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9

Autofluorescence in human and animal tissues

Autofluorescence, also called *primary* or *intrinsic* fluorescence, is the natural fluorescence of a substance. Many fluorescent substances occur in nature, and these are widely distributed in plants, animals, and minerals: examples of particularly strongly fluorescent substances include chlorophyll, collagen, and fluorite. Autofluorescence is important for two reasons: because of its widespread occurrence, autofluorescence has always to be taken into account; and moreover some autofluorescent materials are of sufficient importance to be studied in their own right – such as NADH, ferulic acid and coal.

In general it may be said that autofluorescence in animal and plant tissues is due to aromatic molecules; accordingly fluorescence is to be found (for example) in proteins, coenzymes, and sundry pigments, whereas pure carbohydrates do not fluoresce. Fluorescence in minerals is generally due to the presence of trace amounts of impurities in the crystal structure, or to the presence of organic material.

This chapter deals with autofluorescence in human and animal material; autofluorescence in plants and minerals is dealt with in the next two chapters. Information on autofluorescence in fluorescence microscopy is relatively scanty and therefore I have included some information on gross fluorescence (i.e. as seen with the naked eye) as a guide to what may be expected to be seen in a fluorescence microscope. The early history of the subject is reviewed in Chapter 18.

The number of fluorescent substances of biological origin described in the biochemical literature is very large. Data on many of these were summarized by Wolfbeis (1985). However, only a small proportion of these substances are relevant to fluorescence microscopy: we may exclude those which are only weakly fluorescent (with a quantum efficiency below, say, 0·02), and (in the absence of special technology) we may exclude also those compounds whose fluorescence lies outside the visible range.

Autofluorescence was the first form of fluorescence which was studied by microscopy, and was reviewed in detail by Haitinger (1938, 1959). To most biological fluorescence microscopists, autofluorescence is mainly a nuisance, as it may mimic some specific secondary fluorescence. On the other hand, tissue autofluorescence may serve to show the general tissue pattern, taking the place of a counterstain, to enable microscopists to find their way around the section. These matters were discussed in Volume 1, Chapter 7.

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Table 9.1. *Strongly autofluorescent substances which may occur in animal tissue, arranged in order of increasing excitation wavelength*

Less common alternative fluorescence colours are shown in parentheses.

Substance	Fluorescence colour	Excitation
Proteins	UV to blue	UV
NADH (protein-bound)	Blue	UV
Kynurenine	Blue	UV
Collagen fibres	Blue-green	UV
Elastic fibres	Blue-green	UV
Vitamin A	Blue-green to yellow-green	UV
N-formylkynurenine	Blue	Violet
Riboflavin	Greenish-yellow	Blue
Flavoproteins	Green to yellow	Blue
Ceroid	Yellow	Blue
Lipofuscins	Yellow-brown to orange	Blue
	(Blue)	UV
Porphyrins	Red	Green (blue)
3-Hydroxyanthranilic acid	Blue	UV
N-formylkynurenine	Blue	Violet
Riboflavin	Greenish-yellow	Blue

Autofluorescence must be distinguished from fluorescence induced by fixation with aldehydes, which react with a number of substances to make them fluorescent: this is an induced fluorescence, and is dealt with in Chapter 12.

In biological material, non-specific tissue autofluorescence usually increases during storage of tissues, either in paraffin blocks or as sections. Hence, old biological material is not usually suitable for study by fluorescence methods.

AUTOFLUORESCENCE IN VERTEBRATES

Some of the more strongly autofluorescent components of animal tissues are listed in Table 9.1.

Cytoplasmic autofluorescence

In cells, the major components of autofluorescence are believed to be due to NADH (blue), and flavins (green); the latter is about one-twentieth the intensity of the former (Aubin, 1979). Aubin (1979) studied the non-protein autofluorescence of cells in culture. Aubin's results can be summarized as follows. The autofluorescence is concentrated in discrete cytoplasmic regions, while the nucleus remains dark. The fluorescence can be divided into two distinct spectral regions, one with spectral maxima at Ex 365 nm, Em 445 nm, appearing blue; the other with excitation maxima at 390 and 440 nm and emitting maximally at 520 nm (green). The former is believed to be due to NADH, the latter to flavins, both bound to proteins. The fluorescence intensity of both

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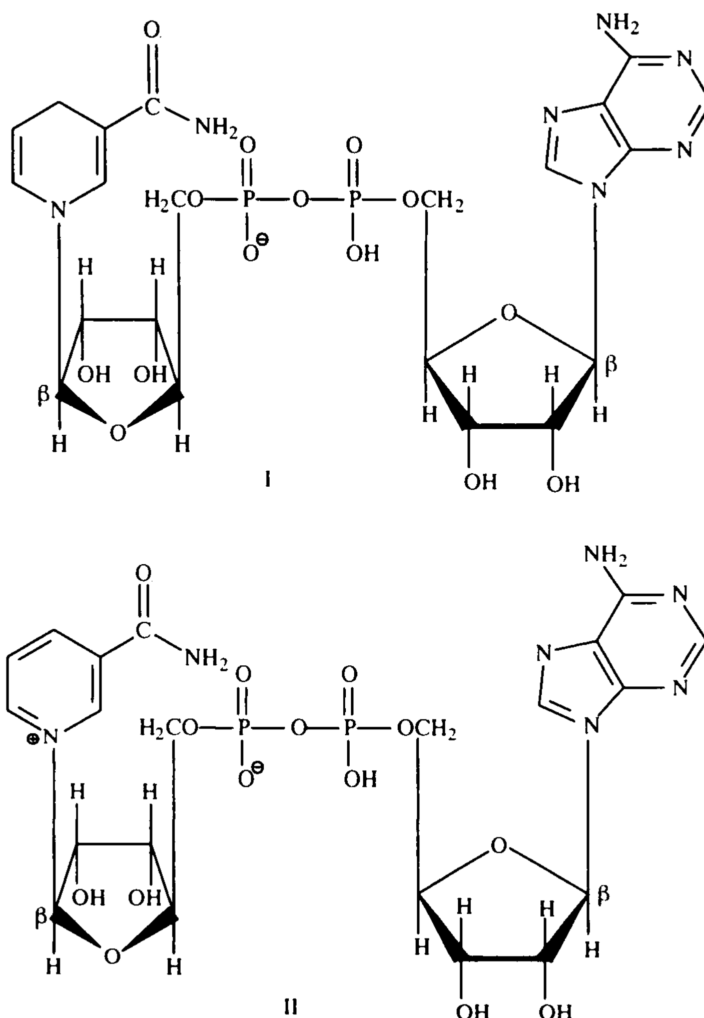
depends on the redox state of the cell. Similar conclusions, that intracellular autofluorescence in the 500–600 nm range is due to flavins, were reached by Benson *et al.* (1979).

NADH (reduced nicotinamide-adenine dinucleotide, I) bound to a mitochondrial component or to a dehydrogenase is believed to be the main source of blue autofluorescence within cells (Chance & Thorell, 1959a; Aubin, 1979). The fluorescence has spectral maxima at about Ex 365, Em 445 nm (Aubin, 1979), and therefore requires ultraviolet (UV) excitation, and a colourless barrier filter. NADH fluorescence was discussed also by Haitinger (1959) and Barenboim, Domanskii & Turoverov (1969). Only the reduced form (NADH) is fluorescent; the oxidized form (NAD^+, II) is non-fluorescent. There is accordingly a change in cellular autofluorescence according to the redox conditions obtaining within the cell. This can be measured by microfluorometry (Chance & Thorell, 1959b). NADH fluorescence has also been measured from the surfaces of a number of organs: cardiac muscle (Chance *et al.*, 1965; Moravec, Hatt &



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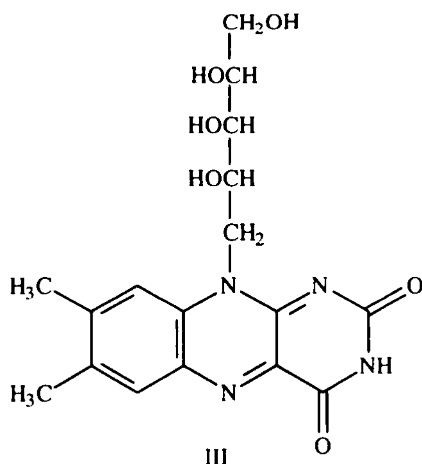
Rost, 1972; Nuutinen, 1984), liver (Ji *et al.*, 1982), and kidney (Franke, Barlow & Chance, 1980). This subject will be referred to again in Chapter 15.

The corresponding phosphates (NADPH, $NADP^+$) have not been specifically studied cytochemically; the fluorescence of NADPH has probably been included with that of NADH.

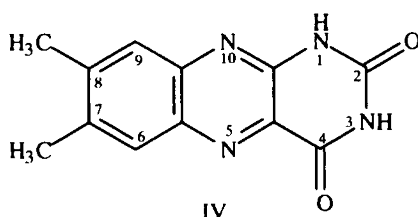
Flavins, bound to proteins (flavoproteins), are believed to be responsible for green fluorescence (Aubin, 1979; Benson *et al.*, 1979) with emission maximum at about 520 nm, and excitation peaks at 390 and 440 nm (Aubin, 1979). Flavins are widely distributed in animal and plant tissues: in general they have a strong yellow-green fluorescence, and are water-soluble. Flavins, like the $NADH/NAD^+$ system, are involved in redox reactions. The oxidized forms are fluorescent. The best known is riboflavine, vitamin B_2 (III). In aqueous solution, riboflavine has maximal fluorescence at 565 nm at pH 6·7 (*Merck index*, 1983). Koke, Wylie & Wills (1981) measured fluorescence attributed to oxidized flavoproteins in isolated heart cells.

Flavins are very sensitive to light. Visible or UV irradiation of riboflavin results in a splitting off of the ribose moiety; in acid or neutral solution, irradiation produces lumichrome (IV), which fluoresces blue, together with varying amounts of a related substance, lumiflavin (V). In alkaline solution only lumiflavin is produced.

Mitochondria show autofluorescence when excited with violet or blue light; this has been applied to the identification of mitochondria-rich cells in epithelia, e.g. to



III



IV

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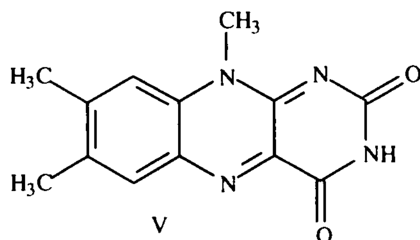

differentiate parietal cells from chief cells in gastric mucosa (Köhler & Frömter, 1985). The fluorescence is believed to be predominantly due to flavins.

Proteins and polypeptides

Proteins and polypeptides usually fluoresce, with emission maximum at about 340–350 nm, if excited by ultraviolet light in the range 250–300 nm, due to the presence of tryptophyl, tyrosyl and phenylalanyl peptide units. As far as fluorescence is concerned, the most important of the amino acids is tryptophan. Tryptophan-containing proteins and polypeptides may show excitation and emission maxima somewhat different from that of tryptophan, or of tryptophyl peptides, depending upon the environment of the protein and due to the presence of other fluorophores. For example, Smith *et al.* (1980) found that dry keratin had an excitation maximum at 290 nm; the appearance of the peak at this position rather than at 280 nm was believed to be due to an inner filter effect of tyrosine in the keratin. Moreover, the fluorescence was observed to have a longer lifetime in the comparatively rigid environment of dry keratin. The fluorescence of proteins has received much attention; see Konev (1967) and Barenboim *et al.* (1969).

The ease with which tryptophyl units in proteins can be excited has led to the widespread use of fluorescence as a tool for probing protein structure and conformation, and for monitoring changes in the protein. However, because the fluorescence of proteins is predominantly UV, it is doubtful whether any significant amount can be observed visually, even given an appropriate barrier filter.

Connective tissues

Connective tissue fibres (collagen, elastic fibres, and basement membranes) are strongly autofluorescent when excited with UV light, e.g. at 365 nm; see Fig. 9.1. Visually, their fluorescence may be confused with that of the formaldehyde-induced fluorescence of catecholamines in small nerve fibres, but they can easily be distinguished by comparison of their excitation spectra (Rost & Van Noorden, unpublished).

Collagen has an emission maximum at about 450 nm (Prenna & Sacchi, 1964), and exhibits difluorescence (Rost, unpublished).

Elastin, the main component of elastic tissue, is a protein which is insoluble, elastic, yellow in colour, and exhibits a brilliant fluorescence when excited by ultraviolet light.

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Its histochemistry was studied by Zanotti (1964). The spectral maxima have been variously measured at about Ex 350 nm, Em 405 nm (Banga & Bihari-Varga, 1974) or Ex 350 nm, Em 428 nm (Thornhill, 1975), or Ex 340 nm, Em 410 nm (Deyl *et al.*, 1980); Prenna & Sacchi (1964) measured a broad emission peak at about 475 nm in elastic fixed with formaldehyde vapour. These properties are consistent with a molecule comprising randomly coiled polypeptide chains joined by covalent cross-links (Partridge, 1962). It is believed that the cross-linkages are associated with two unusual heterocyclic amino acids, desmosine and isodesmosine, discovered in elastin hydrolysates (Thomas, Elsden & Partridge, 1963). Neither the colour nor the fluorescence can be accounted for by the currently documented amino acid composition of elastin, nor by current theories of secondary structure. The fluorescence has been ascribed to a fatty acid and to the desmosines. However, the fatty acid is now known to be an impurity, and the desmosines cannot be responsible (Thornhill, 1972). A group of coloured, fluorescent ampholytes can be freed from elastin by hydrolysis and manipulation under reducing conditions; these have spectral maxima at about Ex 340–350 nm, Em 425 nm under acid conditions, the excitation peak of some changing to 380 nm under alkaline conditions (Thornhill, 1975). Deyl *et al.* (1980) attributed the fluorescence of elastin to

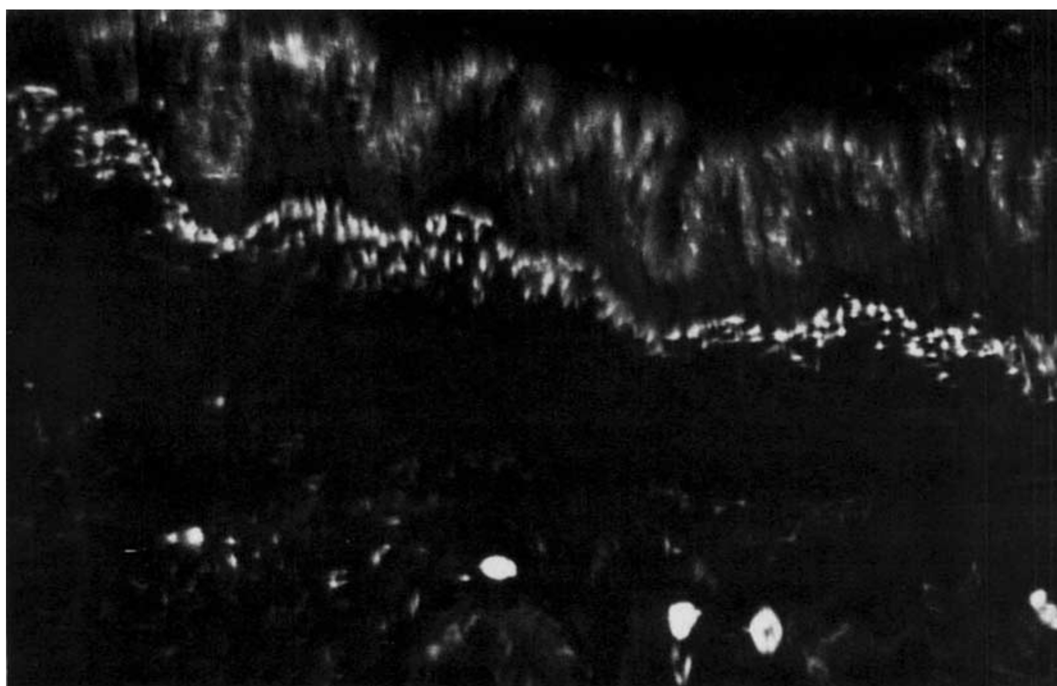

Fig. 9.1. Rat aorta. Field size 0·3 mm × 0·2 mm. The picture shows, from top to bottom: autofluorescence of elastic fibres, formaldehyde-induced fluorescence (FIF) of noradrenaline in sympathetic nerves (see Chapter 12), and FIF of serotonin in mast cells. Freeze-dried, formaldehyde-vapour fixed, Leitz Orthoplan microscope. Preparation by Dr S. Van Noorden, Royal Postgraduate Medical School, London.

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a tricarboxylic, triamino pyridinium derivative, similar to a substance previously described in collagen.

The distinction between elastic fibres and immature or pathologically altered collagen fibres can be a major problem, for example in arteriosclerotic lesions. Immature and incompletely cross-linked collagen fibres may take up 'elastic' stains, and at the ultrastructural level abnormal collagen may lack cross-striations and thus resemble elastic fibres. Puchtler, Waldrop & Valentine (1973) found that elastic fibres could be distinguished by their autofluorescence after treatment with phosphomolybdic acid (PMA) in aqueous solution (for method, see Appendix 5). This method is based on the differing affinity of collagen and elastin for heteropolyacids, because of their differing content of basic amino acids. After treatment with PMA, only elastic tissue remained strongly autofluorescent; while the autofluorescence of collagen, reticulum fibres, basement membranes, pseudo-elastic fibres, and the 'elastic' membranes of small arteries was quenched.

Atherosclerotic human aortae have been studied by Banga & Bihari-Varga (1974). They found two fluorescent substances, of which one was elastin (Ex 350 nm, Em 405 nm) and the other was termed 'atherofluorescent component' (Ex 380 nm, Em 450 nm). They considered that the latter may perhaps be formed as follows: the degeneration and damage of elastin during the atherosclerotic process may lead to the formation of Schiff-base products which react with the peroxidated lipid material which develops in elastin with age. Fluorescence detection of atherosclerotic plaques has been applied to laser angioplasty (Deckelbaum, 1991).

Ocular lens tissue shows autofluorescence, in man and other species (Zigman, 1971). First noted by Stubel (1911), the fluorescence of the human lens is the subject of widespread current interest because of its increase with age (Bleeker *et al.*, 1986) and greatly increased intensity in nuclear senile cataracts (Augusteyn, 1975) and diabetes (Bleeker *et al.*, 1986). The autofluorescence has two main spectral bands, and some minor ones. An ultraviolet fluorescence appears when the lens tissue is excited with light at a wavelength below 300 nm; this fluorescence is characterized by spectral maxima at Ex 296 nm, Em 332 nm. This fluorescence is believed to be due to the presence of tryptophan in the lens proteins (Borkman & Lerman, 1978). Secondly, a visible blue fluorescence appears when the lens is excited at *circa* 340–360 nm, characterized by spectral maxima at Ex 359 nm, Em 435 nm (Lerman *et al.*, 1976). Other fluorescence bands can be produced by excitation at 435 nm (Lerman & Borkman, 1976) and at 647 nm (Yu, Kuck & Askren, 1979). The visible blue fluorescence is not seen in young lenses but becomes apparent and increases with age (Lerman *et al.*, 1976). The blue fluorescence of an intact lens is mainly that of the cortex and outer parts of the nucleus. An unidentified fluorophore (Ex 345 nm, Em 425 nm) was extracted from human lenses by Bessems & Hoenders (1987). A blue-green fluorophore (Ex 407 nm, Em 496 nm) was found by Yu, Bando & Kuck (1983) in the mouse lens. The red fluorescence reported by Yu *et al.* (1979) was found by excitation at 747·1 nm using a krypton ion laser; it occurs in the nucleus of old and brunescent lenses. The emission maximum was measured at 672 nm. Because the cortex of the lens is fully

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transparent at both excitation and emission wavelengths, this provides a means to study changes in the nucleus. Other fluorescence bands, in the orange, have also been reported (Yu *et al.*, 1979).

Bone has a blue autofluorescence, which has been described by Bachman & Ellis (1965).

Teeth have been studied by several workers, of whom the first was Benedict (1928). Human teeth fluoresce blue in UV light, dentine fluorescing more strongly than enamel; the fluorescence is believed to be due to the presence of tryptophan and hydroxypyridium, with peaks at 350 nm and 400 nm respectively (Hoerman & Mancewicz, 1964; Perry *et al.*, 1969; Foreman, 1980). The fluorescence of carious dentine and enamel is weaker than that of normal material in the same tooth (Alfano & Yeo, 1981; Sundström *et al.*, 1985). Dentine and cementum fluoresce red with green excitation, cementum fluorescing more strongly than dentine; the fluorescence of both increases linearly with age (Kvall & Solheim, 1989). Fish teeth were studied by Nishigori, Yamashita & Yamada (1986).

Ivory has in general a white fluorescence. Different species, and different tusks in the same species, may be distinguishable by different fluorescence (Ritchie, 1969). The nature of the fluorescent substances in not known, but it may be surmised that the fluorescence is due at least in part to the presence of collagen and various substances adsorbed to the collagen and to the mineral (hydroxyapatite).

Skin, hair, nails and feathers in various species were studied by Kodrnja (1982). Fluorescence emission maxima were measured, all at about 440 nm. The possum and the tree kangaroo (both of which are Australian marsupials) have fluorescent material adhering to the hair, which in the possum is predominantly 3-hydroxyanthranilic acid and in the tree kangaroo is predominantly a mixture of 3-hydroxyanthranilic acid and kynurenine (Nicholls & Rienits, 1971). Phosphorescence of the keratinized layer of skin was noted by Bommer (1929).

Lipids and related substances

For fluorescence microscopy of lipids, tissues should be fixed in formalin, frozen sections cut and mounted in water (fat solvents will of course remove lipids).

Vitamin A (Retinol, VI) gives a bright green fluorescence, which characteristically fades with great rapidity. This fluorescence, first noted by Querner in 1932 (see Chapter 18) was studied extensively by Popper (1944), particularly in the human and the rat. Strong vitamin A fluorescence was observed in mouse liver after a large dose of the vitamin (Riecken, Gössner & Pearse, 1967). *In vitro*, retinol has excitation maxima between 325 and 328 nm, and corrected emission maxima 475–510 nm depending on the solvent (Wolfbeis, 1985).

Lipid droplets may be demonstrable by fluorescence microscopy. The fluorescence is normally due to substances dissolved in the lipids, which are not themselves fluorescent.

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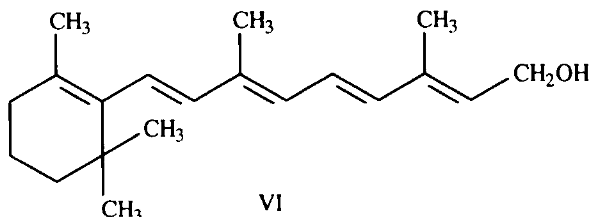

Atherosclerotic plaques have been distinguished from normal arterial wall by spectroscopy of autofluorescence (Kittrell *et al.*, 1985). Two excitation peaks were found at 350 and 480 nm. Excitation at 480 nm produced emission with spectra displaying pronounced differences between normal wall and plaques: normal walls showed emission peaks of approximately equal height at 550 nm and 600 nm, while atherosclerotic plaques showed the same two peaks but with the 550 nm peak always larger than that at 600 nm.

Pigments and related substances

Lipofuscins, also known as 'wear-and-tear' or 'age' pigments, are brown to yellow pigments, of mixed composition, probably derived from lipids by peroxidation. Their presence in cells is one of the most prominent manifestations of cellular aging in a variety of postmitotic cells, both animal and plant. They are commonly seen, for example, in cardiac muscle and liver. Their histochemical characteristics were described by Pearse (1985) and Porto & Hartroft (1969). Lipofuscin fluorescence has been variously reported as red, orange-brown, yellow, or blue (cf. Hamperl, 1953 and Graham, 1968). Eldred *et al.* (1982) reviewed and investigated the colours of lipofuscin, using microspectrofluorometry and chromatograms which they illustrate in colour. They found that lipofuscin granules emit broad-band yellow light when excited with UV.

Lipofuscin, together with melanin, is believed to be a component of neuromelanin. The melanin masks the fluorescence of the lipofuscin component, which, however, can be revealed by bleaching the melanin with hydrogen peroxide (Barden, Aviles & Rivers, 1979).

Autofluorescence of granules has been noted in various organs, particularly in the kidney (Figs. 9.2, 9.3); these latter at least are believed to be lysosomes (Koenig, 1963). The fluorescing material is possibly related to lipofuscin or ceroid; it gives a golden-yellow fluorescence.

Ceroid is a form of immature lipofuscin, and may give a yellow fluorescence. In the central nervous system, a ceroid-type lipofuscin pigment, associated with a disorder known as neuronal ceroid lipofuscinosis, shows a whitish-yellow autofluorescence (Pallis, Duckett & Pearse, 1967); the pigment has an emission maximum around 540 nm (Dowson, 1982).

The retinal pigment epithelium shows fluorescence due to various substances, including lipofuscin. The fluorescence was characterized by Nicolaissen *et al.*, (1984).

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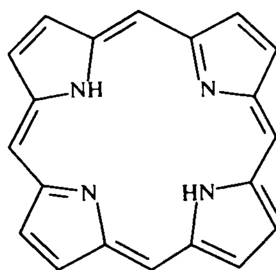
Porphyrins give a red autofluorescence. *Porphine* (VII) is the parent substance of the porphyrins, in which side chains are substituted for the hydrogen atoms in the porphine pyrrole rings. Porphyrins are the basis of respiratory pigments in animals and plants. Examples of porphyrin derivatives are haemoglobin, haematin, vitamin B_{12} (cyanocobalamin) and chlorophyll. Haemoglobin is not fluorescent.

Porphyrins present in liver and bone marrow can be observed by visual examination



VII

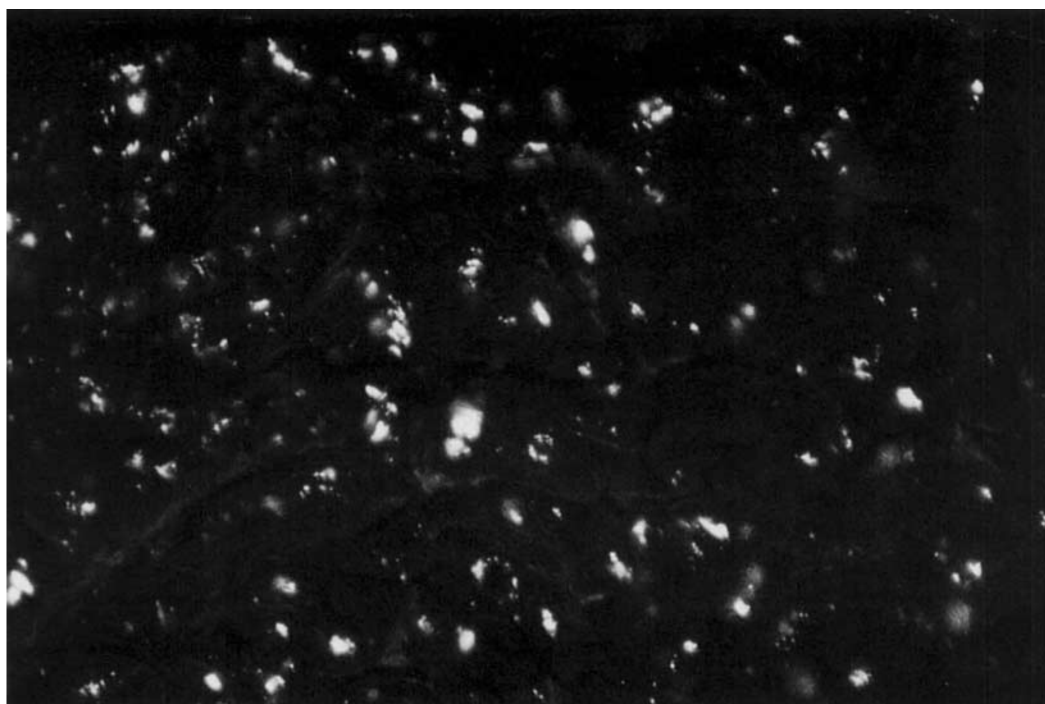


Fig. 9.2. Human heart muscle from left ventricle, showing autofluorescence of lipofuscin ('wear-and-tear' pigment). Field 0·28 mm × 0·42 mm. Freeze-dried, formaldehyde-vapour fixed, treated with 90% isopropanol for 10 min to remove catecholamine fluorescence. Preparation by S. Van Noorden, Royal Postgraduate Medical School, London.

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