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Bibliographic Review

The Pigmented Life of a Redhead

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As a redhead I have had a personal interest in red hair, freckles and sunburns since childhood. An observation of a formaldehyde-induced fluorescence in human epidermal melanocytes initiated my scientific interest in these cells. Prota and Nicolaus demonstrated that oxidation products of cysteinyl dopas are the main components of pheomelanin. Our identification of 5-S-cysteinyl dopa as the source of formaldehyde-induced fluorescence of normal and pathological melanocytes started a series of investigations into this amino acid, enzymatic and non-enzymatic oxidation of catecholic compounds and the metabolism of thiols. All melanocytes with functioning tyrosinase produce cysteinyl dopas and the levels of 5-S-cysteinyl dopa

in serum and urine are related to the size and pigment forming activity of the melanocyte population. The determination of 5-S-cysteinyl dopa in serum or urine is a sensitive diagnostic method in the detection of melanoma metastasis. Some non-specific formation of cysteinyl dopa is present in the body, as demonstrated by 5-S-cysteinyl dopa in individuals with tyrosinase-negative albinism.

Key words: Albinism, Basophil leucocytes, Cysteinyl dopa, Macrophage migration inhibitory factor, Melanin, Phenylketonuria, Tautomerase, Tyrosinase

INTRODUCTION

I was born in 1930 in Skånes Fagerhult, Sweden. I understood at an early age that my hair colour was special and different. I was also freckled. Several sunburns taught me that I was not able to tan as many other children did. I remember my father with a red beard and my mother had brown hair. My brother was blonde and tanned better than I did. My wife Birgitta, whom I met at medical school, had brown hair and freckles. Our three children are redheads of different shades. Later in life I became scientifically interested in the genetics of freckles and red hair (1). Today I realise that my family and I probably have mutations of the melanocyte-stimulating hormone receptor (MC1R) gene (2).

Both my parents were teachers. I considered Medicine as a profession at an early age. A recently published History of Medicine (3) was a gift on my 15th birthday when I left home to begin my studies at Lund's Private Elementary School, where Latin and Greek were my favourite subjects. At the age of 17, I was admitted to the University of Lund.

At medical school I received training in histology for 4 yr under Prof. Gösta Glimstedt who was also a dermatologist.

His clinically-oriented teaching inspired me to apply for a residentship at the Department of Dermatology, where Gösta Hagerman was Professor and Chairman. He was focused on clinical immunology. I became interested in basophil leucocytes since these cells seemed to contain similar substances to mast cells, the key cells in anaphylactic reactions. I modified a method for quantitation of these rare leucocytes and then spent 7 yr exploring basophil leucocytes and their role in anaphylaxis and urticaria, parallel with my clinical training. In that way, I became familiar with some aspects of immunology and histamine metabolism and I made my first study on an enzyme, histidine decarboxylase (4). The histamine group at the Department of Physiology of Lund University, Georg Kahlson, Elsa Rosengren, Håkan Westling and Sven Eric Lindell taught and helped me.

In May 1962, I successfully defended my thesis (5) and became Associate Professor at the Faculty of Medicine at Lund University. After 7 yr of clinical training and simultaneous research it was great to have a summer holiday with

Abbreviations – 6H5MI-2-C, 6-hydroxy-5-methoxyindole-2-carboxylic acid; DHICA, 5,6-dihydroxyindole-2-carboxylic acid; IFPCS, International Federation of Pigment Cell Societies; IPCS, International Pigment Cell Society

my family which we spent in a forest planted by my grandfather 80 yr previously on the west coast of Sweden.

CYSTEINYLDOPA BEFORE CYSTEINYLDOPA

When I returned to Lund after the holiday I met Bengt Falck, a friend from medical school. He was working with Nils-Åke Hillarp at the Department of Histology. Hillarp was an expert on the histology of the adrenergic nervous system and the histochemistry of catechol amines. Hillarp and Falck had developed a new method for the visualization of adrenergic nerves based on the reaction of formaldehyde with noradrenaline. Bengt Falck showed me beautiful preparations of iris with brilliantly green fluorescence of the adrenergic nerves. I was more than willing to collaborate in order to define the adrenergic innervation of the skin with this new method. I took biopsies from my own skin and from the skin of friends. Bengt Falck processed the biopsies according to the Falck-Hillarp method and we performed the microscopic examinations together. The method involved freezing the biopsied tissue in propane cooled by liquid nitrogen, freeze-drying and treatment with dry formaldehyde gas. Catechol amines condense in situ with formaldehyde to form highly fluorescent isoquinoline derivatives.

We found that the adrenergic nerves were present in the arterioles and in the arrector muscles only. However, we observed a formaldehyde-induced fluorescence in certain cells in the basal epidermal layer (6). The cells did not have any connection with the adrenergic nerves, but had delicate dendrite-like processes extending between the epithelial cells (Fig. 1). Their shape and distribution strongly suggested that they were melanocytes. We realized that dopa, being an intermediate substance in melanogenesis, was a compound that fulfilled the requirements to give fluorescence with formaldehyde. We wanted to know if melanocytic tumours contained the same fluorescence as normal melanocytes. They did, and in many naevi and melanomas the fluorescence was much more pronounced than in the normal melanocytes (7). These studies were performed together with another histologist, Hans Olivecrona, and with the plastic surgeon Sten Jacobsson. The colour of the fluorescence in nevi and

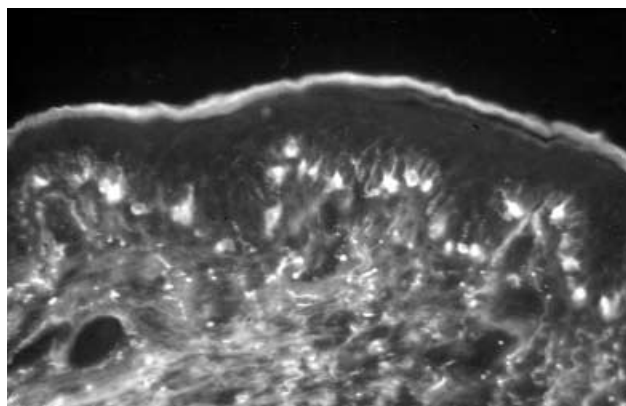


Fig. 1. Formaldehyde-induced fluorescence of cysteinyl-dopa in human epidermal melanocytes as photographed by Bengt Falck and myself in 1962.

melanomas was more in the yellow range than would be expected from a dopa-derived fluorophore. The formaldehyde-induced fluorescence of cytological preparations of melanoma cells had excitation and emission maxima which were at longer wavelengths than those of dopa (8). It would take us a long time before we could perform systematic studies on the fluorescence of this formaldehyde-induced fluorophore (9). In a study on tricolour guinea-pigs we found that melanocytes in the matrix of red hair had much stronger fluorescence than the melanocytes of black hair, an observation that would prove to be significant (10).

5-S-CYSTEINYLDOPA AND MELANOMA

The finding of a formaldehyde-induced fluorescence in melanomas meant that we could get larger quantities of melanin-forming cells for definition of the substance giving fluorescence with formaldehyde. A collaboration with Evald Rosengren was started, which was to become pivotal for our work on the biochemistry of melanocytes (Fig. 2). In the late 1950s Evald Rosengren had, in collaboration with Åke Bertler and Arvid Carlsson, performed studies on catechol amines in the brain. This work became classic and of great importance for the pharmacology of the central nervous system (11, 12). Arvid Carlsson, Nils-Åke Hillarp and Evald Rosengren then worked closely together on catechol amines. When Arvid Carlsson, who was awarded the Nobel Prize in medicine in 2000, became Professor in Pharmacology in Gothenburg, Nils-Åke Hillarp and Evald Rosengren joined him. Evald Rosengren subsequently returned to a professorship in pharmacology at Lund in 1963.

The chemical analysis of melanoma tissue for compounds that could give fluorescence with formaldehyde demonstrated dopa (13), but we had difficulties in finding any correlation between the content of dopa and the histochemically detected fluorescence. Hydrolysis of melanoma precipitates gave additional amounts of dopa, but the melanoma eluates also contained an unknown compound with similarities to dopa. The fluorescence spectrum after oxidation of this unknown



Fig. 2. Evald Rosengren and Shosuke Ito at our laboratory in Lund.

compound differed from that of dopa (14). Attempts to relate the formaldehyde-induced fluorescence in melanomas to dopa peptides gave negative results and we therefore looked for other compounds that could explain the fluorescence of melanocytes and melanomas (15). We had been working for some time with thioethers of dopa which were examined for their fluorescence after oxidation or formaldehyde treatment when studies by Nicolaus and Prota in Naples demonstrated that formation of 5-*S*-cysteinyl-dopa was a first step in the formation of phaeomelanin (16, 17). The reports from Naples intensified and focused our work on thioethers of dopa.

At our clinical department a melanoma patient with red hair exhibited rapidly progressing disease. We obtained excised metastatic tissue for analysis which proved that extracts of the metastasis contained a catecholic compound seemingly identical with the unknown compound we had previously observed (14). With the information available from Naples on cysteinyl-dopa as a precursor of phaeomelanin we concentrated our analytical work on 5-*S*-cysteinyl-dopa. All examinations performed on our catecholic compound extracted from the melanoma-produced evidence for the presence of 5-*S*-cysteinyl-dopa in the tissue. I went to Naples to discuss our data with Nicolaus and Prota. They agreed with our identification of the melanoma catecholic compound as 5-*S*-cysteinyl-dopa. We were ready to report on the presence of this amino acid in the human melanoma tissue (18).

The International Pigment Cell Meeting in Sydney, March 1972, gave us an opportunity to present our findings to a large qualified audience (19). I also had the chance to meet many of the scientists whose work had impressed me when I was studying the literature on melanin and melanoma.

From then on we were devoted to cysteinyl-dopa. A sensitive and highly specific method for 5-*S*-cysteinyl-dopa was developed (20). Evald Rosengren's experience with fluorescence methods for the determination of catechol amines in the brain was of the utmost importance for the rapid progress of our work. Anna-Maria Rosengren, a biochemist, and Gun Agrup, a dermatologist with training in chemistry, joined our group.

With the new sensitive method we were ready to study whether 5-*S*-cysteinyl-dopa was limited to melanomas developing in persons with red hair. This proved not to be the case. We found 5-*S*-cysteinyl-dopa in melanomas of Swedes of all complexions (21). But red hair is not rare in Sweden and we suspected that phaeomelanin formation might be present due to a recessive gene hidden in subjects with other hair colours. My visit to Kampala, Uganda in the winter of 1971–1972 provided an opportunity to demonstrate that 5-*S*-cysteinyl-dopa was present in melanomas of black Africans. My old friend Gunnar Lomholt from Copenhagen had established a Department for Dermatology and Venereology at Mulago Hospital, attached to Makerere University, and he invited me and Niels Hjorth, my Danish colleague, to teach dermatology. I received excellent training in tropical dermatology, leprosy and syphilis and Lomholt brought me in contact with the Tumour Center in Kampala where melanoma patients were treated. We could send melanoma tissue for analysis of 5-*S*-cysteinyl-dopa to our laboratory in

Lund. Melanomas of Africans contained 5-*S*-cysteinyl-dopa. It seemed that cysteinyl-dopa, in addition to being a precursor of phaeomelanin, was of general importance in pigment biochemistry (22).

The method for analysis of 5-*S*-cysteinyl-dopa also made it possible to measure this amino acid in the urine of healthy subjects (23). Increased excretion of cysteinyl-dopa could be a sign of metastatic disease in melanoma patients (24). Some early results from our studies on 5-*S*-cysteinyl-dopa were reported at the pigment meeting at Yale in January 1973 (25).

Our work then proceeded at high speed. The new fluorimetric method for determination of 5-*S*-cysteinyl-dopa facilitated our work. This method also made studies on 5-*S*-cysteinyl-dopa more attractive to other groups, including one in Paris, where Jean Pierre Cesarini, who had visited Lund, was introducing our 5-*S*-cysteinyl-dopa assay for the follow-up of melanoma patients. Michel Prunieras, a pioneer in culturing normal melanocytes and a friend from the early days of the European Society for Dermatologic Research (ESDR), invited me to give a talk in Paris and introduced me to Christian Aubert who was investigating culture conditions of melanoma cells. Aubert was moving his group, sponsored by Institut National de la Santé et de la Recherche Medicale (INSERM), to Marseille. A close link between our groups was established and the 5-*S*-cysteinyl-dopa method proved to be of value for studies on the pigment metabolism of melanoma cells in cultures (26–31). Christian Aubert, his family, and his collaborators, turned every visit to his laboratory in Marseille into a cultural event as well.

In the spring of 1974, I was invited to give the Dowling Oration in London which allowed me to present our work on the histochemistry and biochemistry of 5-*S*-cysteinyl-dopa to English dermatologists (32).

In 1974, when I was president of the ESDR I suggested to our board to invite a guest lecturer from some branch of Science outside Dermatology. The proposal was accepted and Giuseppe Prota was invited as the first Guest Speaker of our society. His presentation was a great success. The subject of pigment, eumelanin and phaeomelanin was presented in a way that was accessible to all. All my contacts with Giuseppe Prota had been very pleasant and I was eager to learn more from him and therefore asked him to join us in Lund for some time. He was willing to come for a 3 months' stay in the summer of 1975. It was a good summer. Giuseppe and his wife Giovanna had three children, one of them a baby, and they liked their home with a small garden close to our laboratory. Giuseppe Prota, Anna-Maria Rosengren, Evald Rosengren and I worked together at the laboratory and Gun Agrup supplied us with clinical material (Fig. 3).

During 3 months we isolated phaeomelanin from melanoma tissue and could determine 2-*S*-cysteinyl-dopa, 2,5-*S*,*S*-dicysteinyl-dopa and trichochromes in urine from melanoma patients (33–35). Our observations on melanins in melanoma tissue generated the concept of 'mixed type melanogenesis' based on our observations on a melanoma melanin that had the characteristics of both eu- and phaeomelanin, as previously defined.

That summer with Giuseppe at the laboratory saw our real introduction into the fascinating world of melanin chemistry, the field in which Nicolaus' laboratory in Naples had been



Fig. 3. Patrick Riley, Giuseppe Prota, Hans Rorsman, and Bengt Larsson planning a pigment meeting in the Netherlands.

leading for many years. In Giuseppe, we had a competent and didactic mentor. We always enjoyed meeting with Giuseppe and Giovanna. The last time Birgitta and I met Giovanna was in 1998 when she and Giuseppe hosted a dinner with their friends and collaborators at their beautiful home overlooking the bay of Naples. Giovanna died in 1999. Giuseppe and I met the last time at the European pigment meeting in Ulm. In January 2003 Giuseppe died.

5-S-CYSTEINYLDOPA AS TUMOUR MARKER IN MELANOMA

Early in our studies on cysteinyldopa we observed that patients with melanoma metastases often excreted large amounts of 5-*S*-cysteinyldopa in urine (24, 25, 32). Christer Hansson, an organic chemist and pharmacologist, joined our group. Shortly thereafter he focused his work on new analytical methods (36, 37). The fluorimetric method was soon replaced by HPLC and electrochemical detection (38) that enabled us to determine cysteinyldopa in serum or plasma. A sensitive method for analysis of the indolic metabolite 6-hydroxy-5-methoxyindole-2-carboxylic acid (6H5MI-2-C) was later developed by Christer Hansson (39).

Studies on 5-*S*-cysteinyldopa as a biochemical marker of malignant melanoma were reviewed in 1983 (40), at which time the analytical methods were further improved by the introduction of a new internal standard, a diastereomer of 5-*S*-cysteinyldopa (41). Bertil Kågedal, Professor in Clinical Chemistry in Linköping, refined the 5-*S*-cysteinyldopa method for routine laboratory use. All determinations of cysteinyldopa in Sweden are currently performed at his laboratory. In a recent study, on the value of 5-*S*-cysteinyldopa determinations in the follow-up of melanoma patients (42) the sensitivity for the detection of stage III–IV melanoma was 83%.

Other reviews on 5-*S*-cysteinyldopa as a marker of melanoma disease have recently been published from Japan and Germany. In the Japanese study (43), the sensitivity of the determination of serum 5-*S*-cysteinyldopa for detection of metastasis was 73% and in the German study (44) increased levels of 5-*S*-cysteinyldopa in plasma were found in

52, 67 and 81% in patients with stage I/II, III and IV melanoma, respectively.

When we had started to use determination of 5-*S*-cysteinyldopa as a biochemical marker of melanoma metastases it soon became evident that confounding factors were present. We, therefore, investigated some circumstances, which clinical observations had suggested to be relevant in evaluating cysteinyldopa values obtained in melanoma patients.

CONSTITUTIVE PIGMENTATION AND 5-S-CYSTEINYLDOPA

Studies in guinea pigs suggested that constitutive pigmentation could influence cysteinyldopa levels in serum. No cysteinyldopa was found in the white hair of tricoloured guinea pigs. The 5-*S*-cysteinyldopa content was 10 times higher in red than in black hair (25). The findings of more cysteinyldopa in red hair than in black hair were confirmed in guinea pigs having uniform colour and we also found that red guinea pigs had higher serum levels of cysteinyldopa than black animals. It was also of note, that white animals had some cysteinyldopa in their serum (45).

Studies on the urinary excretion of 5-*S*-cysteinyldopa in healthy humans were first performed with the fluorimetric method (46). The mean value in men was 100 µg/24 h and in women 78 µg/24 h. Subjects with white hair, who were generally older, had lower excretion values. No significant difference in excretion was found between people with red, blonde or dark hair. The amount of 5-*S*-cysteinyldopa was not correlated to body weight or body surface. In 12 persons from Africa and America with black or brown skin, 10 had 5-*S*-cysteinyldopa excretion values in the same range as persons of Swedish origin, whereas the excretion was higher in two subjects (47). In a later study performed with HPLC methods the excretion of 5-*S*-cysteinyldopa was found to be unrelated to the constitutive pigment type, but the indole metabolite 6-hydroxy-5-methoxyindole-2-carboxylic acid (6H5MI-2-C) was excreted in larger quantities in subjects with genetically dark skin (48).

IS 5-S-CYSTEINYLDOPA AN AMINO ACID SPECIFIC FOR THE MELANOCYTE?

The use of 5-*S*-cysteinyldopa as a tumour marker in melanoma made it important to define if this amino acid could be generated outside the pigment-forming cells. We studied albinism in order to find out if cysteinyldopa also occurred in the absence of functioning tyrosinase.

The urinary excretion of 5-*S*-cysteinyldopa and of the indole metabolite, 6H5MI-2-C, was determined in black and in albino mice. The black animals excreted 32 ng/ml 5-*S*-cysteinyldopa and the albino animals 16 ng/ml. These data indicated that cysteinyldopa formation, i.e. dopaquinone generation, occurs in the body in the absence of tyrosinase activity (49). By contrast, determination of 6H5MI-2-C with Christer Hansson's method showed 21 ng/ml in the black animals but no excretion of 6H5MI-2-C in albino mice.

In a human tyrosinase-negative albino patient we found a low cysteinyldopa excretion in the urine and no excretion of

6H5MI-2-C (50). Two tyrosinase-positive albino patients had normal cysteinyl-dopa and 6H5MI-2-C excretion. Another study on human tyrosinase-negative albinos has also demonstrated 5-S-cysteinyl-dopa formation in the absence of tyrosinase activity (51).

We concluded that 5-S-cysteinyl-dopa is formed in albinism with non-functional tyrosinase, whereas – in contrast, the indole metabolite CH5MI-2-C is not excreted in tyrosinase – negative albinism and this compound seems to be a specific recorder of tyrosinase activity in the melanocyte.

How is cysteinyl-dopa formed in the absence of tyrosinase? Large quantities of dopa are produced in the adrenal medulla and in the nerve tissues by the enzyme tyrosine hydroxylase. Most of this dopa is enzymatically decarboxylated, but some dopa is found in tissues containing catechol amines. A small portion of dopa is oxidized, probably by oxygen radicals, and the dopaquinone formed reacts with thiols. Since glutathione is the dominant thiol in the cells most dopaquinone reacts with glutathione to form glutathionyl-dopa. This compound was found in different organs after injection of dopa in tyrosinase-negative albino animals (52). Glutathionyl-dopa is enzymatically hydrolysed resulting in the formation of cysteinyl-dopa, which is excreted in the urine (53). This pathway is probably important for the elimination of non-specific oxidation products of dopa, whereas cysteine seems to be the thiol reacting with dopaquinone in the case of tyrosinase-catalysed oxidation in the melanosome (54, 55).

UV-INDUCED PIGMENTATION AND 5-S-CYSTEINYLDOPA

In the summer of 1973, we were working intensely with determination of 5-S-cysteinyl-dopa in the urine of patients operated on for primary melanoma; healthy individuals were studied as controls. We found unexpectedly high excretion values in several normal subjects and in melanoma patients without signs of metastases. My own urine was examined some days after a sunny weekend when I had been playing tennis for several hours and we found urinary 5-S-cysteinyl-dopa excretion levels previously observed only in patients with melanoma metastases. We assumed that the raised excretion was due to stimulation of, or damage to, the melanocytes owing to UV-exposure and decided to make a systematic study of the seasonal variation of 5-S-cysteinyl-dopa (56). The summer values were found to be considerably higher than those recorded at other seasons. The inter-individual variation was great in the summer, intermediate in autumn and spring and small in winter.

With the arrival of psoralen UV-A (PUVA) treatment for psoriasis came a good model system for studies on induced pigmentation and 5-S-cysteinyl-dopa metabolism. PUVA treatment was found to induce a several-fold increase of 5-S-cysteinyl-dopa excretion and this increase preceded the start of pigmentation. Peak values were observed after 1 or 2 weeks' treatment at which time the pigment response was pronounced. With continued treatment 5-S-cysteinyl-dopa excretion decreased but remained higher than at the start of the PUVA treatment (57, 58). Treatment with UVB has similar effects (59). The excretion of the indole metabolite 6H5MI-2-C paralleled that of 5-S-cysteinyl-dopa (58). The

time course of 5-S-cysteinyl-dopa excretion during PUVA treatment was similar for serum values of cysteinyl-dopa (60, 61). The serum levels of dopa were not influenced by the PUVA-induced stimulation of the pigment system (60).

RENAL CLEARANCE OF CYSTEINYLDOPA

Early on we were able to detect 5-S-cysteinyl-dopa in the plasma of two melanoma patients with advanced disease and to determine the renal plasma clearance of this amino acid. The 5-S-cysteinyl-dopa clearance values were 43 and 45%, respectively, of the creatinine clearance in the two patients (62). The electrochemical method allowed us to determine serum concentrations of 5-S-cysteinyl-dopa in healthy subjects and confirm the renal clearance previously observed for 5-S-cysteinyl-dopa in melanoma patients. The serum levels of 5-S-cysteinyl-dopa are considerably increased in chronic renal failure. Renal function must be taken into account when evaluating serum or plasma levels of 5-S-cysteinyl-dopa (63).

THE SIZE OF THE MELANOCYTE SYSTEM

Between 1972 and 1974, I was Professor and Chairman of the Department of Dermatology at the University of Gothenburg. At that time Inger Rosdahl started her training in dermatology at the department. Working with George Szabo and Thomas B. Fitzpatrick at Harvard University she became a pigmentologist. After her return to Sweden she created pigment research groups, first in Gothenburg, then in Linköping.

Inger Rosdahl and collaborators had found that UV-irradiation had systemic as well as local effects on the epidermal melanocyte population (64). We discussed whether estimating the total volume of the 'normal' melanocytes might improve the sensitivity of cysteinyl-dopa determinations in melanoma patients by compensating for individual differences in the melanocyte mass. Inger was willing to do the job.

The epidermal melanocyte population density of different areas of the skin, the number of follicular melanocytes of scalp hairs and the mean volumes of the cells were determined. The epidermal melanocytes constitute the dominant part of the 'melanocyte organ'. In the human adult not recently exposed to sunlight, the functionally active melanocytes were calculated to form a tissue of 1–1.5 cm³ (65). Assuming the same pigment metabolic activity of malignant and normal melanocytes a doubled cysteinyl-dopa excretion would indicate a total metastatic volume of several cm³ since some of the 'normal' excretion is due to non-specific oxidation of extra-melanocytic dopa.

TRICHOCHROMES OR TRICHOCHROME PRECURSORS IN URINE?

Giuseppe Prota made us familiar with trichochromes, the simplest phaeomelanin pigments, and we found trichochrome C and B in the urine of some melanoma patients with high excretion of cysteinyl-dopa (35, 66). Trichochrome E and F, previously described in red feathers and hair, were formed as artefacts in the analytical procedure from cysteinyl-dopa present in urine (67, 68).

Trichochromuria is seen only in melanoma patients with high excretion of cysteinyl-dopa. We found that a rare clinical sign in advanced melanoma disease, diffuse melanosis, was linked to trichochromuria. Patients with melanosis and trichochromuria exhibited fine electron-dense granules in lysosomes of dermal histiocytes. It seemed that trichochromes or similar pigments in dermal histiocytes were responsible for the melanosis in these patients (69).

Recent studies have raised the possibility that the trichochrome pigments are generated from cysteinyl-dopa derived precursors such as 3-carboxy-benzothiazine or 2,2-benzothiazine dimers (70). Such compounds may be abundant in the urine of melanoma patients (71). An interesting question relating to these pigments is: are all trichochromes artefacts formed in the analytical procedure?

STUDIES ON TYROSINASE

Mushroom Tyrosinase

It was used in our production of cysteinyl-dopas from the very beginning. We became interested in the function of the enzyme when we were studying the formation and oxidation of cysteinyl-dopas (72). A new product of mushroom tyrosinase, 5-OH-dopa, was detected (73–75) which further deepened our interest in the enzyme (76). The knowledge acquired through studies on mushroom tyrosinase proved to be of great value when we started to work on the human enzyme.

Human Tyrosinase

When we had developed analytical methods that could differentiate between diastereomers of 5-S-cysteinyl-dopa we realized that we could devise a sensitive tyrosinase method with an internal control of non-specific oxidation. By studying the formation of 5-S-L-cysteinyl-L-dopa in comparison with the formation of 5-S-L-cysteinyl-D-dopa in short-time incubations we determined the dopa oxidase activity in the medium of melanoma cell cultures (77, 78). For the isolation of human tyrosinase we needed large volumes of melanoma cells. As always we were helped by Prof. Bengt Källén, our benefactor at the Tornblad Institute, Lund, and his collaborators. Tyrosinase was purified from cultured human melanoma cells and the hydroxylation of tyrosine and the oxidation of dopa were studied (79). We found that L-tyrosine was hydroxylated only in the presence of a reducing co-substrate. L-dopa was most effective as co-substrate but in addition dopamine, dopac and 5,6-dihydroxyindole-2-carboxylic acid (DHICA) functioned as reducing substrates. Ascorbic acid, 5-S-cysteinyl-dopa and 5-OH-dopa were not co-substrates. The fact that DHICA is a reducing co-substrate means that human tyrosinase can function not only as tyrosine oxygenase and dopa oxidase but also as DHICA oxidase. This function has been carefully analysed by the Murcia group (80). Since dopac has been demonstrated to be capable of entering melanin-producing cells (81) this compound may, as reducing co-substrate, be of importance for the initiation of tyrosinase activity. Other candidates for initiating the oxygenase function of tyrosinase

are hydrogen peroxide and/or oxygen radicals, since the reducing co-substrate dopa is formed from tyrosine in the presence of reactive oxygen species (82).

In studies on dopamine and serotonin metabolism in the brain, Evald Rosengren had gained considerable experience in enzyme work. With a group of able collaborators we focused on the isolation of human tyrosinase. The project was successful. 0.21 mg tyrosinase was prepared from 72 mg protein isolated from membranous material of melanoma cells (83). The N-terminal amino acid sequence of the isolated tyrosinase was determined by Edman degradation. I had two famous scientists as teachers in biochemistry at medical school, Prof. Pehr Edman who developed the method for protein sequencing, and Prof. Sune Bergström, who was awarded the Nobel Prize in medicine for his work on prostaglandins. Comparison of the N-terminal amino acid sequence of our isolated human tyrosinase with an amino acid sequence predicted from a nucleotide sequence of a human tyrosinase cDNA clone published by Kwon et al. (84) made it possible to define a signal peptide of 18 amino acids of human tyrosinase.

Cultured melanoma cells also contain soluble tyrosinase. Isolation and amino acid sequencing of this enzyme showed N-terminal identity with the membranous enzyme (85). The soluble form of tyrosinase lacked the C-terminal sequence including the hydrophobic intramembranous part, which explained its solubility.

We used most of the published methods in our studies of tyrosinase but we favoured the method where the 5-S-L-cysteinyl-L-dopa production was measured after incubation with D-L-dopa and L-cysteine. With this method non-specific oxidation can be monitored by comparison with 5-S-L-cysteinyl-D-dopa production. Furthermore the sensitivity of the determination is high which permits a short incubation time, thereby minimizing any later reactions. In high concentration cysteine inactivates tyrosinase but we could demonstrate that incubation of tyrosinase and cysteine together with tyrosine or dopa prevents inactivation of tyrosinase (86). Cysteine in the quantities used in our dopa oxidase method did not inhibit tyrosinase.

CLINICAL STUDIES ON TYROSINASE

Using L-cysteinyl-D-dopa as internal standard with an improved purification procedure tyrosinase could be detected in serum of normal subjects. A seasonal variation of serum tyrosinase activity with lower values in winter than in summer and autumn was observed (87). Increased activity of tyrosinase in serum was found in melanoma patients with wide-spread disease (88, 89).

EFFECTS OF HYDROGEN PEROXIDE AND/OR OXYGEN RADICALS ON TYROSINASE

In a study on the effect of dihydroxyphenyl derivatives on tyrosinase Eszter Karg, a dermatologist from Pécs, Hungary, and PhD student at our laboratory, found that dopac increased the tyrosinase activity of melanoma cells (90). Catalase reduced this effect and we considered the possibility that hydrogen peroxide might mediate the effect of dopac on

tyrosinase. Cultured melanoma cells, exposed to hydrogen peroxide and reactive oxygen species, increased their tyrosinase activity and catalase, added to the medium, prevented increased tyrosinase activity (81).

Inhibition of intra-cellular catalase by 3-amino-1,2,4-triazole either alone or in combination with dopac caused an increase in the tyrosinase activity. The increased tyrosinase activities observed mirrored increased quantities of tyrosinase protein in the cells. The observations indicated that hydrogen peroxide induces an increase of tyrosinase (91).

Clinical studies on the effect of UVA on pigmentation indicate that hydrogen peroxide may also be of importance for the induction of melanogenesis.

In a study on the effect of UVA on pigment precursors Eva Tegner, at the Department of Dermatology, observed that persons lying on their back on sun-beds did not become pigmented in the skin over their scapulae and sacrum where body weight prevented the blood flow by compression. The finding indicated that cutaneous hypoxia, at the time of UV exposure, could prevent pigmentation induced by UVA (92, 93). Hydrogen peroxide applied topically just before irradiation reversed the effect of pressure hypoxia. With this treatment the skin at pressure sites tanned normally after UVA exposure (94).

Thus, hydrogen peroxide and/or oxygen radicals seem to have a dual upregulating role in melanogenesis: 1) initiation of the function of resting tyrosinase; and 2) induction of an increase of tyrosinase.

Tyrosinase, Tyrosinase-Related Protein and Phenylpyruvate Tautomerases

In our studies on tyrosinase we were pleased to collaborate with Alison J. Winder at Oxford University (Fig. 4). She is an expert in molecular biology and had established lines of mouse fibroblasts expressing tyrosinase. Transfected fibroblasts expressing mouse tyrosinase were shown to share several characteristics with melanoma cell lines, including



Fig. 4. Evald Rosengren, Alison Winder, Anna Wittbjer, and Gerd Odh at Skillinge on the east coast of Skåne (Scania). Anna and Gerd were PhD students, who worked on tyrosinase, tyrosinase-related proteins, phenylpyruvate tautomerase and melanin.

increased pigmentation and tyrosinase activity in response to increased cell density (95). The pigmented fibroblasts contained 5-*S*-cysteinyl-dopa and 5-*S*-glutathionyl-dopa and produced a phaeomelanin-like pigment but did not contain detectable eumelanin. Alison Winder also established cell lines expressing both tyrosinase and Tyrp1 by transfecting tyrosinase-expressing fibroblasts with a Tyrp1 expression vector (96). Double transfectants expressing both proteins had a higher steady-state level of tyrosinase than fibroblasts expressing tyrosinase alone and contained elevated levels of melanin intermediates. These cells synthesized more phaeomelanin, and oxidation with potassium permanganate produced some pyrrole-2,3,5-tricarboxylic acid indicating eumelanin. In contrast, cells expressing tyrosinase alone contained only phaeomelanin (97).

We found high levels of *O*-methylated 5,6-dihydroxy indole-2-carboxylic acid in the medium of cells containing tyrosinase and Tyrp1 (96). This fact, taken together with the results of some preliminary studies, suggested that Tyrp1 had L-dopachrome tautomerase function, but further studies on Tyrp1 with specific antibodies donated by Vincent J. Hearing at NIH, disproved this putative function of Tyrp1.

We used D-dopachrome as controls in our studies on Tyrp1 and observed that two proteins had the ability to catalyse the transformation of D-dopachrome into indoles. Both proteins were isolated. The first protein which catalysed the transformation of D-dopachrome into 5,6-dihydroxyindole was previously unknown (98, 99). The other protein proved to be identical with a cytokine, macrophage migration inhibition factor (MIF). This protein catalysed the transformation of D-dopachrome into 5,6-dihydroxyindole-2-carboxylic acid (100). Both proteins were later found to have substrates of great interest (101, 102). One of the substrates, *p*-hydroxyphenyl pyruvate, is a compound formed in the physiologic degradation of phenylalanine and tyrosine. The other substrate, phenylpyruvic acid, is a metabolite found in phenylketonuria (103).

NEUROMELANIN

In 1971, we studied the pigment of substantia nigra and found dopa and dopamine in hydrolysates of the neuromelanin (104). When we later observed cysteinyl-dopa in tyrosinase-negative albino patients and in albino animals (49, 50) we started to look for explanations in the neural system. The analysis of catecholic amines in cervical ganglia of albino rats demonstrated dopa in addition to noradrenalin, dopamine and adrenalin (105). For a study of the possible occurrence of cysteinyl-dopa in sympathetic nerve tissue we turned to a larger animal, the cow, and found substantial quantities of 5-*S*-cysteinyl-dopa in the stellate ganglion (106). This finding demonstrated that oxidation of catecholic compounds and thiol addition may occur in nerve tissues.

Several members of our group who had been working with melanins in our melanoma project became interested in neuromelanin. Ragnar Carstam, a dermatologist at our University Hospital, and I both became pupils in neuroscience. A series of investigations on 5-*S*-cysteinyl derivatives of dopa, dopamine and dopac performed by Bodil Fornstedt in collaboration with Evald Rosengren and Arvid Carlsson

in Gothenburg demonstrated that oxidation of catechols and thiol addition occur in dopamine-rich regions of the brain (107–109). The finding of 5-*S*-cysteinyldopac in human urine by Carstam et al. produced direct evidence for the oxidation of catecholic compounds and thiol addition in the nervous system *in vivo* (110).

Neuromelanin was studied with regard to its monomeric components. The substantia nigra neuromelanin contained both indolic and benzothiazine-derived monomers. Isolated neuromelanin contained 2.3% sulphur and 8.1% nitrogen. The data obtained defined neuromelanin as a mixed type melanin (111, 112). The explanation for the presence of benzothiazine and indole monomers in neuromelanin may be that oxidation of dopamine occurs both in the presence and absence of thiols, but monomers containing indoles may also be formed after oxidation of dopamine in the presence of glutathione (113).

The demonstration of oxidation of dopa, dopamine and dopac in the brain has triggered interest in such events in the pathogenesis of Parkinson's disease and also, more generally, in the ageing of the nervous system.

MEETINGS AND SOCIETIES

Many of the studies referred to in this review were reported at pigment cell meetings in Europe, USA and Japan or at workshops and symposia arranged by the European Society for Dermatological Research sometimes co-ordinated with meetings of the Society for Investigative Dermatology (Figs 5–7). As a board member of scientific societies I learned the value of stable organizations for the effective planning of meetings.

In 1988, I took office as President of the International Pigment Cell Society (IPCS). When I left office 1 November 1990, the IPCS had been transformed into the International Federation of Pigment Cell Societies (IFPCS). The history of the IPCS and the creation of the IFPCS have been summarised (114).



Fig. 5. Myron Gordon Awardees London 1993, Patrick Riley, Hans Rorsman, and Takuji Takeuchi.



Fig. 6. Evald Rosengren, Birgitta Rorsman, Aaron B. Lerner, Millie Lerner, and Hans Rorsman at the pigment meeting in London 1993.



Fig. 7. Hans Rorsman and Fritz Anders as brand-new honorary members of ESPCR at Château Beychevelle in 1997.

I will give a brief account of the main events between 1988 and 1990 based on five volumes of correspondence kept by my excellent secretary Mrs Annette Zaczek, and the Bulletins edited by the Secretary-Treasurer of the IPCS, Madhu A. Pathak, my wise advisor (Fig. 8).

Even before taking office I realized that the arrival of pigment societies in Europe and Japan would make it necessary to modify the function of the IPCS and reconsider its role. In June 1988 Drs Richard A. King and James J. Nordlund organized a pigment meeting at the University of Minnesota Medical School in Minneapolis, Minnesota. At this meeting the Pan American Pigment Cell Society (PASPCR) was formed. There was a need for co-ordinating the interaction of the three new societies and in discussions with Madhu Pathak and the earlier presidents of the IPCS Thomas B. Fitzpatrick, Aaron B. Lerner and Sidney N. Klaus it became clear to me that IPCS should maintain a key role in the future. Amendments in its functions and bylaws had to be made. I called and chaired a meeting in Minneapolis 25 June 1988 to discuss the relationship between IPCS and the pigment societies then existing in Europe, Japan and



Fig. 8. Madhu Pathak at his visit to Lund in 1981, where he lectured at a symposium on cutaneous porphyrias.

America. In attendance were: Giuseppe Prota, President of the ESPCR; Yutaka Mishima, President of the JSPCR; James J. Nordlund, President of the PASPCR; Richard A. King, Secretary-Treasurer of the PASPCR; and Joseph T. Bagnara, Editor, Pigment Cell Research.

The discussion focused on the interaction between the three new societies and on the role of the IPCS in this international co-operation. The recently started journal Pigment Cell Research (PCR) had problems since the publisher realized that the new societies could weaken the finances and the sponsorship of the IPCS. The discussions were intense and the meeting had to be continued on Sunday, 26 June. This second day was closed with general agreement on the necessity of reorganization of the IPCS to meet the requirements of the international pigment cell community. It was understood that we needed to come quickly to terms with how to proceed with the interaction between the IPCS, the regional societies and PCR. I offered to host a new meeting as soon as possible in order to reach definite decisions and initiate activities for the creation of a federation of pigment cell societies. This meeting took place 1 October 1988 in Lund, Sweden. The participants were the same as at the meeting in Minneapolis. Consensus was reached on recommendations to be made to the IPCS council in Kobe 1990 and a committee to review and revise the bylaws was appointed (Mishima, Nordlund, Riley). Decisions were made on interaction, finances, PCR and on a steering committee until the next general assembly of the IPCS.

All agreements reached in Lund were confirmed at a meeting in Venice, Italy, October 1989. Prof. Walter C. Quevedo Jr, Brown University Providence, Rhode Island, contributed greatly to the IPCS in its new status with advice

and assistance in the work of revising its bylaws. The proposals formulated at the Lund meeting and the new bylaws were presented to the IPCS Council Meeting 31 October 1990 in Kobe, Japan. The council voted unanimously to recommend and submit resolutions for the dissolution of the existing IPCS and for the establishment of the International Federation of Pigment Cell Societies (IFPCS). The resolutions were unanimously approved by the General Assembly of the IPCS present on 1 November 1990 in Kobe.

At the time of the Kobe meeting negotiations with regard to PCR had advanced. Joe Bagnara had established contact with Peter Hartmann, senior Vice President at Munksgaard in Copenhagen. I met with Peter Hartmann and a good agreement between Munksgaard and the IFPCS was soon to come.

When I left office 1 November 1990, I felt happy. An international federation for co-operation between pigment cell societies had become established and the future for PCR looked bright.

Much work remained to be done for Madhu Pathak with regard to finances and legalities of the IPCS and the IFPCS. In a letter dated 4 March 1991 Madhu sent me a final report and I again realized how important his activities had been for the IPCS.

OTHER ACTIVITIES

My training in Dermatology started 47 yr ago. Like other branches of Medicine, Dermatology has become highly sub-specialized. An example: in 1972 I wrote as single author a textbook for Swedish medical students with five colleagues as referees. For the latest edition (115) my co-editors and I asked for advice or co-authorship from 53 Swedish colleagues.

In the 1960s, I visited the Department of Dermatology, New York University, lead by Prof. Rudolf L. Baer, where I worked with Leonard C. Harber who educated me in photodermatology. Rudolf Baer and Leonard Harber introduced me to US dermatology and to the cultural life of New York City. Alfred W. Kopf became my teacher in skin oncology and he has ever since continued to advise me on clinical matters.

I have been Professor and Chairman of dermatology departments for 23 yr, first in Gothenburg then at Lund. Between 1983 and 1992 I was chief medical officer of the University Hospital at Lund, i.e. advisor to the CEO and Board of Directors, a demanding and time consuming assignment, which even on some occasions prevented my participation in scientific meetings. This job made me more familiar with the activities of my colleagues at our hospital and gave me great satisfaction.

My clinical interest has been focused on two different fields, both related to my research activities. The first 15 yr were spent on skin diseases of immunologic nature. After our finding of cysteinyl-dopa in melanoma, this tumour and diseases of the pigment system have received most of my clinical attention.

Between 1987 and 2002, I was a member of the International Committee of Dermatology, which represents an umbrella organization for National and international

dermatological societies. The Committee sponsors a dermatologic training center in Moshi, Tanzania, lead by the German dermatologist Henning Grossman. I was treasurer for the Committee and the African project during a 5-yr period.

At Moshi, Barbara Leppard, an English dermatologist, had been running a project in the district of Kilimanjaro, educating albino Africans on sun protection. In 2001, I had the opportunity to meet with Dr Leppard and her patients with albinism. The impact of her programme on the albino population was striking. Recently I have been involved in an educational programme for parents of Swedish children with albinism. Problems with eye-sight are similar in Africa and Sweden but sun protection is, of course, less difficult in Sweden. Swedes and Africans alike are keen to become informed.

My visit to Australia in 1972 initiated my interest in the prevention and early detection of melanoma and other skin cancers. Prof. Ulrik Ringborg, Department of Oncology, Karolinska Sjukhuset, Stockholm, has for a long time co-ordinated a Swedish melanoma study group with members, myself included, from all specialities involved in melanoma prevention, diagnosis and treatment. The Swedish educational programmes on sun protection and early detection of melanoma seem to have resulted in increased awareness of the carcinogenic affects of UV irradiation and a decrease in the mortality rate of patients with melanoma since these tumours are diagnosed and treated earlier than before.

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