1
History of Melanosome Research

Jan Borovanský

1.1
Introduction

Melanosomes were first proposed as specific organelles, unique to pigment cells, in a preliminary publication that appeared on 30 July 1960 [1]. An announcement had been made at the 21st Annual Meeting of the Society for Investigative Dermatology, at Miami Beach, Florida, USA on 13 June 1960 [2] and the news, that the chemical composition and enzyme activities in melanosomes and mitochondria are completely different, was considered to be of such significance that it appeared in a newspaper report (Figure 1.1). Similar data, with an emphasis on terminology, were published in 1963 [3].

This advance was the result of collaborative work between M. Seiji (1926–1982), at that time working at the Department of Dermatology, Harvard Medical School in Boston under the leadership of T.B. Fitzpatrick (1919–2003) (Figure 1.2), and H. Blaschko and M.S.C. Birbeck, with whom Dr Fitzpatrick established scientific cooperation during his tenure of a Commonwealth Fellowship at the Department of Biochemistry, Radcliffe Infirmary in Oxford.

The history of melanosome research can be formally divided into three parts: (i) the pre-Seiji era (prior to 1960), (i) the Seiji era (1960–1982), and (iii) the post-Seiji era (1983–).

1.2
Melanosome Research in the Pre-Seiji Era

The first description of mammalian pigment cells was published by Gustav Simon in 1841 [4] who observed round and stellate pigment cells in the hair bulbs of pig embryos. It was preceeded in 1838 by Purkyně's description of pigment in the cells of the substantia nigra, which not only drew attention to pigment granules, but also noted the rise in their numbers with age [5]. We have to admire these early reports because their authors, armed only with primitive light microscopes, were able to ascertain that melanin was not diffusely distributed in the cytoplasm

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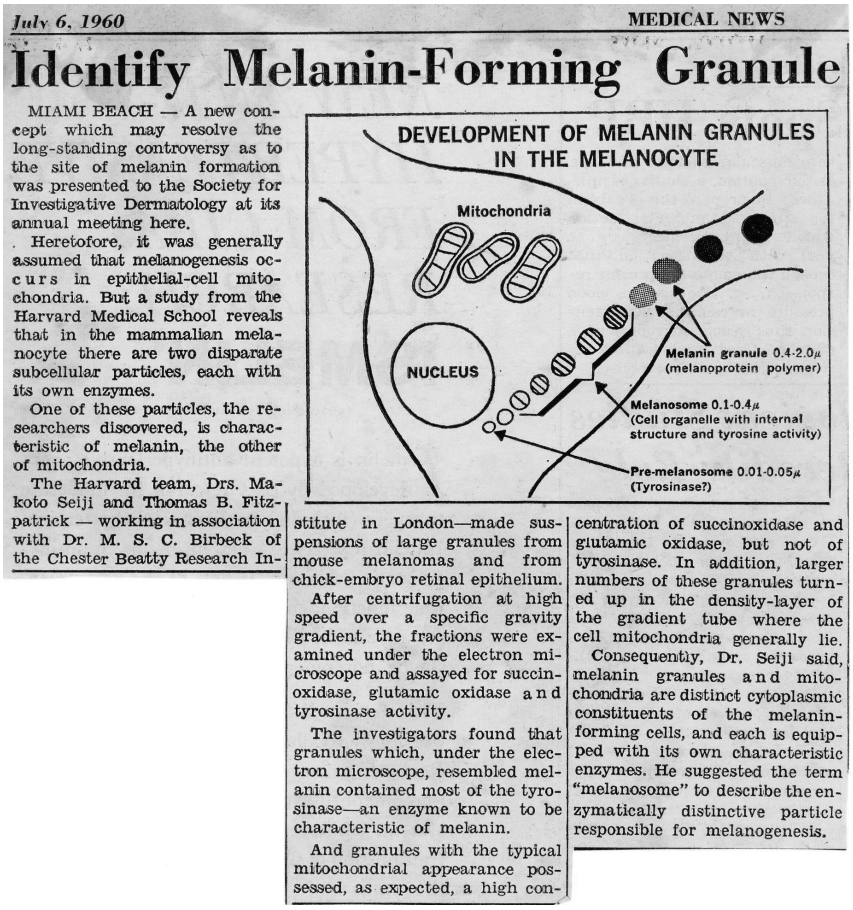

July 6, 1960 MEDICAL NEWS

Identify Melanin-Forming Granule

MIAMI BEACH — A new concept which may resolve the long-standing controversy as to the site of melanin formation was presented to the Society for Investigative Dermatology at its annual meeting here.

Heretofore, it was generally assumed that melanogenesis occurs in epithelial-cell mitochondria. But a study from the Harvard Medical School reveals that in the mammalian melanocyte there are two disparate subcellular particles, each with its own enzymes.

One of these particles, the researchers discovered, is characteristic of melanin, the other of mitochondria.

The Harvard team, Drs. Makoto Seiji and Thomas B. Fitzpatrick — working in association with Dr. M. S. C. Birbeck of the Chester Beatty Research Institute in London—made suspensions of large granules from mouse melanomas and from chick-embryo retinal epithelium.

After centrifugation at high speed over a specific gravity gradient, the fractions were examined under the electron microscope and assayed for succinoxidase, glutamic oxidase and tyrosinase activity.

The investigators found that granules which, under the electron microscope, resembled melanin contained most of the tyrosinase—an enzyme known to be characteristic of melanin.

And granules with the typical mitochondrial appearance possessed, as expected, a high concentration of succinoxidase and glutamic oxidase, but not of tyrosinase. In addition, larger numbers of these granules turned up in the density-layer of the gradient tube where the cell mitochondria generally lie.

Consequently, Dr. Seiji said, melanin granules and mitochondria are distinct cytoplasmic constituents of the melanin-forming cells, and each is equipped with its own characteristic enzymes. He suggested the term "melanosome" to describe the enzymatically distinctive particle responsible for melanogenesis.

DEVELOPMENT OF MELANIN GRANULES IN THE MELANOCYTE

Mitochondria

NUCLEUS

Melanin granule 0.4-2.0μ (melanoprotein polymer)

Melanosome 0.1-0.4μ (Cell organelle with internal structure and tyrosine activity)

Pre-melanosome 0.01-0.05μ (Tyrosinase?)

Figure 1.1 Announcement of the independent status of melanosome in *Medical News* on 5 July 1960.

of pigmented cells, but was present in the form of discrete aggregates [5, 6] (Figures 1.3 and 1.4).

Deciphering the old literature is problematical as authors often fail to distinguish between melanin (the pigment itself), melanoprotein (the natural melanin–protein complex), and melanin granules (the subcellular organelle). If the method of separation is not adequately described, it is difficult to be certain what material was studied and any conclusions can be misleading [8]. The lack of electron microscopic identification of isolated material led to many misinterpretations; for example, the "melanopseudoglobulin" studied by Greenstein *et al.* [9] was later shown to be melanosomes [10] and Bolt's "melanoprotein" [11], widely used in biophysical studies, turned out to consist of damaged melanosomes [12]. Mason *et al.* [10] posed the question of whether melanin granules were particles with a specific structure or consisted of random aggregates of precipitated metabolic

Figure 1.2 Professor Makoto Seiji (left) and Professor Thomas B. Fitzpatrick (right) in 1972.

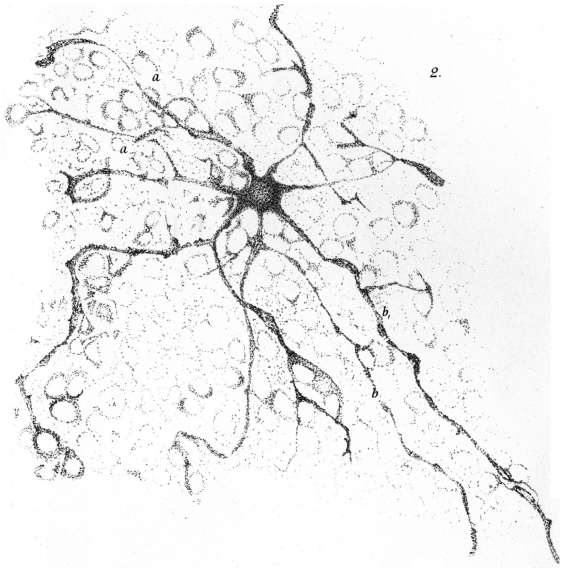

Figure 1.3 "Chromatophore" from donkey conjuctiva [7].

products. The introduction of electron microscopy was able to resolve this matter and Laxer *et al*. [13] were able to discern an inner ultrastructure in isolated melanosomes. The first clear pictures were obtained only in 1956 [14].

An avalanche of papers in subsequent years brought with it enormous amounts of information on the ultrastructure of melanosomes and its changes during

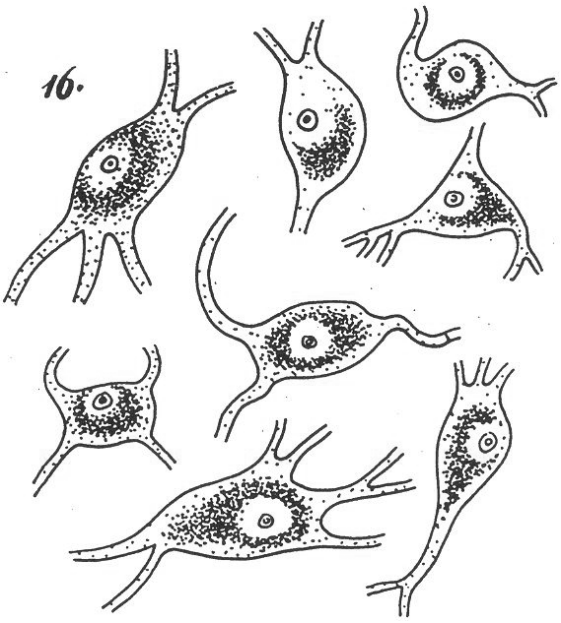

Figure 1.4 Cells of substantia nigra containing neuromelanin [5].

melanosome development (good examples are [15–17]). Other papers (reviewed in [18]) brought together ultrastructural and biochemical data that, in combination, laid the basis for the nomenclature of melanosomal ontogenesis.

By comparison with the morphological data, biochemical investigations of melanosomes were more modest, mainly due to the fact that ultrastructural data were derived from studies of intact cells or tissues, whereas biochemical research used samples prepared by relatively harsh preparative procedures. These samples sometimes consisted of melanins, or altered melanosomes, or their fragments, usually without any check of their nature or homogeneity [18].

The aim of researchers in the nineteenth century was not to prepare subcellular particles or native melanoproteins, but to separate the colored pigment ("Farbstoff" = melanin in the terminology of that time). The presence of protein in the isolated material was considered an unwanted contaminant [19]. Probably the first mild separation protocol was used by J.J. Berzelius [20]. He investigated pigment (melanosomes?) obtained from eye membranes by water extraction, and noticed its insolubility in acids and limited solubility in alkali. Similar mild extraction procedures were used by Landolt [21] and Mörner [22]. The early isolation procedures were reviewed by Waelsch [23]. He studied "natural melanin" from human melanoma metastases and horse choroids, confirmed the presence of protein attached to pigment, and suggested that melanin could be synthesized from the cyclic amino acids present in the protein moiety; this idea has not been

abandoned till now. Herrmann and Boss [24] demonstrated dopa oxidase activity in the fraction of melanin granules from ciliary bodies of cattle eyes, but, as their samples were contaminated with mitochondria, they demonstrated the presence of mitochondrial enzyme markers as well. In 1949, du Buy *et al*. concluded that melanosomes are modified mitochondria typical of pigment cells [25]. It is interesting that du Buy [26] and other authors [27] did not abandon the mitochondrial theory of melanosome origin even in 1963 (i.e., 2 years after the formulation of Seiji's melanosomal concept) and even published their papers in the same volume in which Seiji *et al*. published detailed confirmation of their model [28].

It is interesting that history has disregarded the contribution of Stein [29] who, several years before the work of Seiji *et al*., using a separation procedure of his own, isolated melanin granules from ox choroids and analyzed their content not only of melanin, but also lipids, carbohydrates, RNA, and metals (including the pioneer finding of a high level of zinc), and concluded that the chemical composition of melanin granules is completely different from mitochondria.

The ability of melanin in melanin granules, isolated from Harding-Passey melanoma and from the ink sac of *Loligo opalescens*, to act as a cation exchanger [30], and the demonstration of free radical activity in melanin-containing tissues [31] also rank among the observations of the pre-Seiji era.

1.3
Melanosome Research in the Seiji Era

1.3.1
Terminology of Melanosomes

The demonstration of melanosomes as unique pigment cell organelles possessing developmental stages prompted the introduction of a system of terminology that reflected the characteristics of the various states. Until 1961 the common term for all varieties of these organelles was melanin (or pigment) granule [1, 2]. The first system of nomenclature [2] described three stages in the ontogenesis of melanosomes:

i) Premelanosomes: spherical organelles.
ii) Melanosomes: organelles with an internal structure and tyrosinase activity.
iii) Melanin granules: melanoprotein polymer.

A second terminological system was proposed [3, 26] consisting of three developmental stages plus a final product. Thus:

- Stage I (first stage): biosynthesis of protein.
- Stage II (intermediate stage): biosynthesis of organelle.
- Stage III (late phase): biosynthesis of melanin.
- Final product: melanin granule.

These nomenclature systems introduced a certain degree of confusion, particularly as the term melanin granule had been used to describe pigment granules at any developmental stage. In an attempt to establish a consensus, Fitzpatrick *et al.* [32, 33] circulated a postal questionnaire seeking opinions about the adequacy of the terms in common use in pigment cell research and, with the approval of the participants of the Sixth International Pigment Cell Conference in 1965 in Sofia, Bulgaria, recommended the use of two terms:

- **Melanosome:** a discrete melanin-containing organelle in which melanization is complete as indicated by its almost uniform density by electron microscopy and the absence of demonstrable tyrosinase activity.
- **Premelanosome:** a term applied to all the stages in melanosome biogenesis that precede the fully developed state. Within the restrictions of this general definition, the premelanosomal stage might, at the discretion of the investigator, be subdivided into early, intermediate, and late phases.

The nomenclature in general use today does not adhere to any of the three systems outlined above, but is essentially a system proposed by Toda *et al.* [34–36] reflecting the earlier descriptions of Birbeck [37, 38] which employs the uniform term "melanosome" with a numerical indication (I–IV) of the degree its ontogenetic development.

However, in practice, chaos prevails. While the system of Toda *et al.* is widely – if somewhat erratically – used, some European authors refer, often incorrectly, to the stages proposed in the second system of nomenclature [3, 26] and some American authors tend to cite nomenclature introduced in their previous papers or those of their friends.

1.3.2 Ultrastructural and Histochemical Studies

The concept of subcellular biosynthesis and localization of melanins and melanoproteins in melanosomes was further confirmed by (i) autoradiographic evidence with [^{3}H]dopa and [2-^{14}C]dopa [39–43]., (ii) incorporation of [2-^{14}C]dopa and monitoring radioactivity in subcellular fractions [44, 45]., and (c) isolation of melanosomes and analysis of their chemical composition [46, 47].

Electron microscopy enabled the definition of the basic morphometric data of isolated melanosomes (i.e., their size, shape, and ultrastructural appearance). The most extensive data were published by Hach *et al.* [48, 49]. For discussion concerning the ultrastructural appearances of melanosomes, see Section 12.3 in Chapter 12.

Various pathological states may be manifested by changes in melanosome morphology. Mishima *et al.* [50] considered that melanosome polymorphism, such as changes in size, shape, ultrastructural matrix, the manner of melanin deposition, and the degree of melanosome maturation, as a criterion of molecular pathology that could find practical use in the differential diagnosis of various pigmentary disorders.

In melanoma cells, various irregularities in the architecture of melanosomes are common. Deposition of melanin can be uneven, leading to a bizarre appearance of melanosomes [51–57]; the presence of melanosomes of all stages of development is typical [51, 52]. Melanoma melanosomes also often exhibit defects of their limiting membranes that may lead to leakage of toxic melanin precursors into the cytosol. The pathological consequences of this failure of containment of melanogenic intermediates are discussed in Section 12.4.2. Extracellular deposition of melanin on fibrils resembling melanosomal matrix fibrils has also been observed in melanoma cells [58, 59].

Early ideas on melanosomal biogenesis were summarized in several studies [52, 60, 61].

Fitzpatrick and Breathnach defined a functional unit in human epidermis named the "epidermal melanin unit." This was viewed as a symbiotic relationship between melanocytes and keratinocytes in which each melanocyte supplies approximately 36 keratinocytes with melanosomes [62]. The mechanism of melanosome transport and transfer to keratinocytes was outlined in Mottaz and Zelickson [63]. Szabó *et al.* [64] demonstrated racial differences in the fate of melanosomes in human epidermis.

1.3.3 Biochemical Studies

A prerequisite for classical biochemical studies is the ability to isolate native and pure melanosomes [65]. To this end 17 isolation protocols suggested for the isolation of melanosomes between 1940 and 1973 were critically reproduced [18, 65], and it was concluded that the best samples could be obtained using the procedures described by Stein [29], Doezema [66], and Haberman and Menon [67]. Isolation of melanosomes from keratinous material turned out to be more difficult because the need to release melanosomes from hair required chemical means of tissue disintegration, which always engenders a search for a compromise between sufficient tissue disintegration and minimizing the extent of melanosome modification by the isolation procedure [61, 68]. Ten methods for isolating melanosomes from hair were critically reviewed and half of them, which from the assessment of the isolation conditions seemed to be promising, were reproduced [68]. The best results were obtained with the isolation protocol of Borovanský and Hach [69].

The availability of melanosome samples of adequate quality [18] opened the gate to the subsequent establishment of their basic chemical composition. Melanin and protein moieties were shown to be dominant constituents of melanosomes [46, 47, 61, 70–72]. Isolated melanosomes were reported to contain 5–10% of carbohydrates [29]. Tyrosinase, as a glycoprotein, also brings into melanosomes sialic acids containing *N*-acetylneuraminic acid [73]. In addition to gangliosides mentioned in Section 12.2, many other lipid constituents in melanosomes have been reported including cholesterol and free fatty acids [74], and phospholipids [3, 75]. The level of total lipids was found to vary between 1–5% [29] and 5–11% [76]. Jimbow *et al.* [77] made a complete qualitative and quantitative analysis of lipids and their

Figure 1.5 Professor Kowichi Jimbow, a pioneer in melanosome research, in 1981.

fractions in isolated Harding-Passey and B16 melanoma melanosomes, and found quantitative differences between them (Figure 1.5). The demonstration of the absence of DNA in isolated melanosomes was clear evidence of the difference between melanosomes and mitochondria [78]. Melanosomes are abundant in various metals as described in detail in the Section 12.6. The description of the development of analytical methods including classical chemical techniques such as titration, spectrophotometry, electrochemical and isotope methods, neutron activation analysis, mass spectrometry, and inductively coupled plasma techniques, together with cell biological methods such as histochemistry, autometallography, autoradiography, and microanalytical techniques (using electron, proton, laser, X-ray, and ion beams), and their use in melanosome analyses is comprehensively covered in a review [79].

Nowadays it sounds incredible, but in the 1960s there were doubts as to whether melanosomes merely represented an association of tyrosinase with melanin or whether the organelles contained other proteins as suggested by the electron microscopic appearance. The matter was investigated by electrophoretic studies of isolated melanosomes treated with detergents. Of the many studies summarized in [61], only four used samples of melanosomes that had been checked for purity by electron microscopy [66, 80–82] and these publications unambiguously demonstrated that melanosomes contain several proteins, some of them of brown color and rapid anionic mobility on electrophoresis suggesting their melanoprotein nature [80–82]. The presence of more proteins in melanosomes was later confirmed by comparison of the amino acid composition of tyrosinase with that of melanosome hydrolysates [11]. Matrix proteins were characterized by means of sodium dodecyl sulfate–polyacrylamide gel electrophoresis in melanosomes isolated from Harding-Passey and B16 melanomas and treated with Brij-35 and

guanidine hydrochloride. A simultaneous ultrastructural study revealed that treatment of melanosomes with guanidine hydrochloride induced partial degradation detectable by electron microscopy [83].

A strong stream of research represented studies aimed at demonstrating the presence of enzymes in melanosomes. Naturally, special attention was paid to the melanosomal marker enzyme – tyrosinase (EC1.14.18.1) [1–3, 84–89].

Among the common constituents of melanosomes are acid phosphatase (EC 3.1.3.2.) [90–95] and other lysosomal hydrolases, such as β-galactosidase (EC 3.2.1.23) [75], β-glucuronidase (EC 3.2.1.31) [74, 96], β-*N*-acetyl glucosaminidase (EC 3.2.1.30) [74], cathepsin D (EC 3.4.23.5) [74], and arylsulfatase (EC 3.1.6.1) [97]. The presence of acid phosphatase and other acid hydrolases used to be explained by adhesion of lysosomal enzymes because during isolation melanosomes are contaminated with phagosomes [90, 96] and autophagosomes [94, 97]. However, as removal of superficially bound proteins by detergents [96] or enzymes [74] did not remove the activity of lysosomal enzymes, they seemed to be integral constituents of melanosomes.

Tyrosine-2-oxoglutarate amino transferase (EC 2.6.1.5) and tryptophan-2,3-dioxygenase (EC1.13.11.11) were demonstrated to be a constant constituent of melanosomes from guinea pig skin [98]. The presence of ATPase (EC 3.6.1.3) is not surprising [45]. γ-Glutamyltransferase (EC 2.3.2.2) was demonstrated in melanosomes and premelanosomes of B16 melanoma cells [99]. γ-Glutamyltransferase is thought to have a role both in melanogenesis and in cellular protection against oxidative stress.

Progress in melanosome research was quite rapid. Ten years after the recognition of the melanosome as a unique subcellular particle of pigment cells, the basic biological processes associated with pigmentation were shown to be related to: (i) formation of melanosomes in melanocytes, (ii) melanization of melanosomes in melanocytes, (iii) transfer of melanosomes into keratinocytes, and (iv) transport of melanosomes by keratinocytes, with or without degradation, in lysosome-like organelles [100]. These four processes were partially characterized, and biochemical knowledge of melanosomes reached a level enabling consideration of their function and the possibilities of exploiting these functions in clinical practice [101–105]. A well-balanced review on the melanosome and melanogenesis, describing the situation at the beginning of the twenty-first century, was written by Tolesson [106]. The advent of the techniques of molecular biology has still further accelerated the growth of our knowledge of melanosomes.

1.4
Melanosome Research in the Post-Seiji Era

Professor Makoto Seiji died in 1982. In recognition of his key role and fundamental achievements in melanosome research the Seiji Memorial Lectureship was established in his memory by the International Federation of Pigment Cell Societies to be given every third year at the International Pigment Cell Conferences.

Symbolically, the first Seiji Memorial Lecture was given by Professor T.B. Fitzpatrick at the International Pigment Cell Conference in Giessen in 1983. In parallel with the "epidermal melanin unit" a "follicular melanin unit" was introduced [107].

Modern analytical techniques of high sensitivity make heavy demands on the purity of the melanosomal fractions studied. Hence, the problem of isolation has resurfaced. For the isolation of melanosomes from keratinized structures enzymatic tissue disintegration has been introduced by Arnaud and Boré [108]. However, they used preliminary treatment of hair either with dimethylsulfoxide at 120 °C or treatment of hair under reflux with an aqueous solution of lithium bromide. Such methods are in absolute contradiction to principles of denaturation-free separation. Isolation methods strongly predetermine the quality of the samples obtained, such as surface area-to-mass ratio as demonstrated by Liu and Simon [109]. In 2000, Prota *et al.* developed an isolation procedure based only on enzyme digestion [110]. Melanosomes of various stages could be separately isolated by inserting into the protocol a free-flow electrophoresis step [111]. Percoll gradients were also introduced into melanosome isolation [112].

Tyrosine-induced increase of melanin was shown to influence melanosomal size and shape, especially of those originating from the light skin types [113].

The list of lysosomal hydrolases was extended by the detection of cathepsin B (EC 3.4.22.1) and L (EC 3.4.22.15) [114], and α-mannosidase (EC 3.2.1.24) [99]. After the discovery of the acidic pH of melanosomes [115], the presence both of acid hydrolases and lysosome-associated membrane proteins 1, 2, and 3, and evidence of phagocytotic ability, melanosomes were designated as specialized lysosomes [116, 117] and their existence as specific organelles was endangered. The ranking of melanosomes among lysosome-related organelles [118] was the only appropriate solution, which readily explains the common participation of lysosomes and melanosomes in some pigmentary disorders such as Chediak–Higashi syndrome and Heřmanský–Pudlák syndrome.

The mechanism of melanosome disintegration and degradation has been studied for a long time [119]. There are many histochemical reports of the presence of acid hydrolases, and particularly acid phosphatase, in melanosome complexes and these have been interpreted as implying their presumptive role in melanosome degradation (reviewed in [119, 120]). However, the reaction specificity of acid phosphatase consists of hydrolyzing phosphate esters and there have been no reports to suggest that phosphates play any part in maintaining the aggregation pattern of melanin or melanosome architecture. Acid hydrolases may have a role the degradation of the protein moiety of the melanosome, but melanin seems to be susceptible mainly to redox reactions [119–122] (see also Sections 12.4.1 and 12.4.2).

Immunological techniques have helped a great deal in understanding melanosome structure and biogenesis. Monoclonal antibodies prepared by immunization with melanosomal proteins [123], but especially antibodies prepared by Hearing against synthetic peptides corresponding to the C-termini of melanosomal proteins, have proved to be invaluable tools in melanosome research [124]. In the post-Seiji era the contribution of molecular biological techniques has been enormous and is reflected in Chapters 2, 9, 10, and 11. The group of Professor John

Simon has recently introduced new sophisticated biophysical and chemical techniques into melanosome research (e.g., [109, 125]).

The combined consensus of the current knowledge of melanins and melanosomes that has emerged from the many investigations briefly alluded to above constitutes the material contained within the chapters written by the leading authorities in the field that illuminate this book.

1.5 Other Historical Aspects

The author has been engaged in pigment research since 1968 and this chapter reflects his subjective preferences for the articles taking into account melanosomes as subcellular organelles. Hundreds of articles (and their authors) dealing with the investigation of processes, control factors, and molecular characteristics of melanocytes, which have no direct relation to melanosomes as functional units, can be found in other reviews. The description of pigment cell research along a time axis was monitored in a unique way by Nordlund *et al.* [126] and there are also articles with a geographical emphasis on pigment cell research [127, 128].

The history of melanocyte research, mentioning the first description by Sangiovanni in 1819 [129] of a pigment cell as a "chromatophore" in the squid, was summarized by Westerhof [130] and repeated by Falabella [131]. Brief historical remarks can be found in [132, 133]. Melanoproteins were first defined in 1910 [134], and studied again by Serra [135] and reviewed in [6, 136]. Since the formulation of an exact definition of specific melanosomal proteins the general term "melanoprotein" has been fading. However, the terminology reappears on occasion in descriptions of the manner of melanin attachment to proteins such as Pmel-17. Of course, the history of melanin and the development of knowledge in the field is much longer than the time since it was given its name by Berzelius in 1840 [20], and is covered in considerable depth in the books by Nicolaus [137] and Prota [138], and in several reviews (e.g., [139]). In his book, Nicolaus [137] divides the development of melanin chemistry into three periods. (i) The period of frustration, which started with the studies of Dressler and Pribram in 1856 and terminated with Raper's fundamental work in the 1930s. (ii) The period of uncertainty 1930–?. In 1968, Nicolaus predicted that ever-increasing interest would soon lead to entry into the third period – (iii) the period of elucidation. It is undoubtedly to this era that the articles of this book belong.

The ever-increasing interest in the investigation of melanosomes can be illustrated by data from the ISI Web of Knowledge (Table 1.1).

Until 1960 the term melanosome on the ISI Web of Knowledge did not exist and a slow increase took place up to the period 1981–1985. The decrease in the subsequent period can be explained by the failure to use the term melanosome among the "key words" as investigators concentrated more on the molecular level. A further complication is that the term melanosome has two meanings: (i) a subcellular particle of pigment cells as described in this book and (ii) a dark region

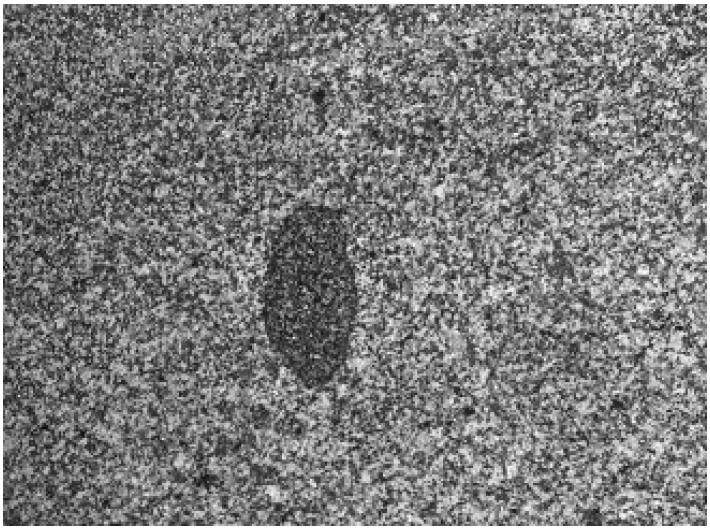

Figure 1.6 Melanosome – a dark region present in migmatite rocks – on the staircase of the Institute of Biochemistry and Experimental Oncology, First Faculty of Medicine, Charles University in Prague. Length = 54 mm.

Table 1.1 Number of entries under the term "melanosome" in the ISI Web of Knowledge (www.isiknowledge.com).

Period (years)	No. entries
1961–1965	4
1966–1970	13
1971–1975	26
1976–1980	29
1981–1985	49
1986–1990	33
1991–1995	131
1996–2000	167
2001–2005	289
2006–2010	343

present in migmatite rocks [140] (Figure 1.6). The entries in Table 1.1 have been adjusted to exclude the geological citations.

Acknowledgments

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