The Role of Iron in Histological Staining

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With two plates (figs. 1 and 2)

SUMMARY

Rapid methods for staining sections with iron sulphide are described which give results comparable with iron haematoxylin.

Nucleic acids and proteins are mainly responsible for the uptake of iron. The staining capacity of different proteins runs parallel with the number of free carboxyl groups they contain. It is concluded that the iron forms non-ionizing complexes with carboxyl and phosphoric acid groups.

The intensity of staining is a measure of the relative abundance of such groups in the proteins and nucleic acids present, and of the concentration density of these substances.

In the staining of nuclei and chromosomes with iron the proteins are at least as important as the nucleic acids.

The iron taken up is mostly in the ferric state but it is partially converted to ferrous iron by reducing substances (chiefly sulphydryl groups) present in many fixed tissues. Iron in the form of iron sulphide or undifferentiated iron haematoxylin therefore acts as a metachromatic stain: a blue colour indicates ferrous iron, a brown colour ferric iron.

It has long been known that nuclear structures have a high affinity for iron. The iron which A. B. Macallum (1908) believed to be a normal constituent of 'nuclein' was shown by Gilson (1892a) and by Wiener (1916) to have been picked up during the manipulations from the traces of this metal present in the vessels and reagents used. The absence of notable quantities of iron in normal nuclei has been confirmed by micro-incineration (Policard, 1934).

Gilson (1892b) made use of this affinity for iron in devising a 'chemical method' for staining nuclei and he describes a somewhat elaborate mixture of iron and nickel salts which gives rather pale staining of nuclear structures. Iron sulphide had been used much earlier by Landois (1865) and Leber (1868), but no differential staining of nuclei was claimed and later reports have been unfavourable (Gierke, 1884).

The present work, which was started with the object of reinvestigating the possibilities of this type of metallic staining, has led to a consideration of the part played by metals, and particularly by iron, in histology. Before dealing with these matters it will be well to describe the methods that have been devised.

Sections were immersed in solutions of the salts of metals which have black insoluble sulphides, rinsed well in running water, and immersed in distilled [Quarterly Journal of Microscopical Science, Vol. 93, part 1, pp. 105-18, Mar. 1952.]
water to which a little ammonium sulphide had been added. Silver, lead, &c.,
gave completely negative results; copper gave almost no staining; nickel and
cobalt were a little better and did give some nuclear staining; but iron was in
a class by itself and gave excellent staining of the nuclei.

TECHNIQUES FOR STAINING WITH IRON SULPHIDE

The procedures finally adopted were as follows:
(i) The sections are immersed in a saturated solution of iron alum for
1 minute, washed well in running water, and immersed in dilute ammonium
sulphide for 15 seconds or longer.
Results: nuclei blue-black; chromosomes intense blue-black; cytoplasm
grey or brown; fibrous tissue pale chocolate brown; Malpighian layer of
mammalian skin blue; red blood corpuscles pale blue-grey. Fig. 1A shows a
whole mount of resting and dividing cells in the fat body of the insect Rhodnius
stained with iron sulphide alone.

If the staining is too dark it may be differentiated by immersing for a few
seconds in 0·25 per cent. iron alum. The section of the heart, epidermis, and
cuticle of Rhodnius in fig. 1D was prepared in this way. Alternatively, after the
initial immersion in iron alum the washed section may be rinsed in 0·1 per
cent. nitric acid for a few seconds before transfer to the ammonium sulphide.

The amount of iron taken up by a given section is constant and never ex-
ceeds a certain maximum. Since iron sulphide is soluble in iron alum it is not
possible to obtain deeper staining by repeating the process; but where heavier
staining is required the following procedures can be used.
(ii) After immersion in ammonium sulphide the section is transferred to a
solution of potassium ferricyanide in which the iron sulphide is at once con-
verted to Turnbull's blue. This is insoluble in iron alum; consequently the
staining process can be repeated and on immersion a second time in ammo-
nium sulphide a double quantity of iron sulphide is formed. This procedure
can be repeated to give the desired depth of staining. Fig. 1E shows a section
of an early rabbit embryo deliberately overstained in this way.
(iii) An alternative method of obtaining darker staining consists in trans-
ferring the section, after treatment with ammonium sulphide and washing in
water, to a saturated solution of cuprammonium sulphate. Within a second or

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Fig. 1

A. Dividing cells in fat-body of Rhodnius (whole mount). Iron alum, ammonium sulphide.
B. Sebaceous gland above and sweat gland below, from human scalp. Iron alum, ammone
nium sulphide, cuprammonium sulphate, ammonium sulphide.
C. Stomach of cat. Staining as in B.
D. Heart with pericardial cells inside, fat-body, epidermis, and cuticle of Rhodnius. Iron
alum, ammonium sulphide, differentiation in dilute alum.
E. Neural tube in early rabbit embryo. Iron alum, ammonium sulphide, potassium ferri-
cyanide (cycle repeated three times), iron alum, ammonium sulphide.
F. Gill of Ostrea. Iron alum, ammonium sulphide, nickelammonium chloride, ammonium
sulphide.
two the iron is displaced by the copper to give the rather pale brown copper sulphide. But the iron is precipitated in situ as the hydroxide so that if the section is rinsed in water after half a minute and re-immersed in the ammonium sulphide, deposits of both iron and copper sulphide are thrown down. This gives a very agreeable sepia colouring well suited for photography. Fig. 1c, showing the stomach of the cat, and fig. 1b, showing parts of a sweat gland and sebaceous gland in the human scalp, were prepared in this way.

The same procedure can be used with any metal which forms a soluble ammine complex with ammonia and is more electronegative than iron. Cobalt and nickel displace the iron more slowly than copper; silver acts rapidly but the ammoniacal solution is unstable, whereas the other salts can be used repeatedly. Cuprammonium sulphate or nickelammonium chloride are the most generally useful. Fig. 1f shows the gill of Ostrea prepared with nickelammonium chloride.

The sulphides of cobalt and nickel are not soluble in strong solutions of the parent salt; by repeated transfer between saturated cobalt nitrate or saturated nickelammonium chloride and the ammonium sulphide solution it is possible to build up the grey-brown staining of the tissues to any required depth.

### Fixatives and Mountants

Most ordinary fixatives can be used. Carnoy (fig. 1, A and D), Bouin (B and C), Heidenhain’s ‘Susa’ (E and F), Zenker’s or Helly’s mercuric chloride and potassium dichromate have all given good results. Osmium tetroxide interferes with the subsequent uptake of iron; Flemming’s or Altmann’s mixtures do not give satisfactory results.

Iron sulphide is readily oxidized in the presence of acid so that the staining fades rapidly in many samples of Canada balsam. This may be prevented by adding a little butylamine to the mountant, and such preparations have remained unchanged for 18 months—although the medium gradually darkens. Euparal with the addition of butylamine also gives permanent preparations. Thick cedar-wood oil ringed with Hazen’s cement has been much used; fig. 1A was photographed a year and a half after mounting in cedar-wood oil. Gurr’s neutral mounting medium, mounting being made directly from absolute alcohol, has proved the most generally useful (fig. 2, A–E).

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**Fig. 2**

A. Closely packed spermatozoa in median vesicula seminalis of Lumbricus. Feulgen’s method.
B. The same. Iron alum, ammonium sulphide.
C. The same. Extraction with 5 per cent. trichloracetic acid at 90° C.; iron alum, ammonium sulphide.
D. Neural tube of early rabbit embryo. Extraction with 5 per cent. trichloracetic acid at 90° C.; iron alum, ammonium sulphide.
E. The same, more highly magnified.
F. Spermatozoa of bull. Normal; iron alum, ammonium sulphide.
G. Spermatozoa of bull. Extraction with 5 per cent. trichloracetic acid at 90° C.; iron alum, ammonium sulphide.
FACTORS INFLUENCING THE UPTAKE OF IRON

Many factors influence the amount of iron taken up by the sections. The following have been considered.

(i) Valency. Ferric iron is taken up much more rapidly than ferrous. M/2 ferric ammonium sulphate was compared with freshly prepared M/2 ferrous sulphate, sections of rat embryo at 8μ being used as test material. In the case of the ferric salt uptake appeared to be complete within about 1 second; there was no perceptible difference in the amount of iron taken up after immersion for 1 minute and 1 second. In the case of the ferrous salt very little iron was taken up in 5 seconds. The amount increased as immersion was prolonged but it did not reach its maximum for several hours. After immersion for 12 hours the depth of staining was about equal to that given by the ferric salt.

(ii) Time of immersion. As seen in (i) the uptake of ferric iron is complete within a second or so. If treatment in saturated iron alum is prolonged for 2 days staining is very slightly darker than after immersion for 15 seconds, when both preparations are examined at once. When examined a week later the difference is more marked; the staining is more permanent in the sections given 2 days’ immersion in the alum. (Perhaps, after very brief immersion, there are some free acid groups remaining which lead to partial fading of the sulphide.)

(iii) Concentration. Dilution of molar ferric chloride in distilled water to M/5 causes a reduction in the depth of staining equal to perhaps 10 per cent. Dilution to M/25 causes a further reduction of about 10 per cent. Staining becomes progressively less at greater dilutions, though even at M/30,000 some iron is absorbed. At each concentration there is a maximum uptake which is soon reached and is not changed by prolonged immersion.

(iv) Temperature. The effect of temperature is complicated by hydrolytic dissociation; the solutions become darker on warming owing to the increased formation of ferric hydroxide. Ferrous salts are much less subject to hydrolytic dissociation. Freshly prepared ferrous sulphate (M/2) gives a greater speed of uptake at 80° C. than at 20° C. but the depth of staining which is finally reached is unchanged. It would appear that high temperature accelerates the uptake but has no effect on the final equilibrium.

(v) Acidity. We have seen (p. 106) that iron is readily removed from the section by treatment with dilute acids before immersion in ammonium sulphide. The uptake of iron is also depressed by increasing acidity. With a given concentration of iron salt the depth of staining is reduced as the pH is lowered; but with high concentrations (M/2FeCl₃) all the constituents of the tissues will stain as usual in N.HCl, although less strongly than in neutral solution. These results have been obtained equally with ferrous iron in which little hydrolytic dissociation occurs. The effect of acidity on staining will be considered in more detail later (p. 113).

These results do not provide conclusive evidence of the nature of the forces by which iron is held in the tissues; but the effects of valency, of temperature,
and of acidity suggest that the metal is held by chemical combination rather than by physical adsorption.

**Substances Responsible for Binding Iron**

The iron taken up by tissue sections is in the 'masked' state. If the sections after immersion in iron alum are washed in a saturated aqueous solution of copper sulphate, only a very small part of the iron is displaced by copper. Sections after treatment with iron alum give almost no colour with neutral potassium ferrocyanide, and sections after treatment with ferrous sulphate give no colour during brief immersion in ferricyanide. Clearly the iron is in some non-ionizing complex, but this complex is readily broken down by ammonium sulphide.

Many biological substances form complexes with iron (Tompsett, 1934), but only those which are non-diffusible and which are present in relatively massive concentration will bind enough iron to be visible in histological sections. The obvious materials in question are nucleic acids, proteins, perhaps carbohydrates and phosphatides.

**Nucleic acids.** If nucleic acid is dissolved in a saturated solution of urea and a little ferric chloride is added, nucleic acid is precipitated and carries the iron down with it so firmly bound that it will give no colour with ammonium thiocyanate (Smythe and Schmidt, 1930). If finely powdered nucleic acid is dusted on a microscope slide lightly smeared with glycerine-albumen and held in place with a thin film of celloidin, it can be subjected to the same staining procedures as the tissue sections. Immersed in iron alum, rinsed in water and copper sulphate, and treated with ammonium sulphide, each particle shows a thin black coating over a colourless interior. Apparently the precipitate on the surface prevents the iron from reaching the centre of the particle. If the nucleic acid is very finely dispersed by allowing the solution in urea to dry on the slide and then covering with celloidin, an intense general staining with the iron is obtained. If the nucleic acid before drying and powdering is mixed with a non-staining protein like salmine (p. 110), this seems to facilitate the entry of the iron and the particles stain throughout.

The importance of nucleic acids in iron staining cannot be in doubt. The part they play has been studied in sections of *Lumbricus*. If these are stained by Feulgen's method after Heidenhain's 'Susa' fixative the abundance of desoxyribonucleic acid in the developing spermatzoa is very striking. In the young spermatids it is in the form of dense granules in the nuclei; later it forms a round homogeneous core to the spermatid with a non-staining cortex. The core gradually elongates to form the head of the spermatozoon and when the ripe sperm come to line the median seminal vesicle their closely packed heads appear in the Feulgen-stained sections as intense red bands (fig. 2A).

If the sections are stained with iron sulphide, the most strongly Feulgen-positive structures stain an intense blue-black; the structures showing a weaker Feulgen reaction stain a paler blue-grey; most of the remaining structures stain varying shades of grey-brown (fig. 2B). In the epidermal and some other
cells there are blue-grey zones around the nuclei, and in the nerve-cells there are blue-grey networks. The nucleoli stain blue-black. Ribonucleic acid is presumably responsible for much of this deep staining.

If the sections are heated in 5 per cent. trichloracetic acid at 90° C. for 15 minutes (Schneider, 1945) to remove the nucleic acids, and are then stained with iron sulphide, most of the blue-grey and blue-black structures will disappear. The bands of ripe sperm show an almost colourless zone where the deeply staining heads were before (fig. 2c). The core of the spermatids is unstained and the nucleoli in the giant nerve-cells and in other cells are markedly paler.

**Proteins.** In many tissues, however, the extraction of the nucleic acids with trichloracetic acid makes comparatively little difference to the staining with iron. In the placenta and embryo of the rabbit, for example, there is almost no reduction in the depth of staining in the nuclei and chromosomes after the nucleic acids have been removed (fig. 2, D and E). Clearly the residual protein in the chromosomes is largely responsible for the uptake of iron. (Kaufmann et al. (1950) showed that the protein framework of the chromosomes stains with acid dyes even better than normally, after removal of nucleic acids.) In the salivary chromosomes of *Drosophila*, although there is some reduction in iron staining after trichloracetic acid extraction, their normal banding can still be clearly seen.

The question arises whether the differential staining of the tissues after removal of nucleic acid is due in part to differences in the capacity of different proteins for binding iron or whether the intensity of staining is simply a measure of the density of the protein.

This has been studied by dusting finely powdered proteins on to microscope slides thinly smeared with glycerine-albumen, covering with a very thin layer of celloidin, denaturing in alcohol, and staining by the same procedure as the tissue sections. It is convenient to apply four different proteins to four small quadrants on the slide so that they receive identical treatment. The depth of staining varies widely, of course, with the size of particle, but it is not difficult to choose for comparison particles of the same size—a few micra in diameter. The method is highly subjective and will only give reliable results where there are large differences in the affinity for iron.

Table 1 summarizes the results. The staining properties differ widely. The most deeply staining are the muscle proteins tropomyosin and myosin from the rabbit; the nuclear proteins from the thymus of the calf, chromosomin and histone, fall little short of these; whereas the cereal seed proteins, zein and gliadin, and the protamine salmine are virtually unstained. Clearly there are large differences in the affinity for iron in the different proteins.

In the spermatozoa of *Lumbricus* after extraction with trichloracetic acid the sperm head is quite colourless in the posterior half; it shows a very slight grey-brown staining in its anterior half. This difference is just visible in fig. 2c, but is conspicuous in the specimens. The deep blue-black staining of the normal sperm head is clearly due wholly to nucleic acid; the protein moiety must be almost all of the non-staining, probably protamine, type. On the
other hand, the apical piece and the middle piece stain quite deeply after removal of nucleic acid (fig. 2c). It is otherwise in the spermatozoa of the bull. Fig. 2f shows a smear of normal bull sperm fixed with Bouin's solution and stained with iron sulphide. Fig. 2g shows a similar film after trichloracetic acid extraction. The head after extraction is much paler (it now stains less than the tail) but it still retains a considerable amount of iron. Perhaps the protein here is of the histone or chromosomin type.

Other substances. Grains of 'soluble starch' retain little iron. They give a uniform pale staining, showing that the iron penetrates rapidly throughout the grain, but very little is held. The chitinous cuticle of insects shows no more staining than can be expected from its protein content. Carbohydrates are probably of little importance in the uptake of iron. Egg lecithin smeared on a slide will bind considerable amounts of iron; phosphatides firmly held by the tissue proteins may play a small part in staining with iron. Guanosine phosphoric acid will bind iron like nucleic acid; the various mononucleotides present in the tissues may contribute to its retention, and so doubtless do other non-diffusible phosphoric acid compounds.

Nature of the Union with Iron

There seems little doubt that iron will be bound by any monesters or diesters of phosphoric acid present in the fixed tissues. It was shown by Fischer and Hultsch (1938) that all such esters will form non-ionized compounds with ferric iron. They consider that the combination with nucleic acid is of this type. Whether these compounds are simple salts or chelate complexes is uncertain. A precipitate will form with diphenylphosphoric acid, for example, where there is little likelihood of chelation. In the case of nucleic acids, however, it would seem that there are many opportunities for the formation of co-ordinate linkages to stabilize the electrovalent bond—for example, with the hydroxyl groups of the sugar.
The binding of iron by proteins has been the subject of much argument. Bechhold (1928) and his co-workers found that a constant equivalent of iron was firmly held by protein and could not be washed out, and that equivalent quantities of the metal and its anion were retained. They concluded that the salt was held in combination by electrostatic adsorption to the amino and carboxyl groups of the protein. Other authors have favoured the view that there is chemical combination between the iron and the protein. As Fischer and Hultzsch (1938) point out, the matter can only be proved when a stoichiometric relation has been demonstrated between the amount of iron in combination and the concentration of some active group in the protein.

It is possible, however, to get some indication of the probable nature of the linkage from the staining experiments. In the first place the free amino groups would seem to play no part. If powdered egg albumen dusted on a slide (or sections of tissues) are soaked in 40 per cent. formalin so as to block the free amino groups and then immersed in equal volumes of formalin and the iron alum solution, there is not the slightest reduction in the amount of iron retained.

On the other hand, if the free carboxyl groups are methylated by immersion for two or three days in methyl alcohol containing N/10 hydrochloric acid (Fraenkel-Conrat and Olcott, 1945), the uptake of iron is markedly reduced. Finely powdered proteins (ovalbumen, chromosomin, histone sulphate) fixed to microscope slides were treated in this way, transferred to 5 per cent. ferric chloride in acetone for half a minute, rinsed in water, and then immersed in ammonium sulphide. Compared with non-methylated proteins similarly stained there was a striking reduction in the quantity of iron taken up. There was a similar great reduction in the depth of staining of the tissues.

Different proteins vary widely in the number of free carboxyl groups which they contain. Table 1 shows the percentage of free carboxyl groups among the side chains of the proteins studied, taken from the review by Tristram (1949). It can be seen that there is general agreement between the affinity of a given protein for iron and the number of free carboxyl groups in the molecule. The only serious exception for which data exist is insulin, which would have been expected to stain much more deeply than it does. The other polar side chains, amino, amide, phenolic, hydroxyl, sulphhydril (as set out in Tristram’s review) show no correlation with the intensity of iron staining.

In the binding of iron by proteins it thus seems highly probable that the initial linkage is an electrovalent bond with free carboxyl groups. But the stability of the complexes of iron with organic compounds depends upon the tendency for iron to form chelate compounds of the type

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\begin{align*}
R-C & \quad OH \quad Fe^{III} \quad O \\
R-C & \quad O \quad Fe^{II} \quad HO
\end{align*}
\]
in which the ionization of the salt linkage is suppressed by the co-ordination of the iron atom with some adjacent group. Ferric iron seems to have a special tendency to link through oxygen. Indeed, the affinity of iron for carboxyl groups is so great that calcium oxalate will actually dissolve in solutions of ferric chloride (Sidgwick, 1950). Ferrous iron will also form complexes of this type but does so much less readily.

It was shown by Smythe and Schmidt (1930) that ferric iron will form non-ionizing complexes with carboxylic acids most readily when there is another carboxyl group or a hydroxyl group immediately adjacent to the first carboxyl. For example, lactic acid or citric acid are effective, \( \alpha \)-hydroxybutyric acid is much more effective than \( \beta \)-hydroxybutyric, whereas there is little tendency to form such complexes with \( \alpha \)-amino acids.

In the case of proteins, therefore, it seems probable that the suppression of ionization of the salt linkage is brought about by co-ordination of the iron with some neighbouring group; perhaps the double-bonded oxygen of another carboxyl group, perhaps more probably the oxygen of an adjacent hydroxyl group.

The formation of such compounds must, of course, depend ultimately upon the steric possibilities of the molecules in question, a matter which has not been critically considered. The same problems arise in connexion with the mordanting of wool preliminary to dyeing. Although the nature of the union of chromium and other metals with the fibre is not exactly known it is believed at the present time to be of the same general type as that outlined above (Race, Rowe, and Speakman, 1946; Bird, 1947).

**Differential Staining of Proteins and Nucleic Acids**

In acid solution iron will be displaced from its salt linkages by hydrogen and the complexes will break down. This will occur more readily with the weaker carboxylic acids than with the stronger phosphoric acids. It is therefore to be expected that at low pH iron will be more firmly retained by those elements in the tissues that are most rich in nucleic acids and phosphoproteins. Indeed, Fischer and Hultzsch (1938), working with very dilute ferric chloride (M/5000), showed that the binding of iron by isolated constituents of the tissues under strongly acid conditions is due wholly to phosphoric acids. The maximum uptake of iron by nucleic acids is attained at pH 1.5, whereas the uptake by proteins continues to increase up to pH 4 or higher.

It was hoped that this might afford a means for the differential staining of proteins on the one hand, and nucleic acids and phosphoproteins on the
other; but the experiment did not prove wholly successful. If powdered casein and ovalbumen are immersed for some hours in M/5000 ferric chloride in 2/3 N. HCl (the solution used by Fischer and Hultzsch (1938)), the phosphoprotein casein gives a uniform pale blue stain with ammonium sulphide, whereas ovalbumen is quite unstained. But when the tissue sections are treated in this way the amount of iron taken up by the nucleic acids is so small that staining is too faint to be of practical value. If the iron concentration is increased to M/2 ferric chloride in 2/3 N. HCl all the tissue constituents stain, though rather faintly. Immersion in saturated iron alum followed by washing out in N/5 citric acid (pH 2-2), N/100 HCl (pH 2) or N/10 HCl (pH 1) gives tolerably good differentiation; the nucleic acid-containing structures in *Lumbricus* retain the iron and stain relatively more strongly. But it is clear that the curves of uptake or loss of iron by protein and nucleic acid in relation to pH are not sufficiently widely separated to give clear-cut differentiation by this means.

**Metachromatic Staining with Iron**

We have seen (p. 106) that iron sulphide gives a different tint in different tissues. This is most evident in sections of the scalp where the fibrous tissue stains chocolate brown, muscle grey-brown, nuclei blue-black, Malpighian layer blue-grey, stratum corneum and the keratohyalin granules of Huxley's layer pure blue, hair unstained. The same is to be seen in the developing 'egg tooth' of the chick, where all the cells of the future 'tooth' are filled with blue-black keratohyalin droplets and contrast sharply with the brown-staining cytoplasm of the cells beneath. In sections of *Lumbricus* most of the tissues stain varying shades of grey-brown, but the sperm heads stain blue-black, spermatids and other nuclei and nucleoli slate-grey, giant nerve-fibres and contents of mucous glands slate-grey.

Ammonium sulphide reduces ferric iron to ferrous sulphide. Since the sections are mostly blue or blue-grey in colour while immersed in ammonium sulphide, and become for the most part grey or grey-brown when washed in tap-water and exposed to the air, it seemed probable that this metachromatic staining results from a variable proportion of ferrous and ferric iron in the different tissues.

This has been tested by immersing the sections in iron alum and then treating with freshly prepared potassium ferrocyanide or ferricyanide. Neither of these salts alone will liberate iron from its combination with the tissues (p. 109), but in the presence of 1 per cent. hydrochloric acid some of the iron at least is set free and the tissues are stained blue. In general the amount of ferric iron present far exceeds that of ferrous: a much more intense blue staining is given by ferrocyanide. But the distribution of the colour after ferricyanide shows clearly that the blue, blue-black, or slate-grey quality in the iron sulphide stain is due to the presence of ferrous iron.

Thus in sections of the scalp treated with *ferricyanide*: the fibrous tissue is practically colourless except for the deep blue nuclei; the darkest blue struc-
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Features are the Malpighian layer, particularly the superficial horny layer, and the nuclei; the keratohyalin granules in the root sheaths are a deep clear blue, Henle’s layer pale blue (fig. 3A); red blood corpuscles pale blue.

When the scalp sections are treated with ferrocyanide: the fibrous tissue is an intense blue; Malpighian layer pale blue, horny layer almost colourless; nuclei are darkly staining; keratohyalin granules glassy and refractile and quite colourless, Henle’s layer colourless (fig. 3B); red blood corpuscles unstained.

Similarly, in sections of *Lumbricus* all those structures which give a slate-grey tinge with iron sulphide stain blue with ferricyanide: nuclei and nucleoli, sperm heads, the core of the young spermatids, the giant nerve-fibres, and the networks in the giant nerve-cells. The muscles also stain blue and so do the contents of the mucous glands.

It is evident that certain of the cell structures, notably the nuclei, still contain reducing substances after fixation which prevent the oxidation of the ferrous sulphide to ferric. These substances possibly vary from one tissue to another. Golodetz and Unna (1909), who observed that the Malpighian layer of the skin would reduce potassium permanganate whereas the fibrous tissue would not, attributed this to the presence of tyrosine in keratin and its absence from collagen. But perhaps it is more probable that sulphhydril groups are responsible. Cystein will give a blue colour instantly in the cold with freshly mixed ferric chloride and potassium ferricyanide, whereas cystine will not, nor will tyrosine, glycine, glucose, or riboflavin. Di- and polyphenols (catechol, pyrogallool) likewise give immediate reduction; so does ascorbic acid; aldehydes (formaldehyde, acetaldehyde) do not.

It has been shown by Giroud and Bulliard (1930) that the soft keratin of the skin and the bulbs of the hair contains fixed —SH groups which give an intense nitroprusside reaction. The material responsible is regarded as the
precursor substance of keratin. When this is fully hardened, as in the hair itself, the sulphur is all in the oxidized -S—S- form and the nitroprusside reaction is negative. Collagen contains no cystine or cystein, which will explain the absence of ferrous iron in the fibrous tissue.

Whether the existence of sulphydryl groups in the side chains of the proteins will explain the presence of ferrous iron in the nuclei, muscles, &c., is less certain; but such groups are widely distributed in fixed tissues (Giroud, 1930) and may well be responsible. A number of proteins were tested by mixing with a drop of dilute potassium ferricyanide, leaving for 10 minutes, and then adding a drop of 0.1 per cent. ferric chloride in 1 per cent. nitric acid. Salmine and gelatine which contain no cystine give no blue colour; β-lactoglobulin gives a faint blue; ovalbumen, chromosomin (from calf thymus), and edestin a darker blue; histone from calf thymus, myosin, and tropomyosin the darkest blue.

The association of ferrous iron with nucleic acids in the cells is interesting. Neither ribonucleic acid nor desoxyribonucleic acid will reduce ferricyanide in the cold. Perhaps the associated histone or chromosomin are responsible, or perhaps some other unrecognized reducing substance is concerned.

**HAEMATOXYLIN, IRON, AND ALUMINIUM**

The differential staining of the tissues by haematoxylin is commonly attributed to differences in the affinity of the different structures for the iron-haematoxylin lake. The present observations suggest, however, that the differential affinity is for iron and the haematoxylin merely serves as an indicator for the varying quantities of iron taken up. Haematoxylin, like other mordant-fixed dyes, forms a very stable chelate compound or lake with iron (Werner, 1908; Race, Rowe, and Speakman, 1946) and further amounts of the dye are possibly added in the form of an adsorption complex (Weiser and Porter, 1928).

If a little haematoxylin (Heidenhain) is added to a solution of ferrous sulphate freshly prepared in boiled distilled water it gives a blue-violet colour; if it is added to a solution that has been kept for some days and contains an appreciable amount of ferric iron a purple colour is produced; with a solution of ferric ammonium sulphate it gives a chocolate-brown colour.

It was interesting, therefore, to examine the colours produced in a section of the scalp immersed in iron alum, placed for a brief period in Heidenhain's haematoxylin and then mounted without differentiation. All those structures (red blood corpuscles, stratum corneum, keratohyalin granules) which, as shown above, contain ferrous iron alone stain blue; structures (such as the Malpighian layer and the nuclei) which contain both ferrous and ferric iron stain purple; whereas the fibrous tissue, containing only ferric iron, stains brown.

The conclusions reached about the uptake of iron by the tissues probably apply equally to aluminium. Like iron, aluminium forms chelate complexes through oxygen (Sidgwick, 1950). In the mordanting of tissue sections it
probably competes for the same groups. Using ammonium sulphide as the indicator for iron, and alizarin as the indicator for aluminium, it can be shown that the relative intensity of staining with the two metals runs parallel. Moreover, the amount of iron taken up from 1 per cent. ferric chloride is reduced if the section has already been immersed in 10 per cent. aluminium sulphate, whereas treatment with iron depresses the subsequent uptake of aluminium.

There is a similar competition between iron and methylene blue (cf. Michaelis, 1947): the uptake of methylene blue by ovalbumen is almost completely inhibited by previous treatment with iron; but these matters have not been studied closely.

CONCLUSIONS

The simple and rapid methods for staining with the sulphides of iron and copper (p. 106) may prove useful for general histological purposes. The monochromatic sepia colouring given with copper is particularly well suited for photography. For low and medium-high powers the results are often equal to those given by iron haematoxylin, but at the highest magnifications they sometimes lack the sharpness and density of iron haematoxylin preparations.

The main interest of the observations lies, however, in the information which they give about the mechanism of the differential staining with compounds of iron. Such staining has the force of a rather crude histochemical test. According to the interpretation advanced in this paper it is a test for free acidic groups (free carboxyl and phosphoric acid groups); and the depth of staining is determined by (i) the relative abundance of such groups in the proteins and nucleic acids present and (ii) the concentration density of these substances in the tissues.

The observations on the staining of chromosomes with iron prove that this is often due as much to the proteins as to the nucleic acids. In some cases the protein seems to be the more important (p. 110) (cf. Stedman and Stedman, 1947).

Furthermore, the relative proportion of ferrous and ferric iron, which is revealed by the blueness or brownness of the colour of the iron sulphide or of the undifferentiated iron haematoxylin stain, is a measure of the concentration of reducing substances (chiefly sulphhydril groups) in the fixed tissues. High reducing power is characteristic of the nuclei and chromosomes, of the telophragma in striated muscle, of red blood corpuscles, and of keratohyalin and soft keratin. Complete absence of reducing power is characteristic of fibrous tissue.

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