Ultrasonic Characterization of Chemical and Physical Structure of Melanosomes in B16 and Harding-Passey Melanomas. R. Kono. National Defense Academy, Yokosuka, Japan  K. Jimbow, H. Takahashi, K. Maeda. Sapporo Medical College, Sapporo, Japan

An ultrasonic measurement of melanosomes was carried out in frequencies from 0.333 MHz to 300 MHz on B16 and Harding-Passey (HP) mouse melanomas, which produce two different forms of melanosomes, i.e., ellipsoidal-lamellar melanosomes in B16 and spherical-granular melanosomes in HP. We found that the structure of the two melanosomes is basically amorphous and co-polymeric in the molecular dimension of a segment composed of five to six zigzag units. Stochastic resonance was, however, markedly different between B16 and HP melanosomes, indicating that the structure of the B16 melanosome is moderately packed, while that of the HP melanosome is more open and similar to that of the sepiamelanosome. A marked difference of particle wave resonance was also found around 200 MHz in the two melanosomes. It was indicated, based on the chemical structure of melanosins proposed by Hempel, that the physical structure of the HP melanosome is a co-polymer in which melanin and protein moieties run parallel to each other but may bind together at the sites of planar groups, while that of the B16 melanosome is a double-helix polymer of melanin and protein moieties with a screw symmetry of $N = 6$. This type of one-dimensional cyclic ordering, commonly known as Born-Karman periodic boundary condition in semiconductive band theory, may be related to the formation of the lamellar structure seen in the B16 melanosome.

Structural Characterization of Sepia Melanosome and Sepia Melanin by Ultrasonic Measurement. R. Kono. National Defense Academy, Yokosuka, Japan  Y. Miyake, Y. Izumi. Hokkaido University, Sapporo, Japan  K. Jimbow. Sapporo Medical College, Sapporo, Japan

Ultrasonic measurement was conducted on sepiamelanosomes and sepiamelanin in water suspension, and their ultrasonic properties were compared to those of the synthetic melamins. It was found that the absorption behavior of sepiamelanosomes is quite similar to that of hydrated diethylamine melanin; it was composed of three terms of spectra: (1) a stochastic resonance at 0.38 MHz characteristic of living systems, (2) the principal relaxation at 56 MHz characterized by amorphous chain polymer due to microbrowian motion of the main C-C bond, and (3) a particle wave resonance at 220 MHz referred to stiff-chain molecule with a sub-resonance showing interchain stacking.

Employing the chemical structure proposed by Nicolaus, term (3) was analyzed for resonance frequency $f_r$. It was indicated that sepiamelanin is amorphous with a middle range of $\sim 50 \text{ Å}$ consisting of a rod-like rigid molecule with a short range of $\sim 10 \text{ Å}$. The sepiamelanosome was also found to be amorphous in structure, in which the protein moiety binds to a rod-like form of melanin and conformation of the protein moiety governs the secondary structure of the melanin moiety. However, the structure of sepiamelanin was more packed than that of sepiamelanosome.

Mammalian Tyrosinase Catalyzes Three Reactions in Melanin Biosynthesis. Ann Korner, John Pawelek. Yale University School of Medicine, New Haven, Connecticut

Tyrosinase (EC 1.10.3.1) catalyzes the oxidation of tyrosine to dopa and dopa to dopaquinone, thus initiating the biosynthesis of melanin. Recently, three additional control points in the Mason-Raper pathway of melanogenesis have been identified. Dopachrome conversion factor accelerates the conversion of dopachrome to 5, 6-dihydroxyindole (DHI). Indole conversion factor accelerates the conversion of DHI to melanochrome. Indole blocking factor inhibits melanogenesis from DHI. Indole conversion factor is found in melanotic cells while the blocking factor is found in amelanotic cells. Exposure of cultured melanocytes to MSH results in the removal of blocking factor and the appearance of indole conversion factor. Dopachrome conversion factor is found in both amelanotic and melanotic cells and is unaffected by MSH. We now report that indole conversion factor is synonymous with tyrosinase. Indole conversion
factor activity co-purifies with tyrosinase isolated from Cloudman S91 melanomas, B16 melanomas, and extracts of skin from new-born brown mice. Highly purified tyrosinase catalyzes the conversion of DHI to melanochrome in a reaction which is stimulated when dopa is a cofactor and which is inhibited by L-tyrosine, phenylthiourea, and diethylthiocarbamate. Conversely, DHI inhibits the oxidation of tyrosine to dopa. The inhibition of the conversion of DHI to melanochrome by L-tyrosine is non-competitive, suggesting that DHI and L-tyrosine bind to separate sites on the enzyme. Thus, tyrosinase has the unusual property of catalyzing three distinct reactions within a single biochemical pathway: the hydroxylation of a monophenol, the dehydrogenation of a catechol, and the dehydrogenation of a dihydroxyindole. The first and third of these reactions require dopa as a cofactor; in the second reaction, dopa is a substrate. It has been demonstrated that the precursors of melanin are toxic to cells producing pigment and that MSH enhances the toxicity of such precursors. Under normal conditions pigment cells are protected from the toxic effects. Overproduction of melanin disrupts the protective mechanism. Our results suggest that the relative concentrations of L-tyrosinase and DHI may control the generation of toxic intermediates.

The Pigmentation of Keronopsis rubra and of the Colorless Mutant K. rubra Strain "albino." N. Laasch, G. Uhlig. Biologische Anstalt Helgoland, Marine Station, Helgoland, Federal Republic of Germany

In May 1979 we received a sample of a clonal culture of Keronopsis rubra from the Groote Schuur Hospital Capetown, South Africa. These cells were descendants of 2,000 cells which had been γ-radiated in 1978. Later, in 1980, from a highly populated mass culture of this strain, a few colorless cells could be isolated and mass-cultivated as well.

Both the strain "rubra" as well as the so-called strain "albino" show negatively phototactical behavior. The phototactical reaction of the "rubra" cells, however, is significantly stronger than that of the colorless "albino" cells, obviously due to their difference in pigmentation.

The pigments of both strains were studied by spectral and chromatographical analysis. With extracts of the red pigment of K. rubra, two absorption maxima at 420 nm and 460 nm were registered. In contrast, no absorption could be measured with aliquot concentrations of "albino" extracts. From chromatographic studies it is suggested that the dominant pigments in the "rubra" cells represent carotenoids. Obviously, in the "albino" cells biosynthesis of carotenoids is severely inhibited.

The Prognostic Significance of Nuclear DNA Content in Malignant Melanoma.
B. Lagerlöf, G. Cewrien, G. Auer. Karolinska Hospital, Stockholm, Sweden

The most important histopathological parameters to predict prognosis of malignant melanomas are tumor thickness and level of invasion. In some cases, however, the course does not follow the pattern predicted by the histopathological parameters; a case with a thin melanoma which is usually cured by the primary excision can develop metastases, and a thick melanoma can behave less malignantly than many thick melanomas do. It is therefore obvious that the ordinarily used histopathological parameters do not fully predict the prognosis of malignant melanomas. The present study was undertaken to elucidate if the pattern of the DNA distribution of the tumors, as an expression of the tumor growth activity, might add additional information concerning prognosis. We have therefore retrospectively analyzed the DNA distribution of four groups of malignant melanomas, namely thin melanomas without metastases and thin melanomas with metastases, and corresponding groups of thick melanomas.

DNA measurements were determined in 4 micro thick sections from archival paraffin blocks. For this the sections were deparaffinized and stained according to a modified Feulgen-staining technique (5 NHCL, 22°C, 60 minutes). The nuclear DNA content was determined in morphologically defined single cells. In each case 100 tumor cells and 50 normal diploid control cells were measured. The results indicate that tumors composed of cells with DNA amounts comparable with that of normal tissue were correlated with a good prognosis independent of the tumor thickness. In contrast, tumor cells with highly increased and scattered DNA amounts were correlated with bad prognosis.

These preliminary results suggest that DNA measurements of malignant melanomas can be
of prognostic value and contribute additional information over and above the information obtainable by means of clinical and histopathological criteria.

**Preparative Purification of \( \beta \)-Melanotropin by High Performance Liquid Chromatography.** D.T. Lambert, A.B. Lerner. Yale University School of Medicine, New Haven, Connecticut

Porcine \( \beta \)-melanotropin (\( \beta \)-MSH) is an octadecapeptide that stimulates both the dispersion of melanosomes in melanophores of poikilothermic vertebrates and the activation of tyrosinase, the enzyme that catalyzes the synthesis of melanin in melanosomes of Cloudman S91 melanoma cells. In the past the hormone has been purified by classical biochemical techniques such as carboxymethyl cellulose chromatography [Lerner, McGuire, New Eng J Med 270:539, 1964]. We report here the further purification of porcine \( \beta \)-MSH by reversed-phase high performance liquid chromatography (RP-HPLC).

When the hormone, previously purified by carboxymethyl cellulose chromatography, is injected onto an analytical Waters C18 \( \mu \)Bondapak column, it elutes as a major peak which is preceded and followed by minor contaminants. Ten passes through the column, using the Waters instrument's recycle mode and isocratic conditions (20 percent acetonitrile in 5 mM sodium phosphate, pH 3.17), show this peak to be a single molecular entity. It is possible to perform small-scale (2-4 mg) preparative purifications using the above conditions or similar conditions in which the sodium phosphate is replaced with ammonium acetate (pH 5.8). Larger-scale (e.g., 50 mg) purifications can be performed using the Waters 1" Semi-Prep column packed with C18 Corasil; elution is performed isocratically with 20 percent acetonitrile in 5 mM ammonium acetate (pH 5.8). The material of the major peak eluting from the HPLC can be lyophilized and then be desalted by applying it to a BioGel P-2 column and eluting it with distilled water. The resulting preparation has both the amino acid composition and the tyrosinase-stimulating activity expected of \( \beta \)-MSH.

**Receptors for MSH on Cloudman Melanoma Cells: Basic Characterization.** D.T. Lambert, A.B. Lerner. Yale University School of Medicine, New Haven, Connecticut

We have recently solved the difficult problem of producing highly purified \( ^{125}\text{I}-\beta \text{MSH} \) possessing full biological activity [Lambert et al: J Biol Chem 257:8211-8215, 1982] and we are now employing this probe to characterize the receptor for \( \beta \text{MSH} \) on Cloudman S91 melanoma cells. For these assays, cells are harvested from monolayer with 1 mM EDTA in Joklik's modified MEM, centrifuged, resuspended in Ham's F10 medium containing 15 mM HEPES and 5 mg/ml BSA, centrifuged, then resuspended in fresh buffer. The cells are then incubated in the presence of the \( ^{125}\text{I}-\beta \text{MSH} \) in a siliconized glass vial in a shaking water bath at 15°C. At an optimal pH of 6.8, approximately 85 percent of the binding of the probe to Cloudman cells can be blocked by a thousandfold excess of unlabeled MSH. Dithiothreitol in the binding buffer, usually around 0.1 mM, does not appear to interfere with binding. DTT is present in the stock solution of \( ^{125}\text{I}-\beta \text{MSH} \) to prevent oxidation of this labile hormone.

Evidence supporting the conclusion that the \( ^{125}\text{I}-\beta \text{MSH} \) is binding to a physiological receptor includes: (1) the label binds specifically to pigment cells, such as murine melanoma cells, but not to fibroblasts; (2) the binding of the labeled hormone is competed against by peptides structurally similar to \( \beta \text{MSH} \) which mimic \( \beta \text{MSH} \) in stimulating tyrosinase in Cloudman cells (e.g., \( \alpha \text{MSH} \) or ACTH), but not be unrelated hormones (e.g., insulin, LHFRH, vasotocin); (3) preliminary analysis by Scatchard plots indicates an affinity constant in the nanomolar range, which is consistent with the half-maximal stimulation of tyrosinase by MSH; (4) the binding is saturable; and (5) the binding is reversible.

**DK/Lm: A Strain of Laboratory Mouse in Which the Lethal Yellow (\( A^\text{L} \)) Phenotype Is Uniquely Expressed.** M. Lynn Lamoreux. Texas A & M University, College Station, Texas D.B. Galbraith, Trinity College, Hartford, Connecticut

DK/Lm is a new inbred mouse strain with over 20 generations of brother-sister mating. The genotype of the DK/Lm mouse at the black-brown locus is b/b and heterozygosity at the
agouti locus (A\textsuperscript{a}) is maintained by matings between yellow and brown mice. DK/Lm-A\textsuperscript{a} mice become sable in phenotype at the first molt, whereas C57BL/6J-A\textsuperscript{a} mice do not. The sable phenotype is defined as that of a mouse whose basic color is yellow (pheomelanin) but whose dorsal aspect is more or less darkened by the presence of non-yellow (eumelanin) pigment. At about six months of age the DK/Lm mouse gradually reverts to yellow in phenotype.

Mice of the two strains were compared. Pigment phenotype was examined grossly and microscopically, before and after the first molt. Mice were weighed weekly. All experimental mice carry the same allele (A\textsuperscript{+}) which affects rate of weight gain, obesity, blood glucose levels, tumor incidence, and pigmentation. The non-yellow control mice of the DK/Lm and the C57BL/6J strains are similar in rate of gain, although the DK/Lm mice are somewhat smaller. There is, however, a significant difference between the yellow and sable A\textsuperscript{a} mice. This result corresponds with observations made by others, with the exception of a unique situation in which the pleiotropic effects are separable.

These observations are discussed and related to hypotheses regarding gene action at the a and e loci. The new strain is offered as a useful experimental animal for study of the relationship between gene action at the agouti locus and the important pleiotropic effects influenced by this locus.

A Method for Culturing Chick Embryo Melanocytes Which Controls the Gene Products Involved in Melanogenesis. K. Langner, W. Oetting, J. Osborne, J. Brumbaugh. School of Life Sciences, University of Nebraska, Lincoln, Nebraska

The tumor-promoting agent, 12-0-tetradecanoylphorbol-13-acetate (TPA), and medium conditioned by Buffalo Rat Liver (BRL-3A) cells provide an excellent means of culturing chick melanocytes. The dissected trunk region of each 72-hour embryo produces an average of 4 × 10\textsuperscript{6} cells (95\% percent purity) in 16 days. A typical experiment of 20 embryos produces 8 × 10\textsuperscript{7} melanocytes. Cultures were fed three parts BRL-3A conditioned medium: one part fresh medium on day 1 after culturing and then on alternate days until termination. On days 5, 7, and 9, 1 × 10\textsuperscript{-7}M TPA was added. On day 10 TPA was removed and regular feeding resumed. During the time in TPA, the cells remained unpigmented as viewed with light and electron microscopy. Twenty-four hours after TPA removal, however, a strong DOPA reaction was seen at the ultrastructural level. After 48 hours pigment was visible with the light microscope. By day 16, 90\% percent of the cells which were pigmented. The melanogenesis which occurs after TPA removal is both cycloheximide and alpha-amanitin sensitive.

Replicate cultures were labeled with \textsuperscript{3}H-leucine while in TPA (day 9) and after removal from TPA (day 13). Homogenates of cells were detergent-extracted and subjected to two-dimensional gel electrophoresis followed by fluorography. The resulting autoradiograms showed that eight protein spots or groups of spots appear when TPA is removed. These are therefore putative melanogenic proteins. Tyrosinase has been tentatively associated with one group of spots. Various mutant genotypes are being tested to see which, if any, of the eight melanogenic proteins are affected.

Supported, in part, by NIH grant GM 18969.

Flow Cytometric Procedure for DNA Ploidy Analysis of Human Melanomas. J.K. Larsen, H.I. Nielsel, I.J. Christensen. The Finsen Laboratory, Finsen Institute, Copenhagen, Denmark

In a previous study DNA ploidy characteristics of human malignant melanoma were analyzed by flow cytometry and compared with histology and clinical course [Søndergaard et al: Virchows Archiv Cell Pathol 42:43–52, 1983.]

In a present study, by adopting the methods described by Vindeløv et al. [Cytometry 3: 317–339, 1983], the resolution and reproducibility of the flow cytometric DNA analysis have been considerably improved. Monodisperse nuclear suspensions were made with Nonidet P40 detergent, the nuclear DNA was stained with propidium iodide and RNase, and the fluorescence intensity of each nucleus was measured in a Becton Dickinson FACS IV cell sorter. For calibration and control of flow system performance hen and trout erythrocyte nuclei were added to each sample as internal DNA reference standards. In the frequency distribution histogram of fluorescence per nucleus the intensities of the individual peaks of
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these substances are incorporated into melanin as false precursors.

We have also found that the sulfur of the thioamides is of crucial importance for this incorporation, which most probably results from a condensation of the sulphydryl groups of the thioamides with quinones produced in the melanin synthesis.

A suitable radiolabeling of the false melanin precursors would make it possible to improve the diagnostic and therapeutic possibilities for malignant melanomas. We have found that iodination of thiouracil (5-iodo-2-thiouracil) does not essentially change its property as a false melanin precursor. The experiments include both whole-body autoradiography and liquid scintillation counting of excised tissues and tumor pieces of mice treated with 125I-thiouracil. In other experiments, melanoma-bearing mice were injected with 125I-thiouracil. The label of the tumors could be detected and followed by gamma scintigraphy. Preliminary results with human malignant melanomas show that 125I-thiouracil accumulates linearly with time of culture. The kinetics of 125I-thiouracil in patients with advanced forms of malignant melanomas is presently being studied by gamma scintigraphy and measurements of concentrations in blood and excised melanoma tissues. The results so far indicate an accumulation in progressing tumor areas.

Nevus Cell Nevi in Humans, A New Histogenetic Concept Based on Transmission Electron Microscopy. P.J. Lea, Electron Microscopy Laboratory, University of Toronto, Ontario, Canada A. Pawlowski. Faculty of Medicine, University of Toronto, Ontario, Canada

Ultrastructural studies were carried out to investigate epidermal and dermal components of human nevi. Forty lesions were obtained from either volunteer donors or were removed for medical and/or cosmetic reasons. The nevi were processed for electron and light microscopy. Serial thin sections (50-90 nanometers thick) were cut with a diamond knife, mounted on single-slot formvar carbon-coated grids, and examined in a Philips EM300 electron microscope. Thick sections (approximately 5 micrometers thick) were also cut, to serve as the light microscopy routine control.

Our findings tend to indicate that nevus cells do not detach from the epidermal-dermal junction. The nevus cells do not drop down into the dermis (“abtropfung”), but remain surrounded by a basement membrane. The depth of invagination into the dermis was determined and the diameters of “nevus nests” were measured. All measurements were made with a micro-computer-based, digital analysis system, developed specifically for image analysis. Electron microscopical findings did not support the present histological classification of nevus cell types. There also appeared to be no relationship between the morphology and the localization of nevus nests and innervation of the skin. Nevus cells and melanocytes were compared, with emphasis on cytoplasmic filaments and autophagic activity. The origin of nevus cells from normal, adult melanocytes has been suggested.

Ultrastructural Evolution in albino Mutant Epidermis of Locusta migratoria L., During the Last Larval Instar and the Beginning of the Imaginal Life. J. Lhonoré. Laboratoire d Histophysiologie fondamentale et appliquée, Université P. et M. Curie, Paris, France A. Bouthier. Laboratoire de Zoologie, Paris, France

Albino mutant of L. migratoria L. shows a general aspect which differentiates it from wild strain: lack of tegumentary pigmentation confers a creamy background shade; after imaginal ecdysis it appears a slightly cuticular melanization.
Ultrastructural studies show that epidermal cells are practically deprived of ommochromes while internal organs accumulate them as in the wild insects. When they exist acridiomatine granules are atypical; electron microprobe analysis reveals a calcium phosphate storage, such as inclusions of spherochystal types, which sometimes replace the ommochrome granules in adult epidermis.

Whatever tegumental areas are studied, epidermal cells show an important accumulation of potassium urate as microcrystals, which amount of variations correspond approximately to those recorded for wild insects during the fifth larval instar and the beginning of the imaginal life. Imaginal ecdysis seems to be accompanied by an increase in inhibition of cuticular melanization, which goes on all four with epidermal acridiomatine synthesis.

**Biosynthesis and Shedding of a Human Melanoma-Specific Oncofetal Antigen gp83 Identified by the Monoclonal Antibody 140.240.** S.-K. Liao, M.J. Khosravi, P.B. Dent, P.C. Kwong. McMaster University, Hamilton, Ontario, Canada

We have produced a murine monoclonal antibody 140.240 which identifies a melanoma-specific oncofetal antigen with an apparent molecular weight of 87 Kd [Liao et al: Int J Cancer 30: 573, 1982]. Immunoprecipitation of cell lysates of 3S-methionine or 3H-glycosamine metabolically labeled (16-18 hours) cutaneous and ocular melanoma cultures, followed by SDS-polyacrylamide gel electrophoresis under reducing or non-reducing conditions showed an identical band of 87 Kd, indicating that the molecule detected is a monomeric glycoprotein, thus designated gp87. Pulse labeling experiments with 3S-methionine showed that after 10 minutes, a single band 87 K (gp83) was noted; gp87 band appeared after one hour. Pulse-chase experiments revealed that by four hours (one-hour pulse and three-hour chase) the 83 K band was replaced by a heavy labeled 87 K molecule. Blocking of glycosylation with tunicamycin (0.5-2 μg/ml) resulted in the appearance of 77 polypeptide (p77). Two-dimensional tryptic mapping indicates that p77, gp83, and gp87 showed a common protein structure. In addition to molecular size, gp87 was different from gp83 in that gp87 was sensitive to neuraminidase. Immunoprecipitation studies showed that, similar to the cellular form, the shed antigen detected in spent medium of melanoma cultures is also a monomeric glycoprotein but with a molecular weight slightly larger than the cellular form. Cellular and shed gp87 had similar tryptic maps, except that two additional peptides were noted in the shed form, indicating certain modifications with shedding. These data suggest that p77 polypeptide backbone is initially synthesized, which is then immediately glycosylated with high mannose-type oligosaccharides giving the intermediate molecule gp83. Sugar chains of gp83 may be sialylated and progressively modified to yield the final mature produce of gp87 without involving major alteration in the backbone polypeptide structure. Further studies are needed to fully elucidate the mechanisms involving the shedding process of gp87.

Supported by MRC and NCI of Canada.

**Human Melanoma Cell Differentiation: In Vitro Modulation by Theophylline of Cellular Morphology, Surface HLA-DR (Ia-Like) Antigen Expression and Susceptibility to Natural Killer Cell Cytolysis.** S.-K. Liao. McMaster University and the Ontario Cancer Treatment and Research Foundation, Hamilton, Ontario, Canada

Malignant melanoma cells undergo differentiation and maturation spontaneously in vitro and in vivo, and as a result of experimental manipulation in vitro. To gain a better understanding of this phenomenon, we studied the effect of theophylline (Th) on cultured human melanoma cells (CaCl 73-36). We observed a dose-dependent inhibition of cell growth with a reduction in plating efficiency of 16, 64, and 99 percent at concentrations of Th of 0.1, 1, and 2 mM, respectively. Th at the concentration of 1 mM was used in subsequent experiments. Th-treated cells showed marked morphological changes consistent with a more differentiated state, including increased dendritic formation and contact inhibition. Quantitative absorption of rabbit anti HLA-DR antiserum with different number of Th-treated and untreated cells followed by testing absorbed and unabsorbed antibody samples was performed to determine surface antigen content. Basal surface HLA-DR antigen content on melanoma cells remained constant during normal culture conditions without Th added. Exposure of melanoma cells to Th
resulted in the disappearance of surface HLA-DR antigens with a concomitant increase of their susceptibility to natural killer cell lysis by five- to eightfold. Removal of Th from the culture medium resulted in (i) morphological conversion to the cell shapes of untreated cultures, and (ii) increase in surface HLA-DR antigen density to the basal level, indicating the reversibility of the Th-induced changes in melanoma cells. It is not known why certain melanomas expressed HLA-DR antigens, while their normal counterparts, normal melanocytes, do not. HLA-DR antigens on melanoma may therefore be considered as markers for a state of differentiation that may be associated with differences in clinical behavior and response to treatment. Furthermore, HLA-DR antigens may be used for defining subsets of human malignant melanoma.

**Melanoma Differentiation: Antigenic and Hormonal Approaches.** A. Libert, G. Ghanem, R. Arnould, A. Vercammen-Grandjean, F. LeJeune. Laboratory of Oncology and Experimental Surgery, J. Bordet Institute, Brussels, Belgium

Our first purpose was to investigate the presence of specific differentiation surface antigens on human melanoma cells cultured in vitro. 19 human melanoma lines, 10 human fibroblasts, and 5 other human tumor cell lines were screened by six monoclonal antibodies (Mabs) including four anti-melanoma, one anti-glioma, and one anti-HLA-DR Mabs, using a $^{125}$I-protein A assay. The reactivity pattern of the four anti-melanoma Mabs showed that they were not directed against antigens strictly restricted to melanoma, for they cross-reacted with gliomas. Thus, they seem to recognize neuroectoderm-associated differentiation antigens. The antigenic distribution of these antigens was visualized by autoradiography. Only a proportion of the cells varying from 11 to 54 percent expressed the relevant antigens.

The second purpose of our study was to assess the receptivity of human melanoma cells to $\alpha$-MSH (melanocyte stimulating hormone). Preliminary results show that 3/5 melanoma lines bound significant amounts of $^{125}$I-MSH, as compared to three fibroblast and three other neoplastic lines used as controls. Competition curves show that the binding occurred through a membrane receptor. Visualization was performed also by autoradiography, showing that a proportion of 50 percent of the cells of the most receptive line were labeled. We can tentatively conclude that human melanoma cells in culture show antigenic determinants and peptidic hormone receptors, which are related to cell differentiation. Only a fraction of the studied cells exhibited these properties.

**Cytogenetic Analysis of Transplantable Hamster and Mouse Melanomas of Different Melanin Content and Growth Rate.** J. Limon, Z. Gibas, M. Babińska, A. Bomirski, B. Beil. Institute of Medical Biology, Medical School, Gdańsk, Poland; Roswell Park Memorial Institute, Buffalo, New York

The chromosome banding analyses of the three transplantable Bomirski hamster melanomas (black melanotic Ma, brown melanotic MI, amelanotic Ab) and of the two B-16 mouse melanomas (melanotic P and amelanotic NP) were performed. Hamster melanomas differ in growth rate, which is highest in the Ab melanoma and lowest in the MI melanoma. All the hamster melanomas have a common origin in a spontaneous pigmented melanoma. The NP melanoma arose by a spontaneous alteration from the P melanoma. Both the mouse melanomas have the same growth rate.

Pigmented melanomas of hamster (Ma) and mouse (P) are characterized by a stable near-diploid number of chromosomes with low degree of variability. On the other hand, the Ab melanoma displays hypertriploidy, and the MI as well as the NP melanomas show hypotetraploidy. Therefore spontaneous changes of pigmented melanomas into less pigmented or pigmentless tumors are accompanied by polyploidization and by the appearance of specific marker chromosomes. The origin of the majority of these chromosomes in all melanomas was established.

No correlation was found between the chromosome number of melanoma cells and their degree of malignancy as measured by the growth rate of the transplanted tumors.

Thus, unexpectedly, chromosome number in the melanomas under study is more closely related to their differentiation level as expressed by pigmentation than to their growth rate.
A Comparison Between the Effects of Single or Fractionated Doses of β-Rays on the Growth of Hamster Melanoma Tumors. E. Link, S. Łukiewicz. Jagiellonian University, Kraków, Poland

Pigmented and non-pigmented Bomirski's hamster melanoma tumors were used in these experiments. Intradermal tumors of different sizes were irradiated with β-rays from a strontium-yttrium applicator. Single doses of radiation or fractionated regimes were used.

The experiments revealed that a single dose of β-rays is more effective than the equivalent dose employing a fractionated regime.

The pigmented tumors were found to be approximately two times more radioresistant compared with the non-pigmented tumors and differed in the rate of regression following irradiation.

It was possible to achieve a β-radiation dose which, when given as a single treatment to either pigmented or non-pigmented tumors of more than 1.5 mm thickness, caused complete regression without causing irreversible damage to the epidermis.

Cytoskeletal Structure and Composition in Goldfish Xanthophores. Sze-cheng John Lo, Robert E. Palazzo, Gary R. Walker, Thomas J. Lynch, John D. Taylor, T.T. Tchen. Wayne State University, Detroit, Michigan

Cytoskeletons (CSKs) from goldfish xanthophores and dermal non-pigment cells have been prepared by extraction with Triton X-100 to allow correlative ultrastructural and biochemical studies. The morphology of the xanthophore CSK differs greatly from that of the non-pigment cells and undergoes striking changes in ogranization during pigment translocation induced by ACTH or cAMP. Despite these structural differences, the major proteins which comprise the CSKs are similar qualitatively and quantitatively in all cases. Differences in minor proteins are seen between cell types and between xanthophores chronically deprived of ACTH (rounded morphology) and chronically stimulated by ACTH (highly dendritico). All of the major cytoskeletal proteins, with the exception of actin, are phosphorylated. The patterns of CSK protein phosphorylation in xanthophores (labeled with 32P-phosphate prior to extraction) differ slightly from that of the non-pigment cells but are unaffected by treatment of the cells with ACTH. The results suggest that minor changes in protein composition are associated with dramatic changes in cytoskeletal structure. Further, carotenoid droplet dispersion or aggregation is not accompanied by changes in the phosphorylation of the stable CSK proteins (e.g., actin or tubulin) nor by a net assembly or disassembly of the cytoskeletal elements.

Large-Scale Isolation and Maintenance of Normal Goldfish Pigment Cells. Sze-Cheng J. Lo, Susan M. Grabowski, Carl R. Clark, Thomas J. Lynch, John D. Taylor, T.T. Tchen. Wayne State University, Detroit, Michigan

We have isolated from xanthic and black moor goldfish xanthophores and melanophores in sufficient quantities for biochemical analyses. The method is based on selective tissue dissociation first to remove epidermal cells and then to produce suspensions of individual dermal cells.

The pigment cells are then separated from the unpigmented dermal cells by density gradient centrifugation, exploiting the differences in buoyant density due to the pigment content of the chromatophores. This procedure produces cells of high purity (70-90 percent) and viability (80-90 percent) and the yield can approach 10^8 cells (xanthophores) in a single preparation. The cells can be maintained for three to four weeks, throughout which they retain the ability to translocate their pigment organelles in response to various hormones and cyclic AMP. There appears to be little mitotic activity in these cultures, which is in marked contrast to the melanized cells whose differentiation can be induced in vitro from dermal non-pigment cells.

The Pigment Organelles Stained with Hot Osmium Tetroxide. S.J. Lo. Division of Biology, National Yang-Ming Medical College, Taipei, Taiwan

In order to reveal the membrane origin of pigment organelles in chromatophores, dorsal skin tissues from frogs (Rana catesbeiana) were incubated in 1 percent osmium tetroxide at 40°C
for forty hours (Friend's reaction) and then prepared for electron microscopy. The observations were as follows. (1) In addition to Golgi complex and nuclear enveloped, the osmium tetroxide reaction productions (OTRP) were present in three different pigment organelles of the chromatophore unit; however, in each type of chromatophore, there were only some pigment organelles containing OTRP. (2) Inside of perinosomes, small vesicles containing OTRP were associated with outer membrane of perinosome whereas inside of melanosomes, the OTRP appeared along the rim of whole membrane. (3) Inside of refractosomes, or reflecting platelets, the OTRP were present in either whole refractosomes or a bar structure of the organelle.

Melanoma Cell Production of an Interleukin 1-Like Thymocyte Activating Factor.
T.A. Lugner, A. Köck, U. Wirth, M. Vetterlein, E.M. Kokoschka, M. Micksche. II. Universitäts-Hautklinik Wien, Inst.f.angewandte und experimentelle Onkologie Wien, Austria

There is evidence that immunological factors may affect the course of malignant melanoma. Thus the occurrence of lymphohistiocytic infiltration is often associated with definite regression of the tumor. In order to determine whether melanoma cells through the secretion of mediators may contribute to these immunological reactions, cell lines of primary melanomas (GT BS) as well as melanoma metastases (KRFM) were established. Supernatants of these melanoma cell lines were tested in the thymocyte co-stimulator assay, which is widely used to detect interleukin 1 activity. All supernatants tested contained significant levels of thymocyte growth-enhancing activity. Additionally the melanoma cell supernatants like IL 1 were directly mitogenic for fibroblasts in culture. In contrast, the supernatants were not able to promote the growth of an IL 2-dependent cell line (CT 6) and therefore did not contain IL 2 activity.

Melanoma cell production of IL 1-like activity was significantly enhanced by stimulation with lipopolysaccharide (LPS), OK 432, silica, hydroxyurea (HU), platiniol, and carmustine (BCNU). The increased factor production was either due to an increased proliferative activity (cisplatinol, BCNU) or blocking of the cell cycle (silica, HU, OK 432). These observations suggest that injurious stimuli that prolong the G1 phase of the cell cycle favor factor production.

Biochemical characterization of the melanoma cell derived factor as determined by HPLC using three TSK 250 columns in series, showed that the activity eluted as two major peaks at 21.000 and 14.000 molecular weight. Therefore the melanoma-derived thymocyte growth-enhancing factor has the same molecular weight as macrophage-derived IL 1.

These results indicate that melanoma cells may interact with the immune system through the production of immunomodulating IL 1-like mediators.

Reverse Transcriptase Activity in Muscle and Melanoma of Xiphophorus. W. Lüke, F. Anders. Genetisches Institut der Universität Giessen, Federal Republic of Germany

Crossings of a spotted platyfish with a nonspotted swordtail result in F1 hybrids that develop benign melanoma. Back-crosses of the F1, hybrids using the swordtail as the recurrent parent result in BC1, 50 percent of which exhibit neither spots nor melanoma, while 25 percent develop benign melanoma and 25 percent develop malignant melanoma. In a preliminary test both normal tissues of nontumorous and tumors fish as well as the tumors showed RNA-dependent DNA polymerase activity. Extensive investigations on muscle tissues could confirm the presence of this enzyme activity: in the microsomal pellet fraction reverse transcriptase activity for poly(2'-O-methylcytidylate)-oligodeoxyguanylate,poly(rCm)p(dG)12-18, was found. This template primer is specific for reverse transcriptase as described by Gerard et al. [Nature 256: 140–143, 1975]. The polymerase activity was partially purified by DEAE cellulose column chromatography. The 0.05 M KCl wash fraction revealed poly(rCm)p(dG)12-18 dependent polymerase activity, higher than that for poly(dC)p(dG)12-18, poly(rA)p(dT)12-18, and poly(dA)p(dT)12-18. A 0.3 M KCl wash showed a poly(rCm)p(dG)12-18 dependent polymerase activity much lower than that of the other template primers used. Endogenous virus polymerase activity was not detected. The results indicate that RNA-dependent DNA polymerase activity is a normal component of the cell in muscle tissue and, probably, in melanoma.

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In Vivo Electron Spin Resonance Spectroscopy of Pigmented Tumors in Mice. S.J. Lukiewicz, S.G. Lukiewicz. National Biomedical ESR Center, Medical College of Wisconsin, Milwaukee, Wisconsin; National Foundation for Cancer Research, Bethesda, Maryland; Jagiellonian University, Krakow, Poland

A novel microwave lumped-circuit structure, called "loop-gap resonator" was introduced in 1982 to electron spin resonance (ESR) spectroscopy by W. Francisz and J. Hyde. Two modified versions of this resonator, especially adapted for the ESR measurements on water-containing samples of large size at the S- and L-bands (2-4 GHz and 1-2 GHz, respectively) have been designed and constructed at the Medical College of Wisconsin by T. Oles of the Jagiellonian University, Krakow, in collaboration with the present authors.

These new advances in the ESR technique made it possible to initiate the in vivo ESR studies on intact biological objects of macrosopic dimensions. In contrast to the first attempts along this line described in 1975 by Feldman et al., the newly elaborated approach is entirely non-damaging and does not require surgical implantation of ESR probes into the body of experimental animals. Instead, the whole animal or its part can be accommodated inside the ESR resonator so that the examination of the paramagnetic properties of the living organism is performed without affecting its normal physiological functions.

A strongly pigmented line of B16 melanoma growing in C57 Bl mice was among the first biological objects investigated using the described in vivo ESR technique. The ESR signals of a number of nitroxide spin labels injected intravenously into the blood of tumor-bearing mice proved to be detectable with an excellent signal-to-noise ratio in the B16 tumors. The kinetics of changes in the amplitudes of these signals could be continuously followed over extended periods of time (up to 72 hours or more). It has been demonstrated that these changes can provide information about the physiological state of tumor cells, and in particular about their redox state, a parameter of great importance for radiotherapy of melanoma.

Phosphorylation of Organelle-Associated Proteins During Pigment Translocation.
Thomas J. Lynch, John D. Taylor, T.T. Tchen. Wayne State University, Detroit, Michigan

During ACTH- or cAMP-induced pigment (carotenoid droplet) dispersion in goldfish xanthophores, the activity of a cAMP-dependent protein kinase is stimulated. The kinase is found in the cytosolic fraction of homogenates separated on sucrose gradients and is therefore not an intrinsic part of the carotenoid droplets. When assayed in vitro, the enzyme requires Mg²⁺ and is stimulated by physiological levels of cAMP (half-maximal at 200 nM). The most prominent substrates for the kinase are a group of peptides (57 kilodaltons molecular weight) which are specifically associated with the carotenoid droplets. The 57 Kd peptides are phosphorylated at multiple sites during early stages of pigment dispersion and are rapidly dephosphorylated at the onset of aggregation. It is proposed that the phosphorylation and dephosphorylation of the 57 Kd peptides are key regulatory events in the translocation of these pigment organelles and may govern the interactions between organelle and cytoskeleton.

Pigment Translocation and Cytoskeletal Function in Normal and Neoplastic Pigment Cells from Goldfish. Thomas J. Lynch, Jiro Matsumoto, Robert E. Palazzo, Gary R. Walker, John D. Taylor, T.T. Tchen. Wayne State University, Detroit, Michigan

The normal goldfish xanthophore and its neoplastic counterpart, the GEM-81 erythrophoroma cell, have been employed in studies of three generally significant cellular processes: intracellular organelle motility, determination of cell shape, and neoplastic transformation of pigment cells. Although superficially distinct, all are accompanied by structural alterations of the cytoskeleton (microfilaments, microtubules, and intermediate filaments), which apparently result from reorganizing these structural elements rather than from gross compositional changes. The possibility that protein kinases affect the interactions of the cytoskeletal elements with each other and with other cellular components is suggested by: (i) the regulation of pigment translocation and cell morphology by cAMP; (ii) the reversible phosphorylation of
pigment organelle proteins during pigment translocation; (iii) the extensive phosphorylation of many cytoskeletal proteins including several found only in the tumor cells; and (iv) the association of protein kinases with the cytoskeletons. Many of these characteristics find parallels in normal and/or neoplastic cells of higher vertebrates, which indicates the relevance of teleost chromatophores to the study of cellular morphology and motility. The ready availability and unique properties of xanthophores and GEM-81 cells permit experiments which are difficult or impossible in other systems.

**Distribution and Organelle Specificity of a Cytoplasmic Antigen Detectable in Human Melanoma Cells with Monoclonal Antibody (MoAb-465.12).** K. Maeda, Y. Akutsu, K. Jimbow, M. Kiyota, H. Takahashi, K. Imai. Sapporo Medical College, Sapporo, Japan

A large number of monoclonal antibodies have been developed to identify melanoma-associated antigens (MAA), and their specificity and tissue distribution have been characterized. Among these, MoAb-465.12(Mo465) is a unique anti-MAA monoclonal antibody that detects, under indirect immunofluorescence test, only a cytoplasmic antigen which is highly restricted to the cells of malignant melanoma (MM) [Wilson et al: Int J Cancer 28: 295, 1981]. As yet, it is not known which cytoplasmic component(s) is reactive with Mo465 within the MM cells. To elucidate the nature of cytoplasmic MAA, this study, using ELISA (enzyme-linked immunosorbent assay) technique, examines the distribution and specificity of Mo465-reactive MAA in the whole MM tissues as well as in the cytoplasmic organelles which are isolated by cell fractionation. Light microscopically, Mo465 bound strongly to the cytoplasmic component(s) that was localized as microgranular within the MM cells. When the cultured human MM cells were fractionated, Mo465 reacted with both soluble and membrane fractions of the MM cells and with the spent culture medium. The specific activity per mg protein was, however, much higher in the membrane fraction, which was solubilized by a non-ionic detergent (Brij-35), than the other two fractions. To further identify the organelle specificity of Mo465-reactive MAA, metastatic tumor of MM was fractionated into Golgi complexes, mitochondria, rough endoplasmic reticulum (ER), smooth ER, microsome, and melanosomes. Mo465-reactive MAA was detected in the fractions of crude homogenate, microsome, and rough ER. Our findings clearly indicate that Mo-465-reactive MAA is localized in rough ER and that it is released into the cytoplasm and shed in vitro into the culture medium. Characterization of cytoplasmic MAA may be useful for monitoring MM in such a way as α-fetoprotein, which is shed in large amounts in body fluid, inasmuch as it will be possible that Mo465-reactive MAA is also shed in vivo into body fluid from the MM cells.

**Clonal Heterogeneity in Goldfish Erythrophoroma Cells as the Basis of Their Multiple Differentiation.** Jiro Matsumoto, J.D. Taylor, T.T. Tchen. Keio University, Yokohama, Japan; Wayne State University, Detroit, Michigan

The objective of this study is to clarify the mechanisms of multiple differentiation shown by erythrophoroma cells in vitro, the permanent cell lines of which were established from spontaneous tumors of brightly colored pigment cells appearing in goldfish. The phenotypes they express upon induction with DMSO and others cover the formation of melanosins, reflecting substance, drosopentin as heterotopic pigments, and dermal skeleton-like structures, all of which are of neural crest origin in the normal ontogeny. Recent development of culture techniques with use of autologous serum has made it possible to induce cytodifferentiation in these cell lines, mostly withholding the ability for proliferation. Induced cells exhibit a variety of morphological, biochemical (pigmentation and others), and physiological (contact behavior and responsiveness to a neurotransmitter, hormones, and light) characteristics which are different among those of different clones but markedly similar among those of the same clone. Based on wide variations and stability of cellular characters induced, it is presumed that the parent populations of these cell lines are composed of a number of subpopulations different with regard to their differentiation programs. The formation of such cellular heterogeneity is considered to be a first step in the manifestation of multiple differentiation seen in neoplastic pigment cells.
Erythrophoromas and Irido-Melanophoromas: Establishment of Cell Lines, Characterization of Cell Properties, and Multiple Differentiation in Vitro. Jiro Matsumoto. Keio University, Yokohama, Japan

Erythrophoromas are tumors of erythrophores that appear spontaneously in the goldfish (Carassius auratus). Irido-melanophoromas are spontaneous tumors of a mixed population of iridophores and melanophores that appear in the croaker (Nibea mitsukurii). Permanent cell lines, GEM from the former and NIM from the latter, were established from biopsy specimens with use of fetal calf serum-supplemented synthetic medium. All cell lines established manifested a criss-cross arrangement and cell mounding before reaching confluency in monolayer culture, and formed colonies in semi-solid soft agar. These properties were taken as an in vitro indication of their neoplastic origin. All of these cells retained the ability to produce pigments characteristic of their original tumors even after long passage in vitro.

Experimental induction of cytodifferentiation caused, in addition to their own marker pigments, the formation of heterotopic pigments, dermal skeleton-like structures, and lentoid body, suggesting the presence of the potentials for multiple differentiation as the neural crest stem cell tumor cells. Clonal heterogeneity present in the parent cell populations of these cell lines was considered to be a basis for this phenomenon. Discussion will include: (1) the usage of conditioned medium for establishing permanent cell lines, (2) the stage of differentiation at which neoplastic transformation occurs, and (3) the stability of differentiated-state neoplastic pigment cells.

Protein Phosphorylation in Normal and Neoplastic Pigment Cells. Jiro Matsumoto, Robert E. Palazzo, Thomas J. Lynch, John D. Taylor, T.T. Tchen. Wayne State University, Detroit, Michigan

The patterns of protein synthesis in normal xanthophores from goldfish and their neoplastic counterparts, the GEM-81 erythrophora cell line, are broadly similar. In particular, several peptides (57 Kd) which are associated with the carotenoid droplets in the xanthophores are retained by the GEM-81 cells despite the absence of these organelles in the tumor cells. When protein phosphorylation is examined, a complex set of differences is apparent, including a nearly complete absence in the tumor cells of the normal pattern of cAMP-dependent protein phosphorylation. In some cases, most prominently the 57 Kd peptides, the unphosphorylated forms of the peptides are present, suggesting that the defect in the tumor cells resides in one or more cAMP-dependent protein kinases. There are also a number of tumor-specific phosphoproteins present in the GEM-81 cells, some of which are dependent on cAMP for phosphorylation. It is of interest that the most prominent of the cell-specific phosphoproteins are not retained with the cytoskeletons when labeled cells are extracted with Triton X-100 despite striking morphological differences between the normal and tumor cells' cytoskeletons.

The Histogenesis of Melanoma with an Adjacent Component of Superficial Spreading Type. V.J. McGovern. The University of Sydney, New South Wales, Australia

An analysis of 665 melanomas with an adjacent superficial spreading component has shown that in 61 percent the superficial spreading component was melanoma in situ (MIS) while in 39 percent it consisted either wholly or partly of premalignant melanocytic hyperplasia (PMH) which is a melanoma precursor.

In thin lesions the adjacent component was predominantly MIS. In lesions thicker than 1.5 mm, PMH was the predominant adjacent component. Thick lesions had much more mitotic activity than thin lesions.

It is proposed that the majority of thin lesions commenced as melanoma in situ while the majority of thick lesions commenced in a pre-existent lesion. The epidemiologic implications will be discussed.
Protein Phosphorylation and the Mechanism of Action of MSH. John McLane, John Pawelek. Yale University School of Medicine, New Haven, Connecticut

Melanotropin (MSH) enhances pigmentation and alters proliferation of Cloudman melanoma cells in culture. cAMP-dependent protein kinases may regulate the response of the cells to MSH, indicating that protein phosphorylation/dephosphorylation reactions are involved. Accordingly, we have examined patterns of protein phosphorylation in Cloudman cells treated with MSH. Cells were cultured in the presence or absence of MSH/MIX (methylisobutylxanthine) for four days prior to lysis with Triton X-100. The cell lysates were incubated briefly with $^{32}$P-ATP in the presence or absence of cAMP. $^{32}$P-labeled proteins were then analyzed by gel electrophoresis and autoradiography. Cells which were grown in the presence of MSH/MIX showed an increased phosphorylation of a protein having a molecular weight of 62 Kdaltons, but a decreased level of phosphorylation of proteins with molecular weights of 47, 54, and 71 Kdaltons. These changes in phosphoproteins following exposure of cells to MSH/MIX were also observed when cells were exposed to dibutylryl cAMP. However, when cAMP was added directly to the reaction mix, an additional protein of 50 Kdaltons was phosphorylated. This protein was unaffected by exposure of the cells to MSH/MIX. That is, exposure of intact cells to MSH/MIX activated protein phosphorylation systems that were not affected by direct addition of cAMP to cell lysates. Conversely, addition of cAMP to cell lysates activated a protein kinase that was not affected by exposure to cells to MSH/MIX. This implies that the cell has several mechanisms to regulate phosphokinases and/or their substrates, and that not all cAMP-sensitive protein kinases in Cloudman cells are activated by MSH. This experimental approach should be of value for understanding how MSH regulates multiple cellular functions in melanoma cells.

Dopa Metabolites of in Vivo and in Vitro Metastatic Hamster Melanoma. C.W. Mehard. University of California-San Francisco, San Francisco, California

Metabolites of dopa (3,4-dihydroxyphenylalanine) occur in the urine of melanoma patients and tumor-bearing animals and are diagnostic indicators of the metastatic disease. These metabolites originate in the melanocytes of the tumor and their amounts may change with tumor growth or spread. Biochemically determining the tissue site of metastatic melanoma through the analysis of the tumor-related metabolic excretion products found in the urine is an interesting possibility. This study addresses this possibility by comparing melanogens from in vivo and in vitro tissue-specific metastatic melanomas. Detection of these metabolites, collectively termed melanogens, was done by cation exchange column chromatography with post column colorimetric detection. Melanogens from the urine of animals inoculated with tissue-specific melanoma clones were compared with the melanogens found in the growth media of the in vitro tumor clones. Melanogens are labile, however, and in serum containing culture media are readily oxidized due to tyrosinase activity. Therefore, the cell culture media (DME-H21) contained a serum substitute or Nu-serum with less than 2 percent serum and was supplemented with thyroxine, insulin, glutamine, and non-essential amino acids. The amounts of melanogens released by the in vitro tumor clones were low, requiring an enhancement of concentration by organic solvent extraction prior to chromatographic analysis. The culture media from liver, lung, and testes tissue specific melanoma clones produced fewer melanogens than those observed in the urine of tumor-bearing animals. The results suggest that there may be biochemical modifications occurring within the host tissue, blood system, or within the bladder which alters the melanogens released by the in vivo tumor since there are more melanogens observed in the tumor-bearing animals than found in the cell culture media or from tumor cell extracts.

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Photobiological Effects of Eumelanins and Pheomelanins. I.A. Menon, S. Persad, N.S. Ranadive, H.F. Haberman. University of Toronto, Toronto, Canada

Eumelanins and pheomelanins have different chemical structures and physical and chemical properties. Some of the photobiological properties of these melanins are described here.
Eumelanin (BHM) and pheomelanin (RHM) were isolated from human black and red hair, respectively. The effects of irradiation of Ehrlich ascites carcinoma cells in vitro with a mercury vapor lamp emitting discontinuous radiation between 320 and 700 nm in the presence of BHM and RHM were compared. Irradiation in the presence of BHM or RHM produced more cell lysis than in the absence of either melanin; however, irradiation in the presence of RHM produced more lysis than in the presence of BHM. Irradiation in the presence of RHM also decreased the growth of the cells when injected into mice, whereas the irradiation in the presence of BHM or in the absence of either melanin did not affect the growth. Irradiation of RHM is known to produce more superoxide than that of BHM. The cell lysis by irradiation in the presence of RHM was decreased by nitroblue tetrazolium chloride. Therefore the cell lysis appears to be mediated by superoxide. The cell lysis was enhanced by the presence of 1 mM cysteine in irradiation medium, and this lysis was prevented by catalase. The cell lysis was decreased when the cysteine concentration was increased to 5-20 mM. When mixtures of cysteine and RHM were irradiated, cysteine was oxidized and the superoxide formation was enhanced. These results show that both BHM and RHM act as photosensitizers and the photosensitizing activity of RHM was significantly more than that of BHM. The photosensitization is mediated by superoxide and hydrogen peroxide formed during the irradiation. These results appear to be related to the higher sensitivity of light-colored skin to radiation-induced damage and carcinogenesis.

Raman Spectroscopic Study of Crustacean Chromatophore Pigments. J.C. Merlin, M.L. Delé, J. Barbillat, P.Y. Noel, C.N.R.S., laboratoire de Spectrochimie Infra rouge et Raman, Université des Sciences et Techniques de Lille I, Villeneuve d'Ascq, France; Laboratoire de Zoologie, Université de Paris VI, Paris, France

Localization and identification of crustacean pigments have been previously reported only at the organism or tissue level. The method based on use of resonance Raman spectroscopy, together with the Raman microanalysis technique, allows vibrational spectra from samples as small as a few μm², and concentrations of 10⁻⁸M (carotenoids) to be obtained. Hence, detection of pigments at the cellular level can be made.

Erythrophores and xanthophores contain high levels of carotenoids which exhibit very intense resonance Raman spectra similar to that of astaxanthin. Some modifications in the spectra appear when the carotenoids are complexed with proteins.

White pigment from Caridea was found to be difficult to study because of its fluorescence and its frequent association with carotenoids. In general, fluorescence was not observed in the case of carotenoid only, but was characteristic of the spectra of white pigment constituents. Its spectrum was compared with those of authentic isoxanthopterin and riboflavin.

The spectra of the black pigment from various melanophores, which were compared with those of pure melanin from cephalopods, exhibit wide bands.

Crustacea studied belong to Brachyura, Anomura, Caridea, Isopada, and Mysidacea.

Biology and Chemosensitivity of Clonogenic Human Melanoma Cells Grown in Semisolid Agar. F.L. Meyskens, Jr. Cancer Center, University of Arizona, Tucson, Arizona

Growth of cells in semisolid medium into colonies has been a useful operational measure of stem cells. This subpopulation of cells represents the key cellular subunit responsible for the growth and regrowth of tumors after subcurative therapy. We have extensively studied clonogenic tumor cells from biopsies of metastatic melanoma over the past five years. We have demonstrated that the cells in colonies are malignant melanocytes using a combination of special stains, transmission electron microscopy, and karyology. Additionally, we have shown that the clonogenic cells are stem cells, as defined by replating of cells from colonies in agar and growth of colonies into tumors after injection into nude mice. The response of clonogenic melanoma cells to radiation follows a log-linear decrease in survival with D₀'s ranging from 2.0 to 3.80 Gy; in some cases a shoulder is evident. This characterization of features of clonogenic human melanoma cells indicates that the biological foundation is sound, suggesting that responses of clonogenic melanoma cells in vitro to biological or therapeutic modulators accurately reflect in vivo reactions. We have compared the effect of therapeutic agents on
clonogenic cells \textit{in vitro} to the \textit{in vivo} clinical response. In a retrospective study clinical response was accurately predicted in 12 of 19 trials (63 percent) and clinical failure in 25 of 29 cases (86 percent). In a prospective trial with 34 patients (48 trials) clinical sensitivity was predicted with 42 percent resistance with 86 percent accuracy. The predictions for clinical progressions are quite encouraging and suggest that the assay can be used to screen out ineffective drugs. The lower frequency for sensitivity may be related to pharmacological distributions of the drugs not measured \textit{in vitro} and tumor heterogeneity, although the contribution of these factors is not presently known.

Malignant Melanoma: What Influences Patient Survival and the Development of Metastases? F. Meyskens, Jr., D. Berdeaux, T. Moon, D.H. Franks. University of Arizona, Cancer Center, Tucson, Arizona

Univariate and multivariate analysis of prognostic factors in patients with malignant melanoma were performed for (1) overall survival, (2) development of distant metastases, and (3) survival after a metastasis had occurred. From 1973 to 1982, 374 patients with melanoma with a median follow-up of 56 months were evaluated. Overall survival was best predicted by multivariate analysis containing the variables (1) stage of disease ($p < .0001$), (2) sex ($p = .002$), and (3) level of invasion for stage I patients ($p = .01$). Development of a distant metastasis was best predicted by (1) presence of a satellite lesion ($p = .005$), (2) deeper Clark's level of invasion ($p = .001$), and (3) no family history of melanoma ($p = .046$). Survival once a distant metastasis occurred was best predicted by (1) number of high-risk (brain, bone, or viscera not including the lung) metastases ($p < .0001$) and (2) an age/sex interaction ($p = .02$).

| No. Metastases | No. Patients | Median Survival |
|----------------|--------------|-----------------|
| 0              | 107          | 15 months       |
| 1              | 53           | 7 months        |
| $\geq 2$       | 21           | 3 months        |

| Predicted Median Survival After Metastases (Months) |
|-----------------------------------------------|
| Male                                         |
| $\leq 50$                                    |
| 0 High-Risk Metastases                       |
| 0                                            |
| 1                                            |
| $\geq 2$                                     |

| Female                                      |
|---------------------------------------------|
| $\leq 50$                                    |
| 0                                            |
| 1                                            |
| $\geq 2$                                     |

These results indicate that stratification factors should be included in clinical trials comparing different treatments for metastatic melanoma.

A Prospective Trial of Single-Agent Chemotherapy for Metastatic Malignant Melanoma Directed by \textit{in Vitro} Colony Survival in a Clonogenic Assay. F. Meyskens, Jr., L. Loescher, T. Moon, S. Salmon. University of Arizona, Cancer Center, Tucson, Arizona

Different types of assays have been used to predict sensitivity of cancers to chemotherapeutic agents. We have used the effect of therapeutic agents on clonogenic growth in agar to discriminate between active and inactive agents for malignant melanoma. We report a prospective study of single-agent chemotherapy for metastatic malignant melanoma. Forty-five separate \textit{in vitro}/\textit{in vivo} correlative trials were conducted in 34 patients. A number of agents were used in these evaluations including actinomycin D, m-AMSA, bisantrene, mitoxantrone, BCNU, velban, vindesine, 5-fluouracil, MGBG, VP-16, interferons, tamoxifen, and 13-cis-retinoic acid. At the "cut-off" concentration a colony survival less than 30 percent was desig-
nated as “sensitivity” and greater than 30 percent as “resistance.” Clinical sensitivity was designated as complete, partial, and mixed responses. The correlations for these 45 trials were:

| In Vito (CR + PR + MR) | S  | R  | p   |
|------------------------|----|----|-----|
|                        | 7 (47%) | 8  | 15 | 0.02 |
|                        | 4  | 26 (87%) | 30 |
|                        | 11 (24%) | 34 | 45 |

These results suggest that this clonogenic assay can be used to predict clinical resistance with a high degree of accuracy (87 percent). Prediction for clinical response was 47 percent which was significantly better compared to our response rate (CR + PR + MR) in 56 patients (No. trials = 86) with metastatic melanoma treated with single-agent chemotherapy (but not in vivo directed) between 1977 and 1982 in which the response rate was 20 percent (p = .02). These results indicate that this clonogenic assay may also help select useful agents for clinical trial.

Self-Renewal Capacity of Clonogenic Cells from Biopsies of Metastatic Human Malignant Melanoma. F. Meyskens, Jr., S. Thomson. University of Arizona, Cancer Center, Tucson, Arizona

A procedure was developed to measure directly the self-renewal capacity of clonogenic cells from biopsies of metastatic human malignant melanoma. A culture of colony-forming cells was performed with bilayer agar in microtiter wells. The number of live tumor cells from biopsies of melanoma tissue was determined and was used to calculate plating efficiencies. Sequential photography showed that cells did not migrate in agar, thereby documenting that all the cells within colonies were direct descendants of clonogenic cells. A calibrated, pneumatically controlled micropipet attachment to a micromanipulator was used to quantitatively remove melanoma colonies without removing adjacent cells or agar. Plucked primary colonies were mechanically disaggregated into single cells; viability was greater than 95 percent as determined by trypan blue dye exclusion. Dose-related formation of secondary colonies was observed after replating of cells from pooled primary colonies. Cells from individual colonies were replated and secondary colonies formed. A key question is whether the size of a colony relates to its self-renewal capacity. Replating of clusters and colonies by size indicated that even the smallest group of cells was capable of self-renewal. However, the data did suggest that clonogenic cells which divided fewer than three times had less proliferative capacity after replating. These techniques allowed a simple and direct assessment of the self-renewal capacity of colony-forming melanoma cells.

L-Dopa and Dopamine Oxidase Activities in Human Substantia Nigra. M. Miranda, D. Botti, A. Bonifiglie. Institute of General Biology, University of L'Aquila, L'Aquila, Italy

The degeneration or dysfunction of the dopaminergic neurons of mesencephalon or dopamine depletion in this area results in extrapyramidal disorders, such as Parkinson's disease, chronic manganese poisoning, and tardive dyskinesia induced by neuroleptic drugs. A common feature of Parkinson's disease or postencephalitic parkinsonism is a depigmentation of substantia nigra. Evidence has been presented that melanin activates tyrosine hydroxylase in vitro [Nagatsu et al: Biochim Biophys Acta 523:47-52, 1978] and that L-epinephrine activates tyrosinase [Miranda, Botti: Gen Pharmac, in press]; this suggests a possible interaction between melanin and catecholamine syntheses. The mechanism of synthesis of substantia nigra melanin is unknown, though this is of the dopamine type.

In this work evidence is presented that human substantia nigra contains a protein fraction which oxidizes dopamine and L-dopa to melanin by a phenylthiourea-sensitive reaction.

Substantia nigra (pars compacta mostly) was, after autopsy, minced in 0.01 M Na-PO₄ buffer, pH 7.0 (1:10 w/v) at 0°C and homogenized. The homogenate was centrifuged at low gravi-
Dynamics of Functioning Macromolecules in Depigmenting Melanoma Cells Induced by Glycosylation Inhibitors in Vitro. Y. Mishima, G. Imokawa. Kobe University School of Medicine, Kobe, Japan

Carbohydrate moieties of glycoproteins in various cellular membranes play a role in the cellular or intracellular recognition process. We found that glycosylation inhibitors, glucosamine (Glc) or tunicamycin (TM), are specific inhibitory modulators for melanogenesis. When added to cultured B-16 melanoma cells, these inhibitors induced a loss of pigmentation, accompanied by distinct biochemical and ultrastructural aberrations in their melanogenic compartments. Electron microscopy reveals that these newly depigmented melanoma cells form uniquely altered melanosomes, although their number is not greatly reduced. Analysis of these melanosomes showed selective aberrations, including deformity, bulging, segregation, and irregularly concentric lamellar structures within the melanosomes. Tyrosinase decreases in the melanosome-rich fraction, but not in the total activity of the remaining fractions. Electrophoresis of tyrosinase in the 30,000 g supernatant demonstrates an increase in the T3 soluble tyrosinase, while a disappearance of membrane-bound tyrosinase, Tm, is seen in the small- and large-granule fractions. Initial melanogenesis can be induced as a reversed recovery process by removing glycosylation inhibitors in a synchronized fashion.

Our evidence can be summarized as follows. Tyrosinase is synthesized by ribosomes of the rough ER where glycosylation is initiated shortly after synthesis of tyrosinase. Further glycosylation and processing are carried out within GERL. Glycosylated T3 is transported from GERL into premelanosomes by budded-off coated vesicles. For this process, the carbohydrate moieties of T3 play an integral role as a signal for the intracellular recognition process. Premelanosomes, as another TM-sensitive glycoprotein, may possess a selectively accepting function for T3, carrying coated vesicles. Coated vesicles, fusing into premelanosomes, transmit their matured T3, tyrosinase and thus exhibit three sequential stages of melanogenetic status as shown by their strong, weak, and finally non-positive dopa reaction, which is conversely parallel to the reaction of the premelanosome interiors.

Production, Excretion, and Regulatory Factors of 5-S-Cysteinyldopa Genesis in Melanoma Cells: Implications for Mixed Eu- and Pheo-Melanogenesis. Y. Mishima, M. Ichihashi, M. Mojamdar. Kobe University School of Medicine, Kobe, Japan

5-S-cysteinyldopa (5-S-CD) formed by the addition of cysteine to tyrosinase-catalyzed dopaquinone is a key intermediate of pheomelanogenesis. Since its synthesis, excretion, and further oxidation to pheomelanin would be in a dynamic equilibrium within pigment cells, we have been investigating the factors that regulate these functions in cultured melanoma (Mm) cells, animals bearing Mm, and human subjects with Mm and other pigmentary disorders. Mm cells have been found to secrete large quantities of 5-S-CD when cultured in the presence of both dopa and glutathione at 10^{-5}M concentration. Similarly Greene's melanotic Mm bearing hamsters excrete significantly higher concentrations of 5-S-CD in their urine after 5g dopa (ip)/hamster as compared to controls. Further, a definite relationship between tumor size and the amount of 5-S-CD excreted in the 24-hour urine in the hamster has been found. Based on this and 5-S-CD content in Mm, we have also developed an equation for estimating the size of this Mm. In human 50 mg dopa loading (iv)/subject enhances significantly the urinary excretion of 5-S-CD in Mm bearing subjects as compared to non-Mm subjects. The percentage increase in urinary 5-S-CD levels after dopa loading is a better criterion for estimation of Mm metastasis and progression for evaluation of operation and/or chemotherapy. Some of the generalized vitiligo patients excrete high levels of 5-S-CD in their urine despite almost total depigmentation. The premelanosome fractions of hamster and human Mms have been found to be rich not only in tyrosinase but also in γ-glutamyl transpeptidase and 5-S-CD, and to in-
crease 5-S-CD levels after *in vitro* incubation with dopa and glutathione. Thus pheomelanogenesis could also be occurring in the premelanosomes. Ultrastructural differentiation of pheomelanosome from eumelanosome as seen in genetically pheomelanic strains appears to be not universally distinctive in mixed melanogenic melanoma cells and possibly also melanocytes. The possibility that mixed melanogenesis takes place commonly within the same premelanosomes cannot be excluded.

**Role of Functioning Sub-Units within Melanogenic Compartments During Initial Melanogenesis After Release from Glycosylation Inhibition.** Y. Mishima, G. Imokawa. Kobe University School of Medicine, Kobe, Japan

Glycoprotein synthesis in the melanogenic subcellular compartments of pigment cells plays an integral role in eumelanogenesis. Contrary to previous concepts of initial melanogenesis in a single functional segment of Golgi, we found recently that tyrosinase (Ty) synthesized by ribosomes is condensed and activated in the GERL-coated vesicles (CVs), acquiring the capacity to form dopa-melanin *in vitro* through its glycosylation. After maturation of Ty from T₁ to T₃, and to Ty, with sequential changes in their carbohydrate moieties, CVs acquire a transporting function to the premelanosomes (PMs), which possess, as glycoprotein, a specific Ty-accepting function. Glycosylation inhibitors such as glycosamine and tunicamycin can induce selective ultrastructural aberration of PMs and loss of T₃, Ty, resulting in pigment loss of cultured melanoma cells. Extending this release from inhibition enables us to analyze initial melanogenesis with synchronized timing. Evidence to support these proposals follows. During inhibition, dopa positivity becomes high in GERL and CVs but low in PMs due to interrupted Ty transfer. T₃, Ty is reformulated in GERL and condensed in CVs. This is rapid compared to the recovery of Ty transfer and of accepting function of premelanosomal (PMal) glycoprotein. Thus, in the first 24 hours GERL and CVs are not only strongly dopa-positive but also melanized in living melanoma cells. This phenomenon thereafter disappears. Intra-PMal CVs exhibit three phases of melanogenic status and their dopa reaction is moderately to scarcely positive in an approximate reverse relationship with their surrounding PMal interiors. This relationship between intra-PMal CVs and interiors is approximately parallel with the degree of existing melanization in contrast to extra-PMal free CVs and GERL which never melanized yet are strongly dopa-positive. This finding suggests that intra-PMal CVs contain mostly T₃, Ty which is transported to PMal interiors; then melanin polymers can be formed there. 48-72 hours after removal of inhibitors the interrelation of Ty glycosylation, transfer, and PMal acceptance is re-normalized; thereafter melanization can occur only within PMs in living melanoma cells.

**The Effect of Bivalent Metallic Cations on Mammalian Tyrosinase.** K. Miyazaki, N. Ohtaki. Tokyo Medical and Dental University, Tokyo, Japan

Mammalian tyrosinase, which catalyzes the first two steps in the biosynthesis of melanin, is generally associated with the intracellular particles of melanocyte, while the other tyrosinases, including those found in mushrooms, *Neurospora*, potatoes, insect pupae, and the like, are in a soluble state. In addition, the molecular weight of mammalian tyrosinase is much greater than that of other origin. Mammalian tyrosinase synthesized *in vivo* is well known to be inactive until it is transformed to melanosomes, which are gradually melanized and become uniformly dense particles, finally losing tyrosinase activity. Bivalent metallic cations play an important role in cell membrane functions and structures. It would be of great importance to examine the effect of those cations on purified mammalian tyrosinase. In the present paper, we used mammalian tyrosinase solubilized and purified from Harding-Passey mouse melanoma described before [J Invest Dermatol 57:81, 1971]. The activity of tyrosinase was measured colorimetrically, using PIPES buffer instead of conventional phosphate buffer. Those cations inhibit the tyrosinase in different ways. For example, calcium chloride inhibits the tyrosinase 40 percent in 5 mM and 33 percent in 1 mM concentrations, respectively. In contrast to calcium ions, magnesium chloride inhibits the activity about 10 percent in both concentrations.
14C-Tyrosine Uptake and Ultrastructure of Tyrosinase-Positive Oculocutaneous Albino Hairbulbs. M. Mizoguchi, Y. Kawa, H. Sato, Y. Hori. Teikyo University and Tokyo University, Tokyo, Japan

Pathogenesis of tyrosinase-positive oculocutaneous albinism (TPA) is unknown. Our previous work has shown that intensity of pigmentation and structure of melanosomes vary among the patients with this disease.

To determine possible correlation between tyrosinase activity and intensity of pigmentation, 14C-tyrosine hairbulb uptake, which is dependent on tyrosinase activity, was investigated by the method of King. Fresh anagen hairbulbs from four TPA patients were incubated for 24 hours with 14C-tyrosine, dopa, cyclohexamidine, catalase, and antibiotics. Normal Japanese brown hairbulbs and white hairbulbs were used as the control. The structure of the hairbulb melanocytes was also investigated under an electron microscope.

14C-tyrosine uptake levels of hairbulbs from TPA patients were lower than those of normal brown hairbulbs, but greater than those of white hairbulbs, though different among patients. Ultrastructural studies showed that intensity of melanization in melanosomes varied among patients. However, the intensity of melanization in melanosomes was not correlated to the hairbulb uptake of 14C-tyrosine.

From these findings, it can be considered that tyrosine uptake might be partly related to the intensity of pigmentation, but some other factors may play a role in it.

Internalization of 125I-βMSH by Cloudman S-91 Melanoma Cells: An Electron Microscopic Autoradiographic Study. G. Moellmann, E. Kuklinska, D. Lambert, J.M. Varga, A.B. Lerner. Yale University School of Medicine, New Haven, Connecticut

We have shown previously that an electron-dense, biologically active hormonal conjugate, MSH-Ferritin-FITC (MFF), is internalized by MSH-responsive murine melanoma cells. The ferritin label was traced from the cell surface and Golgi-associated vesicles to premelanosomes, suggestive of a pathway that links the intracellular destination of MSH with the hormonal stimulation of tyrosinase. Native ferritin or ferritin-FITC alone were not internalized and had no biological activity.

MFF contains several hormone molecules in close proximity and might function as a multivalent ligand causing cross-linking of receptors and artifactual internalization. We, therefore, incubated Cloudman melanoma cells with unlabeled, radiolabeled βMSH, which is also biologically active [Lambert et al: J Biol Chem 257:1982]. Our autoradiographic studies show that 125I-βMSH is internalized on a time schedule similar to that of the ferritin-labeled probe. The time course of uptake appears to reflect the predilection of receptor availability for the G1 phase of the cell cycle in Cloudman melanoma cells. Addition of excess unlabeled MSH virtually abolished the internalization of the radiolabeled MSH.

In addition to cytoplasmic localizations of radioactivity, we found autoradiographic grains in nuclei. We, therefore, re-examined cells labeled with the higher-resolution ferritin probe (MFF) in the absence of electron-dense counterstains and discovered small clusters of ferritin within euchromatic regions.

We conclude from these studies that Cloudman melanoma cells that respond to MSH internalize the hormone into both cytoplasmic and nuclear compartments. The two techniques used to reach this conclusion complement each other.

Intracellular Differentiation of Melanogenic Compartments for Eumelanin and Pheomelanogenesis in Melanoma Cells. Manoj Mojamdar, Masayuki Tsuji, Masamitsu Ichihashi, Yutaka Mishima. Kobe University School of Medicine, Kobe, Japan

Bio-melanins are now considered to be a mixed type of melanins where the color depends upon the degree of eumelanin and pheomelanin synthesized within the pigment cells in genetically determined proportions. However, intracellular differentiation of melanogenic compartments for the synthesis and processing of mixed melanogenesis remains to be clarified.
We have therefore investigated the intracellular and interrelated dynamics of γ-glutamyl transpeptidase (γ-GTP), tyrosinase, and cysteinyl dopas (Cys-dopas) during mixed melanogenesis in Greene's melanotic melanoma. In contrast to tyrosinase which becomes the active form in the GERL through glycosylation [Imokawa, Mishima; Cancer Res 42:1994, 1982], Golgi has been found to be rich in γ-GTP. Among the melanogenic compartments in melanoma cells, the pre-melanosome fraction has been found to contain substantial quantities of both γ-GTP and tyrosinase. On the other hand, the melanosome fraction has been found still to contain some tyrosinase but no detectable γ-GTP. Also the premelanosome fraction has been found to contain higher levels of Cys-dopas than the melanosome fraction. Further, the isolated premelanosome has been found to synthesize large amounts of Cys-dopas after incubation with dopa and glutathione while the melanosomes are unable to form Cys-dopas under similar conditions. Thus γ-GTP present in the premelanosomes appears to convert glutathione to cysteine, which then reacts with tyrosinase-catalyzed dopaquinone to form Cys-dopas. These findings indicate that while tyrosinase is condensed and activated in the GERL and transferred to the premelanosomes, γ-GTP present in the Golgi is also transferred to the premelanosomes. Thus the degree of eu- and pheomelanogenesis may depend upon the genetically predetermined levels of these two enzymes transferred into premelanosomes from differentiated sub-cellular membrane compartments.

Cytological Modifications of Pattern-Differentiation of the Retina Under the Action of White Light in the Opilionids (Arachnida). A. Muñoz-Cuevas. Laboratoire de Zoologie (Arthropodes). Muséum National d'Histoire Naturelle, Paris, France

The differentiation of pigment granules (ommochrome, ommins) in the retina of the Opilionids shows four different stages, Muñoz-Cuevas [1980a,b]:

First stage: formation of saccules from the smooth reticulum
Second stage: matrix elaboration inside the saccules
Third stage: elaboration and secretion of pigment from the Golgi apparatus
Fourth stage: filling of the saccules and formation of two types of granules, I and II

Under the action of white light (1.500 Lux) this pattern of pigment differentiation is modified. The action of light occurs after the first stage, including important modifications:

a. The Golgi apparatus is activated by the light and its formation and maturation faces are strongly distended.
b. The morphology of the intermediate stages is modified, aberrant shapes occurring.
c. The sequence of the two types of granules I and II is accelerated.
d. The saccules issued from the smooth reticulum of the second type granules are unusually expanded.
e. The pigment migrates toward the apex of the retina.

Effect of Rhodizonic Acid on the Lag Period of Tyrosinase. Sandra Naish, P.A. Riley. University College School of Medicine, London, England

It is known that the oxidation of monophenolic substrates by tyrosinase is characterized by unusual kinetics in the form of an induction or lag period during which the oxidation rate accelerates. If oxygen is not limited, the maximum velocity is reached toward the end of the reaction and, above a level of about 1 mM, the induction period increases with the substrate concentration.

The kinetics of the induction period have been investigated employing mushroom tyrosinase. The product formation was measured spectrophotometrically in experiments with 4-hydroxyanisole as the monophenolic substrate. These indicated that the induction period is modified by the addition of rhodizonic acid to the incubation mixture. Further investigations of this effect suggest that the catalytic action of tyrosinase is dependent on the availability of a suitable electron acceptor species such as a quinone with the appropriate stereochemistry.

Giant Melanin Globules (So-Called “Macromelanosome”). H. Nakagawa, Y. Hori, S. Sato, T.B. Fitzpatrick. Harvard Medical School, Massachusetts General Hospital, Boston, Massachusetts. Yamanashi Medical School, Yamanashi, Japan

The term “macromelanosome” was originally proposed to describe what was believed to be a special large type of melanosome. We now believe this term is unsatisfactory as these giant
organelles are not, in fact, large melanosomes but may represent end stages of an autophagic process that occurs in some melanocytes. We propose the term giant melanin globule (GMG) for the present. To elucidate the nature and origin of GMG, electron microscopic studies were carried out in several dermatologic disorders, including café-au-lait macules from neurofibromatosis (14 patients), x-linked ocular albinism (two patients), nevus spilus (two patients), melanocytic nevi (two patients), and dysplastic nevi (one patient). The findings were almost identical among these disorders. Our main results indicated (1) the most remarkable characteristics of GMG are (a) the pleomorphism of their internal structure and (b) the polymorphism of their size. (2) GMG are possibly formed through an autophagic process and represent unique forms of cytolsosomes resulting from the fusion of compound melanosomes with primary and/or secondary lysosomes. (3) GMG are transferred from melanocytes to keratinocytes and to dermal macrophages and rarely to Langerhans cells, in any of their developmental stages. After transfer, GMG can fuse with other heterolysosomes and probably increase in size in these cells. Our proposed mechanisms involved in the formation of the GMG are as follows: at an early stage the melanosomes are aggregated within the cytoplasm of the melanocyte and an autophagosome is formed. This autophagosome fuses with primary and/or secondary lysosomes, acquiring hydrolytic enzymes and forming an autolysosome. Within the autolysosome, phagocytized melanosomes are subjected to the action of a large number of lytic enzymes, resulting in the complete breakdown of the protein-lipid portion of melanosomes. Ultimately, indigestible or slowly digestible substances (i.e., melanin moieties) microvesicles remain to form the GMG.

Lipoxigenase Activity of Pityrosporum. M. Nazzaro-Porro, S. Passi, M. Picardo, R. Mercantini. St. Gallicano Dermatological Institute. Rome, Italy
A. Breathnach. St. Mary's Hospital Medical School, London, England

It has been shown previously that, in culture, Pityrosporum is capable of oxidizing unsaturated fatty acids with formation of dicarboxylic acids. C9-C12 dicarboxylic acids are competitive inhibitors of tyrosinase and of other oxidoreductases; in vivo, they are toxic for hyperactive or malignant melanocytes but they have no effect on normal cells.

Re-investigations, carried out by spectrophotometry, thin layer chromatography, high pressure liquid chromatography, and mass spectrometry, showed that Pityrosporum, cultured with a sebum-like mixture, has a high lipoxigenase activity and that lipoperoxidation involves not only unsaturated fatty acids but also squalene and cholesterol. During the process, lipid free radicals, lipoperoxy radicals, lipo-hydroperoxides, cyclic endoperoxides, aldehydes, and so on are formed, all products which are known to be highly reactive and then cytotoxic.

These metastable intermediate products of lipoperoxidation might account for fluorescence, probably due to interaction of aldehydes with amino groups of proteins, and for damage to melanocytes [Breathnach A, et al: Giorn It Derm 110:457, 1975] with subsequent hypochromia, present in Pityriasis versicolor.

Dicarboxylic acids per se represent non-radical, stable by-products of lipoperoxidation of unsaturated fatty acids, and this might explain their lack of toxicity on normal cells.

The Role of Calcium and Magnesium on Pigment Translocation in Melanophores of Oryzias latipes. S. Negishi, M. Obika. Keio University, Yokohama, Japan

Pigment aggregation in melanophores of a teleost Oryzias latipes is mediated by α-adrenergic receptors. In varieties of cell types, stimulation of α-adrenergic receptors is reported to produce cellular responses through the elevation of intracellular free calcium concentration. In erythrophones of Holocentrus ascensionis, it has been demonstrated that the pigment translocation is mediated by the change in cytoplasmic free calcium level. Our recent observation on the subcellular localization of calcium in Oryzias melanophores at dispersed and aggregated states also suggests that the influx of calcium is actually taking place during the process of melanosome aggregation. In the present experiment, we investigated the role of calcium and magnesium on melanosome movements. The external and intracellular calcium concentrations were manipulated by the aid of ionophore A23187. Isolated melanophores were cultured for two to three days before use. Cells maintained in calcium-free saline in the presence of EGTA remained dispersed without showing any responses to epinephrine. When calcium was introduced into the medium together with ionophore at 4 μM, melanosome aggregation was
elicited in a dose-dependent manner. Melanosome aggregation was most effectively produced when the concentration of calcium in the ambient medium was in a range between 8 and 50 μM. Minimum effective dose was around 5 μM, and concentration higher than 100 μM produced irreversible pigment dispersal. Pigment aggregation produced by calcium was readily reversed by perfusing the cells with calcium-free saline in the presence of EGTA and magnesium.

Comparison of DNA Ploidy Characteristics of Human Melanoma Cells as Found in the Tumor and After Cultivation in Vitro. H.I. Nielsen, A.M. Madsen, I.J. Christensen. The Finsen Laboratory, The Finsen Institute, Copenhagen, Denmark

Cultures of human melanoma cells are being established routinely in our laboratory for several purposes. First we wish to obtain more information about the individual tumors (e.g., tyrosinase activity, reaction to antibodies, and motility of the cells), second we need the cells for studies on the effect of hormones and drugs. It is, however, important to know if the cells we study are comparable to the cells originally found in the patient's tumor. We therefore as far as possible make DNA determinations at regular intervals by means of flow cytometry.

So far eight primary tumors and three metastases were examined in this way. The cell populations of all of these tumors changed during cultivation. The tumors contained a total of 11 diploid (one each), two other euploid and 21 aneuploid populations. During cultivation (2–26 passages) two diploid, two other euploid, and 16 aneuploid populations were lost, while eight euploid (no diploid) and one aneuploid population were formed.

This shows that normal, diploid cell populations are surprisingly stable in culture, while aneuploid populations are not. New populations formed in culture are normally euploid, containing double the amount of DNA of already existing populations. The results indicate that there is a selection of cells by the cultivation, and it calls for circumspection when one tries to infer the in vivo situation from in vitro results.

Cultures of Normal Human Melanocytes. H.I. Nielsen, P. Don. The Finsen Laboratory and The Fibiger Laboratory, Copenhagen, Denmark

Melanocytes of normal origin were isolated from adult human skin and culture in vitro. Separation of the epidermis from the dermis by trypsin floating proved to be superior to collagenase treatment in providing viable cultures of melanocytes with a minimum of fibroblast contamination. Centrifugation on a discontinuous, rather than a continuous, Percoll gradient was more efficient in separating the epidermal cell types. The majority of the melanocytes were often found in one particular layer, and most of the viable keratinocytes usually in the sediment. None of the layers, however, produced a uniformly high percentage of melanocytes when cultured in vitro in regular medium with fetal calf serum or in adult human skin fibroblast-conditioned medium. When Percoll gradient-separated cells were seeded in conditioned medium where fetal calf serum had been replaced by horse serum and to which polyamines were added, purer melanocyte cultures were obtained. In subsequent experiments the isolation process was simplified by seeding non-separated epidermal cell suspensions in medium with a low Mg" and Ca" content, which mainly inhibited the settling of keratinocytes. After 24–48 hours, such cultures were grown in medium with normal amounts of ions. Any contaminating keratinocytes present were lost upon subculture. Melanocytes were identified by their dendritic morphology, ultrastructure, reaction to cholera toxin, and at times by their pigment production after treatment with melanocyte-stimulating hormone. Pure cultures of adult human melanocytes of normal origin have been cultivated for over 43 weeks (ten passages).

DNA Determination of Human Primary Melanomas and Melanoma Metastases by Flow Cytometry. H.I. Nielsen, I.J. Christensen, K.T. Drzewiecki, K. Hou-Jensen, J.K. Larsen, K. Wallevik. The Finsen Laboratory, The Finsen Institute, Copenhagen, Denmark

The DNA content of the cells of tumors of 52 melanoma patients has been examined by means of flow cytometry.

Generally metastases contain a higher number of cell populations. In 15 percent of the primary tumors, but never in metastases, we find only one population of cells (diploid). In 43
percent of the primary tumors and 23 percent of the metastases two populations are found. A content of five or more populations, however, is found in 30 percent of the metastases, but only 15 percent of the primary tumors.

The DNA index defined as the corrected ratio between the DNA content of the population in question and the DNA content of a material of normal nevi was calculated. We almost always find diploid cells in the tumors, and in primary tumors not seldom only diploid cells.

Although some of these might be contaminating non-malignant cells, it is concluded that malignant melanoma cells can be diploid, as well. Nevertheless, the presence of aneuploid cell populations appears to be characteristic of most malignant melanomas and their metastases. There is a difference in the amount of DNA (the DNA index) found in primary tumors and metastases. Relatively more primary tumors have a DNA index between 1.1 and 1.2, while relatively more metastases have a DNA index in the range 1.5–1.9, and only metastases have an index above 2.2.

A comparison of flow cytometry data and tumor thickness, diameter, level of invasion, clinical stage, pigmentation, nucleolar size, number of mitoses, and lymphocyte infiltration showed no correlation.

Wherever possible, more than one sample was taken from each tumor. In this way a heterogeneity within the larger tumors was demonstrated.

**Action of Serotonergic Neuromediators on Erythrophores from Processa edulis (Crustacea, Caridea).** P.Y. Noël. Laboratoire de zoologie, Université Paris VI, Paris, France

Serotonin is known to induce in vivo red pigment dispersion in chromatophores of different crustacea such as *Palaemon, Cambarellus,* and *Uca.* Since it gives no effect in vitro it is supposed to stimulate only the release of a red pigment-dispersing hormone from the central nervous system.

Serotonergic neuromediators were tested both in vivo and in vitro on red pigment dispersion within epidermal erythrophores from the prawn *P. edulis.* Serotonin injected to normal and eyestalkless prawns (1 μg/g) has a powerful dispersing effect. In vitro most of the serotonergic neuromediators disperse red pigment: tryptamine and N,N-dimethyl-tryptamine (100 μM) disperse completely, serotonin is the most potent and still has a maximum dispersed effect at μM concentration; melatonin is less effective but induces some red pigment dispersion (100 μM); on the other hand, tryptophane (mM) has no effect.

Preliminary experiments on other shrimps established also that serotonin (mM) in the same conditions disperses in vitro red and white pigments of *Solenocera membranacea,* white pigment of *Palaemon xiphias,* and black pigment of *Philocheras trispinosus,* but pigments of many other species do not react. So the in vitro dispersing action of serotonin is not restricted to *Processid prawns' red pigment,* but could be a more general effect in crustacea, at least in some species. Thus, this monoamine can represent a pigment-dispersing hormone and may act directly on pigment cells.

**Chromatophores' Direct Photosensitivity.** P.Y. Noël. Laboratoire de zoologie, Université Paris VI, Paris, France

Invertebrates and poikilothermic vertebrates' chromatophores are controlled by hormones and sometimes by nerves. They can also react directly to physical stimuli such as light or temperature, even in vitro (physiological color changes).

It is known that yellow and red pigments retract under strong light in a cnidarian and several crustaceans. White pigment disperses with light in various crustaceans. Dark pigments disperse with light in sea urchins, crabs, reptiles, and some fish and amphibians. Light-induced retraction is known to occur in some tadpoles' fins and fish embryo culture.

It has been shown on sea-urchin melanophores and shrimp erythrophores that a thin beam of light can induce pigment retraction only in part of a cell, even if pigment is not present there. So the protoplasm itself seems to be photosensitive and photoreactive.

Most chromatophores are mainly ultraviolet-sensitive; some can react, however, to green or blue light; photosensitive molecules, in those cases, are unknown yet. Light can induce activation of photodiesterase or other enzymes, leading to pigment granule translocation by proper internal mechanisms.
Homeothermic vertebrates' melanophores react under illumination, but without pigment migrations (morphological color changes).

Some problems still exist; for instance, it is not known why some chromatophores and not others react to light in the same species. New types of experiments (electrophysiology, photochemistry), must be done for a better understanding of chromatophores' direct photosensitivity.

**Direct Photoreactivity of Crustacean Chromatophores.** P.Y. Noël. Laboratoire de zoologie, Université Paris VI, Paris, France

Pigment migrations in crustacean chromatophores are mostly controlled by hormones. Scarcce contributions have shown that light can directly induce pigment dispersion within leucophores from the prawn *Leander serratus* and melanophores from the crabs *Sesarma reticulatum* or *Uca pugilator*, and pigment retraction in erythrophores from the crab *Macropipus vernalis* and the prawn *Processa edulis*.

27 species of crustacea were investigated here as to direct photoreactivity. Pieces of integument containing one or several chromatophores were incubated *in vitro* in diluted seawater, and kept alternatively one hour in the dark or the light (0 = 5,000 1 ×).

Then most chromatophores showed a weak reaction or no reaction at all. It was therefore observed that transparent yellow pigment from the prawn *Palaemon xiphias* and red pigment from the prawn *Processa robusta* retracted with light and that whitish pigment from the shrimps *Lysmata seticaudata* and *Hippolyte inermis* dispersed with light. The same individual could have the same type of chromatophore that did or did not react to light, according to its localization. For instance, erythrophores from *P. robusta* scaphocerite reacted to light on the internal edge, but not those on the external one.

Direct photoreactivity is not evident in most adult crustacean chromatophores; only pigment cells that possess extensive internal mechanisms for pigment migrations can show it. Ultrastructural studies might reveal that appropriate structures are in fact lacking. Most crustacean chromatophores might be photosensitive, however, but the present experiments cannot demonstrate it.

**Ultrastructural Study of Caridina Chromatophores (Crustacea, Caridea).** P.Y. Noel. P.N. Laboratoire de Zoologie, Université Paris VI, Paris, France

R. Nagabhushanam, S. Kandharkar. Marathwada University, Aurangabad, India

The freshwater shrimp *Caridina* possesses essentially white, red, blue, and yellow pigments in its tegumental chromatophores; these are found in epidermis, between cuticle-producing cells and basement membrane. Different types of chromogranules appeared to be present. Red pigment from *C. weberi* is found as small (80 × 220 nm) granules, with a matrix of high electron density and no bounding membrane, while blue pigment granules are much bigger (200-400 × 600-4,000 nm) with a medium electron density matrix and a bounding membrane. *C. rajadhari* red pigment granules are similar to those from *C. weberi* (90 × 120 nm) with a weaker electron density; other medium-sized granules (100 × 600 nm) with a medium density and that might have a bounding membrane seem to represent blue pigment. Other cell organelles such as mitochondria and microtubules are scarce. These results are compared with other data on Caridean chromatophores.

**In Vitro Action of Adrenergic Neuromediators on Crustacean Chromatophores.**

P.Y. Noël. Laboratoire de zoologie, Université Paris VI, Paris, France.

Adrenergic neuromediators are known to affect pigment migrations *in vivo* in quite a variety of crustacean chromatophores. Actually, no neuromediator is known to be effective *in vitro*. Since we established recently that serotonin induces red pigment dispersion *in vitro* in the prawn *Processa edulis*, it was decided to seek for similar reactions among adrenergic neuromediators.

Small pieces of tegument containing chromatophores from different species were incubated in 75 percent seawater containing various concentrations of neuromediators (0.1 μM to mM). *Processa edulis* red pigment is dispersed *in vitro* by (10 μM or less) dopamine, noradrenalin
(NA), adrenalin, tyramine (T), octopamine, and synephrine (S). The more potent are synephrine (μM) and octopamine (0.05 μM). Precursors such as tyrosine and dopa (mM) have no effect.

mM NA, T, and S were tested under the same conditions on chromatophores (E = erythrophores; L = leucophores; M = melanophores) from ten other species with the following results (− = no effect; +D = dispersion; +R = retraction): *Processa acutirostris* (E + D), *P. robusta* (E + D), *P. macrorynchus* (E + D), *Palaemon xiphias* (E −), *Periclimenes amethysteus* (E −; NAL −; TSL + R), *Hippolyte inermis* (E −), *Thoralus cran- chi* (E −), *Lysmata seticaudata* (E −), *Philocheras trispinosus* (NAM −; TSM + R), and *Leptomysis mediterranea* (NATE + D).

According to the great variability of responses observed, depending upon species, individuals, and chromatophore localization, wider experimentation and more detailed analysis are needed for interpretation of those results.

**Proliferation of Melanocytes in Murine Epidermis Induced in Vivo by Melanocytotoxins. A Possible Role for Metabolism of Arachidonic Acid (AA) in the Regulation of Pigment Cell (PC) Proliferation.** J.J. Nordlund. University of Cincinnati, Cincinnati, Ohio  A.E. Ackles. VA Medical Center, West Haven, Connecticut

Only a few physical or chemical agents, e.g., ultraviolet light (290-320 nm) and DMBA, have been shown to stimulate PC to proliferate. We have studied the effects of two melanocytotoxins, monobenzyl ether of hydroquinone (MBEH 20 percent − Benuquin) and 4-tert-butyphenylpyrocatechol (PTBC in ethanol) on the population densities of keratinocytes (K), Langerhans cells (LC), and PC in murine pinnal epidermis. Daily applications of the agents increased the population density of K, LC, and PC. The number of dopa-positive cells in epidermal sheets treated for 14 days increased from 240/mm² to 420/mm² after MBEH treatments and from 240/mm² to 330/mm² after PTBC. Autoradiographic studies on vertical sections of treated tissue labeled with tritiated methylthymidine showed that more K- and dopa-positive PC were labeled than in diluent-treated controls. Applications of AA (2 percent in 50 percent DMSO) caused similar increases in the population densities of PC and K. Autoradiographic studies in AA-treated epidermis showed more K and PC were labeled with tritiated methylthymidine than in the control epidermis. The changes induced by MBEH, PTBC, and AA were blocked by concomitant topical applications of indomethacin (0.1 percent). These observations suggest that the proliferative effects of these chemicals are mediated by AA metabolism. In a separate experiment, murine epidermal sheets were labeled in cultures with tritiated AA. After extensive washing, the epidermal sheets were incubated in PBS containing TPA. By thin-layer chromatography, we found that untreated epidermal sheets produced prostanoids, predominantly PGD and thromboxanes, but also PGE, PGF2α, and 6-keto PGF1α. Treatment of the epidermal sheets in vivo with MBEH, PTBC, or AA accelerated the metabolism of AA along the cyclo-oxygenase pathway. We suggest that the para-substituted phenolic melanocytotoxins activate AA metabolism in the epidermis. The proliferative effects of these compounds may be mediated by one or more of the AA by-products.

**Arachidonic Acid (AA) and Prostanoids in Suction Blister Fluid (SBF) from Vitiligo Lesions (IS) and Uninvolved Skin (US).** J.P. Ortonne. Hôpital Pasteur, Nice, France  A. Civier, B. Shroot. CIRD Sophia Antipolis, Valbonne, France  J.P. Poirier. Hôpital Pasteur, Nice, France  C.H. Hensby. CIRD Sophia Antipolis, Valbonne, France

It has been recently suggested that in vitiligo (V) patients, an as yet ill-defined external stimulus causes the excess production of prostanoids in epidermal cells. This excess production could result in an excess release of free radicals (as a by-product of PG synthesis) which in patients who develop V could lead to the destruction of pigment cells due to their having a lower capacity to handle these free radicals.

In order to test this hypothesis, PGE2, PGD2, PGF 6. oxo. PGF1α, and AA were measured by gas chromatography and mass spectrometry in the SBF from 11 patients (age 16-69 years) with typical V lesions and in 37 healthy volunteers. Exudates from US, IS, and normal skin of healthy volunteers (NS) was obtained at room temperature.
Our data supports the opinion that in IS, where no underlying inflammation is present, there are elevated levels of AA and various prostanoids. The levels observed in V lesions are comparable with those observed in other lesions associated with inflammation. Furthermore, in US, the levels of these compounds were similar to those observed in NS.

Our results show that elevated levels of AA and prostanoids are found in IS not associated with macroscopic evidence of inflammation, suggesting their excess epidermal production in the V lesions. Whether this feature is the primary defect or a secondary event in the course of V remains to be established.

**Motile Iridophores Play the Leading Role in Damselfish Coloration.** N. Oshima, M. Sato, T. Kumazawa, N. Okeda, H. Kasukawa, R Fujii. Toho University, Franbashi, Chiba, Japan

In a few species of damselfish, we found simple dermal chromatophore units consisting of iridophores and melanophores. Forming a compact single layer, the iridophores lie just under the subepidermal collagenous lamella. The dendritic processes of underlying melanophores were inserted into the space among the iridophores. Each iridophore is an ellipsoidal cell with a nucleus around the top of the cytophasm, and contains a number of piles of reflecting platelets. These piles dispose radiately from the nucleus. Electron microscopic observations revealed that the distance between platelets is strikingly uniform. Being probably a guanine crystal, each platelet is enveloped with a cisternal membrane. We found that the spectrum of light reflected from the iridophore shifts in response to electrical stimulation of chromatophore nerves, catecholamines, adenosine and so on [Fujii, et al: this Conference]. Studies on isolated skin preparations revealed that the peak of the spectrum stands within the near ultraviolet region in the physiological saline, i.e., the iridophores are almost transparent. The nervous and catecholamine stimuli induced the shift of the peak toward yellowish green, through violet, blue, and green. A progressive decrease in the toxicity of bathing media also caused the shift of the spectral peak, leading to the brightness of the cells. These observations led us to conclude that the inimitable integumental coloration of the damselfish is mainly caused by the piles of guanine platelets in the iridophore, through a multilayered thin-film interference phenomenon, partially influenced by the state of melanophores. When the distance between the crystals is short, e.g., as in the physiological saline, the cell reflects only ultraviolet rays, thus being practically invisible. If the distance is increased, it brightens, reflecting violet through yellowish green rays of light. Finally, we wish to emphasize here that the unique motile activity of the iridophore of the present material is of an entirely new type among the cell movements known and described hitherto.

**Cytoskeletal Protein Kinase Activities in Pigment Cells.** Robert E. Palazzo, Jiro Matsumoto, Thomas J. Lynch, John D. Taylor, T.T. Tchen. Wayne State University, Detroit, Michigan

We have described morphological differences in the Triton-insoluble cytoskeletons (CSKs) of normal xanthophores, GEM-81, erythrophoroma cells, and fibroblast-like dermal non-pigment cells from the goldfish. In addition, the organization of the xanthophore CSK and, to a lesser extent, that of the GEM-81 cells are altered by treating the intact cells with cAMP. We have evidence for the presence of cAMP-dependent protein kinase (cAdPK) in all these cells but have seen little or no effect of cAMP on the phosphorylation of the cytoskeletal proteins. To pursue these observations further, we have tested the capability of the Triton-insoluble CSKs to support protein-phosphorylation in vitro. In this system: (i) the fidelity of protein phosphorylation compared with that in intact cells is high in all three cases, suggesting the association with the CSK of the kinase(s) responsible for phosphorylating all major cytoskeletal proteins. (ii) No cAdPK is detectable in the CSKs of the GEM-81 or non-pigment cells. (iii) The xanthophore CSK contains peptides which resemble the 57 Kd carotenoid droplet phosphoproteins and which are phosphorylated in vitro in the presence of cAMP. (iv) The phosphorylation of certain peptides is exaggerated in vitro, suggesting preferential association of substrate and kinase within the CSK and/or the removal of modulating factors by Triton extraction.
Non-Enzymic Oxidation of Cysteinyldopa Catalyzed by Metallic Ions. A. Palumbo. Stazione Zoologica, Naples, Italy  G. Misuraca. University of Naples, Italy  G. Nardi. Stazione Zoologica, Naples, Italy  G. Prota. University of Naples, Italy

In the course of a study on the effect of 5-S-cysteinyldopa (cysdopa) on biological systems, we found that the rate of autoxidation of this catechol amino acid was markedly enhanced by certain metallic cations, e.g., Ca**, Mg**, Zn**, present in the medium. Following this observation, the ability of cysdopa to form metal complexes was studied spectrophotometrically by recording the variation of its ultraviolet spectrum as a function of the amount and type of the metallic ion added.

In this paper the properties of these complexes are reported with special reference to their behavior toward oxidation under near physiological conditions. As a rule, the rate of oxidation was found to be primarily dependent upon the nature of the metal ion used. Thus, for example, in the presence of Ni, Fe, Al, and Pb, which form strong coordination complexes with cysdopa, no oxidation is observed, whereas, under the same experimental conditions, the Zn, Co, Cd, or Cu complexes readily autoxidize with formation of yellow or red pigments, identified as benzothiazinederivatives by chemical and spectral analysis. These and other experiments, which will be described, provide evidence that metallic ions such as Zn and Cu may have a profound influence in the oxidation behavior and presumably on the metabolism of cysdopa in vivo.

Analysis of the Synthetic Dopa-Melanin ESR Spectrum. M. Pasenkiewicz-Gierula, R.C. Sealy. Institute of Molecular Biology, Jagellonian University, Kraków, Poland; National Biomedical ESR Center, Medical College of Wisconsin, Milwaukee, Wisconsin

Synthetic DOPA-melanin was obtained by auto-oxidation of DOPA. Its ESR spectrum is due to the presence of orthosemiquinone-type monomers in the polymer. The number of "paramagnetic monomers" in the melanin sample, as well as magnetic parameters of the ESR spectrum, depends, among other factors, on pH.

ESR spectrum of the frozen aqueous suspension of DOPA-melanin at Q-band (35 GHz) is asymmetric in the pH range 3-12, and the asymmetry increases with increasing pH.

Detailed analysis of DOPA-melanin ESR spectra based on computer simulation indicates that the asymmetry of the melanin spectrum may be caused by two factors: (1) the anisotropy of the melanin g-tensor—which increases with increasing pH probably due to the pH-induced changes in the unpaired electron delocalization over "paramagnetic monomer"; (2) superposition of at least two lines each arising from "paramagnetic monomer" at different protonation states. The intensity ratio of the superimposed lines depends on pH.

The fact that for each protonation state of "paramagnetic monomer" the shape function is Gaussian would suggest that the "individual" ESR line is an envelope of several hyperfine lines arising from the unpaired electron hyperfine interaction with adjacent hydrogen atoms.

Effect of Depigmenting Phenolic Substances on Mitochondrial Respiration. S. Passi, M. Nazzaro-Porro, M. Picardo. Institute of Dermatology San Gallicano, Rome, Italy  A. Breathnach. St. Mary's Hospital Medical School, London, England  P. Riley. University College, London, England

Depigmentation of skin by phenolic compounds is known to be due to tyrosinase-induced semiquinone radicals which lead to liperoxidation and melanocyte lysis. It is also known that within the cell cytochrome P 450 system is capable of oxidizing phenols with radical formation [Bachur N, et al: Cancer Res 38:1745, 1978]. As other free radical inducers in the cell are represented by respiratory chain enzymes, we tested the effect of depigmenting phenols on isolated rat liver mitochondria.

Studies were carried out by O2 electrode, spectrophotometric methods, and high performance liquid chromatography.
The results showed that respiration was inhibited, in decreasing order, by isopropylcatechol, terbutylcatechol, 4-hydroxyanisole, monobenzylether of hydroquinone, and paracresol. Experiments on submitochondrial particles showed that the inhibition concerned the following enzymes: NADH dehydrogenase, succinic dehydrogenase, hydroCOQ : cyt C oxido-reductase.

These findings could suggest that enzymatic systems different from tyrosinase, viz., Cyt P 450 system and respiratory chain, might be involved in the depigmenting activity of phenolic substances. They might also account for the toxic activity of some of these substances (4-hydroxyanisole, hydroquinone, etc.) on cells other than melanocytes.

Histomechanical Considerations on the Process of “Dropping Off” in Nevocellular Nevi. E. Paul, H. Gerhard. Universität Giessen, Federal Republic of Germany

M. Ishii. Osaka City University, Medical School, Osaka, Japan (Scholar of Yamada Science Foundation at Universität Giessen)

It is generally suggested that the growth of nevus cell nevi starts from the epidermis. This process was investigated in cases of recurrent nevi after shave biopsy by means of fluorescence-histochemical (FIF) and electron-microscopical methods. Since the time of traumatization was known, we tried to reconstruct the course of this process. Shave biopsy was followed by an increase in the number of melanocytes in the newly grown epidermis over the wound. They accumulated in a palisade manner in the basal layer of the epidermis. Thereafter, nest-like conglomerations of dendritic cells could be observed in the basal layer of the epidermis. Through their increase in size these nests assume a globular shape, and they displace the neighboring keratinocytes. These cells are heavily deformed and elongated because they remain in contact with both the basal membrane and the adjacent keratinocytes at the opposite pole. It might be possible that mechanical power resulting from the deformation of the keratinocytes forces the cell nest to evade into the dermis. During the stage of “dropping off” the nevus cells are elongated. Further morphological characteristics indicate that the cells are more likely to reach the dermis passively. The present results suggest that the dropping cell nest is finally separated from the tip of a rete ridge. Our studies support the hypothesis of Unna in the model of recurrent nevus; however, they do not give evidence of the actual stimulus to nevus growth.

Observations of the Development of Melanoma by Means of Private Photo Series. E. Paul. Universität Giessen, Federal Republic of Germany

The use of modern cameras with high-quality objectives and relatively fine-grained films allows big enlargements of the original negatives from private photographs. Amateur photographs that accidentally show the tumor-bearing area may be most suitable for reconstruction of the dynamics of the tumor growth. They also give evidence of the duration of tumor development. In many cases there has been a surprisingly exact determination of the shape and size of the “mole” that later turned out to be a malignant melanoma. Some photo series have documented the development of melanomas over periods between ten and twenty years, from small pigment spots to the definite tumors. In addition, the photo series have demonstrated very slow changes in size and shape during the early phases of development, which would not be thinkable in benign processes and which are only observed in malignant lesions. The pigment spots that initially did not allow a clinical diagnosis of melanoma were probably malignant from the very beginning. However, they were often misinterpreted as “nevi” by both patients and physicians. Only in rare cases there must have been pre-existent nevus cell nevi because remnants were histologically demonstrable beside or beneath the tumor cells.

Diphosphophenylalanine: A New Compound Exhibiting Selective Toxicity Toward Melanoma Cells. J. Pawelek, M. Murray, D. Chandler. Yale University School of Medicine, New Haven, Connecticut

Cloudman melanoma cells in culture are killed when exposed to diphosphophenylalanine (DPP) and the killing is enhanced several-fold when the cells are pre-exposed to MSH. DPP is a highly soluble compound which is stable to boiling and wide fluctuations in pH. The synthesis is straightforward and yields of greater than 90 percent can be obtained using dopa as starting material. Brief exposure to alkaline phosphatase results in hydrolysis of the phosphate
moieties and production of dopa. DPP does not inhibit tyrosinase activity. Tyrosinase does not catalyze the conversion of DPP into melanin unless the DPP is treated with alkaline phosphatase. When equimolar amounts of dopa and DPP were incubated in the presence of horse serum, the dopa was converted to melanin but the DPP was not, indicating that the phosphate moieties remain intact in the presence of serum. However, in the presence of cultured melanoma cells, DPP is converted into melanin, indicating that the cells are able to produce dopa from DPP. At neutral pH, DPP can be dissolved in aqueous solutions at concentrations approaching 0.5 M. Labeled DPP was prepared from 4C-dopa and injected into mice with Cloudman melanomas. Most of the 4C-label was excreted in the urine; however, there was significant and selective incorporation of 4C into tumors. It is not yet clear whether DPP affects the growth of melanomas in mice, but we showed that mice can tolerate injections of at least 50 mg/day (0.75 g/kg) for prolonged periods with no noticeable side-effects. The absence of side-effects combined with the solubility, stability, and selective uptake by tumors makes DPP a potential candidate for use in melanoma therapy. Additionally, we are investigating whether 3P-labeled DPP shows enhanced cytotoxicity compared to its nonradioactive counterpart.

**MSH, Isobutylmethylxanthine, and DBcAMP Each Induce Increases in Tyrosinase Activity in the G2 Phase of the Cloudman Melanoma Cell Cycle.** J. Pawelek, A. Bergstrom. Yale University School of Medicine, New Haven, Connecticut

In culture, Cloudman S91 melanoma cells respond to melanotropin (MSH) with increases in cAMP levels, tyrosinase activity, and melanin content, accompanied by changes in morphology and rates of proliferation. These processes seem to be mediated, at least in part, by cAMP-dependent protein kinases. It was reported earlier that the increases in cAMP and tyrosinase activity are restricted to the G2 phase of the cell cycle and that receptors for MSH are accessible predominantly during this phase. Recently the validity of the cell cycle studies was questioned. It was suggested that the use of colchicine to obtain synchronized cultures might have produced artifacts since colchicine appears to prolong the G2 phase in Cloudman cells. We felt that this criticism was valid, and we undertook the current studies which approach the problem in a different manner. Using a centrifugal elutriator, we isolated cells from non-synchronized cultures on the basis of their stages in the cell cycle. With this technique, cells are separated from one another by differential sedimentation and can be collected rapidly, gently, and in numbers suitable for assays of enzymatic activities. Cells were exposed to MSH, isobutylmethylxanthine (MIX), and or DBcAMP for various times. They were then separated on the basis of their position in the cell cycle by centrifugal elutriation, and tyrosinase activity was assayed. We found that the earlier conclusions were correct: MSH caused an increase in tyrosinase activity in the G2 phase of the cycle. We also confirmed a previous finding that DBcAMP, unlike MSH, increases tyrosinase activity throughout the cycle. However, with the increased resolution provided by the elutriator, we also demonstrated a stimulation of tyrosinase activity both by DBcAMP and MIX which was specific to the G2 phase. In addition, we found that as the cells pass from mitosis to early G1, there is a transient but marked depression in tyrosinase activity.

**Early Pattern Formation During Embryogenesis in Xiphophorus.** Ralf Uwe Peter, Manfred Schartl. Genetisches Institut, Giessen, Federal Republic of Germany

Prenatal behavior of melanophores, xanthoerythrophores, and iridocytes has been investigated in the embryos of wild-type pigmented fishes and two mutants, affecting (a) pigment cell differentiation (golden g/g), and (b) melanin synthesis (albino a/a).

Light microscopical studies of whole mounts and paraffin sections, scanning electron microscopy of in vitro cultured embryos, and grafting experiments revealed:

- Melanophores, xanthoerythrophores, and iridocytes differentiate at different times.
- Formation and differentiation of pigment cells is cell autonomous.
- Cellular size, forms, and density of distribution are determined by the surrounding tissues.
- Blood vessels are frequently covered by pigment cells, whereas peripheral nerves are not.
- Pigmentation of the yolk sac in late embryogenesis, of parenchymous and central nervous tissues, and of the musculature is entirely confined to supplying blood vessels.
We conclude that pigment cells and their presumed precursors migrate and distribute themselves along vascular rather than neural pathways.

Quantitative Determination of Melanin in Pigmented Cells by Electron Spin Resonance Spectroscopy. B. Pilas, T. Sarna. Institute of Molecular Biology, Jagellonian University, Kraków, Poland

The amount and type of melanin present in pigmented cells is considered to be one of the factors that determine photo- and/or radiosensitivity of the cell.

We have developed a new, specific method for quantifying melanin in pigmented systems based on the observation that the electron spin resonance (ESR) spectrum of melanin free radicals, under strictly controlled conditions, provides a sensitive indicator of the concentration of melanin in the sample. We determined the sensitivity and specifically of the ESR approach for melanin determination in model systems containing either synthetic dopa-melanin or natural melanin isolated from bovine eyes. In these samples the intensity of the dark ESR signals, recorded at liquid nitrogen temperature, in the form of icicles 0.4 cm in diameter and 2 cm long, showed a linear dependence on melanin concentration over the range of concentrations: 0.002—1.0 mg/ml. The intensity of the ESR signal of the tested samples can be enhanced four to five times after illumination from visible light. This procedure improves both the sensitivity and specificity of the method since paramagnetic contaminations randomly present in some samples usually do not show any significant photosensitivity of their ESR signals.

The accuracy of the ESR method was tested on samples containing 0.25—25 × 10⁴/ml B16 melanoma cells isolated from mouse tumors with substantial variation in the degree of their pigmentation. The results of the ESR method compared favorably with spectrophotometric and gravimetric determination of melanin, particularly in case of lightly pigmented material. The accuracy of the ESR procedure is not affected by the presence of other cellular components.

Influence of Selenium on Activity of Glutathione Peroxidase in Experimental Melanoma. B. Procházková, I. Obrusnik, J. Duchoň. Faculty of General Medicine, Charles University, Prague, and Institute of Nuclear Research, Řež, Czechoslovakia

Selenium (Se), as integral constituent of the active site of glutathione peroxidase (EC 1.11.1.9.), contributes to physiological destruction of H₂O₂ and organic peroxides. In the last decade a certain amount of hope has been put into the possibility of the therapeutic influence on tumorous disease with antioxidants.

In our experiments in the past we administered Na₂SeO₃ (2 ppm of the body weight weekly intraperitoneally) to hamsters bearing M-melanoma (Bomirski). We found the elevation of glutathione peroxidase (GSH-px) activity in body tissues and prolongation of survival time of experimental animals from three weeks (untreated animals) to seven weeks (treated animals). The content of Se was determined using the neutron activation analysis method. The activity of GSH-px was determined using the modified method of Paglia and Valentine [J Lab Clin Med 70:158, 1967].

In order to reduce the risk of Se toxicity we performed an analogous experiment using organic Se compounds (Se cysteine and Se methionine). We found that these organic compounds were not so effective as Na₂SeO₃, as far as the elevation of activity is concerned. They also did not influence the survival time of animals.

Accumulation of Se in melanoma tissue and, especially in melanosomes, was similar to the inorganic form of Se. So far it is difficult to explain the mechanism of action of Se on the survival time of animals; however, it seems that it is not based on interaction with melanoma itself.

Interactions of Yellow (A⁴) and Light (B⁴) Genes Revealed by MSH-Induced Conversion of Coat Color in Mice. W.C. Quevedo, Jr. Brown University, Providence, Rhode Island M.E. Hadley. University of Arizona, Tucson, Arizona

Lethal yellow (A⁴/-, B⁴/-) mice do not exhibit the premature death of follicular melanocytes found in their nonagouti (eumelanin) counterparts. Repeated injections of N1e⁴,
D-Phe]-α-MSH bring about a eumelanization of hairs regrown in manually depilated skin of lethal yellow-B¹ (pheomelanic) mice. When sufficient amounts of the α-MSH analogue are administered, large clumps of eumelanin, i.e., the remains of abnormal melanocytes prematurely dislodged from hair bulbs, are found within black hairs. The absence of programmed melanocyte death in lethal yellow-B¹ mice appears to be directly related to the absence of eumelanogenesis rather than to some other action of the A² gene. The evidence to date suggests that death and dislodgment of follicular melanocytes in B¹ mice may result from the release of cytotoxic intermediates of eumelanogenesis and/or of lysosomal hydrolases which are normally confined safely within membrane-limited vesicles.

**Explanation for the Lag, Requirement of 3, 4 Dihydroxyphenylalanine, and Inhibition by Excess Tyrosine of Tyrosinase from Melanosomal Fraction of Murine Melanoma and Absence of These Properties for the Highly Purified Enzyme.**

A. Ramaiah, E. Vijayan, A. Bhatnagar, Ch. Chaya Devi. All India Institute of Medical Sciences, New Delhi, India

1. Tyrosinase of melanosomal fraction from murine melanoma exhibits lag in cresolase activity, requirement of 3, 4 dihydroxyphenylalanine, and inhibition by excess tyrosine similar to the properties of tyrosinase from other sources.

2. The lag exhibited by the enzyme in the melanosomal fraction was shown to increase with increasing concentration of tyrosine and, if rate after the lag was taken, there was no inhibition by excess tyrosine.

3. The enzyme after purification to high specific activity by one-step procedure of affinity chromatography exhibits none of these properties. Therefore the explanation given earlier in the literature for these properties of the enzyme as due to the competition of tyrosine for the activator site of 3, 4 dihydroxyphenylalanine on the enzyme is perhaps not valid.

4. Addition of melanosomal fraction to the highly purified enzyme produces inhibition, which increases with increasing concentrations of tyrosine, and the inhibition could be seen in the first minute after addition of the melanosomal fraction to the purifier enzyme; the extent of inhibition remains unaltered up to 60 minutes. These results rule out both modification of enzyme during purification or accumulation of metabolites of tyrosine by the melanosomal fraction as responsible for tyrosine concentration-dependent lag in tyrosinase in the melanosomal fraction and its absence in the purified enzyme.

**Lipid Peroxidation During Irradiation of Fatty Acids and Cells in the Presence of Melanins.**

N.S. Ranadive, I.A. Menon, S. Persad, H.F. Haberman. University of Toronto, Toronto, Canada

It has been reported that irradiation of pheomelanin from human red hair (RHM) produced more superoxide than the irradiation of eumelanin from black hair (BHM). Superoxide is known to peroxidize unsaturated fatty acids. The present paper describes the peroxidation of lipids during the irradiation of arachidonic acid (AA), linoleic acid (LA), and Ehrlich ascites carcinoma cells in the presence of these melanins. Irradiation was carried out using a Westinghouse mercury vapor lamp. The formation of diene conjugation was studied by ultraviolet absorption spectrophotometry. Irradiation of AA and LA in the presence of RHM produced dienes (as shown by the increase in the absorbance at 233 nm) in significantly larger amounts than the irradiation in the presence of BHM or in the absence of either melanin. The formation of diene conjugates was, however, much more prominent when these fatty acids were irradiated in the presence of hematoporphyrin (HP). Thin-layer chromatographic analysis carried out using ³H-labeled AA suggest that mono and dihydroxy fatty acids are formed during irradiation of AA with RHM. The formation of similar products was also seen when AA was irradiated in the presence of HP. When Ehrlich ascites carcinoma cells labeled with ³H-AA were irradiated, peroxidation products of AA could be detected by TLC analysis of lipid extracts of the cells irradiated with RHM. The cells irradiated in the absence of melanin did not show detectable amounts of peroxidation products. These results suggest that irradiation in the presence of RHM produces peroxidation of lipids in the cell membrane. The relationship between lipid peroxidation and cell lysis is, however, still a matter of speculation.
Physiological Specificity and Structure-Activity Relationships of Crustacean Chromatophorotropins. K. Ranga Rao, O.J. Semmes, R.M. Sattelberg, J.P. Riehm. University of West Florida, Pensacola, Florida

Crustacean red pigment concentrating hormone (RPCH: <Glu-Leu-Asn-Phe-Ser-Pro-Gly-Trp-NH$_2$>) and a structurally related insect neuropeptide, the adipokinetic hormone (AKH: <Glu-Leu-Asn-Phe-Thr-Pro-Asn-Trp-Gly-Thr-NH$_2$), can bring about pigment aggregation in one or more of the different chromatophore types in decapod crustacea. The cellular specificity of action of these peptides varies with the species tested. Thus synthetic RPCH and AKH trigger pigment concentration both in the erythropores and leucopores of the brown shrimp Penaeus aztecus, whereas their actions are directed specifically toward the erythropores in the case of the fiddler crab Uca pugilator. The latter erythrophore-specific action in Uca is demonstrable by testing these peptides (AKH/RPCH) either singly or in conjunction with a synthetic pigment-dispersing hormone (PDH/DRPH: Asn-Ser-Gly-Met-Ile-Asn-Ser-Ile-Leu-Gly-Ile-Pro-Arg-Val-Met-Thr-Glu-Ala-NH$_2$). Both AKH and RPCH are able to antagonize (suppress) the dispersing effect of PDH on the erythropores of Uca, but are unable to affect the PDH-induced dispersion in the leucopores and melanophores of this crab.

Studies on the influence of N-terminal sequence deletions and of certain chemical treatments on the melanophore pigment-dispersing effect of the octadecapeptide PDH show that the methionine residues and the N-terminus are important for full activity and that phenylglyoxal modification of Arg-13 leads to an increase in activity; the latter effect is thought to result from protection of the peptide from proteolysis in vivo. For additional evaluation, synthetic PDH analogues with replacement of Arg-13 by norvaline and those with replacement of Met-4 and/or Met-15 by norleucine are being prepared.

The New Mexico Skin Cancer Project. J.C. Redman. Albuquerque, New Mexico

Patterned after the Queensland Melanoma Project of Dr. Neville Davis and his Australian colleagues, the New Mexico Skin Cancer Project was founded in June 1977. The goals of the Project are: the prevention, early recognition, and early treatment of cutaneous malignant melanoma.

Sponsored by the Johnson & Johnson Baby Products Company, the Project conducts seminars about malignant melanoma for the public and for the healing professions. Recognized authorities in the field of melanoma, from the United States and from other countries, are featured speakers.

Posters, leaflets, and a “mole booklet” are distributed to groups and individuals. Radio and television talks about the nature of malignant melanoma are given.

An important part of the recent educational efforts of our Project has resulted from the work of Dr. Eberhard Paul and his photodocumentary investigations concerning the growth dynamics of malignant melanoma, and from the innovative public and professional educational efforts of Professor Dr. med. Leonhard Illig, both of Giessen. Dr. Paul's photos have made a significant impact on both the American public and the American professions in terms of the need to remove isolated pigmented lesions of the skin before obvious change takes place. Professor Illig's educational efforts have stimulated increased interest in getting the “melanoma message” to as many high-risk people as possible.

The apparent effectiveness of our educational efforts was realized after a survey of malignant melanomas of the skin removed in Albuquerque, New Mexico, in 1980. Whereas the population of Albuquerque and the rest of the county in which it is the largest city rose 32.9 percent between 1970 and 1980, the number of melanomas removed between 1971 and 1980 increased more than 200 percent. A noticeable increase in the annual number of melanomas removed began in 1977, the first year of existence of the New Mexico Skin Cancer Project.

The PUVA Lentigo: Light Microscopic and Ultrastructural Features, and an Analysis of Predisposing Factors. A. Rhodes, T. Harrist, R. Stern, H. Nakagawa, K. Momtaz-T, J. Melski, J. Parrish, T. Fitzpatrick. Harvard Medical School, Boston, Massachusetts

Eleven PUVA-induced pigmented macules (PM) on buttocks (ten) or groin (one) of seven psoriatic adults four to six years after starting PUVA were compared to eight sun-induced
pigmented macules (SM) on sun-exposed upper back and five specimens of light-protected buttock skin (LPS) from 12 non-psoriatic control subjects who had not received PUVA. Unlike SM, many PM were darkly and irregularly pigmented. In a blind histologic assessment using routine and L-dopa incubated tissue sections, both PM and SM were "lentigines." In contrast to solar lentigines and LPS, melanocytes in PUVA lentigines were more often hypertrophic and cytologically atypical. Transmission electron microscopy revealed melanocytes in PUVA lentigines to have longer and more numerous dendrites, more active melanogenesis, close apposition of Langerhans cells to melanocytes, and cytoplasmic and melanosomal alterations (including giant pigment granules). Compared to solar lentigines and LPS, there was a significant shift to large, predominantly single melanosomes in keratinocytes of PUVA lentigines.

In 1,380 psoriatic adults treated with PUVA and followed prospectively, buttock lentigines were noted in 53 percent of patients at the final examination (an average of 5.7 years after starting PUVA). The frequency and severity of buttock lentigines at the final examination was positively associated with the total number of PUVA treatments received and age at starting PUVA ≥ 35 years, and negatively associated with skin types V&VI. According to a regression analysis, buttock lentigines appeared to persist in some patients even after PUVA had been discontinued for one to two years or longer. Given that PUVA lentigines are characterized by proliferations of hypertrophic and sometimes cytologically atypical melanocytes, and that these lentigines may persist after PUVA is discontinued, it is recommended that individuals who develop pigmented macules while on PUVA be monitored continually for melanocytic dysplasias and melanoma.

A Transmission Electron Microscopical and Freeze-Etch Study of Malignant Melanoma in Fish. Rüdiger Riehl. Institut für Zoologie II, Universität Düsseldorf, Federal Republic of Germany Manfred Schartl. Genetisches Institut, Universität Giessen, Federal Republic of Germany

Melanotic malignant melanomas (MM) in *Xiphophorus* (Teleostei: Poesiliidae) were studied by transmission electron microscopy (TEM) and freeze-etching (FE). The conventional TEM technique showed clear advantage in the demonstration of internal architecture of organelles, whereas FE had considerable potentialities in respect to the visualization of membrane surface specializations.

MM of *Xiphophorus* exhibits tightly packed pigment cells with prominent dendritic processes and interdigitations of their plasma membranes. The most impressive feature of MM cells is the occurrence of large, lobulated nuclei with numerous nuclear pores and some nuclear pockets. Abundant spheroidal or ellipsoidal melanosomes (diameter 200-650 nm) and vesicular structures are distributed throughout the cellular dendrites, whereas the perinuclear cytoplasm is free of melanosomes. The membrane surrounding the melanosomes carries particles with a random distribution. A further characteristic feature of melanoma cells in fish is the occurrence of melanosome complexes ("compound melanosomes"). These melanosome complexes consist of a few to numerous melanosomes which are enveloped by a separate membrane. Pinocytotic vesicles could be demonstrated with distinct differences in frequency and distribution patterns, indicating differences in the metabolic activities of the cells in the same melanoma. Intercellular junctions are lacking in the MM cells.

Some Aspects of the Regulation of Melanogenesis in Cultured Melanocytes. P.A. Riley. University College School of Medicine, London, England

One of the consistent features both of primary and established cultures of melanocytes *in vitro* is the progressive loss of pigmentation among the proliferating cells. This phenomenon seems to be more prominent if the melanocytes are separated from other cells, such as keratocytes, with which, *in vivo*, they are normally in functional contact. Progressive amelanosis in pigment cell cultures may be due to interruption or modification of a number of stages involved in the synthesis and distribution of melanin or to loss of stimulating signals generated by complex processes in the interaction between melanogenic cells and the recipients of the pigment. The extent to which hypopigmentation in culture is the result of these factors will be reviewed.
Functions of Human Tyrosinase. H. Rorsman, B. Jergil, Ch. Lindbladh, E. Rosengren. University of Lund, Sweden

Tyrosinase (E) was isolated from cultures of human malignant melanoma cells. The enzyme hydroxylated tyrosine (T) only in the presence of co-substrate. L-dopa (D) was the most effective co-substrate, 5-S-cysteinyldopa was ineffective, while 5,6-dihydroxyindole-2-carboxylic acid had a certain effect.

The function of tyrosinase can be described by the following equations:

I. a. $E_{ox} + D \rightarrow E_{red}D$
    b. $E_{ox}D \rightarrow E_{red} + DQ + 2H^+$
II. a. $E_{red} + O_2 + D \rightarrow EO_2D$
    b. $EO_2D + 2H^+ \rightarrow E_{ox} + DQ + 2H_2O$
II'. a. $E_{red} + O_2 + T \rightarrow EO_2T$
    b. $EO_2T + 2H^+ \rightarrow E_{ox} + D + H_2O$

I + II describe the dopa oxidase function, I + II' the combined dopa oxidase and tyrosinase hydroxylase function. Reactions II and II' are mutually competitive.

The Size of the Melanocyte System in Man. I. Rosdahl, H. Rorsman. Universities of Göteborg and Lund, Sweden

The size of the melanocyte system in man was estimated by assuming that all pigment-producing melanocytes were assembled to a single compact organ. Two microscopical procedures were used to assess the melanocyte population density in different areas of the skin. The melanocyte cell volume was estimated by measuring the maximal three axes of the cell. In addition the sum of the dendritic volume was calculated. Our estimates indicate that the epidermal melanocytes constitute the dominant part of the "melanocyte organ." In the adult man not recently exposed to sunlight, the functionally active melanocytes form a tissue mass of 1.0–1.5 cm$^3$. Other melanocytes, such as those in the mucous membranes, the follicles, and the eyes constitute only a small portion of the total melanocyte cell mass.

Effect of Cepharanthine on Cell-Type Conversion of B16 Mouse Melanoma Cells. A. León Rudas, H.B. Tamate, S. Sato, T. Takeuchi. Tohoku University School of Medicine, and Tohoku University, Sendai, Japan

A cell line, conv, isolated from B16 mouse melanoma cells is shown to exhibit cell-type conversion. Under usual culture condition, the cells are characterized by rounded and spindle-shaped cell morphology and are not highly melanotic but with high tyrosinase (dopa oxidase) activity. When the cells are seeded to form colonies on plastic culture dishes in Eagle's MEM supplemented with 10 percent bovine calf serum, two kinds of cell types always appear. One is cytochemically dopa-positive and spindle-shaped (S-type cell) with the same phenotypes as those of the parental cells. The other is dopa-negative and fibroblast-like (F-type cell) containing no melanosomes. It has been assumed that the conversion from S-type to F-type occurs with a high frequency under the above condition. This phenomenon seems to be useful as a model system for the study of cell differentiation, for the cell type conversion involves both the differentiative functions of melanocytes and a drastic change in their cell shape.

In the previous study, we demonstrated that a serum factor was responsible for the induction of the conversion and that the factor was heat-labile 740 kd serum protein [Sato et al: 1983]. In the present study, we have studied the effect of cepharanthine, a bisocoumarine alkaloid, which has been shown to stabilize cell membrane. Cells of the line conv 2F6 were plated at a density of 500 cells/dish on glass dishes containing MEM supplemented with 10 percent bovine calf serum. The next day, the media were replaced by fresh media with or without cepharanthine of various concentrations. On day 10 cells were fixed with PLP and were then subjected to the dopa reaction and Giemsa staining to identify the types of colonies. The results indicated that cepharanthine at the concentration of around 0.1 μM induced the cell-type conversion from S-type to F-type in the melanoma cells.
Reversible Depigmentation of Mouse Melanoma Cells by Tumor Promoters. H. Saeki, A. Oikawa, H. Fujiki, T. Sugimura. Tohoku University, Research Institute for Tuberculosis and Cancer, Sendai, Japan; National Cancer Center Research Institute, Tokyo, Japan

In the presence of tetradecanoylphorbol acetate (TPA) or teleocidin (TEL), another potent tumor promoter of a quite different chemical structure and of microbial origin, in culture media, melanogenesis in melanoma cells B-16-S3 delays one or two days [Fisher et al: 1982].

In the present study we found that another cultured melanoma cell line, B16-C1M, lost its tyrosinase activity by treatment with 0.1 μM TPA or dihydroteleocidin B, a hydrogenated derivative of TEL, while the rate of proliferation was not affected. Unesterified phorbol had no effect. Cells retaining almost no tyrosinase activity after the treatment restored the activity gradually upon subculture in the absence of the drugs. In the subsequent (second) subculture, the activity was fully restored.

It seems likely that the reversible change in melanogenic phenotype is not due to selections of amelanotic and melanotic subclones but due to the reversible repression and de-repression of the enzyme synthesis in a single clone.

Stimulation of Melanogenesis by Lysosomotropic Agents. Hisaaki Saeki, Atsushi Oikawa. Research Institute for Tuberculosis and Cancer, Tohoku University, Sendai, Japan

Lysosomotropic agents (LTAs) such as ammonia, methylamine, ethylamine, and imidazole, when added to culture of mouse melanoma cells B16-C1M, stimulated melanogenesis. This stimulation was not observed with tetraethylammonium chloride, a non-lysosomotropic alkylamine analogue.

When tyrosinase activity of cells was expressed by TyH, defined as tyrosine hydroxylase activity exerted by cell homogenates under the defined conditions, cells cultured in medium containing LTA showed a stimulated TyH. Addition of LTA to the reaction mix for TyH assay or that to a culture medium with cycloheximide did not stimulate TyH, indicating the effect was due to an increase in the amount of active tyrosinase.

When the tyrosinase activity was measured as TyC, defined as the activity exerted by living cells in culture, LTA stimulated TyC much more than TyH, resulting in an increase in TyC-to-TyH ratio, a measure of action efficiency of the enzyme in living cells under the specified culture conditions. Chloroquine, another potent lysosomotropic agent, stimulated TyH but not TyC and melanogenesis. The reason is unknown.

Within a pH range up to pH 7.8, higher than the optimal pH of tyrosinase, a similar stimulation of TyC-to-TyH ratio was observed at higher pH.

A possibility that melanosome has a mechanism analogous to lysosome in intravesicular pH control will be discussed.

Melanogenesis and Eye Color in Rats of Different Pigmentation. B. Sahlmann, P. Stanka. Arbeitsgruppe für Mikromorphologie, Institut für Anatomie der Ruhr-Universität Bochum, Federal Republic of Germany

By electron microscopy and by use of the DOPA reaction it can be shown that in the black eyes of the black hooded rat (BDE/Han) melanogenesis in the pigment epithelium of the fundus ends in the fifth week postpartum (pp) but that melanosomes are retained. Melanogenesis continues in the other pigmented ocular structures.

The lighter eye color of the beige hooded rat (E3/Han) derives from the smaller size and number of melanosomes in the ciliary body, iris, and choroid, and from the disappearance of melanosomes from the pigment epithelium of the fundus after the sixth week pp.

In albino rats (OM/NHan) the premelanosomes disappear from the pigment epithelium of the fundus after the fifth week pp. However, they can be found in other ocular structures in adult rats.

Our results reveal that melanogenesis in the pigment epithelium of the fundus is completed after the fifth week pp, in contrast to other ocular structures, and that this is independent of eye color.
Nitroxides as Redox Probes of Melanins: Changes in Redox Equilibria During Irradiation. T. Sarna, W. Korytowski, R.C. Sealy, J.S. Hyde. Jagiellonian University, Krakow, Poland; National Biomedical ESR Center, Medical College of Wisconsin, Milwaukee, Wisconsin

The interaction of nitroxide free radicals and their reduced products (hydroxylamines) with synthetic and natural melanins has been studied using ESR spectroscopy to measure changes in radical concentrations. (i) Reduction of nitroxide occurs in the dark and is completely reversible. The nitroxide can be completely regenerated by one-electron oxidants. The kinetics of the process depend strongly on radical charge and pH; for positively charged nitroxides the rate is much faster than for neutral or anionic radicals. At pH 10 the rate is about 20 times faster than at pH 5. (ii) Oxidation of hydroxylamine (from electrolytic reduction of nitroxide) can also be demonstrated. Thus melanin can participate in both oxidation and reduction processes, and the data allow the estimation of the equilibrium constant for the redox equilibrium. (iii) The position of this redox equilibrium is shifted during irradiation with visible or UV light. Rapid reversible reoxidation of hydroxylamine is apparent, together with a slow irreversible reduction of nitroxide. These processes mimic melanin radical production and oxygen consumption in the nitroxide-free system. Irreversible reduction of nitroxide is inhibited by oxygen. (iv) Results depend strongly on the type of melanin used; the behavior of eumelanins and pheomelanins is quite different, indicating differences in the redox equilibria of these polymers.

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Oxidation of Melanins by Visible and UV Light: Action Spectra for Eumelanins and Pheomelanins. T. Sarna, R.C. Sealy, I.A. Menon. National Biomedical ESR Center, Medical College of Wisconsin, Milwaukee, Wisconsin; Clinical Science Division, University of Toronto, Canada

Melanins undergo oxidation when irradiated with UV or visible light in aerobic systems. During this process oxygen is reduced to hydrogen peroxide. Using a spin-probe electron spin resonance approach, we have made quantitative measurements of oxygen consumption in eight different melanin systems. The melanins studied include both natural and synthetic pigments: eumelanins, pheomelanins, and synthetic melamins from dopa and cysteynlyldopa.

All melanins studied behave in a similar manner. Action spectra for pheomelanins closely resemble those for eumelanins. Both are unaffected by the presence of protein. Quantum yields for oxygen consumption are low for irradiation with visible light, but become moderate for UV irradiation (typically of the order of 0.1 percent at 300 nm). Eumelanins and pheomelanins appear to be equally susceptible to photo-oxidation. The mechanism of the photo-oxidation and implications of those findings are discussed.

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Growth Stimulation by Insulin of Harding-Passey Melanoma Cells Cultured in Serum-Free Medium and Growth Inhibition by Hydrocortisone or Dexamethasone in Medium With or Without Serum. D.O. Schachtschabel, M.-T. Vogel, H.B. Leising. Institut fuer Physiologische Chemie, Philipps-Universität, Marburg, Federal Republic of Germany

Harding-Passey melanoma cells (HPM-73 line) in monolayer culture (cell density ca. 0.5 x 10⁴ per cm²) exhibited little proliferative activity in a defined serum-free modified MEM medium (enriched with non-essential amino acids, pyruvic acid, linoleic acid, adenosine, putrescine, transferrin, albumin, vitamin B₁₂, and trace elements). Addition of fetal calf serum (FCS) or insulin alone resulted in stimulation of DNA synthesis and cell multiplication. Addition of 1–5 μg/ml insulin was about as growth-stimulatory as supplementation with 10 percent FCS. Already 1 ng/ml insulin exerted a significant enhancement of cell multiplication. Hydrocortisone or dexamethasone (10⁻⁴·10⁻⁷ M) inhibited growth stimulation by insulin or serum. Melanin content was slightly increased by hydrocortisone or dexamethasone treatment.

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Studies on the Mode of Action of Testosterone on Pigment Cell Tumors in *Xiphophorus and Girardinus*. A. Schartl, M. Schartl, F. Anders. Genetisches Institut der Universitaet Giessen, Federal Republic of Germany

Depending on the genotype of the fish, treatment with testosterone may lead to (a) induction of melanoma and pterinophoroma, (b) enhancement of tumor development, or (c) tumor regression. The promotion of pigment cell differentiation is shown to cause these divergent phenomena. (a) In genotypes in which a genetic block in pigment cell differentiation normally prevents the expression of a derepressed tumor gene, tumors are induced. (b) In genotypes in which tumors mainly consist of undifferentiated stem cells, testosterone treatment causes an increase of tumor cells and thus an acceleration of tumor growth. (c) In genotypes in which tumors consist mainly of incompletely differentiated, still dividing cells, testosterone causes a terminal differentiation of these cells, which are removed by macrophages, thus resulting in a retardation of tumor growth, and tumor regression.

To investigate whether these effects are due to a specific testosterone or to an unspecific steroid action, fish were treated with a variety of steroids and analogues. Only androgens showed all three effects on tumor development, while estrogens only caused tumor regression. Gestagens and glucocorticoids were inactive. Thus a specific testosterone action is concluded. To determine if testosterone acts indirectly by influencing the hormone system of the fish or directly by interacting with the pigment cell, receptor studies were performed. These showed the presence of a high-affinity testosterone receptor in normal skin and in spontaneous and testosterone-induced melanoma in increasing concentration. This points to a direct interaction of testosterone with the pigment cell.

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Tumor Formation in *Xiphophorus* Following Treatment with Initiating and/or Promoting Agents. C.-R. Schmidt, A. Schartl, A. Herbert, A. Anders. Genetisches Institut der Universitaet Giessen, Federal Republic of Germany

In *Xiphophorus* neoplastic transformation of pigment cells depends on the expression of the oncogene Tu. Melanoma does not develop if Tu is controlled by regulating genes (R). The R-genes can be eliminated by hybridization or impaired by mutation, permitting Tu to mediate melanoma formation. In order to distinguish between initiating and promoting effects of carcinogens, two groups of different genotypes of *Xiphophorus* have been bred. Group A: The R-genes were eliminated except for a prominent one (R-Mel) which is linked to Tu. Impairment of this R by mutation initiated melanoma formation. Group B: All R-genes including R-Mel were eliminated or impaired, respectively. Melanoma formation, however, was inhibited by introduction of a genetic block in cell differentiation which prevents precursor cells from entering the differentiation stage competent for neoplastic transformation. Agents which exert promoting effects on cell differentiation may push the precursor cells to the competent stage. Thus neoplasia develops.

We treated these two groups of tester fishes with N-methyl-N-nitrosurea (MNU), N-ethyl-N-nitrosurea (ENU), methyltestosterone, 12-O-tetradecanoylphorbol-13-acetate (TPA), phenobarbital, and cyclamate. MNU and ENU induced tumors by initiating potencies in fishes of group A (5 percent–18 percent), but exerted also slight promoting effects in group B. Methyltestosterone, TPA, phenobarbital, and cyclamate induced tumors only in fishes of group B (20 percent–100 percent), indicating that they are promoters. These substances did not show any initiating effect in group A. The results show that, by using these tester strains, one can determine whether an agent is a tumor initiator, promotor, or both.

Supported by BMFT.

The Behavior of Phenoloxidase-Containing Cells (POC) of the Connective Tissue and Blood on Glucocorticoids. H. Schmidt, Utta Wagner. Martin Luther Universität, Halle (Saale), DDR

White rats contain phenoloxidase (PO) activity possessing cells in their connective tissue and bone marrow. Under physiological conditions these do not participate in the formation of pig-
5-Azacytidine Induces Melanin and Plasminogen Activator in Amelanotic Melanoma Cells in Culture. S. Silagi, L.H. Graf, Jr. Cornell University Medical College, New York, New York

An amelanotic clone of B16 melanoma (B78H1), almost devoid of plasminogen activator (PA) activity, was pulsed for 24 hours with 5-azacytidine (AzaCr), a nucleoside analog which is incorporated into the DNA and blocks its methylation. Final concentrations of 1, 2, and 5 μM AzaCr were effective in inducing melanin in foci of 3–12 cells at a frequency of 1.5 × 10⁻² by days 8–12 after return to normal medium. No pigmented cells were seen in >10⁶ untreated cells. Selective scraping of pigmented foci for six passages over a two-month period resulted in enrichment for pigmented, dendritic cells until they comprised >90 percent of the population. During the same period, amelanotic cells were also passaged separately by scraping. Each group was then trypsinized non-selectively for seven to ten passages, and PA activity was determined by 125I-fibrin assay. Both melanotic and amelanotic cells had high PA activity, 400–800 percent greater than the parental untreated clone. One pigmented AzaCr pulsed cell line has been growing in culture for over two years and retains a pigmented dendritic morphology and high PA activity. The PA species is the tissue activator as it is in untreated PA-producing clones.

Total genomic DNA from parental clone B78H1 was heavily methylated relative to that of more differentiated clones, as measured by analysis with restriction enzymes HpaII and MspI. A single 24-hour pulse with AzaCr caused rapid and irreversible decrease in methylation. This correlated with induction of differentiated functions, i.e., the synthesis of melanin and PA and the change in morphology from an epithelial type cell to a dendritic melanocyte.

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Chemiluminescent Monitoring of Oxidative Damage During the Extraction of Pheomelanin. D. Slawinska, J. Slawinski, M.R. Chedekel. The Johns Hopkins School of Hygiene and Public Health, Baltimore, Maryland

Standard procedures for isolation of pheomelanin (PM) from pigmented tissues use strong alkali (0.1–1N NaOH) under aerobic conditions. These harsh conditions may alter the structure of the pigment and result in the production of artificial properties. In order to quantify the extent of such reactions, the chemiluminescence (CL) accompanying oxidative degradation of PM was measured under various conditions. Synthetic PM was prepared from L-dopa and L-cysteine by the standard tyrosinase-catalyzed model polymerization reaction. Dilute solutions of PM (OD₄₅₀nm <3), which do not significantly attenuate self-absorption of emitted photons, were subjected to auto-oxidation at pHs from 6.8 to 14. Low-level CL was measured using high sensitivity single-photon counting equipment. For augmentation of ultra-weak CL in the pH range 6.8–9, chemiluminojenic probes such as lucigenin and luminol at concentrations of 10–50 M were used. At pH values higher than 9, the rate of oxidative degradation of PM was high enough (10⁻⁴M⁻¹s⁻¹) to measure intrinsic CL of PM alone (I = 10⁻⁴—10⁹ photons s⁻¹). It was found that alkali pretreatment of synthetic PM leads to detectable CL even when the pigment has been reprecipitated with acid solutions, indicating an accumulation of PM decom-
position products. Evidence will be presented that hydrogen peroxide is the major contributing species to the oxidative damage of PM, and these reactions are significantly affected by the concentration of hydroxide ion. CL can be minimized at pH < 10 if the solutions are handled in the dark under a blanket of nitrogen. The ratio of the CL intensity I, measured under such conditions to the CL intensity I measured in the presence of light and air is proposed as a sensitive and rapid assay for evaluation of the degradation of PM.

**Spontaneous Remelanization of Feathers in Adult Amelanotic Dam Line Chickens.**

J. Robert Smyth, Jr., Susan J. Lamont. University of Massachusetts, Amherst, Massachusetts  Raymond E. Boissy. Yale University School of Medicine, New Haven, Connecticut  Milton L. Boyle III. University of Massachusetts, Amherst, Massachusetts

The mutant DAM chicken line is characterized by a vitiligo-like amelanosis originating as a spontaneous, postnatal destruction of both feather and uveal tract melanocytes. Our previous studies indicate the amelanosis is associated with a basic pigment cell defect, resulting in melanosomal abnormalities and a hyperactive immune system which appears to eliminate melanocytes prior to their migration into the developing feather epithelium. Subsequently, 3-4 percent of adult females with complete integumental amelanosis show the ability to melanize new developing feathers. Such remelanization is variable and temporary with amelanosis again evident before feather growth is completed. It has also been found that the incidence of remelanization is approximately 50 percent in adults following feather molt associated with termination of egg lay. This period is associated with marked hormonal changes (i.e., sex steroids and gonadotropins) and stress. It is hypothesized that the stress would depress immune system function, probably due to increased corticosteroids, and thereby temporarily reduce immune system surveillance allowing remelanization. With this in mind, cessation of egg production and extensive feather molt was induced by food and water deprivation in a group of totally amelanotic females. Immune response levels were then monitored throughout the stress-molt procedure by measuring levels of circulating antibody five days after immunization with sheep erythrocytes. Following initiation of the stress procedures, the mean antibody level of the birds which remelanized after molting was significantly lower than that of birds which remained amelanotic. Light and electron microscopic studies of remelanizing feather barb ridges showed histopathologic changes similar to those observed during the initial loss of pigment.

**Time to Death Is Inversely Related to Tumor Thickness in Clinical Stage I Melanoma.**

A. Sober, C. Day, R. Lew, M. Mihm, T. Fitzpatrick, A. Kopf. Harvard Medical School and Massachusetts General Hospital, Boston, Massachusetts; New York University, New York, New York

Primary tumor thickness is generally acknowledged as the best single predictor of overall prognosis for patients with clinical stage I melanoma. Less well appreciated is the notion that primary tumor thickness is also an excellent predictor of time to death. An inverse relationship appears to exist between primary tumor thickness and time from diagnosis to death, the thickest tumors being associated with the shortest survival.

| Thickness vs. % Survival | 25 Mos. | 50 Mos. | 75 Mos. | 100 Mos. |
|--------------------------|---------|---------|---------|----------|
| <0.85 mm                 | 100%    | 99%     | 99%     | 99%      |
| 0.85 - 1.69 mm           | 98%     | 94%     | 93%     | 93%      |
| 1.70 - 3.64 mm           | 92%     | 82%     | 70%     | 55%      |
| >3.65 mm                 | 65%     | 43%     | 38%     | 38%      |

Multivariate analyses (Cox models) have been used to determine parameters associated with early (<24 months), intermediate (24-60 months), and late (>60 months) death. Early death was most highly associated with ulcerated level 5 tumors (71 percent dead by 24 months). Thickness was less highly associated during this period with only 32 percent of patients with lesions >3.65 percent having died. For the intermediate time interval, the presence of visceral...
metastases at 24 months was associated with lethal outcome in 17/17 patients. Others at high risk were those with non-visceral recurrences (74 percent mortality) and those with the thickest tumors (>3.65 mm—42 percent mortality). Of note, late death in patients bony and visceral disease-free at 60 months was not associated with the thickest tumors (those destined to die in this group had already succumbed) but rather with the next lower risk group (1.70–3.64 mm) in which all deaths occurred (27 percent mortality from 60–100 months). Whether patients with even thinner tumors will recur and die at even greater delay cannot be determined from this data set until longer follow-up becomes available. Thickness of the primary tumor appears to reflect how far each tumor has progressed down the natural history pathway.

The Differentiation of the Outer Layer of the Optic Cup with Regard to Melanogenesis in the Black Hooded Rat (Electron Microscopic Cytochemical Investigation). P. Stanka, B. Sahlmann, G. Bargsten. Institut für Anatomie der Ruhr-Universität Bochum, Federal Republic of Germany

The electron microscopic cytochemical investigation of the black hooded rat (BDE/Han) at different ages shows the following variations of differentiation in the three regions of the outer layer of the optic cup:

In the fundus, melanogenesis is completed in the fifth week postpartum. After this time neither premelanosomes nor tyrosinase activity can be demonstrated. Melanosomes remain and they have an average diameter of 0.5 ± 0.03 μm.

The region of the ciliary body exhibits more dense pigmentation. Tyrosinase activity and premelanosomes indicate the continuation of melanogenesis. In addition to the normal melanosomes with an average diameter of 0.75 ± 0.05 μm, some round, loosely structured giant melanosomes can be found.

Melanogenesis also continues in the iris. The melanosomes, which are particularly numerous here, have an average diameter of 0.6 ± 0.02 μm. A few giant melanosomes also occur here. In the region of the Mm. dilator and sphincter pupillae cells of the pigment epithelium are differentiated to smooth muscle cells. While tyrosinase activity continues the occurrence of filament bundles and the lysosomal degradation of melanosomes can be observed.

Selective Cytotoxicity of Monoclonal Antibody Fragments Linked with 6-Mercaptopurine to Human Melanoma Cells in Vitro. Th. Stieffel, K.E. Theurer. Forschungslaboratorien für Organo- und Immunotherapie, Ostfildern, Federal Republic of Germany

Monoclonal antibodies directed against glycoprotein 97/100 of melanoma cell surfaces are separated enzymatically by pepsin digestion into F(ab)2 and Fc fragments. The identification of these fragments was performed by immunoelectrophoretic techniques. The cytotoxic purine antagonist 6-mercaptopurine was then coupled by electrophilic addition to the antibody fragments. The covalent character of the binding between 6-mercaptopurine and the antibody fragments could be demonstrated using 14C-autoradiography. The antigenic specificity of the conjugates was determined by immunoprecipitation.

Human melanoma cells, treated with these antibody fragments in vitro, show strong inhibition rates of 3H-thimidine uptake within eight hours compared to non-coupled 6-mercaptopurine. The DNA synthesis was significantly reduced to about 10 percent of the untreated control depending on the amount of antibody 6-mercaptopurine. Diploid skin fibroblasts, on the contrary, were attacked more by free 6-mercaptopurine than by the antibody-bound preparation. These cytotoxic findings could be related to the uptake of 14C-marked 6-mercaptopurine into the diploid (fibroblasts) and heteroploid (melanoma) cells.

Reinvestigation on the Formation of Dopa-Melanin. L. Stravs, H. Wyler. Institut de chimie organique, Université de Lausanne, Lausanne, Switzerland

The autoxidation of Dopa leading to melanin was reexamined with particular attention to the "non-polymeric" portion remaining in about 50 percent yield. Using different chromatographic techniques, a number of products were isolated. Their characterization and structures will be presented and their formation discussed.
Effect of UV Light on *Xiphophorus* Tester Strains for Carcinogens. J. Stute, A Wiskemann, A. Anders. Universität Hamburg, and Genetisches Institut der Universität Giessen, Federal Republic of Germany

Certain hybrids of *Xiphophorus* that contain accessory copies of an ubiquitous oncogene are highly susceptible to neoplasia, especially to melanoma. Neoplastic transformation of the pigment cells can be triggered by mutagenic (e.g., X-rays, N-methyl-N-nitrosourea, N-ethyl-N-nitrosourea, diethyl nitrosamine) and promoting (e.g., low temperature and high salinity of the water, methyltestosterone, 12-0-tetradecanoylphorbol-13-acetate) carcinogens. Three tester strains have been bred which are sensitive to (a) mutagenic carcinogens, (b) promoting carcinogens, and (c) both mutagenic and promoting carcinogens. We studied the carcinogenic effect of both UV-A and UV-B.

Philips TL 09 with a special filter was used for UV-A, and Philips TL 01, for UV-B. The fish were irradiated with 10-20 Wh/m² UV-A over 14 hours a day for 14 weeks, and with 0.1-1 Wh/m² UV-B for 1-14 hours. The fish of the tester strains that survived UV-B treatment reacted eight weeks after treatment with alterations of the pigmentation that resembled a promoting effect rather than a mutagenic effect.

Supported by BMFT.

The Efficacy of An *in Vitro* Short-Term Test and the NUNU Mice Model to Chemotherapy of Human Malignant Melanoma. J. Stute. University of Hamburg, Federal Republic of Germany

80 lymph nodes of human malignant melanomas were tested by an *in vitro* short-term test to determine the resistance of the tumors to chemotherapy since the beginning of 1980. The test was developed by Volm et al. of the German Cancer Research Centre in Heidelberg. Mechanically isolated tumor cells were incubated with the respective cytostatic agent in a waterbath for three hours. Radioactively labeled nucleic acid precursors were added during the last hour of the incubation. The incorporated activity was measured in a liquid scintillation counter. Results: 85 percent of the tumors were resistant to dacarbazine and 90 percent to vindesin-sulphate. 40 of these lymph nodes were transplanted on to NUNU mice. 26 (65 percent) showed growth of the tumors. Only 4 (15 percent) had a growth delay after treatment with dacarbazine. This means partial remission. The short-term test gave the same results. In comparison with the therapeutic results this test shows an accuracy of 75 percent with regard to sensitivity and 95 percent with regard to resistance of chemotherapy. The testing with NUNU mice estimates proliferation-dependent action of cytostatics and agrees readily with the short-term test.

Effects of Growth Factors and UVB on Human Epidermal Melanocytes in Pure and Enriched Cell Cultures. G. Szabo, E. Flynn, G. Bue, L. Wilkins. Laboratory of Electron Microscopy, Harvard School of Dental Medicine, Boston, Massachusetts

Pure and enriched epidermal melanocyte cultures have been obtained using both adult and neonatal human skin and have been maintained for over four passages, representing 13-14 cumulative population doublings. Growth of human melanocytes was greatly enhanced in a growth factor supplemented medium consisting of Medium 199 plus epidermal growth factor (10 ng/ml), triiodothyronine (10^{-6}M), hydrocortisone (5 x 10^{-4}M), insulin (10 \mu g/ml), transferrin (10 \mu g/ml), bovine brain extract (150 \mu g/ml), and 7S nerve growth factor (100 ng/ml), which reduced the serum requirement to the 2.0 percent level. Cell number and autoradiographic assays indicated that cholera toxin (10^{-4}M) added to the growth factor supplemented medium resulted in a measurable mitogenic stimulus. Comparable melanocyte growth was not achieved in medium without growth factors even when serum was added to a 10 percent level. Human melanocytes grown *in vitro* displayed a characteristic dendritic morphology and were dopa-positive. Ultrastructurally, these cells contain normal melanosomes in various stages of melanogenesis. By lowering initial epidermal call inoculation density, melanocytes were isolated in greatly enriched populations. Elimination of contaminating keratinocytes was aided by their preference for a more complex extracellular matrix such as
fibronectin, which melanocytes apparently do not need for maximal attachment and growth. The ability of melanocyte cultures to respond in vitro to known melanogenic stimuli was tested by irradiation of cell cultures with UVB. Incorporation of radiolabeled dopa into melanin/cell was found to increase twofold by 10 mJ/cm² and tenfold at a dose of 50 mJ/cm². This human epidermal melanocyte cell culture system provides an excellent experimental model for further investigations into the cell biology and photobiology of melanocytes and melanogenesis.

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Effects of UV (Excimer) Laser Radiation on Human Melanocyte Cultures. G. Szabo, O. Tan, J. Parrish, G. Bue, E. Flynn, R. Garcia. Laboratory of Electron Microscopy, Harvard School of Dental Medicine, and Massachusetts General Hospital and Harvard Medical School, Boston, Massachusetts

Human epidermal melanocyte cultures have been used to examine the cytostimulatory and cytotoxic effects of the intense ultraviolet radiation produced by excimer laser (351 nm). Melanocyte cell cultures were carried in 96-well microtiter plates at sub-confluent cell densities. Before exposure to UV laser radiation, wells were washed free of media, using PBS. A broad dose response curve was developed, using exposures of 0.01, 0.1, 0.8, and 1.0 J/cm², given in 20 nanoseconds. Wells were exposed one at a time while adjacent wells were masked. Cultures were exposed in wells placed over a black target or over a reflective foil background so as to minimize or maximize any backscatter effects. For comparative purposes, cultures of normal human dermal fibroblasts and of a human bladder tumor cell line were similarly treated. After UV exposures, cells were either fixed immediately for light and electron microscopic, qualitative and quantitative, analysis, or were refed with growth medium for fixation at later time intervals of 1, 6, 24, and 48 hours. All cells irradiated at doses greater than 0.1 J/cm² were found to be extensively damaged within one hour of exposure. Exposures of 0.1 J/cm² caused selective damage to pigmented cells only, and only in those cells that were exposed over a reflective background for backscatter augmentation. Doses of less than 0.1 J/cm² had subtler effects and at later time periods. Pigment cells surviving initial damage in culture gave evidence of a delayed pigment stimulatory response to UV radiation. In summary, an experimental system has been developed for testing effects of high intensity UV radiation on cultured cells. Under certain specific conditions, pigment cells appear to be selectively affected by the UV radiation as compared to normal and neoplastic unpigmented cells. With the establishment of threshold and dose response parameters for immediate and delayed cytotoxic effects, we are now examining the stimulatory melanogenic effects in this system.

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Fine Structural Changes of Melanogenesis in Intraepidermal Atypical Melanocytic Hyperplasia of Dysplastic Nevus. H. Takahashi, T. Horikoshi, K. Maeda, K. Jimbow. Sapporo Medical College, Sapporo, Japan

Dysplastic nevus syndrome (DNS) is a clinical and histopathological entity in which patients with DNS are at increased risk of melanoma and cells of dysplastic nevus (DN) are at great risk of malignant transformation. Histological features of DN have recently been well characterized [Elder et al: Am J Dermatopath 4:455, 1982; Rhodes et al: Lab Invest 46:69A, 1982]. Among these, intraepidermal atypical melanocytic hyperplasia is the most characteristic feature of DN [Greene et al: Lancet ii: 1024, 1980; Rhodes et al: Clin Res 30:265A, 1982]. As yet, the melanogenesis in atypical melanocytes of DN is still unclarified. This study characterizes the fine structure of melanogenesis in atypical melanocytes seen in DN which is diagnosed by light microscopy. Among 198 cases of pigmented nevi biopsied recently in our clinic, two cases were diagnosed as DNS. The intraepidermal atypical melanocytes of DN revealed distinct differences from typical nevocellular nevus (NCN). Beside nuclear atypia, dysplastic melanocytes possessed hypertrophic and well-developed dendritic processes. The cytoplasm and dendrites were full of melanogenic organelles and melanosomes which were, however, markedly deranged. The deranged melanogenesis was characterized by formation of numerous spherical-granular
(or microvesicular) melanosomes that were mostly unmelanized. Only on a rare occasion, the ellipsoidal-lamellar granules of normal melanosomes were seen. Occasionally the mosaics of spherical-granular and ellipsoidal-lamellar melanosomes were seen. Only certain foci were melanized in these mosaic melanosomes. Autophagic accumulation of melanosomes was rare. It was indicated that the melanogenesis of intraepidermal atypical melanocytes in DN is distinct and quite different from that of NCN. Ultrastructural characterization of deranged melanogenesis in DN may give a clue to define, besides nuclear atypia, the subcellular changes occurring in the melanocytes during malignant transformation.

**Genetic Studies on Tyrosinase by the Use of Monoclonal Antibodies.** T. Takeuchi, S. Sato, H. Yamamoto, J. Suzuki, K. Ishikawa. Tohoku University, Sendai, Japan; Yamagata University, Yamagata, Japan

Mutation at a structural gene results in a change in the nucleotide sequence of the DNA and consequently involves a change in the amino acid sequence of the encoded protein. Although genetic variations in proteins including enzymes can be detected at the level of their functions, preference of the variants is more precisely demonstrated by the use of electrophoresis. This physicochemical technique has been almost the sole effective means in the studies on isozymes and protein polymorphism before the recent progress in the direct analysis of nucleic acid sequence. Similarity and dissimilarity in protein structure have also been studied by the application of immunology leading to, for example, in genetics, the concept of CRM, cross-reacting material, produced by a mutant gene. With a heterogeneous mixture of antibodies, however, it was difficult to detect the dissimilarity in protein structure, although the similarity could easily be found. Fortunately, development of the technique of hybridoma has enabled us to obtain individual antibodies, monoclonal antibodies, specific for each immunogen of a protein and to demonstrate both similarity and dissimilarity in structure among variant proteins. We have made an effort to apply this sensitive tool to the genetic studies on tyrosinase, the key enzyme for melanogenesis, and attempted to raise monoclonal antibodies against tyrosinase isolated from mouse melanoma. A priori, the probability of obtaining monoclonal antibodies against mouse tyrosinase by sensitizing BALB/c mice would be low because they possess CRM of tyrosinase. We report some results of experiments with different animals and mice with different genetic backgrounds.

**Studies on Melanocyte Differentiation by Use of Monoclonal Antibodies Against Tyrosinase Isolated from Mouse Melanoma.** T. Takeuchi, S. Sato, J. Suzuki, K. Ishikawa. Tohoku University, Sendai, Japan; Yamagata University, Yamagata, Japan

In order to demonstrate immunological differences among tyrosinase proteins produced by animals with different genotypes, monoclonal antibodies were prepared by use of the hybridoma method. The monoclonal antibodies were also employed for the studies on ontogeny of tyrosinase protein during melanocyte differentiation.

Tyrosinase was purified from Harding-Passey mouse melanoma according to the method of Miyazaki and Seiji [1971]. A rat was immunized by injecting it, intraperitoneally, on day 0 and day 21 with 100 µg tyrosinase in Hanks BSS together with Freund's complete adjuvant. The booster injection was carried out on day 42 with 120 µg tyrosinase in BSS. Three days later, spleen cells were obtained from the rat and were fused with cells of P6 myeloma line in the presence of 50 percent polyethylene glycol 1500. Hybrid colonies selected in HAT medium were transferred to fresh medium and then cloned.

Whole embryos of C57BL/6J mice were frozen-sectioned. The sections were treated with the supernatant of the hybridoma cells for 24 hours at 4°C. They were then rinsed and were subjected to FITC-labeled anti-rat IgG.

Specific positive reaction was found in two of the hybridoma lines on the cells located mostly in dermis of 15.5-day embryo. Since these cells were shown to possess dendrites, they seem to represent melanoblasts. Non-specific positive reaction was also detected on cartilage cells in some hybridoma lines.
Gene Control on Pheomelanogenesis in the Mouse. H.B. Tamate, T. Takeuchi. Yamagata University, Tamagata, Japan; Tohoku University, Sendai, Japan

In the house mouse, the agouti (a) and extension (e) loci control the type of melanin produced in hairbulb melanocytes. Geschwind et al. reported that the lethal yellow (A\textsuperscript{Y}a) mice produced eumelanin in response to melanocyte-stimulating horome (MSH), whereas the recessive yellow (e/e) animals did not. To analyze the mechanism of gene control on pheomelanogenesis, comparative studies on the mode of gene action of the a and e loci were performed.

Skin explants were excised from the dorsum of 7.5-day-old neonates of B6-A\textsuperscript{Y} (A\textsuperscript{Y}a,E/E) or B6-e(a/a,e/e) strains. The explants were cultured in the presence of MSH or dibutyryl cyclic AMP (DbcAMP), and the type of melanin in hairbulbs was examined. Eumelanin production was induced in the A\textsuperscript{Y}a explant by treating it with MSH, whereas the e/e explants did not respond to MSH. On the other hand, eumelanin production was induced by DbcAMP in both genotypes. In the hairbulb melanocytes of the DbcAMP-treated e/e explants, typical eumelanosomes were observed.

Another difference in the melanogenic expression between the lethal yellow and the recessive yellow mice was detected in epidermal melanocyte population by dopa histochemistry. In the epidermis of A\textsuperscript{Y}a mice, very few dopa-positive melanocytes were observed. In the e/e epidermis, on the other hand, numerous dopa-positive cells were observed.

These results suggest that the a and e loci are acting on pheomelanogenesis in a different manner. Also suggested is a possibility that the e locus controls a mechanism involved in the function of MSH receptor.

The Mechanism of Cryosurgery on the Dermal Melanocytoses. T. Tezuka, H. Yamazaki. School of Medicine, Kinki University, Osaka, Japan

It is well known that cryosurgery is the effective treatment for nevus Ota if the color of the lesion is gray-brown, but not for blue lesions. The purpose of this investigation is to clarify this mechanism biochemically and electron microscopically, which may develop the new therapy for the deep melanocytoses and malignant melanoma.

Methods: The skins of several patients of nevus Ota and the scrotal skins of C-57 black mice as the animal model of nevus Ota, were frozen by a piece of dry ice for seven seconds and this procedure was repeated. The skins were taken at 30, 60, 120 minutes, 6, 12, 24, 48 hours, and 3, 5, 7 days after this procedure for both electron microscopic and biochemical analyses.

Results: At 30 minutes after the procedure, the cell membrane of the dermal melanocytes, melanophages, and mast cells were broken and a number of melanosomes and mast cell granules were dispersed in the collagen fibers which were not membrane-limited. At 60 minutes, no such free melanosomes were observed. After three days, both melanocytes and melanophages were under vacuolar degeneration and many melanin-laden macrophages were seen in the lymphatic vessels in the papillary dermis. Melanin granules became apparent in the regional lymph node. However, the dermal melanocytes were normal in shape, but decreased in number at five days after the procedure. The activity of the SH proteases was slightly increased at 12 hours, but that of plasmin was not increased at all.

Conclusion: The dermal melanocytes were directly damaged by the freeze and the vacuolar degeneration seen in melanocytes and melanophages could possibly be caused by proteases which were activated and released into the tissue by the freeze. The dispersed melanosomes were removed by two routes: (1) with crust, (2) being carried to the regional lymph node. As the result, the tissue melanin content was decreased.

Physiological Modulators Involved in Diamine Oxidase (DAO) Activity in Human Melanoma Cells Differing in Their Tumorigenicity in Nude Mice. N. Thomasset. INSERM U.218, Lyon, France G.A. Quash. INSERM U. 51, Lyon, France J.F. Doré. INSERM U.218, Lyon, France

Previous kinetic studies from this laboratory showed that the activity of DAO which converts putrescine into \(\gamma\)-aminobutyraldehyde in the degradative pathway of the polyamines, is increased in the highly tumorigenic lines (M\textsubscript{D}Dau, M\textsubscript{D}Beu) and that the Km value of DAO for putrescine is about three times greater in the highly tumorigenic cell lines (14 \(\mu\)m) than in the poorly tumorigenic cell lines (4,5 \(\mu\)m) M\textsubscript{D}GeB, M\textsubscript{D}Dor). To determine whether physiological
metabolites were involved in these altered kinetic data, the transamidinase pathway leading to guanidinoacetate and methyl guanidine, an inhibitor of DAO, was examined. In the poorly tumorigenic cell line M1Dor, methyl guanidine accounted for 93 percent of the metabolites formed from guanidinoacetate while in a highly tumorigenic cell line (M2Dau), methyl guanidine accounted for 44 percent.

Since methyl guanidine has been shown to be a non-competitive inhibitor of DAO, the decrease in activity of this enzyme in M1Dor may be due to the increased production of methyl guanidine through this pathway.

It is likely that the activity of DAO in melanoma cells results from interaction between inhibitors and activators since we obtained some evidence for the presence of dialyzable, non-competitive, enzymic activator in the cellular extract of melanoma cells.

The contribution of these physiological metabolites to the modification of the kinetic properties of DAO and their involvement in the malignant growth of melanoma cells is discussed.

Experimental Models to Define Heterogeneity of Malignant Melanomas in Vivo and in Vitro. W. Tilgen, D. Dzarlileva, B. Hennes, M. Engstner, S. Matzku. Deutsches Krebsforschungszentrum, Heidelberg, Federal Republic of Germany

Primary tumors, derived cell cultures, and xenografts of malignant melanomas (MM) were analyzed to study the maintainance and changes of biological characteristics on the cellular level and to delineate the validity of experimental models for pharmacological trials.

Tumor behavior was followed by immunohistology (IH) and immunoelectron microscopy (IEM) using monoclonal antibodies (MA) as well as flow cytometry (FC) and chromosomal analysis (CA). Chemosensitivity was tested in vitro and in xenografts. Melanomas in nude mice were localized with radiolabeled MA.

Ultrastructurally, melanomas exhibited distinct differences in cell morphology and pigmentation. While characteristics were well preserved in cell culture over long periods of time and even after nude mouse passage, xenografts sometimes were amelanotic. IH showed different antigenicity between the MM when tested with MA. IEM of cell cultures revealed a membrane-associated antigen and, hence, MM transplanted in nude mice could be visualized by radioimmunolocalization. In one MM FC demonstrated a distinct stem line deviation which stayed stable in vivo and in vitro. CA substantiated this finding by a modal chromosome number of 56 and three stable marker chromosomes. Cell lines and xenografts showed different susceptibility against cytostatics as demonstrated morphologically and by FC. Instability of antigen expression after treatment was pointed out by MA.

This thorough follow-up gave evidence that reliable answers to clinical questions can be provided by in vitro systems or xenografts only when applying a panel of complementary analytical methods.

The Golgi Apparatus May Play a Role in the Processing of Tyrosinase. Y. Tomita, A. Hariu, C. Kato, M. Seiji. Tohoku University School of Medicine, Sendai, Japan

In mammals, melanin formation occurs only in melanosomes within the melanocyte, where tyrosinases are most highly concentrated; however, tyrosinase activity has been demonstrated not only in melanosomes but also in microsomes.

Using a combination of radioisotope tracer techniques and immunoprecipitation, microsomal tyrosinase was confirmed as a precursor to melanosomal tyrosinase. The data indicate that it takes about one hour for de novo synthesized tyrosinase to arrive at, and be deposited within the melanosome in the Harding-Passey mouse melanoma. The Km value of melanosomal tyrosinase for tyrosine was 0.6 mM, but that of the microsomal enzyme was 1.3 mM. The Km for tyrosine of microsomal tyrosinase was lowered to that of the melanosomal tyrosinase after incubation of the microsomal enzyme with the Golgi fraction isolated from mouse melanoma. Further, the molecular weight of microsomal tyrosinase appeared to increase after incubation with the Golgi fraction, i.e., microsomal tyrosinase showed a slower mobility in SDS polyacrylamide gel electrophoresis after incubation with the Golgi fraction as compared to before incubation.

These data suggest the possibility that microsomal tyrosinase is processed in the Golgi area during transfer to the melanosome in the melanocyte.
Stereological Analysis of Lipofuscin in the Central Nervous System of *Torpedo M*.: Correlation with Superoxide Dismutase Distribution. E. Aloj Totâro, F.A. Pisanti. Università di Napoli, Italy

The distribution of lipofuscin varies in the different areas of the central nervous system of *Torpedo m*. a batoid salemchian that produces electric discharge; lipofuscin is particularly abundant in the electric lobes.

We have recently determined superoxide dismutase (SOD) activity, an enzyme that neutralizes the toxic action of the superoxide radicals, in the central nervous system of *Torpedo m*. The results of this experimental analysis showed that SOD concentration was inversely proportional to the presence of lipofuscin.

In the present work we have used an automatic image analysis system to more accurately assess the presence of lipofuscin in various areas of brain of *Torpedo m.*, and our results confirm the correlation between high lipofuscin content and low SOD activity.

Himalayan Tyrosinase is Not Temperature-Sensitive. D. Townsend, R. King, P. Guilley. University of Minnesota, Minneapolis, Minnesota

Previous work in our laboratories had suggested that the c-locus is not the structural locus for the enzyme tyrosinase as had been previously suggested. The c-locus mutant Himalayan is temperature-sensitive in regard to pigment synthesis, and it has been suggested that tyrosinase is the sensitive component. We have compared the C57BL/6J standard black mice with an isogenic strain containing the single Himalayan mutation and found the activity and stability of tyrosinase to be nearly identical. The tyrosine hydroxylase activity of the Himalayan mutation is only 3 percent that of the standard C57BL/6J; however, the thermal denaturation constant (K/min) at 60°C of 7.02 ± 0.97 and the slope of the Arrhenius plot, energy of activation −18.3 ± 0.9 kcal/mole, were nearly identical to the standard C57BL/6J with a thermal denaturation constant of 6.73 ± 0.76 and an energy of activation of −19.3 kcal/mole. These results show that tyrosinase is not the temperature-sensitive component, and that temperature sensitivity occurs prior to synthesis of the active enzyme, supporting our previous findings that the c-locus is not the structural locus for tyrosine.

The Kinetics of the Conversion of Tyrosine to “Melanin” in Several Mouse Mutants. D. Townsend, R. King, L. Zeeman. University of Minnesota, Minneapolis, Minnesota

We have used a hairbulb explant from various mouse mutants, in order to determine the influence of extracellular tyrosine on the synthesis of melanocyte melanin. The mice tested were isogenic or congenic with the standard C57BL/6J and included: lethal yellow, brown, beige, chinchilla, dilute, light ear, pale ear, and pink eye. Hairbulb explants from 14-day postplucked mice were harvested, washed with saline, and incubated with 14C-tyrosine for various periods of time. The explants were then washed with buffer and the tyrosine incorporation into melanin determined by TCA precipitation, digestion with NaOH, and scintillation counting. Hairbulbs from all mice strains will maintain a linear conversion of tyrosine to an insoluble polymer for at least 20 hours. The Km of tyrosine incorporation for this reaction is approximately 7.5 × 10⁻⁴ Molar for all mice strains and is 500 times higher than the Km for tyrosine hydroxylation (1.4 × 10⁻⁴ Molar). These results strongly suggest that a saturable transport component for tyrosine exists in the melanocyte membrane.

Studies of the Pink-Eye Mutation in C57BL/6J Mice. D. Townsend, R. King, B. Cutler. University of Minnesota, Minneapolis, Minnesota

The biochemical defect of the pink-eye mutation is unknown, but we have investigated several of the known components of melanin synthesis in this mutation. The hairbulb tyrosine hydroxylase and dopachrome oxidoreductase activity of the pink-eye mutation is nearly identical to that of wild type (≈200 moles/hour/mg protein and 7.0 μmoles/hour/cm², respectively). The Km of tyrosine by pink-eye hairbulbs for the overall conversion of tyrosine to melanin as well as for pink-eye tyrosinase is the same as the standard C57BL/6J (0.75 mMolar and 1.4 μMolar, respectively). Electron microscopic studies have also shown that the pink-eye
melanosome is structurally equivalent to the standard C57BL/6J. Yet the incorporation of the 14C-tyrosine into melanin occurs at about half the rate of the standard C57BL/6J black mouse. These data suggest that the defect in the pink-eye mutation does not involve tyrosinase or dopachrome oxidoreductase but is likely to be at a step distal to the conversion of dopachrome to 5,6-dihydroxy indole.

The Distribution Patterns of p97 Antigen on the Cell Surface of an Established Melanoma Cell Line by Means of the Ferritin-Labeled Lectin. A. Uno, I. Ando, T. Saida, Y. Hori. University of Tokyo Faculty of Medicine, Tokyo, Japan

The cell surface plays a major role in cellular functions. Sugar residues in membrane composition especially are often related to the antigen of human cancer cells. It has also been found that various antigens on human melanoma cell lines are closely related to glycoproteins. We studied ultrastructurally the distribution pattern of p97 antigen on the cell surface of an established melanoma cell line by means of the ferritin-labeled lectin. p97 is a 97,000 molecular weight cell surface glycoprotein, which is present in most human melanomas. It was demonstrated biochemically by Brown that the terminal sugar residue of p97 antigen is composed of sialic acid. We therefore examined if the distribution patterns of antigen on the melanoma cell surface could be observed ultrastructurally by using wheat germ agglutinin (WGA) which specifically binds to sialic acid. In the present study, we used a human melanoma cell line which had been established in our laboratory. This melanoma cell line was derived from axillary lymph node of a 42-year-old male patient. The primary lesion on the back of this patient had shown spontaneous regression. The presence of p97 antigen on the surface of this melanoma cell was confirmed by one PAP method using monoclonal antibody (Hybritech, Inc.)

Methods: Experiment A, melanoma cells were incubated with monoclonal antibody specific for p97, and then were fixed in Karnovsky’s mixture, reacted with ferritin-WGA conjugate, osmicated, and processed for electron microscopic observation. Experiment B, melanoma cells were only reacted with ferritin-WGA conjugate without addition of monoclonal antibody. For a control study, three lectins which bind to the other saccharid determinants were used.

In this investigation, difference in the number of ferritin particles was confirmed between melanoma cells labeled with monoclonal antibody (experiment A) and those not incubated with monoclonal antibody (experiment B).

Lectin-Binding Sites and Lectin-Binding Patterns of Human Pigment Cells. A. Uno, Y. Hori, T. Saida, K. Oohara, Y. Seki, Y. Inoue, A. Kukita. University of Tokyo Faculty of Medicine, Tokyo, Japan

The important cell surface components of all cell types are proteins, glycoproteins, lipids, glycolipids, and glycosaminoglycans. Some of these components have been implicated as antigens or other important surface recognition structures, and there have been many reports on the modifications, for example, of complex carbohydrates in tumor cells. The most prominent complex cell surface carbohydrates are the glycosaminoglycans which make up the "glycocalix" outside the integral membrane zone. Lectins are protein- (or glycoprotein-) agglutinins extracted from plants. They bind specifically to certain saccharide determinants and can be labeled to various markers such as ferritin, isotope, and peroxidase.

To investigate membranous structures and to clarify the differences among complex carbohydrates in cell membranes of several kinds of human pigment cells, lectin-binding sites and lectin-binding patterns in membranous structures were observed in human epidermal melanocytes, dermal melanocytes of blue nevus, pigmented nevus cells, and melanoma cells by using ultrastructural histochemical technique and horseradish peroxidase- and ferritin-labeled lectins—concanavalin A (Con A), ricinus communis agglutinin (RCA), and wheat germ agglutinin (WGA).

Specimens were fixed in 2.5 percent glutaraldehyde-2 percent paraformaldehyde mixture, reacted with peroxidase-lectin conjugate and then with 3,3'-diaminobenzidine (DAB) for cytochemical detection of peroxidase activities, osmicated, and processed for electron microscopic observation. Cultured pigment cells were fixed in the same mixture, reacted with ferritin-lectin conjugate, osmicated, and processed for electron microscopic observation.

Con A binding sites were observed on the plasma membrane, the membranes of endoplasmic
Autoregulation of Tieghem, M. Brussels, Laboratory of Arizona Superpotent Melanotropins Purification of Tyrosinase 428 Prague, heim, J. Woods, M. E. Hadley, B. C. Wilkes, V. J. Hruby. University of Arizona, Tucson, Arizona

α-Melanotropin (α-MSH), a tridecapeptide, synthesized and secreted by the pars intermedia of the pituitary, controls integumental pigment production in many vertebrate species. Studies of the action of α-MSH on skin melanogenesis in mammals have been limited to but a few in vitro and in vitro models. α-MSH used at extremely high concentrations for prolonged periods will stimulate follicular melanogenesis in the mouse. Superpotent melanotropins that are resistant to enzymatic inactivation both in vivo and in vitro have now been synthesized in our laboratory. We report here that these α-MSH analogs provide powerful probes for studying pelage pigmentation and related hair growth phenomena. A single injection of [Nle⁴, D-Phe⁶]-α-MSH or Ac[Nle⁴, D-Phe⁶]-α-MSH₄₁₈₁₁ₙH₄ into the mouse (C57BL/6J.A/J) can induce a band of melanogenesis in all body hairs if delivered at a specific time in the hair growth cycle. At a similar concentration an injection of the native hormone, α-MSH, is ineffective. Repeated injections (nine to ten days) of the analogs cause complete melanogenesis of all new hairs in plucked areas of the skin. Most important, we have noted melanin synthesis in response to our melanotropins is sex-dependent. Females are more responsive to melanotropin stimulation. Patterns (waves) of pelage pigmentation can be induced, and it is possible that our melanotropins may be used to permanently mark (by melanin production) all body hairs at a particular time of the hair cycle. Superpotent melanotropins with prolonged (enzyme resistance) biological activity may then provide tools for detailed studies of mammalian hair growth and the possible relationship of gonadal steroids to melanogenesis.

Purification of Tyrosinase Isozymes and Their Survival in Rat Serum. J. Vachtenheim, J. Duchoň, B. Matouš. Faculty of General Medicine, Charles University, Prague, Czechoslovakia

The two isozymes of tyrosinase (EC 1.14.18.1) have been purified from pigmented hamster melanoma: sialylated (T₁) and asialylated (T₂). Asialo-T₁, tyrosinase was also prepared by the neuraminidase treatment. These three isozymes migrated with Rᵢ values 0.50, 0.38, and 0.34, respectively, in polyacrylamide gel electrophoresis. The isozymes were further injected intravenously into rats and dopa oxidase activity in rat serum was followed at various time intervals. It was clearly seen that while T₁, tyrosinase remained in circulation and its activity in serum decreased slowly during one hour, T₂ and asialo-T₁ tyrosinase rapidly disappeared from circulation in a few minutes. Asialofetuin inhibited the removal of asialo-T₁ tyrosinase, suggesting that desialylated tyrosinase is trapped into the liver via common hepatocyte receptors for asialoglycoproteins.

Tyrosinase thus shares, as can be judged from these observations, the pathway for the removal from plasma known for the majority of serum glycoproteins. The removal of sialic acid and exposure of terminal galactosyl residue is decisive for the clearance from circulation. Supporting this, tyrosinase isolated from sera of melanoma-bearing hamsters exhibited positive dopa oxidase band in polyacrylamide gel electrophoresis that corresponded to the T₁ form of tyrosinase.

Autoregulation of Final Differentiation in Human Malignant Melanocytes. N. Van Tieghem, M. Fooij, F. Legros, J. M. Prevost. Laboratory of Microbiology and Laboratory of Physiopathology, Faculty of Medicine, Université Libre de Bruxelles, Brussels, Belgium

Permanent cell lines from human metastatic melanomas have been established. Some cultures are fully differentiated in terms of melanin production. Other cultures consist of fibroblast-like cells. The differentiated melanoma cell lines are maintained using culture condi-
tions where tryosine and serum concentrations are controlled. All of the differentiated cultures were found to be receptive to α-MSH. Fibroblast-like cells showed detectable receptors in seven out of 23 cultures. Tyrosinase activation followed by inhibition of DNA synthesis with concomitant detection of retroviral markers could be induced by the hormone.

Some cell lines lost hormonal receptivity during subculture and they did not show any growth inhibition in response to the hormone. These cells were examined in a protein-A radioimmunoassay for surface α-MSH and α-MSH specific receptors. The assay revealed that these cells have various levels of α-MSH-like molecules and hormonal receptors on the plasma membrane, whereas other melanoma cells and other tumor cells lack surface MSH-related substances. These molecules sufficiently mimic the hormone by cross-reacting immunologically and interacting at the receptor level. Moreover, serum-free conditioned medium obtained from non-receptive cells was able to react with hormone-receptive cells.

These cells might be expected to have an escape mechanism against normal terminal differentiation (leading to cell death) through blockage of their own melanin-stimulating hormone receptors by secretion of substances that act indirectly as mitogens.

This may reflect an inappropriate later expression of differentiation inhibitors that were required by the melanoblasts during normal early embryogenesis when they migrate into the cutis before reaching the epidermis where they differentiate into melanocytes.

Establishment of Primary Cultures of Malignant Pigment Cells and Role of Culture Medium’s Composition for Expression of Specific Biological Properties. N. Van Tieghem. Laboratory of Microbiology, Faculty of Medicine, Université Libre de Bruxelles, Belgium

To obtain successful malignant pigment cells cultures, one must keep in mind that:
1. During the melanization process, pigment cells produce substances that are autotoxic.
2. Pigmented cells are killed when exposed to excess tyrosine or dopa in the culture medium.
3. Among the naturally occurring amino acids, only tyrosine, tryptophan, and dopa are toxic to pigmented cells.
4. The toxicity of these compounds is proportional to the amount of tyrosinase activity within the cells.

For these reasons HAM F10 medium that is low in tyrosine concentration (1.81 mg/l) was chosen to initiate primary cultures.

Melanoma specimens direct from the surgery were transported to the tissue culture laboratory in sterile flasks containing HBSS, 10 percent FCS, penicillin 100 U/ml, amikacin 50 μg/ml. Tumor tissue was minced into cubes of 1 mm³ and placed in 75 cm³ plastic tissue culture flasks (NUNC). Different areas of the tumor were sampled and cultured separately to obtain at least one culture in which the melanocytes were present in a larger number than the fibroblasts or fibroblast-like cells. The cultures were incubated in HAM F10 medium supplemented with penicillin 100 U/ml and streptomycin 100 μg/ml, at 37°C in humidified atmosphere supplied with a constant amount of 5 percent CO₂ in air. Cultures were trypsinized when needed either to be subcultivated or to insure a better dispersion of the cells and to free them from macrophages and contaminating fibroblasts. Pure pigmented malignant melanocytes were isolated through their capacity to form colonies. The cells are used in studies on karyology, MSH-receptors, growth, and pigmentation as compared to normal fibroblasts isolated in MEM medium.

Antimitotic Agents and Melanosome Migration in the Melanophores of a Teleost Fish. M.A. Visconti, A.M.L. Castrucci. Instituto de Biociências, USP, São Paulo, Brazil

The effects of cytochalasin B (2.10⁻⁴M), colchicine, and vinblastin (10⁻², 10⁻⁴, and 10⁻⁶M) on melanosome migration of *Papillicromis ramirezi* melanophores were studied. Scales were incubated first in reserpine 4.10⁻⁴M for 15 minutes and then in the anti-mitotic drug for 30 minutes. Their effects on the aggregation or dispersion time of melanosomes were checked employing norepinephrine 10⁻⁴M and theophylline 10⁻⁴M, respectively. Cytochalasin B had no effect on melanosome movements. Colchicine 10⁻⁴M delayed the aggregation and accelerated the dispersion time. At 10⁻⁴ and 10⁻⁶M it had no effects on melanosome movements. Vinblastin
10^{-5}M induced, per se, aggregation on 30 percent of the melanophores. When these scales were transferred to theophylline these melanophores dispersed move slowly than the control, and when transferred to norepinephrine, the aggregation was blocked. Vinblastin 10^{-4}M significantly delayed the aggregation induced by norepinephrine in 50 percent of the melanophores and blocked this process in the other. The dispersion of these aggregated melanophores, induced by theophylline, was not altered. Vinblastin 10^{-5}M had no effects. As the incubation time in colchicine does not seem long enough for the depolymerization of the microtubules, the effects induced by the drug are probably due to their interaction with the plasma membrane or the stimulus on the AMP cyclic production. The results obtained with vinblastin suggest the involvement of microtubules in the melanosome translocations, mainly the aggregation. If microfilaments are also involved in the melanosome movements, they must not be actin or actin-like, since cytochalasin B had no effects on pigment granule displacements.

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**Pigments and H_2O_2 Production: Consequences on the Normal and Transformed Cells' Metabolism.** Monique Vuillaume, Max Goyffon, Martin Best-Belpomme. Laboratoire Zoologie E.N.S., Laboratoire LERAI M.N.H.N., Laboratoire Biologie cellulaire Université Paris, Paris, France

A significant fraction of the biological reduction of oxygen occurs by a pathway involving intermediates such as H_2O_2, which is a dangerous reactant.

We showed that:
1. pterobilin which is synthesized and laid down in insect integuments reduces O_2 into H_2O_2;
2. hemocyanin (blood oxyphoric pigment of scorpions) has a catalatic activity thus showing the scorpion's natural radioresistance to ionizing rays;
3. the energy of H_2O_2 disproportion by catalase is used to produce ATP;
4. in *Xeroderma pigmentosum* or skin UV-dependent cancers we demonstrated an increasing accumulation of UV-H_2O_2 production and a parallel breakdown of catalytic activity. When catalytic activity becomes undetectable the epitheliomas are becoming apparent.

We analyzed these results in terms of a possible biological model of the disproportion of hydrogen peroxide cell production. So, this toxic product is immediately disproportioned by catalase and the energy liberated may be used in the metabolism.

In case of dysfunction of "H_2O_2-catalytic activity" we obtain pathological cell phenomena.

**Establishment and Characterization of Four Cell Lines Derived from Hereditary Melanomas in Xiphophorus Fish Hybrids.** Y. Wakamatsu. Yoshida College, Kyoto University, Kyoto, Japan. A. Oikawa. Research Institute for Tuberculosis and Cancer, Tohoku University, Sendai, Japan M. Obika. Keio University, Yokohama, Japan T. Hirobe. Iwate University, Morioka, Japan K. Ozato. Yoshida College, Kyoto University, Kyoto, Japan

Hereditary melanoma in interspecific hybrids of *Xiphophorus* fish is one of the *in vivo* systems in which the presence of cellular oncogenes and the regulatory mechanisms of their expression have been elucidated by Mendelian genetics. As material to analyze this system by molecular genetics, four melanoma cell lines, including two sub-lines, were established and characterized in morphology, cytochemistry, biochemistry, growth properties, chromosomal constitution, and transplantability. Original melanoma tissues were obtained from old F_1 hybrids and cultured in Eagle's MEM containing 10 percent FCS. Cells of four cell lines were amelanotic and grew in a criss-cross fashion. In regard to cell differentiation, three of these cell lines were composed of melanoblast-like cells and one contained many melanocyte-like cells in addition to melanoblast-like cells.

**Melanoblast- and Melanocyte-Like Cell Lines Derived from Platyfish-Swordtail Hybrid Melanomas.** Y. Wakamatsu. Yoshida College, Kyoto University, Kyoto, Japan A. Oikawa. Research Institute for Tuberculosis and Cancer, Tohoku University, Sendai, Japan M. Obika. Keio University, Yokohama, Japan
T. Hirobe. Iwate University, Morioka, Japan  K. Ozato. Yoshida College, Kyoto University, Kyoto, Japan

Four melanoma cell lines and sublines (PSM-1a, PSM-1b, PSM-2, and PSM-3) were established from nodular melanomas in old F, interspecific hybrids between commercially available platyfish (Xiphophorus maculatus) carrying the Sp gene and swordtails (X. helleri). These cell lines were characterized in their morphology, cytochemistry, biochemistry, growth properties, chromosomal constitution, and transplantability. PSM-1a, PSM-2, and PSM-3 were similar to each other. They were composed of amelanotic bipolar cells which grew in a criss-cross fashion, and showed a low tyrosinase activity. The cells had numerous vesicles containing amorphous substances and showing a weakly positive dopa reaction, and exhibited a positive combined dopa-premelanin reaction. These vesicles seemed premelanosomes. Thus, these cells were considered to be melanoblast-like cells.

PSM-1b was considerably different from these cell lines. This cell line was composed of amelanotic dendritic cells which grew in a criss-cross fashion, and showed a high tyrosinase activity. They contained many premelanosomes with inner striated filaments. An apparently positive dopa reaction was found in these premelanosomes and Golgi-associated vesicles and cisternae. Thus, these cells were considered to be melanocyte-like cells.

Monoclonal Antibodies Against Pigment Cell Cytoskeletal Proteins. Gary R. Walker, Jiro Matsumoto, Thomas J. Lynch, John D. Taylor, T.T. Tchen. Wayne State University, Detroit, Michigan

It has been established that the GEM-81 erythrophoroma line contains all the major cytoskeletal proteins present in the normal goldfish xanthophore from which the tumor cells arose. We have therefore used Triton-insoluble cytoskeletal preparations from GEM-81 cells as antigens and raised monoclonal antibodies against them. When hybridoma cultures are screened by immunoblotting replicas of SDS gels, the most prominent antibodies are those directed against the major structural proteins such as actin and tubulin. However, antibodies are also produced against at least fifteen of the minor cytoskeletal peptides. Most antibodies so produced cross-react with their respective antigens in normal xanthophores and can therefore be used as immunocytochemical probes of the distribution of these proteins in the normal and neoplastic cells and in the normal xanthophores during pigment translocation. A number of monoclonal antibodies recognize more than one cytoskeletal peptide, suggesting that common antigenic determinants are shared by several cytoskeletal proteins.

Production and Characterization of Monoclonal Antibodies to Xanthophore Cytoskeletal Proteins. Gary R. Walker, Thomas J. Lynch, John D. Taylor, T.T. Tchen. Wayne State University, Detroit, Michigan

Monoclonal antibodies are valuable not only as immunocytochemical probes but as highly specific intracellular inhibitors. We have used a line of erythrophoroma cells (GEM-81), derived from goldfish xanthophores, to produce monoclonal antibodies against the Triton-insoluble cytoskeletal proteins. Since the major proteins are shared by the tumor cells and normal xanthophores, the GEM-81 line provides a convenient source for this material. Furthermore, the cytoskeletal preparations are directly adaptable to a solid-phase assay (ELISA) used throughout the procedure to detect antibody-producing hybridoma colonies and clones. A total of sixty-six hybridoma colonies have been produced, most of which contain antibodies directed against three to ten cytoskeletal antigens. Colonies are selected for cloning by a qualitative immunoassay, bi-directional immunoblotting, the results of which can be related to the peptide patterns seen on two-dimensional electrophoresis gels.

Association of 125-I Labeled α-MSH to Human Melanoma Cells and Skin Fibroblasts. K. Wallevik, H.I. Nielsen, The Finsen Laboratory, The Finsen Institute, Copenhagen, Denmark

The specificity of binding of α-MSH to pigment cells as well as its possible internalization by endocytosis is not only essential for the understanding of the physiology of α-MSH but also of interest for the treatment of malignant melanoma. Both the direct effect of α-MSH on the
growth and differentiation of melanoma cells and the possible use of \( \alpha \)-MSH as a carrier for cytostatic drugs need to be thoroughly investigated.

\( \alpha \)-MSH, labeled with \(^{125}\text{I}\) by the iodogen method, associate during the first 24 hours linearly to melanoma cells with an "endocytic clearance" of 125-200 fl/cells/hours. The rate of association to fibroblasts is only 35 fl medium cleared of \(^{125}\text{I}\) \( \alpha \)-MSH/cell/hour. However, there is no significant difference in the rate of cell association of 4\(^\circ\) and 37\(^\circ\)C and there is no competition for "binding sites" with non-labeled \( \alpha \)-MSH.

When cells exposed to \(^{125}\text{I}\) \( \alpha \)-MSH for 24 hours are transferred to medium containing no \( \alpha \)-MSH, the radioactivity is dissociated from both cell types with a half-life of less than 15 minutes.

After prolonged exposure of the melanoma cells to \(^{125}\text{I}\) \( \alpha \)-MSH we could not demonstrate the labeled hormone inside the cells by autoradiographic techniques and there is only a non-significant proteolytic decomposition of the labeled hormone, down to its amino acid constituents.

The present experiments indicate neither specific binding of the \(^{125}\text{I}\) \( \alpha \)-MSH to the melanoma cells nor internalization by endocytosis. The nature of the linear association with time of the \(^{125}\text{I}\) \( \alpha \)-MSH to both melanoma cells and fibroblasts cannot be explained by the available experiments.

**12 Cases of Malignant Melanoma on Small Congenital Nevus.** F. Weidner. Dermatology Hospital, Stuttgart, Federal Republic of Germany O.P. Hornstein. University Hospital, Erlangen, Federal Republic of Germany

Report on ten males and two females (out of 298 melanoma patients; i.e., 0.4 percent) demonstrating malignant melanoma on small congenital nevus (diameter of nevus below 1 cm: one; between 1 and 3 cm: five; between 3 and 10 cm: five; and one of multi-spotted type). Anatomical localization due to sex distribution, mainly on the trunk. Age distribution from 29 to 79 years.

Congenital nature of the nevi was suspected by patient's history and confirmed by histology; in each of the cases there was an isolated horizontal band of subepidermal nevus cells. Vertical orientation in periphery of pilosebaceous follicles, eccrine ducts, vessels, and/or nerve bundles was seen in five cases, three belonging to nevi larger than 3 cm in diameter. According to the literature, deep, dermal, or subcutaneous infiltration by nevus cells cannot be expected in small congenital nevi, whereas this is usually the case in giant congenital nevi. Different from common skin melanoma, histological tumor type disclosed relatively more NM (6) than SSM (4), and 2 UCM. Malignancy exceptionally was found related to the epidermis, superimposing the dermal nevus band or penetrating it from upward. The nevi did lack any cellular inlamation, as did also three of the melanomas.

Our experience proves that malignant transformation in small congenital nevi (below 10 cm diameter) is not so rare as might be suggested, and that preventive measures should also include small congenital nevi of multi-spotted type.

**RNA Proteoglycolipid from Melanoblastoma Serum Transforms Human Bone Marrow Reticulum Cells.** A.J. Wieczorek. Medizinische Poliklinik der Universität München, Federal Republic of Germany

RNA proteoglycolipid is a pleomorphic serum particle floating in a sharp opalescent band between the low-density and the high-density (HDL) lipoproteins in KBr density-gradient ultracentrifugation [Wieczorek, 1983]. It represents a complex of 27S RNA and proteolipid-bound glycosphingolipids and occurs in various types of neoplasia including melanoblastoma.

If human bone marrow cells are incubated in RPMI 1640 medium, enriched with 20 percent serumless medium of Neuman and Tytell and 15 percent fetal calf serum in Falcon plastic flasks, reticulum cells will settle on the plastic and grow in monolayer. Subconfluent monolayers of these cells were incubated with RNA proteoglycolipid at a concentration of 1 \( \mu \)g RNA per ml medium for two days, after which the medium was changed every two days. Within one week a dramatic change in the morphology of the cells was noticed, including polymorphism of cell shape, nuclei and nucleoli, and a rise in the proliferation rate.

This phenomenon was reproducible with RNA proteoglycolipid from three cases of melanoblastoma and suggests that the particles are involved in the development of metastases.
Fine Particle Content in Synthetic L-Dopa Melanins. T. Wilczok, K. Stepień, D. Vučelić. Institute of Medical Chemistry and Physics, Sosnowiec, Poland; Institute of General and Physical Chemistry, University of Belgrade, Yugoslavia

Melanin samples synthesized from L-dopa/1 mg/ml, total volume 500 ml, aerated by air, 0.067 M Na₂/KH₂PO₄, buffer, pH 8.0/incubated at a temperature of 20-22°C, were centrifuged at 50,000, 90,000, 120,000, and 192,000 g by use of a Beckman LS-75 centrifuge at 4°C, washed twice with de-ionized distilled water, centrifuged repeatedly at given g, then suspended in small quantities of water, and used for measurements. Infrared spectra, slow thermal NMR relaxation, DSC, EPR measurements as well as pyrolysis-gas chromatography-mass spectroscopy were carried out, as was Mössbauer spectroscopy of melanin. Fe complexes were performed. It was shown from the obtained data that, depending on size and shape of the melanin particles isolated from the heterogeneous suspension, the physicochemical characteristics do not show large and significant changes, but the Py-GC-MS data show quantitative differences in monomer compound content between the analyzed samples: slow increase of thermal relaxation times and differences in Fe²⁺, Fe³⁺ content in melanin. Fe complexes, free of FeOH₃ were shown when analyzed by the use of Mössbauer spectroscopy. When aggregated during precipitation or obtained after evaporation of solvent followed by dialysis against water, melamins behaved like granules with local crystalline or paracrystalline centers [Shin-Shin Chio, 1977] with a super-paramagnetic character. These findings support the idea that fine particles present in melanin granules are responsible for the observed relaxation phenomena and that during melanin synthesis the fine particles present in the solution aggregate into large granules which affect the measured parameters.

Morphoquantometric Analysis of Melanosomes from Eyes of Guinea Pigs and Cattle. T. Wilczok, B. Atfasik, M. Krystek, K. Stepień, R. Wacławek. Institute of Medical Chemistry and Physics, Sosnowiec, Poland

Melanosomes from inbred black guinea pig eyes, left and right, separately, were isolated from the iris and ciliary body and from choroid together with retinal pigment epithelium by centrifugation, first at 150 g and later at 1,700 g. When cattle eyes were used the retinal pigment epithelium was separated from the choroid and the melanosomes isolated as described by Borovansky, Hack, and Duchon [1977]. The size and shape of the isolated melanosomes were described by nine morphometric parameters and the internal extinction distribution and local absorption by twelve extinction parameters by the use of the computing scanning microscope Morphoquant. The statistical significance of each parameter was calculated from 300 single measurements and histograms were prepared for comparison and analysis. It was shown that analyzed melanosomes when isolated from eyes of prenatal fetus, after breeding, during the first month and after 3, 5, 6, 9, 12, 16, and 20 months of life, demonstrate large differences during the lifetime and also differences depending on the origin. The largest differences in both size and shape as well as in internal extinction distribution were shown in the early steps of development, mainly in melanosomes isolated from choroid and retinal pigment epithelium. Also the left and right eye of the same individual show differences in size distribution of melanosomes evident when histograms are compared. The differences are more significantly expressed in morphometric than in internal extinction parameters.

X-Linked Micromelanosomal Ocular Albinism. C. Witkop, R. King. University of Minnesota, Minneapolis, Minnesota M. Efron. Columbia, South Carolina R. Lebovitz, R. Pauli. University of Wisconsin, Madison, Wisconsin

Two extensive kindreds showing X-linked inheritance of ocular albinism were investigated in which affected males showed normal pigmentation of hair and skin and moderate tanning, but had congenital nystagmus, photophobia, absent fovial reflex, marked hypopigmentation of the fundi, blue-grey diaphanous irides with a cartwheel accumulation of pigment on transillumination, and best corrected visual acuities of 20/100 to 20/150. In contrast to previously described X-linked ocular albinism [O'Donnell, et a; Arch Ophthalmol 94:1883, 1976] in which both hemizygotes and heterozygotes showed macromelanosomes in skin biopsies, serial light and ultra-thin EM sections had no macromelanosomes. Melanocytes contained small (X 0.27 µm Range 0.20-0.30 µm by X 0.11 µm Range 0.08-0.17 µm) micromelanosomes and kera-
Enzymatic Sciences, University Wittenberg.

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tinocytes contained complexes (X 0.34 μm Range 0.26–0.60 μm by X 0.28 Range 0.18–0.39 μm) containing 12 to 14 micromelanosomes. In one kindred, partially reproduced here, tyrosinase-positive oculocutaneous albinism also segregated.

The effects of various combinations of the X-linked micromelanosomal gene and the tyrosinase-positive oculocutaneous albinism gene on melanosomal morphology and hairbulb tyrosinase activity are illustrated.

Two-Step Regimen for Individualization of Malignant Melanoma Chemotherapy.
K.-D. Wozniak, W. Wohlrab, R.-P. Zaumseil. Martin Luther University, Halle-Wittenberg. German Democratic Republic

The clinical course of malignant melanoma frequently shows generalized metastasizing in a short time, and thus places, at present, the adjuvant therapy after removal of the primary tumor in the center of clinical and experimental examinations. Considering that each tumor possesses a different sensibility against the various cytoplastic drugs, the determination of the individual situation of the single tumor is decisive in the form of the therapy. Based on the pre-therapeutic test of sensibility of tumor cells and the proliferating date of melanoma cells, we apply chemotherapeutic drugs in a two-step regimen for individualization of adjuvant chemotherapy of human malignant melanoma. The clinical results of this regime of individualization of malignant melanoma chemotherapy will be discussed.

Enzymatic and Immunological Evaluations of Tyrosinase Expression in Pigment Mutants of the Fowl. H. Yamamoto, A. Churilla, J. Brumbaugh. School of Life Sciences, University of Nebraska, Lincoln, Nebraska

It has been difficult to obtain suitable quantities of tyrosinase from normal tissues in higher animals. Except for cytochemical observations, little was known about tyrosinase in the fowl. This study characterized tyrosinase and evaluated its expression, at a molecular level, in various pigment mutants of the fowl.

Comparison of tyrosinase activities in regenerating feathers from the pigment mutants showed various specific activities, including some showing no activity. Sex-linked albinos (s♂) had the highest specific activity, even greater than wild type, so birds of this genotype were used for enzymatic purification. About 80 percent of the total activity from cell homogenates was localized in the insoluble fraction. At least nine different forms of tyrosinase were present as displayed by isoelectric focusing.

Trypsin-cleaved, enzymatically active tyrosinase was purified from the sal mutant. The molecular weight was estimated at 66,000 daltons. Treatment with neuraminidase indicated that the enzyme is a glycoprotein.

Antiserum and monoclonal antibody against the purified enzyme were prepared. These antibodies allowed us to categorize our pigment mutants into three groups. Group I mutants are like wild type and are tyrosinase-positive and cross-react with the anti-tyrosinase antibodies. These mutants apparently do not alter the enzyme. Group II mutants are tyrosinase-negative and do not cross-react with the antibodies. Such mutants apparently severely alter the enzyme or do not synthesize it. Group III mutants are tyrosinase-negative but apparently cross-react with the antibodies. Such mutants make an immunologically recognizable molecule which has lost its enzymatic activity.

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Control of Melanogenesis in the Interspecific Hybrid Cells and Reconstituted Cells.
N. Yanai, T. Takeuchi. Tohoku University, Sendai, Japan

In order to investigate the control of melanogenesis in melanocytes, hybrid cells and reconstituted cells were formed from chick embryonic cells and mouse B16 melanoma cells.

Hybrid cells were formed by fusing various types of chick embryonic cells with slightly pigmented mouse melanoma cells. Reconstituted cells were formed by fusing chick cytoplasts with mouse melanoma nucleoplasts. The cytoplasts were derived from the retinal pigment epithelium, retinal neurocytes, and heart fibroblast cells. The nucleoplasts were derived from less or highly pigmented mouse melanoma cells. In the case of highly pigmented melanoma cells, almost all reconstituted cells expressed tyrosinase activity more than the parental cells, whereas in the case of less pigmented melanoma cells, about 80 percent of hybrid clones and reconstituted cells exhibited the suppressive tyrosinase activity. It seems that chick cytoplasmic factors are not effective on the control of melanogenetic function of highly melanotic nuclei, while they suppress the nuclei derived from slightly pigmented melanoma cells. Variety of chick embryonic tissues led to a little difference in melanogenic function. Therefore, the melanogenic function is likely to be generally donated by the nuclei, but is also affected by foreign cytoplasmic factor. Since the melanin content of hybrid cells and reconstituted cells was not paralleled by the tyrosinase activity, there seems to be a regulative mechanism of melanogenesis in addition to the control mechanism of tyrosinase activity.

Effect of Chlorpromazine or Resochin (Chloroquine) on the Growth and Some Lysosomal Enzymes Activity of B16 Melanoma in Mice. M. Zarach-Krutysza, G. Drewa, Z. Zbytniewski. Medical School, Gdańsk, Poland

183 male, inbred mice were transplanted subcutaneously with B16 melanotic melanoma tissue. Mice were divided randomly into three groups and from the fourth day after the melanoma transplantation were injected once daily during the following 15 days as follows: (1) chlorpromazine, 10 mg/kg body weight; (2) chloroquine diphosphate, 10 mg/kg body weight; (3) equivalent volumes of saline. 20 days after the melanoma transplantation mice were sacrificed, tumors were weighed, activity of arylsulphatase, β-glucuronidase, acid phosphatase, and cathepsin D were assayed in the tumors' homogenates.

Chlorpromazine inhibits by 54 percent and chloroquine by 64 percent the melanotic melanoma growth. Activity of arylsulphatase, β-glucuronidase, and cathepsin D significantly increased in both experimental groups. The activity of acid phosphatase was significantly higher in the chlorpromazine-treated group and lower in the chloroquine-treated group as compared to the control group.

Effect of Retinoic Acid on the Growth and Dissemination of Melanotic and Amelanotic Melanoma in C57BL Mice. Z. Zbytniewski, A. Kanclerz, G. Drewa. Medical School, Gdańsk, Poland

63 inbred, male C57BL mice were transplanted subcutaneously into the tail with B16 melanoma tissue. Beginning one day after the tumor transplantation, during the following 30 days mice were given once daily intraperitoneally 1 mg/kg body weight of retinoic acid dissolved in O1. Lini. Control group I was given intraperitoneally O1. Lini and control group II saline only. 31 days after the tumor transplantation, tails with melanomas were amputated and weight of tumors was estimated. 28 days later mice were sacrificed and pulmonary and extrapulmonary metastases were estimated.

The mean weight of the melanotic melanoma in the retinoic acid-treated group was by 46.5 percent, and in the control O1. Lini-treated group by 40.5 percent lower than in the control saline-treated group. The incidence of pulmonary and extrapulmonary metastases did not differ significantly between the experimental and both control groups.

Retinoic acid inhibited the amelanotic melanoma growth by 25.9 percent. The mean weight of the amelanotic melanoma in the O1. Lini-treated group was inhibited by 74.3 percent. There were no lung metastases in the O1. Lini-treated group, whereas in the retinoic acid-treated group the incidence of metastases was as high as in the control saline group.
Our results suggest that retinoic acid inhibits the growth of primary melanotic and amelanotic melanoma in mice. It seems that the most potent agent which inhibits the growth as well as the spreading of melanomas is Oleum Lini per se.

**The Multifunctional Aspect of Pterins: Pigments, Cofactors, and Regulators of Cell Proliferation.** I. Ziegler. Institut für Toxikologie und Biochemie der Ges.f.-Strahlen-und Umweltforschung, München, Federal Republic of Germany

Some anabolic pterins (e.g., sepiapterin, drosocin) are known to occur as pigments and are bound to ribonucleoprotein-containing granules. For instances, those pigment granules or "pterinosomes" are found in Drosophila eyes or in amphibian skin. Some crystalline pterin catabolites (e.g., xanthopterin, leucopterin) are typically deposited in integumental structures as are the wings of Pieridae.

Throughout the animal kingdom the cofactor role of tetrahydrobiopterin for aromatic amino acid hydroxylation is well established.

Recently it has been shown that pterins are regulators of cell proliferation in that they fulfill the criteria for lymphokines [Ziegler et al: Chemistry and Biology of Pteridines. Berlin, de Gruyter, in press]. They are produced during hematopoietic cell proliferation and especially in leukemic cell cultures, at a high rate. Vice versa, they specifically regulate the activation of lymphocytes. Tetrahydrobiopterin, dihydrobiopterin, and sepiapterin are co-stimulators during Con A-induced T-cell activation, whereas xanthopterin and isoxanthopterin are inhibitors of cell proliferation. Thus the control system is governed by the equilibria present between the various anabolic and catabolic steps. Kinetic studies indicate that their target is RNA synthesis.

Syntopically, pigmental pterin represents the final goal at adult stage after having passed cell regulatory functions during developmental stages with high rates of cell proliferation.

**The Yellow Pigment Granules (Cytosomes) of Molluscan Tissues and Their Possible Role in Facultative Anaerobiosis.** I. Zs.-Nagy. University Medical School, Debrecen, Hungary  
D.A. Holwerda, D.I. Zandee. State University of Utrecht, Utrecht, The Netherlands

Many molluscan tissues, especially the nerve cell somas, contain yellow pigment granules called cytosomes. The cytosomal pigment is lipochrome-containing carotenoids. A correlation has been observed between the degree of pigmentation of the nervous system of molluscs and their anoxic tolerance: the species having no cytosomes in the nervous system are unable to survive anoxic conditions, whereas those with strongly pigmented nerve cells can tolerate complete environmental anoxia for day- to week-long periods at room temperature without any essential damage (facultative anaerobes).

Characteristic ultrastructural, histochemical, and biochemical alterations have been observed in the cytosomes of several molluscs during their anaerobic incubation. Details of findings have been summarized earlier, suggesting a mechanism of anaerobic energy production in the cytosomes called anoxic endogenous oxidation [Zs.-Nagy: Internat Rev Cytol 49:331-337, 1977]. This hypothesis assumes that the terminal electron acceptor function of molecular oxygen is replaced during anaerobiosis by some kind of internal electron acceptor which may be searched for among the unsaturated fatty acids of the cytosomal lipid pool. Numerous indirect evidences support this assumption.

Recently a series of biochemical experiments were performed on the cytosomal tissue fractions of the ganglia of *Anodonta cygnea* in order to reveal more characteristics of the cytosomal system and learn more about its role in the anaerobic ways of energy production during facultative anaerobiosis.