact with and are structurally related to HA which are alien to normal cells of the tumor-bearing host. Conflicting results have been obtained with regard to the relationship of human melanoma associated antigens (MAA) to HA and β2m. Using the technique of KBr ultracentrifugal flotation, McCabe et al. (JNCI 60:773, 1978) were able to separate HA from MAA (as assessed by skin tests) in material shed by cultured melanoma cells. Spent medium from cultures of melanoma (CaCL 73-36) maintained for 72 hours, in serum-free medium was concentrated 20-fold and applied to KBr gradient (d = 1.23 g/ml). After centrifugation at 160,000 x g for 48 hr three fractions were collected, dialysed against PBS, and tested by quantitative absorption for MAA&HLA-A10 both of which are known to be present on this cell line, using monkey anti-MAA (Liao et al: Cancer Res 39: 183, 1979) and an alloanti-serum against HLA/A10.* Both MAA and HA activity were enriched in the upper fraction; the 50 percent absorption concentration (AD₅₀) for MAA was 7.5 μg (upper) vs. >140 μg (lower) and for HLA-A10, 8.0 μg (upper) vs. >140 μg (lower). To determine if MAA are physically associated with β2m, concentrated shed material from melanoma cultures was applied to a Sepharose 4B-rabbit anti-β2m IgG adsorbent. The bound material was eluted with 3M KSCN, dialysed and tested for MAA, β2m, and HLA-A10 activity in quantitative absorption experiments. The AD₅₀ of the eluted material for anti-β2m was 0.67 μg, for anti-HLA-A10, 11 μg, and for anti-MAA, 8 μg, indicating that MAA bound to the anti-β2m affinity column. These results confirm the original report of Thomson et al. (Brit J Cancer 37:753, 1978), on the association of β2m with MAA and lend further support to the suggestion that tumor associated antigens are structurally if not functionally similar to HA. *Serological assays were done by mixed hemadsorption.
Randomized Clinical Trials on Surgical Treatment of Melanoma of the Skin. N. Cascinelli, S. Orefice, M. Vaglini, F. Preda. W.H.O. Collaborating Centres for Evaluation of Methods of Diagnosis and Treatment of Melanoma, Istituto Nazionale per lo Studio e la Cura dei Tumori, Milano, Italy

553 stage I melanoma patients entered a randomized prospective multicentric clinical trial to evaluate the efficacy of immediate node dissection versus node dissection delayed at the appearance of regional node metastases. Survival was very similar in the two treatment groups; no subset of patients (age, sex, maximum diameter, histologic characteristics of primary) had benefit from immediate dissection.

Stage II melanoma is a high risk disease; after radical surgery the value of adjuvant treatment has been tested in 702 patients treated at random with surgery alone or combined with immuno or chemotherapy or both.

Preliminary results of this trial show a longer disease-free period in patients submitted to combined treatment, but survival does not seem to be improved by adjuvant treatment so far.

Two prospective randomized clinical trials recently started on stage I melanoma patients: the first one to evaluate the optimal resection margins in melanoma not thicker than 2 mm, the second one to assess the therapeutic efficacy of BCG given by intralymphatic route in melanomas of lower extremities thicker than 2 mm.

Prognostic Factors in Melanoma Patients with Microscopic Metastases to Regional Lymph Nodes. C.L. Day, A.J. Sober, M.C. Mihm, A.W. Kopf, T.B. Fitzpatrick, R.A. Lew. Massachusetts General Hospital, Boston, MA, and New York Univ. Medical Center, New York, NY

16 variables were tested for their value in predicting recurrent disease and death in 46 clinical stage I melanoma patients with metastases to regional nodes. A Cox multivariant logistic regression analysis determined that thickness of the primary lesion was the best predictor of recurrent disease and death (p < 0.001). 20 patients with primary lesions >3.6 mm and positive elective regional dissections (PERND) had a five-year survival by life table analysis of only 18 percent. 26 patients with primary lesions <3.5 mm and a PERND had a five-year survival of 72 percent (p < 0.001 compared to the thick group). The following five variables were also useful prognostic indicators in this group of patients: (1) level of invasion (<0.005), (2) lymphatic invasion (p <0.005), (3) regression (p <0.01), (4) lymphocytic response (p <0.05), (5) mitotic rate (p <0.05). The following ten variables were not useful prognostic indicators: (1) sex, (2) age, (3) location, (4) histologic type, (5) number of positive nodes, (6) node percentage, (7) node metastases size, (8) adjuvant therapy received, (9) incontinuity vs discontinuous node dissection, and (10) ulceration. These findings have direct immediate application to both ongoing melanoma adjuvant therapy studies and to the elective regional node dissection controversy.

Ultrastructural Studies of Melanosis Cutis Secondary to Metastatic Malignant Melanoma. S. Sato, T.B. Fitzpatrick. Massachusetts General Hospital, Harvard Medical School, Boston, MA

Association of diffuse cutaneous melanosis with metastatic malignant melanoma is a rare event. The pathogenesis of this generalized hyperpigmentation of the skin is not known, although Silberberg et al. (Arch Dermatol 97:671, 1978) and Konrad et al. (Br J Dermatol 91:635, 1974) have reported the light and electron microscopic findings. We wish to present our ultrastructural observations of a case of melanosis cutis secondary to metastatic malignant melanoma.

In the present study, the skin biopsies were obtained from an area free from metastatic tumor, and the morphological events attributable to the condition were: (1) the presence of accumulated electron opaque material in macrophages in the dermis, and (2) an increased number of melanosome complexes in the basal and suprabasal layers of the epidermis. Present within the phagocytic vacuoles of macrophages was particulate material of granular appearance which was uniform in size and measured approximately 500 A in diameter. Clumps of this particulate material revealed variable patterns of distribution and condensation, but lacked any characteristic internal substructure comparable to melanosomes, either in formative or degradative processes. It was assumed that the degree of melanotic darkening of the skin could be correlated with the increasing number of macrophages containing the phagocytized material. No malig-
nant melanoma cells were identified in the dermis. The Buffy coat preparation revealed no circulating melanoma cells in the peripheral blood.

The pathogenic mechanisms leading to melanosis cutis, due to malignant melanoma, are still not known. The present results support a previous concept that one or more melanin precursors are oxidized and accumulate within macrophages.

**Endocrine Influence on Nevi and Melanoma.** P.K. Chaudhuri, M.J. Walker, C.W. Beattie, H.A. Briele, T.K. Das Gupta. Univ. of Illinois, Chicago, IL

Clinical evidence suggests a possible role of steroid hormone on the biologic behavior of human melanoma and benign nevi. As an initial step to evaluate this effect we assayed the estrogen receptor in nevi from melanoma patients and normal population, melanoma tissue, as well as pigmented and nonpigmented skin including areola and cafe-au-lait spots. Histologic examination was performed on all tissue specimens. The results are as follows:

| Specimen                  | N  | Incidence (%) | Amt Bound | Kd     |
|---------------------------|----|---------------|-----------|--------|
| Nevi-normal person        | 15 | 0/15 (0)      | —         | —      |
| Skin                      | 14 | 0/14 (0)      | —         | —      |
| Nevi-melanoma patient     | 22 | 9/22 (40)*    | 86.3      | 1.79 x 10^-6M |
| (No active disease)       | 35 | 12/35 (35)    | 74.6      | 1.43 x 10^-6M |

* p < 0.001 compared to benign nevi-normal person and skin-normal person.

To evaluate the effect of steroid hormone on human melanoma 5 x 10^6 melanoma cells were inoculated in athymic mice and treated with either estradiol or progesterone for 33 days and their growth was compared to the control group.

| N  | Treatment | Incidence | Tumor Latency | Final Tumor Growth Weight | Incidence of Metastasis |
|----|-----------|-----------|---------------|--------------------------|-------------------------|
| 13 | control   | 100       | 4.5 ± .2      | 3.9 ± 3                  | 8.0                     |
| 10 | 0.1 ugm E2 | 80        | 5.0 ± .6      | 0.9 ± 2*                 | 0                      |
| 10 | 1 ugm Prog| 100       | 4.8 ± .4      | 2.1 ± 0.3*               | 0                      |

*p < .05 compared to the control group. No difference of tumor growth was observed in receptor negative cell line in a similar experiment.

The above experiment suggests a possible role of steroid hormones such as estrogen and progesterone on the biologic behavior of benign nevi and melanoma.

**Experimental and Clinical Investigation of 5-S-Cysteinyldopa in Malignant Melanoma in Vivo: Production, Excretion and Regulatory Factors.** Masamitsu Ichihashi, Manoj Mojamdar, Yutaka Mishima, Kobe University School of Medicine, Kobe, Japan

Melanoma patients are known to excrete large quantities of 5-S-cysteinyldopa in their urine. However, the relationship between 5-S-cysteinyldopa excretion and duration of melanoma, volume of melanoma, type of melanoma, etc. are unknown and are explored in the present report. In general, melanoma patients were found to excrete increasingly larger quantities of 5-S-cysteinyldopa with the progression of melanoma. In melanoma subjects, dopa administration has resulted in a dramatic increase in 5-S-cysteinyldopa excretion in contrast to control subjects who continued to excrete normal levels of this compound. Similarly hamsters bearing melanomas have been found to excrete larger quantities of 5-S-cysteinyldopa as compared to control hamsters. When dopa was given intraperitoneally, melanoma-bearing hamsters were found to excrete significantly larger quantities of this compound as compared to control hamsters. The urinary excretion of 5-S-cysteinyldopa has been found to be higher in amelanotic melanoma as compared to melanotic melanoma in hamsters. However, dopa loading test has revealed that the enhancement in 5-S-cysteinyldopa excretion is much less in amelanotic melanoma than in melanotic melanoma. Radioactive dopa and glutathione incorporation into various subcellular fractions as well as into 5-S-cysteinyldopa and melanin reveals the dynamic nature of 5-S-cysteinyldopa in various types of melanogenesis and its usefulness in the clinical detection of melanoma metastasis.
Melanogenuria. J. Duchoň. Faculty of General Medicine, Charles University, Prague, Czechoslovakia

By the term "melanogenuria" we mean the elevated excretion of s.c. "urinary melanogens", i.e., of all specific compounds occurring in the urine of melanoma patients (and of animals bearing melanomas as well) in higher level than in normals. Most of these compounds are unstable and can change to dark, secondarily formed, "urinary melanin" by oxidation. Until now, at least 20 of such compounds (of phenolic and indolic nature) have been identified in melanoma urine. According to the result of the Thormählen test they can be divided into two principal groups: Thormählen positive melanogens (TPM) and Thormählen negative melanogens (TNM). An unsubstituted pyrrol ring of the indole nucleus is the condition for a positive result of the test.

It is generally believed that urinary melanogens are precursors (or metabolites of these precursors) on the tyrosine-to-melanins biosynthetic pathway. To date, however, the detailed mechanism of their formation has not been exactly elucidated in all types of urinary melanogens.

The clinical significance of urinary melanogens depends on: (1) their specificity: the elevated level of indolic melanogens (including TPM) and of 5-S-cysteinyldopa seems to be most specific for melanoma; (2) the frequency of their occurrence: the elevated level of urinary melanogens occurs in 20-30 percent of all melanoma patients only; (3) the mutual relationship between the stage and type of the disease and the corresponding analytical data: the elevated excretion of urinary melanogens depends first of all on the stage or progress of the disease. However, there are many other factors which may have influence on the excretion of urinary melanogens.

4 Hydroxy Anisole (40 HA) in the Treatment of Recurrent Malignant Melanoma.  
B.D.G. Morgan, P.A. Riley, T. O'Neill. University College Hospital and Mount Vernon Hospital, London, U.K.

The effects of 40 HA on melanocytes in tissue culture and on Harding Passey Mouse melanoma have suggested that it would be effective in the treatment of human malignant melanoma.

The protocol and results of preliminary clinical trial using intravascular infusions of 40 HA are described. The only significant side effect of the drug was the formation of methaemoglobin. Complications of the technique were associated with the hazards of intra-arterial administration. The limited volume that can be administered by this route imposes restrictions on the dose as the drug has a relatively low solubility in saline solution.

Regression was noted in several cases of recurrent malignant melanoma treated with intra-arterial 40 HA.

An indication of the potential effectiveness of the treatment was given by the tyrosinase activity of tumour material obtained at excision.

The Effect of Tuftsin, A Hormone-Like Peptide, on Cloudman S-91 Melanoma in Vivo. G.F. Babcock, R.D. Noyes, K. Nishioka. The Univ. of Texas System Cancer Center, M.D. Anderson Hospital and Tumor Institute, Houston, TX

Tuftsin (L-Thr-L-Lys-Pro-L-Arg) is a hormone-like peptide which is a component of human IgG. It has been shown to stimulate several parameters of the immune response in vitro. This project was designed to study the effect of tuftsin in vivo using the Cloudman S-91 melanoma colony assay as a model system. Mice given various concentrations of tuftsin three times weekly, starting one day after tumor inoculation, had significantly fewer colonies (fivefold reduction \( p < 0.005 \)) in their lungs than the saline treated controls. A significant effect was noted when the mice received from 1 to 1000 \( \mu g \) (each dosage/recipient). This reduction in colony number was not observed when the mice received only a single treatment of tuftsin at any concentration tried. The levels of tyrosinase, in the serum of tuftsin-treated mice bearing melanoma in their lungs, were compared with those receiving only saline. Mice in the tuftsin-treated groups had lower levels of serum tyrosinase than the controls. This decrease in the tyrosinase levels could be correlated with the decrease in tumor burden in the tuftsin-treated group.

Preliminary attempts have been made to determine the mechanism of action of tuftsin in the melanoma colony assay. Purified populations of tuftsin-treated alveolar macrophages from DBA/2 became cytotoxic in vitro for the Cloudman S-91 melanoma cells. This effect was
shown to occur even when the macrophages were exposed to tuftsin for only one hr. In addition to its immunopotentiating properties, no side effects were noted in mice receiving dosages as high as 10,000 \( \mu g \) every other day for up to six weeks. These results suggest that tuftsin is a potential immunotherapeutic agent for the treatment of melanoma and that tyrosinase represents an excellent marker to monitor the effects of various therapeutic agents on melanoma. Supported by Grant No. CA-16672, CA-25381, and CA-27330 from NCI, DHEW.

**Purification and Some Characterization of Melanocyte Chalone from Mouse Melanomas.** K. Ishikawa, J. Suzuki, K. Tsutsumi, I. Watanabe, Y. Tomita, A. Hariu, C. Mizuno, M. Seiji, Yamagata Univ. Sch. Med., Yamagata, Dev. Physiol. Path. Natl. Inst. Radiological Sci., Chiba, and Tohoku Univ. Sch. Med., Sendai, Japan

Ethanol precipitate (70–95 percent) of the water extract of Harding-Passey mouse melanomas contains a melanocyte specific and species non-specific suppressant of cell proliferation (melanocyte chalone). By gel filtration on Sephadex G-100 column followed by DEAE-Sephadex A-25 coloum chromatography, a fraction containing melanocyte chalone is obtained (ME IV\(_2\)). ME IV\(_2\) contains at least two components, one of which shows inhibition on protein biosynthesis in vitro, the other of which shows inhibition on RNA biosynthesis in vitro. These inhibitors are very heat stable. ME IV\(_2\) can be separated further by Bio-gel P-2 column chromatography, which gives three discrete fractions (ME IV\(_2\)-A, B, C). ME IV\(_2\)-A inhibits protein biosynthesis, but does not inhibit RNA synthesis. The main components of this fraction seem to be several kinds of polypeptides with low molecular weight. ME IV\(_2\)-B inhibits RNA synthesis but does not inhibit protein synthesis except at high concentration. The main component of this fraction seems to be mono- or dinucleotide with a net charge of -2 on DEAE-Cellulose column in the presence of 7 M urea. On the other hand, ME IV-C strongly inhibits RNA synthesis but does not inhibit protein synthesis. The nature of this component is not known.

**In Vivo Murine Melanoma Responses to Chemotherapy with Selected Melanocytolytic Agents.** Walter Chavin. Wayne State Univ., Detroit, MI

Melanocytolytic agents effectively and selectively kill normal melanocytes. Several agents kill mammalian melanoma cells in culture. The limited in vivo melanoma chemotherapy studies provide some insight. The present report deals with in vivo effects of two melanocytolytic agents, hydroquinone (HQ) and \( \beta \)-mercaptoethanolamine (MEA) upon murine melanomas.

The B-16, Cloudman S-91, and Harding-Passey (HP) melanomas of NIH origin were grown in C57 BL/6j, DBA/2j, and BALB/c female mice, respectively. Tumors were transplanted as a brei injected subcutaneously. Nine control or drug injections (SQ) at 24 hr intervals were used. The tumor bearing and normal mice received either no treatment, saline vehicle, 0.1 LD\(_{50}\), or 0.5 LD\(_{50}\) doses.

The three melanoma models responded differently to the tested agents. Hydroquinone was most effective upon HP melanoma and least effective upon the B-16 melanoma. The number of HP melanoma takes was reduced greatly and host survival increased. At the same dose levels and time intervals, neither B-16 melanoma take or host survival was affected by HQ. The response of the S-91 melanoma was intermediate to the responses of the B-16 and HP melanomas. MEA as tested appeared to be ineffective as a chemotherapeutic agent. However, the MEA effects were rather interesting as they reversed the positive chemotherapeutic action of HQ upon the HP melanoma. Correlation of the in vitro and in vivo effects of these agents upon the murine melanoma models suggests that the primary mechanism of action is upon DNA. The variation in in vivo melanoma response to the melanocytolytic agents is analogous to the variation reported in vitro. Thus, the murine melanoma models represent different test systems. Although melanoma take is decreased with chemotherapy in some models, the rationale for tumor cell escape from such treatment remains to be clarified. Nevertheless, it is clear from the significant and positive inhibitory responses to treatment with melanocytolytic agents, that these agents may be helpful in the management of some types of malignant melanoma.

**Cell Proliferation in Melanocytic Nevi after UV Irradiation.** Helmut Pullmann. University of Cologne, Federal Republic of Germany

After UV therapy of dermatoses we observed a darkening and increasing size of preexisting melanocytic nevi.
This observation was documented photographically and measured planimetrically in 50 patients; the differences were significant. After excision of moles at the end of treatment we found atypical melanocyte hyperplasia in some cases.

Therefore we were interested in the influences of UV light on proliferative activity of nevus cells. 15 melanocytic nevi of patients treated with systemic PUVA or Selective Ultraviolet Phototherapy were compared with 18 nevi of unirradiated skin. The melanocytic nevi were classified in nevoid lentigo, junctional, compound, and dermal nevus. The 3H-thymidine labelling indices (3H-I) were determined in melanocytic nevi, the adjoining epidermis, and in the dermal infiltrate cells surrounding the moles. In moles of non-irradiated skin the proliferative activity was largely quiescent (3H-I: 0.31 ± 0.10). UV-irradiation (PUVA: total doses of 20-28 J/cm²; SUP: total doses of 10-15 J/cm²) induced an increase of up to factor 6-7 (3H-I: 1.96 ± 0.52). The increase was statistically significant with p < 0.0025. Enlargement of replicative activity was also found in the adjoining epidermis by a 3H-I of 3.3 ± 0.6 in non-irradiated skin in contrast to 6.2 ± 0.4 in UVL-exposed skin. In addition the cells of the dermal infiltrate surrounding the moles were stimulated after UV-irradiation (3H-I: 1.14 ± 0.27 in comparison to 0.34 ± 1.1 surrounding non-irradiated moles). This may be due to an increased antigenity of nevus cells and in consequence to a stimulated cellular immune response.

Antigenicity of Premelanosome and Specific Melanoma-Cytotoxic Activity Demonstrated by Lymphocytes of Melanoma-Bearing Hamster. Akiharu Tamaki, Yutaka Ishii. Kobe University School of Medicine, Kobe, Japan

The conversion of active melanocytes into α-dendritic cells which exhibit no premelanosome formation was previously described in the vitiligo lesion (Mishima, Kawasaki, Pinkus, 1972). In later stage, α-dendritic cells also decrease. On the other hand patients with melanoma-associated vitiligo are known to exhibit better survival. These suggest possible immunological relationship between vitiligo and melanoma. Purposes of present study are to determine immunological responses of lymphocytes obtained from melanoma-bearing hamster to the premelanosome fraction and their cytotoxic effect on pigment cells. The antigenicity of premelanosome has been studied utilizing the lymphocyte stimulation assay. Cytotoxic effect of premelanosome-reactive lymphocytes on the cultured melanoma cells has also been studied. Tumor-associated antigen is detected in the homogenate and the Large Granule fraction isolated by differential ultracentrifugation from Greene's melanotic melanoma. The premelanosome fraction has been found to exhibit antigenicity in the range of 1.4 to 6.4 as expressed by stimulation index. It has been observed that lymphocyte blastogenesis is moderately suppressed by the use of serum from melanoma-bearing hamsters as compare to that of normal hamster serum. The presence of blocking factor-like substances in the serum is suggested. The lymphocytes of Greene's melanoma-bearing hamster which exhibit positive stimulation index are found to possess cytotoxic effect on the cultured Green's hamster melanoma cells, but not on the B-16 melanoma cells. However, the serum of melanoma-bearing hamster and of normal hamster has not exhibited cytotoxic effect in the same system.

Characterization of Melanoma-Associated Antigen (MAA) in B16 Murine Melanoma and Establishment of MAA-Radioimmunoassay. C. Nishio, K. Jimbow, Y. Ishii. Sapporo Med. Coll., Sapporo, Japan

Recently it has been shown that melanoma cells in humans and murines are immunogenic and possess the antigen(s) demonstrable by syngeneic and xenogenic antisera. The purpose of our present studies with serological and immunochemical approaches is to (a) provide evidences that xenogenic antiserum (AMS) to B16 melanoma possesses the properties which can distinguish MAAs from murine xenoantigens and histocompatibility complex antigens, and (b) develop the radioimmunoassay for measurement of MAAs in tissues as well as in serum, using the xenogenic antiserum and purified MAAs.

MAAs were purified by affinity chromatographies of Con A and AMS from 3MKCl extract of B16 melanoma. AMS was developed in rabbits by an immunization with B16 melanoma cells. MAAs defined by AMS were glycoproteins and their mol wt were estimated around 94,000 and 68,000 daltons. The AMS absorbed by virus infected fibroblasts (Freund and xenotropic) reserved a specific immunofluorescence against cultured melanoma cells. MAA-radioimmunoassay showed that MAAs level circulating in B16 melanoma-bearing mice are
parallel to the tumor weight. MAAs were not detected in the tissue extracts of normal and fetal mice. Thus, our radioimmunoassay system using xenoantiserum has provided a basis for establishment of immunodiagnosis in human malignant melanoma.

Clinical Review of Primary Cutaneous Malignant Melanoma in Jordan. Brig. General Dr. O.Y. Oumeish. King Hussein Medical Center, Amman, Jordan

During the period between June 1969 and February 1980, 1,121 cases of cutaneous malignant tumors were seen in different medical centers in Jordan, of these tumors, primary cutaneous malignant melanoma was diagnosed on clinical and pathological bases in 47 cases; this represents 4.1 percent of the total number of cutaneous malignancies and 2.1 percent of the biopsies done for cutaneous tumors. Accordingly, the incidence of malignant melanoma in the population of Jordan is 1.4/1000,000. The age of the patients, sex ratio, the distribution of the lesion and types of melanomas with the etiological factors are described in this report.

Tyrosine Uptake Studies in Normal and Tyrosinase-Positive Oculocutaneous Albino Hairbulbs. Richard A. King. Univ. of Minnesota, Minneapolis, MN

The primary defect in Tyrosinase-Positive Oculocutaneous Albinism (TPA) is unknown. Previous work in our laboratory has shown normal kinetic and electrophoretic characteristics for TPA tyrosinase. To determine substrate availability, tyrosine uptake by normal and TPA hairbulb melanocytes has now been investigated. Fresh anagen hairbulbs are incubated in 14C-tyrosine, dopa, cyclohexamide, catalase and antibiotics for 24 hours, washed, and counted as whole hairbulbs. Normal hairbulbs are from brown, black, blond, and red hair. White hairbulbs are used as control.

TPA hairbulbs take up 14C-tyrosine at levels similar to control hairbulbs. 14C-tyrosine hairbulb uptake is a metabolic process and is inhibited at low (4°C, 21°C) temperatures, and by increasing concentration of cold tyrosine. The uptake is dependent on tyrosinase activity and is inhibited by DDC and PTU. Preincubation of TPA hairbulbs in 0.05-0.5 percent Triton X-100 in buffer reduces the 14C-tyrosine uptake whereas similar pretreatment of normal hairbulbs does not change or increase the uptake. TPA does not appear to have deficient melanocyte substrate uptake. There may be an abnormal Triton-sensitive tyrosinase binding in TPA melanocytes that accounts for the reduced uptake in TPA hairbulbs after Triton pretreatment.

Atypical Tyrosinase Positive Albinos. M. Mizoguchi, Y. Ishibashi, H. Hu, M. Iijima, Y. Hori, A. Kukita. Teikyo Univ., Tokyo, Japan

In order to study the pathogenesis of tyrosinase positive albinism, we have studied five cases of this disease, especially on melanization of the hair-bulbs before and after incubation with L-tyrosine or L-dopa light and electron microscopically. Moreover, the electron microscopic autoradiographs of the hair-bulbs after incubation with 3H-L-tyrosine were investigated. As a result, it was found that three cases are categorized to the ordinary tyrosinase positive albinos but the other two cases are not. This paper deals with these two cases who showed different findings from ordinary tyrosinase positive albinos.

[Case 1] A 19-year-old man who had white hair and white skin at birth has reddish brown hair and rather white skin as a Japanese at present. His hair-bulbs contained spherical, unevenly pigmented melanosomes resembling those seen in hair-bulbs of red hair or yellow mutant albinos. In contrast to yellow mutant albinos, their hair-bulbs produced pigment after incubation with L-tyrosine. Electron microscopic autoradiographs of this patient showed many grains on melanosomes than normal controls.

[Case 2] A 29-year-old man is phenotypically very much resembling a tyrosinase negative albino. No pigment was found in hair-bulbs of this case before incubation under light microscope. Under electron microscope, most of melanosomes were stage 1 or 2, and it was hard to find stage 3 melanosomes. It takes much more incubation time in tyrosine to find ordinary structured stage 3 and 4 melanosomes than other cases. Electron microscopic autoradiographs showed grains but less than Case 1 and normal controls.

From these findings it is assumed that tyrosinase positive albinism may not be homogenous and can be divided into subclasses.
A Case of Oculocutaneous Albinism with Storage Pool Deficiency of Platelet. (A Variant of Hermansky-Pudlak Syndrome?) Hideo Yaoita, Takashi Ohmi, Shuichi Naito. Univ. of Tsukuba, Ibaraki, Japan

Since Hermansky and Pudlak reported two unrelated cases of albinism associated with hemorrhagic diathesis and pigmented reticular cells in the bone marrow in 1959, more than 29 cases of this disease have been reported. To our knowledge, however, no cases with tyrosinase-positive reaction in melanocyte and with various cutaneous manifestations have been reported as Hermansky-Pudlak syndrome. Our case is a 14-yr-old female. Past history: in her babyhood she had blond hair and brown irises. She suffered from pustules or blisters for 4 mo. after her birth, which remained as scars. Nasal bleeding has been mentioned since she was five years old. Status presence: She has brown hair, brown irises, scars like Noble's, irregular and dark brown scars on her head, hands, abdomen, and thighs. She also has congenital conductive hearing loss, nystagmus, cataracta, retinitis, and oligodontia. Orthopedic and psychiatric examination showed normal results. Laboratory examination: storage pool deficiency of platelet (+) STS (-), TPHA (-), phenylketouria (-). Chromosomal-analysis: normal female type, DOPA (+), tyrosinase (+). Pigmented reticular cells could not be found in her bone marrow. Ultrastructural studies on skin and blood cells could not reveal remarkable abnormalities in melanocytes and platelets except a slight decrease in the number of dense bodies in platelets. Family history shows no albinism, hemorrhagic diathesis, epidermolysis bullosa, syphilis, or incontinentia pigmenti. We still don't know the relationship between albinism and the storage pool deficiency. However, no abnormal melanosomes or premelanosomes were found in both dermal and epidermal cells including melanocytes.

The Pigmentary Disorder in a Family with Hermansky-Pudlak Syndrome. E. Frenk, F. Lattion. University of Lausanne, Switzerland

Giant melanosomes occur frequently and for yet unknown reasons in several hyperpigmentary conditions; they were also found in the eye and normal skin of x-linked ocular albinism. We here report their presence in great numbers in albinotic skin of two sisters from a large Swiss kindred with Hermansky-Pudlak syndrome, and autosomal recessive trait. The two sisters had a fair sun-sensitive skin, albinotic eye changes, nystagmus, and astigmatism. Their hair colour was white during childhood, blond-brown after puberty. Incubation of hairbulbs in tyrosine or dopa increased the proportion of pigmented hairbulbs from 26 percent to 62 percent. The epidermal melanocyte population was weakly dopa-positive, its density within normal range. The most striking feature was the presence of numerous giant melanin granules. Electron microscopically, the melanocytes and keratinocytes contained a variable, but generally small number of melanosomes, mostly of stages 2 or 3, with either a lamellar or granular internal structure. Round giant melanosomes were found in melanocytes, keratinocytes, and occasionally in dermal macrophages; they usually measured up to 2-3 μm in diameter. They were composed of either a fully melanized central core with a granular-vesicular periphery or an agglomeration of melanosomes of normal size and small, dense, globular bodies.

The association of this melanosomal disorder with platelet dysfunction and ceroid storage typical of Hermansky-Pudlak syndrome may provide new insights into the mechanisms leading to formation of giant melanosomes.

Translocation of Melanosomes from the Epidermis to the Dermis—Incontinentia Pigmenti Histologica. S. Nagao, S. Iijima. Fukushima Medical College, Fukushima, Japan

Although histological pigmentary incontinence is seen in many dermatoses, there are few reports concerning how melanosomes translocate from the epidermis to the dermis. Such observations have been done only in two dermatoses: Bloch-Sulzberger's syndrome and Riehl's melanosis. Studying 31 cases of seven different dermatoses showing marked pigmentary incontinence with the electron microscope, we found that this translocation occurs in four different ways.

Materials and Methods. Materials were taken from pigmented lesions of nine cases of Riehl's melanosis, five of macular amyloidosis, five of lupus erythematosus, three of Bloch-Sulzberger's
syndrome, six of pigmentatio macularis multiplex idiopathica, two of lichen planus, and one of lichen planus-like drug eruption. The biopsied specimens were processed routinely for light and electron microscopy.

Results and Discussion. Our interest was focussed on how melanosomes pass from the epidermis to the dermis through the basal lamina. We found four different ways of melanosomal translocation. (1) The Schwann cells enter the epidermis and engulf melanosomes. Then they transport the melanosomes through the processes of their own to the dermis. (2) Macrophages enter the epidermis where they phagocytise free melanosomes and return to the dermis. In the cases of (1) and (2) the phagocytosis of melanosomes by these principally dermal cells occurs within the epidermis. (3) Dyskeratotic cells containing melanosomes drop from the epidermis to the dermis, and then transform to the colloid bodies with melanosomes in the dermis. (4) Degenerated epidermal cells leak melanosomes to the intercellular spaces of the epidermis and these freely located melanosomes translocate to the dermis through the interrupted basal lamina. Melanosomes, which reach the dermis by the last two ways, may be phagocytised by various dermal cells such as macrophages, Schwann cells, mast cells, and endothelial cells.

Ultrastructural Studies on the Development of Pigmentary Incontinence. S. Masu, M. Hosokawa, A. Sato, M. Seiji. Tohoku University School of Medicine, Japan

The developmental process of pigmentary incontinence in Riehl's melanosis, lichen planus, and macular amyloidosis was studied under E.M. The pigmentary incontinence seen in common among these pigmentary disorders appears to be formed in a similar way. The first step: The degeneration of keratinocytes appears to be the initial visible change in pigmentary incontinence. Tonofilaments aggregate and form filamentous masses, nuclei shrink and become electron dense, and melanosomes are intermingled in the filamentous masses. The condensed nuclei and other cytoplasmic organelles disappear. The second step: Macrophages migrate from the dermis and appear to make contact with filamentous masses which contain melanosomes. Then, these macrophages are thought to selectively phagocytise melanosomes because, during the next stage, most melanosomes are observed in the cytoplasm of the macrophages rather than in the filamentous masses. Some filamentous masses are also phagocytised by macrophages. The third step: It is assumed that these phagocytised melanosomes and filamentous masses are eventually transferred within macrophages to the dermis. The filamentous masses which have not been phagocytised may be somehow excluded from the epidermis by macrophages into the dermis. In the final stage, in the dermis, all melanosomes are found in the macrophages as melanosome complexes. On the other hand, filamentous masses are mainly present free in the papillary areas and only a few are phagocytised in the macrophages. However, it is not clear why many filamentous masses remain free in the dermis for a longer period of time. Both the filamentous masses (so-called Civatte bodies) and amyloid found in the dermis were shown to be derived from the epidermal fibrous component by the indirect immunofluorescent method using anti-keratin (epidermal fibrous protein) antibody. The free Civatte bodies seen in the dermis may transform into amyloid.

Ultrastructure of Incontinentia Pigmenti Achromians. Masaaki Morohashi, Ryoichi Igarashi, Tetsuo Maeda. Toyama Medical and Pharmaceutical University, Toyama, Japan

The present work was undertaken to elucidate the mechanism of depigmentation in incontinentia pigmenti achromians. Emphasis was placed on the nerve-melanocyte relationship in the process of depigmentation.

The patient was an 11-year-old Japanese girl with bizarre, irregularly shaped, depigmented lesions on the trunk and extremities. The biopsy specimens were taken from the center (depigmented) and peripheral (hypopigmented and normal color) sites of the involved area. In the central area, melanocytes containing melanosomes were rarely present, although numerous clear cells which do not contain either melanosomes nor Langerhans cell granules were seen in the basal cell layers. Most of them were considered to be melanocyte since they were attached to the basal lamina with hemidesmosomes. In the peripheral area, some melanocytes showed aggregation and degradation of the melanosomes within the membrane-bound vacuoles (presumably autophagic vacuoles). Other melanocytes, however, showed no apparent sign of
degeneration. But most of these showed a decrease in the number and size of their melanosomes.

Numerous nerve endings, which were enveloped with Schwann cells, were observed either close to or in direct contact with the basal lamina of the epidermis in the central and peripheral areas. Many of these nerve endings in the peripheral area were associated with the melanocytes in the basal layer of the epidermis.

**Ultrastructural Observations of Melasma.** S. Sato, M.A. Pathak, N.P. Sanchez, J.L. Sanchez, T.B. Fitzpatrick. Harvard Medical School, Boston, MA, and Univ. of Puerto Rico, San Juan, PR

Melasma (chloasma) is an irregularly patterned, brown or ashen-gray hypermelanosis occurring usually in habitually sun-exposed areas of skin such as the forehead, periorbital margins, cheeks, rami of mandibule, upper lip, and neck. Little is known about the ultrastructural pigmentary changes in melasma. This study, carried out in 13 Hispanic patients (biopsies of normal and melasma skin), represents the first attempt to examine the epidermal and dermal pigmentary changes using electron microscopy. The principal alterations were seen in the lower epidermis. The melanocytes were increased in number and appeared highly dendritic. The interfollicular melanocytes in the basal layer of the epidermis were usually seen protruding into the dermis. Their well-developed perikarya were found to contain abundant fully melanized melanosomes, cytoplasmic organelles, and anastomosing dendritic processes. The melanosomes transferred to the keratinocytes were accumulated in the basal and suprabasal layers of the epidermis. They were preferentially distributed around or on top of the keratinocytes' nuclei. The pigment granules were distributed for the most part in the form of single non-aggregated melanosomes with minimal evidence of their degradation or the formation of large melanosome complexes. Although vascular changes were infrequently present in the lower epidermis in certain biopsies, the integrity of the cellular architecture, cellularity, and the ultrastructure of the dermo-epidermal junction appeared normal. Melanophages were invariably seen but they were not prominent, and only a small number of macrophages phagocytizing melanosome complexes were scattered in the loose connective tissue of dermis. No evidence of inflammatory reaction or pigmentary incontinence were obtained. The morphological features of this pigmentary disorder appear to be related to an increase in number and activity of type-specific melanocytes (formation, melanization, and transfer of melanosomes) brought about by exposure of skin to the sun.

**Light and Ultrastructural Study of Minocycline-Induced Hyperpigmentation of the Skin.** M.C. Mihm, Jr., N.P. Sanchez, S. Sato, G.F. Murphy, T.B. Fitzpatrick. Massachusetts General Hospital, Harvard Medical School, Boston, MA

Minocycline administration is known to cause blue or bluish-black circumscribed hyperpigmentation to develop over the lower extremities, in the oral cavity, and in atrophic scars. Histochemically, iron and melanin deposition are demonstrable in the dermal macrophages that contain pigment granules. The present light and ultrastructural study was undertaken to evaluate the nature of the pigment granules in the macrophages so that the etiology of this condition can be more closely understood. Histologically there was hyperpigmentation of the epidermis and increased numbers of pigment-laden macrophages (melanophages) in the superficial and deep dermis. Iron deposition in perivascular array was confirmed with iron stain. Sodium bromide split, dopa incubated biopsy specimens revealed minimal increase in melanocytes in affected pigmented skin. The pigmented cells were highly dendritic and dopa-positive with ovoid, triangular to tetragonal hypertrophic perikaryon.

Ultrastructurally the macrophages in the upper dermis of the lesional skin were found to primarily phagocytize melanosomes in the form of melanosome complexes. Those seen in the deeper dermis were mainly collecting electron-dense, fine particulate matter, identified morphologically as hemosiderin, in phagocytic vacuoles. Frequently, a small number of melanosomes were also encountered within the phagocytic vacuoles. It appears that in minocycline-induced hyperpigmentation there occurs initial diapedesis of erythrocytes from the dermal blood vessels, followed by accumulation of hemosiderin in the macrophages. An X-ray microanalytical study of the electron-dense particulate matter, judged to be hemosiderin, is being carried out.