Minerals

Iron and calcium are the most important minerals which are capable of being demonstrated histochemically in biological tissues. Other metallic ions, such as potassium and sodium, are present in greater concentrations in tissues, but cannot yet be satisfactorily demonstrated by histochemical means. Other minerals are normally present in such small quantities that they cannot be demonstrated either histologically or histochemically. Iron, calcium and copper are important endogenous minerals; the others dealt with in this chapter are largely exogenous and usually gain access to the body by inhalation into the lungs or by implantation into the skin as foreign material. The most common cause of this illegal entry is as a result of industrial exposure in the mining and metal industries.

Iron

Iron can be found in the body in many forms, most of which can be demonstrated histochemically . Some of the stored iron in the body is found in loose combination with protein in the form of a golden brown pigment called haemosiderin. Another storage form of iron is ferritin, in which iron is bound to a protein called apoferritin. The iron can be separated from the protein by reducing agents such as hydrosulphite. Other iron in the tissues is much more strongly bound to protein (e.g. in haemoglobin, myoglobin) and the iron is not available for demonstration; treatment for a short time by 100 vol hydrogen peroxide may release sufficient iron for demonstration by the Perls' reaction. Particulate iron, usually in the form of an inert iron oxide, may be found in the lungs in industrial disease such as 'haematite (ironoxide)-miners lung' and 'mirror polisher's lung' where fine iron oxide particles are inhaled over many years. Iron in this form isnot directly demonstrable by Perls' or related methods, but haemosiderin and ferritin are invariably found in association with the iron oxide.

Calcium

Calcium is the most important cation in bone, and exists there in the form of bone salts, mainly crystalline hydroxy apatite (hydrated calcium phosphate, with traces of carbonate, citrate and other ions). Methods to demonstrate calcium are widely applied to sections of undecalcified bone in the diagnosis of some types of bone disease, particularly in distinguishing between the two bone diseases, osteoporosis and osteomalacia. In certain disorders of calcium metabolism, excessive calcium is deposited in insoluble form in tissues which normally do not contain calcium, and

the deposition of calcium is common in many degenerative and chronic inflammatory diseases (e.g. atherosclerosis and tuberculosis). Calcium in its insoluble form produces a blue-black calcium lakewith haematoxylin, and therefore is easily seen in a routine haematoxylin and eosin section. Unfortunately this is not specific for calcium but serves to draw attention to the possible presence of calcium in asection. Calcium also forms an orange-red dye-lake with the dye Alizarin Red S; this can be used to identify calcium in tissue sections but is not specific for calcium, since other cations produce coloured lakes with Alizarin Red S. The specificity can be increased by performing the reaction within the p H range 6-3-8-5. A traditional way of demonstrating calcium is by the Von Kossa silver reduction method. This method in fact demonstrates phosphates and carbonates, and not calcium; since most of the phosphates and carbonates present are those of calcium, the method is usually regarded as a fairly specific demonstrator of sites of calcium. Calcium also exists in the body as soluble salts (e.g. calcium chloride) and as ionized calcium in combination with proteins. Calcium in these forms rarely requires demonstration.

Copper

Copper is present in many tissues in concentrations too small to be detectable by histochemical means. In the disease called Wilson's disease ('hepato-lenticular degeneration') there is a disorder of copper metabolism leading to excessive deposition of copper in liver and in the basal ganglia region of the brain. The diagnosis can be established in life by demonstrating excessive copper in a needle biopsy of the liver. The method of choice is the Rubeanic Acid technique *{see Figure 13.4).* Early methods for copper used fresh solutions of haematoxylin (Mallory and Parker, 1939); copper forms a blue lake with haematoxylin. Unfortunately many other metals form lakes with haematoxylin and so the method lacks specificity. *Silica* Silica particles are commonly inhaled by miners of coal and many metal ores, since most ores co-exist with silicaceous rocks. The inhaled silica dust excites a marked fibrous reaction in the lung tissue, leading to the crippling industrial disease, silicosis. The silicaparticles are often mixed with other particles, for example coal dust in coal miner's pneumoconiosis and red iron oxide in haematite miner's lung. There is no histochemical method for the positive identification of silica, but the particles can be demonstrated by their birefringence in polarized light, and by microincineration methods *(see* page 304). A special

form of silica is asbestos, which exists in the form of long thin crystalline fibres. Asbestos fibres may be inhaled by workers in asbestos mines and factories, and by workers in any of the many industries which use asbestos. Prolonged exposure to asbestos can lead to crippling fibrosis of the lung ('asbestosis'), and even slight exposure to certain types of asbestos may eventually induce a type of cancer in the pleural lining of the lung. Asbestos fibres themselvescannot be demonstrated histochemically, but once in the lung the fibres become coated with a smooth protein sheath which contains iron. The 'asbestos body' so formed appears brown in unstained and in haematoxylin-and eosin-stained sections *{see Figure 13.*5), and the iron in the protein sheath can be well demonstrated by the Perls reaction.

Lead

Lead poisoning is now uncommon but used to occur following the ingestion of lead-based paints, usually in children who gnawed toys and cots painted with lead-based paints. Adults working in industries involving lead e.g. the battery industry, occasionally developed lead poisoning. In such cases the excessive lead content of the body can be confirmed by the chemical estimation of lead in the serum, and can be demonstrated histochemically within the tissues, particularly bone. Lead can be demonstrated in tissues by the SulphideSilver method and by the Rhodizonate method. The Sulphide-Silver method has low specificity since many other heavy metals will show the positive reaction. The Rhodizonate method is probably more specific.

Beryllium

Beryllium can gain access to the body as a result of industrial exposure. Engineers working with beryllium may get minute beryllium particles implanted in the skin, but a more common route of entry is by inhalation into the lung. Beryllium can be identified in tissues by the Naphthochrome Green B method, and the Solochrome Azurine method. Many beryllium salts fluoresce and it is often worth examining an unstained section in ultra-violet light; beryllium deposits often emit a characteristic bluish white fluorescence .

Aluminium

Aluminium usually gains entry to the body by inhalation of particles in industrial exposure. It can be identified by the Naphthochrome Green B method, although this is not specific.

Silver

This metal may be found in the skin, alimentary tract and other organs in silver workers; it gives the skin a peculiar slate-grey appearance ('argyria'). In sections the particles appear as dark brown to black granules. Silver can be demonstrated in tissues by the DimethylaminobenzylideneRhodanine method.

Carbon

Carbon is the most common exogenous element found in biological tissues. It is inhaled in particulate form in large amounts by coal miners, smokers and city dwellers. It is phagocytosed by macrophages in the lung alveoli, and is then carried to the regional lymph nodes. Carbon is very inert and cannot be demonstrated histochemically.

HISTOCHEMICAL METHODS

Perls 9 Prussian Blue reaction for iron

This technique, first introduced by Perls (1867), is one of the classical methods of histochemistry. The method can be applied to either paraffin sections or frozen sections. A solution of potassium ferrocyanide and dilute hydrochloric acid is applied to the section. The acid liberates loosely bound iron and the ferric ions then react with potassium ferrocyanide to form ferric ferrocyanide. This is an insoluble blue compound and is located at the site of the reaction.

Alizarin Red S

This technique used by Dahl (1952) and McGee-Russell (1958) is a dye-lake reaction. This means that the dye has the ability to form a lake with the calcium. This type of reaction is not specific for calcium, as other metals may also form a lake with the dye. Haematoxylin, Nuclear Fast Red and Alizarin Red S are all capable of forming lakes with calcium at a suitable pH. The best results are obtained with Alizarin Red S. This dye forms a dye-lake with calcium phosphates and carbonates. The length of the staining time is critical, for if the stain is left too long, the orange-red precipitate diffuses over the section and it is difficult to see

the true deposits. If large amounts of calcium are present, the staining time must be greatly reduced.

Von Kossa method for calcium salts

This technique, first described in 1901, is a metal substitution technique, the calcium in the calcium salt being replaced by a different metal. The method is essentially a means of demonstrating insoluble carbonates and phosphates, but since most of the insoluble carbonates and phosphates in the body are their calcium salts, the method can be regarded as demonstrating sites of calcium. The basis of the reaction is that when a solution containing silver nitrate is applied to a section containing one of these insoluble calcium salts, the calcium is replaced by silver. Sites of calcium salts are marked by a dense black precipitate. Fixation for this technique should be with fixatives containing no free acids and at a neutral pH.

Rubeanic Acid method for copper

The Rubeanic Acid method was first introduced by Okamoto and Utamura (1938), and modified by Uzman (1956) and again by Howell (1959). Howell's modification is given in Method 92. This method can be used to demonstrate copper when the metal isfound in excessive amounts in tissue sections. The original method was described for tissue blocks but Howell's modification allows it to be applied to sections. A dilute rubeanic acid solution in alcohol will give a dark green precipitate of copper rubeanate when applied to sections containing copper.

Rhodizonate method for lead salts

The method is based upon the reaction between lead and the chelating agent, sodium rhodizonate. Lead salts appear red. The method is applicable to decalcified bone.

Solochrome Azurine method for aluminium and beryllium

This metal chelation technique demonstrates aluminium and beryllium; both stain deep blue. The two metals can be differentiated, if necessary, by pretreatment of the section with an alkali which removes aluminium salts but leaves beryllium unaffected.

Naphthochrome B method for aluminium and beryllium

The compound Naphthochrome Green B was used to demonstrate beryllium in tissue sections by Denz (1949). Pearse (1960) modified the technique, which is capable of demonstrating aluminium, iron and calcium as well as beryllium. These metals will react with Naphthochrome Green B to produce a dye-lake. The pH of the dye solution is extremely critical. Beryllium and Naphthochrome Green B produce a deep apple-green colour at pH 5-0. At this pH, calcium is soluble and consequently will not be shown, while iron and aluminium are only lightly stained. If Naphthochrome Green B is used at a slightly alkaline pH, 7*2-7-4, beryllium will stain weakly, while aluminium will produce a deep-green colour.

Paradimethylaminobenzylidene-Rhodanine method for silver

This metal chelation technique leads to the production of a reddish brown deposit of silver rhodanate. The main disadvantage in this technique is that the silver rhodanate is not absolutely insoluble and some diffusion is almost inevitable.

Demonstration of carbon

Carbon is inert and cannot be demonstrated histochemically. It is sometimes necessary to distinguish between carbon and other black pigments such as melanin in a tissue section. Carbon is resistant to the action of Mallory bleach and acids; other black pigments are removed by this treatment.

Inorganic constituents staining methods

Constituent	Staining method	Birefringenc	Site
Iron	Perls	No	Many possible
Calcium	Von Kossa, Alizarin Red S	No	Many possible
Copper	Rubeanic acid	No	Liver
Silica		Yes	Lungs, lymph glands, skin
Lead	Rhodizonate	No	Bone; many other possible sites
Beryllium	Naphthochrome B	No	Lungs, liver, skin
Aluminium	Naphthochrome B	No	Lungs, skin
Silver	Rhodanine	Yes	Intestine, skin