QUESTIONS AND ANSWERS
Questions

Chapter 1

Answers can be found on page 14.

1. What type of preparation would you make in order to investigate the populations of cells in the following?:
   (a) the mesentery of the rat
   (b) the articular cartilage of the head of the human femur

2. A frozen section of skin is stained with an oil-soluble dye in order to demonstrate lipids. What would be an appropriate procedure for making a permanent microscopic preparation of the stained section?

3. If alcohol that has been used to dehydrate a fairly large piece of animal tissue is mixed with water, the resulting liquid is turbid. Why?

4. Some staining methods are applied to whole blocks of tissue that are subsequently embedded, sectioned, cleared, and mounted. To what artifacts would this type of technique be especially prone? How could the artifacts be minimized?

5. It is sometimes necessary to remove the coverslip from a section mounted in a resinous medium. Devise a reasonable procedure by which such a section could be made ready to stain in an aqueous solution of a dye.

Chapter 2

Answers can be found on page 14.

1. Deduce the differences that you would expect to see between the appearances of stained sections of animal tissue fixed in:
   (a) formalin, diluted ten times with water immediately before use
   (b) an isotonic solution containing 4% formaldehyde and buffered to pH 7.4

2. Why is it preferable to use Bouin’s fluid rather than an aqueous solution of picric acid alone as a fixative?

3. Before the discovery of the fixative properties of glutaraldehyde, osmium tetroxide (in buffered, isotonic solutions) was the best available fixative for specimens to be examined in the electron microscope. In light microscopy, however, OsO₄ is of limited value as a fixative. Why should this be? Why is OsO₄ useful as a fixative for peripheral nerves but almost useless for central nervous tissue in light microscopy?

4. Glutaraldehyde, although it is an excellent fixative for electron microscopy, is not often used to fix tissues that are to be examined with a light microscope. Why? For what purposes would you expect glutaraldehyde to be the fixative of first choice in light microscopy?

5. What fixative procedure would you use in order to preserve the following?:
   (a) RNA
   (b) neutral fat
   (c) the Golgi apparatus
6. Cetylpyridinium chloride (CPC)

\[
H_3C(CH_2)_4CH_2-N\text{Cl}^-\cdot H_2O
\]

has been used as a fixative for a specific histochemical purpose. What type of substance would you expect to be insolubilized by CPC? Many morphological features are distorted after fixation in CPC. Explain why.

Chapter 3

Answers can be found on page 15.

1. Following decalcification in acidic mixtures, tissues are often placed overnight in 5% sodium sulphate. This is sometimes said to be to neutralize any acid remaining in the specimen, with the effect of preventing swelling during subsequent processing. Is this justification of the use of sodium sulphate correct?

2. Sections are required of a bone-containing specimen fixed in Bouin’s fluid. What would you do before embedding the specimen in paraffin wax? Give reasons for all stages of the procedure. How would the procedure differ for a piece of Zenker-fixed tissue?

Chapter 4

Answers can be found on page 15.

1. Sections of a formaldehyde-fixed specimen are required for a histochemical study of carbohydrate-containing lipids. Which embedding procedures would and would not be satisfactory, and why?

2. Explain how a piece of tissue is dehydrated by (a) dioxane (b) 2,2-dimethoxypropane. Devise a reasonable practical procedure for the use of dioxane in taking a specimen 5 mm³ from Zenker’s fixative into paraffin wax.

3. Paraffin sections of material fixed in formaldehyde are to be stained by a histochemical method for arginyl residues which involves the use of a strongly alkaline reagent. How would you handle the slides between removal of the wax and immersion in the alkaline solution?

4. Euparal is a semi-synthetic resinous mounting medium containing gum sandarac and paraldehyde. It is miscible with absolute ethanol or xylene. Its refractive index is 1.48. For which of the following purposes would it be reasonable to use euparal?
   (a) Sections stained with a cationic dye.
   (b) Sections in which occasional cells contain a stable, insoluble, coloured histochemical end-product.
   (c) Unstained whole mounts of small multicellular animals or plants.
   (d) Sections stained with fat-soluble dyes.

5. Take two slides bearing paraffin sections of a tissue fixed in a mercuric-chloride-containing mixture. Process one but not the other, as described in Section 4.4.1 of Histological and
Histological and Histochemical Methods, fifth edition. Stain both slides by one of the methods described in Chapter 6. Observe the effect of failing to remove mercurial deposits.

6. Stain some paraffin sections of any tissue with (a) polychrome methylene blue, (b) haematoxylin and eosin; and (c) nothing. Mount in DPX, glycerol jelly, and Apathy’s medium (nine slides altogether). The staining solutions are described in Chapter 6. Examine the slides immediately, after 1 or 2 hours and after 1 or 2 weeks. Account for the changes that occur. If you do anything foolish, such as mounting from xylene into an aqueous medium, take note of the results and do not do it again.

Chapter 5

Answers can be found on page 16.

1. Which of the following compounds are coloured and which could be used as dyes?

(a) Dipicrylamine hydrochloride
![Dipicrylamine hydrochloride](image)

(b) N-diphenylbenzidine dihydrochloride
![N-diphenylbenzidine dihydrochloride](image)

(c) Mepacrine dihydrochloride
![Mepacrine dihydrochloride](image)

(d) Chlorpromazine hydrochloride
![Chlorpromazine hydrochloride](image)

(e) Menaphthone
![Menaphthone](image)

2. The compound 3,4-benzpyrene
![3,4-benzpyrene](image)

Can be used as a fluorochrome. Is it a dye? What would you expect it to stain when applied to sections as a saturated solution in water that is already saturated with caffeine? (The caffeine serves only to enable 3,4-benzpyrene to be dissolved in an aqueous medium.)

3. Weigert’s iron–haematoxylin contains 500 mg per ml of haematoxylin and 0.58 mg per ml of FeCl₃.6H₂O (MW 270). This solution stains nuclei. Overstaining does not occur and the sections usually do not require differentiation.

If sections are immersed in 5% iron alum, NH₄Fe(SO₄)₂.12H₂O (MW 482) and then stained in 0.5% ripened haematoxylin, they go completely black (Heidenhain’s method). Differentiation in 5% iron alum, if carefully controlled, permits the demonstration of many structures other than nuclei.

Explain the differences between the Weigert and Heidenhain methods of staining.
4. Urea readily forms hydrogen bonds with other substances. If aqueous solutions of some dyes are saturated with urea they lose their ability to stain sections. Which of the following dyes would you expect to be ineffective in a urea-containing solution?:
   (a) Toluidine blue O
   (b) Eosin
   (c) Methyl blue
   (d) Oil red O.
   Give reasons for your answers.

5. The binding of dyes by electrostatic forces can be suppressed by including a high concentration of a neutral salt such as NaCl or MgCl₂ in the staining solution. Which of the following dyes would you expect to be inhibited by salts?:
   (a) Neutral red
   (b) Procion brilliant red M2B
   (c) Chlorazol black E
   (d) Alcian blue?

6. What structural features of a dye molecule confer the ability to bind metal ions? Draw possible structures for dye–metal complexes of chromium(III) with (a) brazilein, (b) alizarin red S.

7. Dissolve about 10 mg of basic fuchsine in 100 ml of water. Add 4% aqueous sodium hydroxide in aliquots of 0.5 ml. What happens and why?

8. Add single drops of ripened 5% alcoholic haematoxylin to:
   (a) slightly alkaline water
   (b) slightly acid water
   (c) water containing approximately 5 mg per ml potassium alum (KAl(SO₄)₂.12H₂O)
   (d) water containing approximately 5 mg per ml iron alum (NH₄Fe(SO₄)₂.12H₂O) or ferric chloride (FeCl₃.6H₂O).
   For (c) and (d) follow the addition of the haematoxylin with addition of two or three drops of 4% sodium hydroxide.
   Observe the colours of the different mixtures. What do you deduce from these observations about the properties of haematein and of its complexes with Al³⁺ and Fe³⁺?

9. Mix equal volumes of aqueous solutions of:
   (a) 1% neutral red with 1% fast green FCF
   (b) 1% neutral red with 1% toluidine blue
   (c) 1% toluidine blue with 1% eosin
   (d) 1% eosin with 1% fast green FCF.
   Account for the observation that precipitates form with (a) and (c), but not with (b) and (d).

10. Dissolve 50 mg of fast blue B salt (tetraazotized o-dianisidine) in 30 ml of water at 4°C. Dissolve 30–40 mg of thymol in 2–3 ml of 95% ethanol. Pour the thymol solution into the solution of the tetrazonium salt and add three or four drops of 4% aqueous sodium hydroxide. Mix well and leave at 4°C for 20 min. Allow the reddish-brown precipitate to settle and decant off as much as possible of the supernatant liquid. Add 20 ml of ethanol to the precipitate, which will dissolve.
    What is the probable structural formula of the dye you have made? Thymol is 2-isopropyl-5-methyl phenol (MW 150). In view of its structure and the properties you have observed, how might this dye be used as a biological stain?
Chapter 6

Answers can be found on page 17.

1. What differences would you expect to see between two similar paraffin sections of an autonomic ganglion: one stained with H&E, the other by the azure-eosin method?

2. Why is Mayer’s haemalum stable for several weeks, whereas Weigert’s iron–haematoxylin deteriorates after a few days?

3. Which of the following dyes would be suitable counterstains for paraffin sections in which the nuclei have been stained with iron–eriochrome cyanine R?
   (a) neutral red
   (b) erythrosine
   (c) methyl green
   (d) methyl blue
   (e) orange G
   (f) Bismarck brown Y

   (See Chapter 5 for notes on these dyes.)

4. When a specimen has stood for several months in an aqueous formaldehyde solution, it is often very difficult to obtain sufficiently intense staining by eosin when paraffin sections are subjected to the H&E procedure. Suggest a reason for this.

5. What result would you expect if you stained a paraffin section of a specimen fixed in osmium tetroxide (see Chapter 2) with toluidine blue at pH 4.0?

6. Making use of the dyes discussed in this chapter, devise a staining method that would colour nuclei but not cytoplasmic RNA red, and cytoplasm and collagen in some shade of green.

7. Discuss the chemical mechanisms involved in the staining of:
   (a) nuclei by dye–metal complexes
   (b) nuclei by cationic dyes
   (c) cytoplasm by anionic dyes.

Chapter 7

Answers can be found on page 17.

1. How much information could you obtain about the cells contained in a blood-film stained with alum–haematoxylin and eosin?

2. Why are Romanowsky–Giemsa ‘neutral stain’ powders dissolved in methanol and then considerably diluted with water immediately before using?

3. Why is it necessary to stain sections of formaldehyde-fixed tissues with an azure–eosin combination at a lower pH than that required for blood films?

4. What factors determine the shapes and sizes of cells as seen in films and smears? If suspended cells are fixed prior to deposition on the slide, how will their shapes and sizes differ from those of cells fixed after deposition?

5. Prepare some blood films and stain them with Leishman’s or Wright’s stain. Identify the various types of leukocyte. Stain a film with toluidine blue at pH 4 (see Chapter 6) after preliminary fixation in methanol. Does the result suggest any special purpose for which blood films might be stained with a thiazine dye alone?
Chapter 8

Answers can be found on page 18.

1. What are the chemical differences between collagen and elastin? Do these differences account for the stainability of the two substances by different techniques?

2. Discuss the role of phosphotungstic or phosphomolybdic acid in histological techniques for the differential staining of cytoplasm and collagen.

3. Devise a staining procedure whereby nuclei, muscle, collagen and elastin would all be differently coloured.

4. Lison (1955) stained sections of rat’s liver with a trichrome mixture containing orange G and aniline blue. The percentage of yellow nuclei varied with the thicknesses of the sections as follows:

   - 4 μm  0%
   - 6 μm  2.5%
   - 8 μm  16.2%
   - 10 μm 30.1%
   - 12 μm 37.7%
   - 14 μm 43.5%

5. The nuclei that were not yellow were blue. How can these observations be explained? (Hint: the diameter of a nucleus in a fixed, dehydrated and embedded piece of liver is approximately 6 μm.)

6. De-wax and hydrate some paraffin sections of an animal tissue. (Almost anything other than brain is suitable; for best results the tissue should have been fixed in a mixture that does not have formaldehyde as its only active ingredient.) Immerse sections in 2% aqueous phosphomolybdic acid for 15 minutes. Wash in water and then immerse in a freshly prepared 0.5% aqueous solution of stannous chloride (SnCl₂·2H₂O) for 5 minutes. (If you haven’t got any stannous chloride, expose the sections to UV or sunlight until they change colour.) Wash, dehydrate and mount in a resinous medium. What structures in the tissue are stained blue? What chemical reactions produce the colour?

Chapter 9

Answers can be found on page 18.

1. How would you proceed to carry out the Feulgen reaction on sections of a specimen that has been fixed in glutaraldehyde?

2. Using sections of Carnoy-fixed central nervous tissue as test objects, how would you determine (a) whether a sample of DNase is substantially free of RNase, (b) whether a sample of RNase is substantially free of DNase?

3. What types of dyes are suitable for staining the nucleoprotein of the nuclei of mammalian cells? What would be the effect of prior extraction of DNA upon the results of such staining? (Hint: read Section 9.1 and have a look at Chapter 10.)

4. Why do enzymes such as RNase and DNase work more rapidly and more completely on sections of tissue after fixation in alcohol-acetic acid mixtures than after fixation in aqueous formaldehyde or glutaraldehyde? (Hint: the fixative actions of alcohol, acetic acid and glutaraldehyde are discussed in Chapter 2).

5. How would you determine that AgNOR staining does not demonstrate the RNA of nucleoli?
6. Immunohistochemical staining reveals the presence of immunoglobulins in the lesions of many diseases. What could you do to find out whether the immunoglobulin is synthesized in or bound by the cells of the affected organs?

7. Stain paraffin sections of lymphoid and nervous tissues by the methyl green–pyronine method. Apply control procedures to identify cytoplasmic RNA in the presence of other basophilic materials.

8. All the nuclei of an organism’s somatic cells contain the same amount of DNA. Stain some sections by the Feulgen method or with a basic fluorochrome selective for DNA. Why do some nuclei become more intensely coloured than others?

Chapter 10

Answers can be found on page 19.

1. The cytoplasmic granules of the Paneth cells of the intestine are stained strongly by eosin in the H&E and the azure–eosin techniques. How would you set about determining which cationic groups are responsible for the acidophilia of the granules?

2. What would you expect to happen when a section of tissue is treated with a proteolytic enzyme of broad specificity such as trypsin? Could such an enzyme be used to prove that an object seen in a routinely stained section is composed of protein?

3. What would be stained by 0.1% azure A at pH 1.0 in a tissue containing cartilage (whose matrix is rich in sulphated proteoglycans), after the following treatments?:
   (a) nothing
   (b) methylation for 24 hours at 60°C
   (c) sulphation in ether-sulphuric acid reagent.

4. How may azo dyes be formed at the sites of (a) aromatic amino acids, (b) amino groups, (c) ketones?

Chapter 11

Answers can be found on page 19.

1. Which mucosubstances are stained by (a) the periodic acid–Schiff reaction, (b) fluorescein labelled concanavalin A?

2. Hydroxyl groups of sugars can be sulphated (i.e. converted to sulphate esters) by treating sections with sulphuric or chlorosulphonic acid. What effect would you expect this procedure to have on the pattern of staining by thionine or toluidine blue?

3. What substances (if any) would you expect to be PAS-positive in a section which had been
   (a) acetylated
   (b) acetylated and then saponified
   (c) methylated
   (d) methylated and then saponified?

4. What substances (if any) would you expect to stain with alcian blue (pH 1.0 or 2.5) after the following?:
   (a) acetylation
   (b) acetylation followed by saponification
   (c) methylation
(d) methylation followed by saponification
(e) mild acid hydrolysis

5. Which proteoglycans and glycoproteins can bind fluorescently labelled aprotinin? How would the pattern of staining be affected by prior treatment of the sections with (a) amylase, (b) hyaluronidase, (c) neuraminidase?

6. Which of the histochemical techniques discussed here are suitable for the demonstration of glycogen? Describe exactly how you would proceed to demonstrate glycogen in the cells of a piece of liver or muscle freshly removed from an animal.

7. To what extent are chemical blocking procedures useful for confirming the specificity of binding of a fluorescently labelled lectin believed to be specific for the following?:
   (a) terminal α-L-fucose
   (b) N-acetylgalactosamine

How else can one ascertain the specificity of a labelled lectin used as a histochemical reagent?

8. With which (if any) of the methods described in this chapter would you expect to obtain a positive reaction with chitin? How do you account for the fact that the exoskeletal tissues of invertebrates are commonly PAS-positive?

9. A glycoprotein secreted by mucous cells of mammalian submandibular glands bears disaccharide chains with the structure
   \[ \text{NANA} (\alpha-2\rightarrow6) \text{D-Gal} (\beta-1\rightarrow) \text{-PROTEIN} \]

How much of the information contained in this formula could be obtained by histochemical analysis?

10. Reid et al. (1988) developed the following sequence of reactions for the histochemical demonstration of glycoproteins with O-acyl substitution at C2 or C3 of hexose sugars:
   Periodate oxidation → Borohydride → Saponification → Mild periodate oxidation → Borohydride → Periodate oxidation → Schiff’s reagent.

If the section initially contains glycoproteins with PAS-positive neutral sugars and variously substituted sialic acids, what is oxidized by each of the three treatments with periodate?

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Chapter 12

Answers can be found on page 20.

1. How would you determine whether a structure stained by Sudan black B in 70% ethanol was composed of triglycerides or of phosphoglycerides?

2. Tristearin is a neutral fat in which glycerol is esterified by three molecules of stearic acid. Which of the following methods would, and which would not give a positive reaction with tristearin? Give reasons for your answers.
   (a) Sudan IV at room temperature
   (b) Sudan IV at 70°C
   (c) Osmium tetroxide
   (d) The plasmal reaction

3. Answer question 2 for triolein, in which glycerol is esterified by three molecules of oleic acid.

4. Myelin, in frozen sections of nervous tissue, commonly gives a direct positive reaction with Schiff’s reagent. Why? Describe exactly how you would proceed to demonstrate the presence in myelin of (a) plasmalogens, (b) fatty acid amides (ceramides and sphingomyelins).
5. It has been shown by Malinin (1980) that different types of intracellular lipid inclusions may be distinguished from one another by red staining with Nile blue at various temperatures from 19 to 65°C. What does this tell you about the mechanism of coloration?

6. What histochemical evidence could be obtained to support the contention that lipid droplets in the adrenal cortex contain cholesterol and steroid hormones rather than neutral fats or phospholipids?

7. In metachromatic leucodystrophy there are accumulations in the brain of sulphatide in which the fatty-acid residues are largely saturated. Using histochemical methods, how would you distinguish these pathological deposits from the following?:
   (a) proteoglycans
   (b) glycoproteins
   (c) cerebrosides
   (d) gangliosides
   (e) neutral fats
   (f) any unsaturated lipids

8. If wax is not adequately removed from paraffin sections, birefringent crystal-like bodies are seen in the tissue, within nuclei. Using histochemical methods, how would you show that these objects are composed of a hydrocarbon rather than of a naturally occurring lipid, lipoprotein, or mineral material? See Nedzel (1951) for more information about this artifact.

Chapter 13

Answers can be found on page 21.

1. No satisfactory method has ever been devised for the histochemical localization of sodium ions. Why should this be?

2. Devise a simple technique for the histochemical demonstration of chloride in animal tissues, based on the use of the reactions:
   (a) \( \text{Ag}^+ + \text{Cl}^- \to \text{AgCl(s)} \)
   (b) \( 2\text{AgCl} \to 2\text{Ag}^0 + \text{Cl}_2(\text{g}) \) (light)

   What factors would limit the accuracy of localization of chloride ions by this method?

3. How would you proceed to show that a brown pigment found in sections of a tissue contains iron?

4. Classify the types of technique for histochemical demonstration of metallic ions according to the types of reagents used. What factors limit the chemical specificity and the accuracy of localization of metals?

5. Examine the structural formula of bromo-PADAP (Section 13.8.1, Note 3). Why is this compound coloured? Suggest a structure for its metal complexes. (Information that will help the reader to answer these questions can be found in Chapter 5.)

6. Some large cells, stained with H&E, have brown granular material in the cytoplasm. How would you demonstrate that the material was melanin, not lipofuscin or phagocyted debris from an old haemorrhage?
Chapter 14

Answers can be found on page 21.

1. What factors limit the accuracy of localization of an enzyme by a technique that is known to be chemically perfectly specific?

2. The final coloured deposit formed in a histochemical method for an enzyme is sometimes seen to be in the form of well-defined intracytoplasmic granules. Explain why this result, though aesthetically satisfying, does not necessarily prove that the enzyme of the living cell is identically distributed.

3. In histochemical methods for dehydrogenases, hydrogen and electrons are transferred from the physiological substrate to a tetrazolium salt, which is thereby reduced to an insoluble pigment. Identify, in general terms, the substrate, the products of enzymatic action, the trapping agent, and the final product.

4. A section is incubated in 0.1 M calcium chloride together with a substrate that releases phosphate ions under the influence of an enzyme. How many phosphate ions would have to be released in order to precipitate calcium phosphate, \( \text{Ca}_3(\text{PO}_4)_2 \) in the following?:
   (a) an organelle with a volume of 1.0 \( \mu \text{m}^3 \)
   (b) a spherical cell 8 \( \mu \text{m} \) in diameter

   The solubility product \( [\text{Ca}^{2+}]^3[\text{PO}_4^{3-}]^2 \) is \( 2.0 \times 10^{-29} \). Avogadro’s number, \( N_A = 6.022 \times 10^{23} \) molecules per mole. Assume that the enzymatically liberated phosphate ions do not diffuse outside the organelles or cells in which they are formed and ignore any effects of pH and of formation of other phosphates of calcium.

Chapter 15

Answers can be found on page 22.

1. Explain in general terms the methods by which the cellular localizations of hydrolytic enzymes are determined. What factors limit (a) the chemical, and (b) the topographical accuracy of such techniques?

2. What are the differences between reversible and irreversible inhibitors of carboxylic esterases? How are the differences reflected in the histochemical utilization of inhibitors?

3. In sections of nervous tissue it was found that the cells of a certain nucleus had the following histochemical properties:
   (a) Strong reaction with 5-bromoindoxyl acetate as substrate in the presence of 10\(^{-5}\) M diethyl-\(p\)-nitrophenyl phosphate (E600)
   (b) Strong reaction with butyrylthiocholine as substrate; inhibited by 10\(^{-5}\) M eserine, 10\(^{-5}\) M E600, 10\(^{-5}\) M ethopropazine or 10\(^{-7}\) M diisopropylfluorophosphate (DFP)
   (c) Weak reaction with acetylthiocholine as substrate, inhibited by the same agents that inhibited the hydrolysis of butyrylthiocholine.

Which enzyme (or enzymes) is (are) likely to be responsible for these histochemical findings? What further investigations are indicated?

4. Thiamine pyrophosphatase (TPPase), an enzyme found in the Golgi apparatus, releases phosphate ions from the substrate, thiamine pyrophosphate (TPP). The phosphate can be trapped by calcium ions. TPP is also hydrolysed to some extent by alkaline phosphatases, but the latter are inhibited by cysteine. TPPase can act over a wide pH range. Calcium salts are colourless, but the \( \text{Ca}^{2+} \) ion can be replaced by cobalt, which has an almost colourless phosphate but a black sulphide. \( \text{Co}^{3+} \) cannot, however, be included in the substrate mixture, because it would inhibit the enzyme.
On the basis of the above facts, devise an incubation medium for TPPase and explain how the sites of enzymatic activity could be made visible under the microscope.

5. It is possible to determine the histological localization of the enzyme deoxyribonuclease by placing cryostat sections on a film made from a solution of DNA in aqueous gelatin and incubating for 1 hour. Because of the ways in which the section and film are mounted, it is then possible to separate them and obtain two slides, one bearing the section and the other bearing the substrate-film.

How would the sites of DNase activity be detected? What control procedures would be necessary in order to establish the specificity of the method?

Chapter 16

Answers can be found on page 22.

1. In the following chemical reactions, what is oxidized and what is reduced?
   (a) The reaction at the cathode in the electrolysis of molten sodium hydroxide:
   \[ \text{Na}^+ + e^- \rightarrow \text{Na}^0 \]
   (b) The copper-plating of an iron nail immersed in a solution of cupric sulphate:
   \[ \text{Cu}^{2+} + \text{Fe}^0 \rightarrow \text{Cu} + \text{Fe}^{2+} \]
   (c) The conversion of an unsaturated lipid to a saturated one:

2. Examine the formula for the formazan of nitro blue tetrazolium. Why is this compound coloured (yellow)? Is it a dye?

3. What are the oxidizing and reducing agents in the reaction that is accelerated by the presence of catalase? (Section 16.5.3)

4. If, in the histochemical method for succinate dehydrogenase, one used a tetrazolium salt with an oxidation-reduction potential of +1.0 V, the specificity and accuracy of localization might be lower than when nitro blue tetrazolium was used. Why?

5. Compare the rationales of histochemical methods for (a) NAD\(^+\)-linked dehydrogenases; (b) flavoprotein dehydrogenases. Justify the inclusion of all constituents of the incubation media and indicate how the specificity for the substrate can be determined in each case.

6. In a histochemical test for enzymatic oxidation of a substrate \(XH_2\) to \(X\) by an enzyme requiring NAD\(^+\), the following results were obtained in a tissue containing two cell types, A and B.
   (a) With substrate, NAD\(^+\) and nitro-BT: reaction in mitochondria of type B cells
   (b) With NADH and nitro-BT: strong reaction in mitochondria of both cell types
   (c) With substrate and nitro-BT but no coenzyme: moderate reaction in mitochondria of type A cells; nothing in type B
   (d) With nitro-BT but no substrate or coenzyme: no reaction in mitochondria of type A or B cells

   What are the probable localizations of
   (i) \(XH_2\cdot\text{NAD}^+\) oxidoreductase
   (ii) NADH-diaphorase?
   (iii) What other enzyme has been detected, and in which cell type?
7. In ultrastructural cytochemical methods for dehydrogenases, the ferricyanide anion is sometimes preferred to a tetrazolium cation as an artificial electron acceptor (see Benkoel et al., 1976). The phosphate-buffered incubation medium contains, in addition to the substrate and coenzyme, potassium ferricyanide, sodium citrate and cupric sulphate. What are the functions of these ingredients? (Hint: see Chapter 15, Section 15.2.4.) What is the electron-dense product of the histochemical reaction?

8. Dissolve 10 mg of \(N\)-dimethyl-\(p\)-phenylenediamine (= \(p\)-amino-\(N\)-dimethylaniline) or its hydrochloride, and 10 mg of \(\alpha\)-naphthol in a few drops of alcohol. Add the solution to 10 ml of phosphate buffer (pH between 7 and 8). Pour 5 ml of the solution into a small Petri dish or watchglass, and add some small pieces of freshly removed tissue. Do a control experiment with about 1 mg of sodium azide or potassium cyanide added to the other 5 ml of incubation medium. After a few minutes the specimens in the medium without the \(\text{N}_3^-\) or \(\text{CN}^-\) go blue. Explain the observations.

9. Carry out the method for monophenol oxidase on cryostat sections of skin, with appropriate controls. The skin must not be from an albino animal. Account for the different distributions of the enzyme and of the melanin already present in the skin.

Chapter 17

Answers can be found on page 22.

1. A chromaffin granule in a cell of the adrenal medulla contains about 22,000 catecholamine molecules (see H. Winkler & E. Westhead, 1980; Neuroscience 5: 1803–1823). If these were all noradrenaline, could an individual granule be seen with the formaldehyde-induced fluorescence method? (For sensitivity of the technique, see Section 17.3.1. Avogadro’s number, \(N = 6.02 \times 10^{23}\) molecules per mole.)

2. What substances are responsible for the background staining in sections prepared by the azo-coupling method for argentaffin cells?

3. The mast cells of all mammals contain histamine and a sulphated proteoglycan. In rats and mice, the mast cells also contain serotonin. What histochemical methods would provide this information?

Chapter 18

Answers can be found on page 23.

1. Which of the staining methods discussed in this chapter would be suitable for the following?:
   (a) Study of the extents of the dendritic trees of neurons
   (b) Examination of groups of neuronal cell-bodies and tracts of axons in the brain stem, at low magnification
   (c) Comparison of the lengths and thicknesses of astrocytic processes in normal and pathological brains?

2. Why is osmium tetroxide unsuitable for staining myelin sheaths in the central nervous system for light microscopy but highly suitable for electron microscopy?

3. Why does the developer used in Holmes’s method for axons contain sodium sulphite?

4. If, in processing an exposed black and white photographic film, you accidentally fixed it (in sodium thiosulphate) when you should have developed it, what might you do to obtain printable negatives?
5. What metal-staining methods are available for the demonstration of unmyelinated axons in tissues such as the skin or viscera? Evaluate the advantages and disadvantages of each technique.

6. What substances in fungal hyphae account for stainability by Grocott’s hexamine–silver method?

7. If a negative result is obtained in a section stained for spirochaetes by the Warthin–Starry method, can you be certain that the bacteria are absent and that it is not a somewhat untrustworthy technique that has failed?

Chapter 19

Answers can be found on page 24.

1. Why are antisera labelled by conjugating them with fluorochromes rather than with ordinary dyes?

2. Suggest reasons why alcohol and picric acid are more suitable fixatives for antigens than formaldehyde and glutaraldehyde.

3. Devise an immunohistochemical method suitable for localizing HRP in the root of the horseradish plant.

4. It is possible to localize antigens in tissues by a direct method using a fluorescently labelled antiserum. Why is this simple technique usually unsuccessful? How can the direct method be made useful?

5. What are the advantages of the indirect over the direct fluorescent antibody techniques?

6. Devise an immunohistochemical method for the localization of an antigen ‘A’ making use of the following:
   (a) purified ‘A’
   (b) antiserum to ‘A’
   (c) a covalent conjugate of antigen ‘A’ with an enzyme such as HRP

   Discuss the value and limitations of the method (described by D.Y. Mason & R.E. Sammons, 1979; *J. Histochem. Cytochem.* 27: 832–840).

7. When a tissue has been fixed in glutaraldehyde, widely distributed non-specific staining by the unlabelled antibody-enzyme (PAP) method is observed in addition to the specific staining of antigenic sites in the sections. This non-specific staining is suppressed if the sections are incubated with non-immune goat serum before applying the primary antiserum. Explain.

8. In the unlabelled antibody-enzyme method it is necessary to use a high concentration of the secondary antiserum but a low concentration of primary antiserum. Why is a high concentration of secondary antiserum necessary if adequate quantities of PAP are to be bound in the third stage of the technique?

9. In an attempt to stain a hormone in sections of an endocrine gland, the following reagents were applied sequentially to sections:
   (a) mouse monoclonal antibody to the hormone
   (b) goat antiserum to dog γ-globulin
   (c) rabbit peroxidase-antiperoxidase (PAP)
   (d) histochemical detection of peroxidase

   Why did this procedure fail to demonstrate the hormone?

   Using the same primary antiserum, what could be done to obtain a successful immunohistochemical result?
Answers

Chapter 1

1. (a) A whole mount, with the mesentery stretched and spread over a slide.
   (b) Wax- or nitrocellulose-embedded sections of a fixed, decalcified specimen, or sections
      of plastic-embedded undecalcified material.

2. Before and after staining avoid solvents that are more hydrophobic than that in which the
dye is dissolved (typically 70% alcohol). An aqueous mounting medium (see Chapter 4) must
be used.

3. The used alcohol contains fatty material dissolved from the specimen. The fatty substances
become insoluble when the concentration of water in the solvent is increased.

4. The outside of the block is likely to be overstained and the middle understained. The effect
can be reduced by using small pieces of tissue. If possible, the staining solution should be one
that deposits colour very slowly, so that the penetration of the reagents occurs more rapidly
than the coloration of the tissue.

5. Immerse the slide in xylene or toluene until the mounting medium is dissolved. Remove the
coverslip. Treat with the solvent until all mounting medium is removed (no turbidity when
slide is immersed in alcohol). Pass through graded alcohols to water. Removal of a coverslip
and resin can take several days.

Chapter 2

1. If fixation is brief (less than a few hours), the freshly diluted unbuffered formalin will not
depolymerize, so penetration and chemical fixation will be inadequate. In the centre of the
block there will be ruptured cells, osmotically damaged by the most rapidly penetrating
ingredient (water). Shrinkage spaces will be present around cells throughout the specimen.
These develop because the proteins have not been fixed (toughened) enough to resist
defformation in the dehydrating alcohols. With adequate fixation in formaldehyde (12 hours
or more) there is much less physical damage. Buffered formalin is fully active as soon as
it is made. Its isotonicity prevents osmotic damage in the centre of the specimen, and the
duration of fixation can be brief, though some days are needed for full structural stabilization
of the tissue.

2. See Table 2.1. Picric acid alone has several undesirable effects on the tissue. Formaldehyde
is needed to cross-link the precipitated proteins, and acetic acid to prevent shrinkage and to
enhance preservation of chromatin.

3. Slow penetration, blackening, and adverse effects on cutting properties make OsO$_4$ useful
only for tiny specimens that are to be cut into very thin sections. In electron microscopy, this
is what is done. In peripheral nerves, OsO$_4$ blackens myelin sheaths, which are composed
entirely of membranes. Central nervous tissue contains high concentrations of membrane
everywhere, so it is uniformly blackened by OsO$_4$, and structural details are not easily seen
by light microscopy. Fixation in osmium tetroxide also precludes the use of many stains.

4. The amount of cross-linking is so great that the specimens become brittle. Unless the
specimen is tiny, fractures develop during dehydration and paraffin embedding. Frozen
sections can be cut without difficulty, however, and cellular structure is well preserved. The
free aldehyde groups remaining in the tissue have to be taken into account when staining (see Section 2.4.9 and Chapter 10, Section 10.10.6).

5. (a) Modified Carnoy and AFA probably are the best. Neutral buffered formaldehyde is equally good for RNA though structure is somewhat damaged in paraffin sections. Strongly acid fixatives should be avoided or used only for a few hours.

(b) Aqueous formaldehyde or formal-calcium.

(c,d) Aqueous formaldehyde, formal-calcium, Helly and Zenker are all satisfactory. Non-aqueous fixatives cause structural distortion and extraction of much lipid. Osmium tetroxide is useful for small peripheral nerves. Much of the lipid of myelin is protein-bound, so it is not extracted by organic solvents (see Chapters 12 and 18).

(e) Any fixative is suitable.

(f) Aldehydes, especially glutaraldehyde, combine with amines and should be avoided. Strong oxidizing agents (CrO₃, OsO₄) are also avoided because they cause oxidative deamination.

(g) Carbodiimides and chromium-containing fixatives combine with carboxyl groups and should therefore not be used.

(h) No fixation is permissible until after the histochemical method for the enzyme has been carried out.

6. Large anions, which form insoluble ion-pairs with the CPC cation. Glycosaminoglycans (Chapter 11) are the most important substances in this class. As a surfactant, CPC damages membranes and other lipid-containing structures, and it competes with cationic dyes used to stain the tissue.

### Chapter 3

1. Na₂SO₄ would not neutralize acid. It would restrain swelling of inadequately fixed material immersed in water, but a poorly fixed specimen will already have been damaged by the decalcifying solution. 5% (w/v) Na₂SO₄.10H₂O is approximately isotonic with mammalian extracellular fluid.

2. If very little bone is present, Bouin alone may decalcify the specimen. Wash thoroughly in 70% alcohol to remove picric acid, and proceed with dehydration, clearing and embedding. If more decalcification is needed, transfer the washed specimen to one of the decalcifying agents described in this chapter, and follow the instructions. For a Zenker-fixed specimen it is necessary to wash out all free dichromate with water (not alcohol) before immersion in formic acid, which would reduce free Cr(VI) to Cr(OH)₃ (see Chapter 2). If the decalcifier is nitric acid or EDTA, the washing could be done later, because these substances are not reducing agents. Mercury deposits must also be removed from Zenker-fixed material (see Chapter 4) before staining.

### Chapter 4

1. Embedding in wax, nitrocellulose or plastic would extract the lipid. Frozen or cryostat sections must be cut. Gelatin embedding may be needed to provide support for the specimen.

2. (a) The dioxane replaces the water in the specimen. After fixation in Helly or Zenker, wash in running water until all excess dichromate is removed. The specimen may be brought gradually (e.g. through 30%, 70%, 90%) to 100% dioxane, or may be transferred from water to the first of 4 changes of 100% dioxane. Dioxane mixes with molten wax, so the specimens are then put in the first of 3 changes of wax and are infiltrated and blocked out in the usual way.
(b) DMP reacts with water. The products of reaction are methanol and acetone, which are miscible with most clearing agents (see Table 4.1).

3. Coating with nitrocellulose would be advisable. The slides have to be manipulated carefully, one at a time.

4. Euparal is unsuitable for (a) and (d) because the solvent will extract some or all of the dye. It is suitable for (b) and (c) because the low refractive index makes unstained structures visible.

5. Mercury deposits look like sand spilt upon the section. The distribution of the dirt is not related to structural features but it is clearly in the tissue, not on the surface of the section or slide.

6. Water or alcohol in a resinous medium appears as colourless droplets. Xylene in an aqueous medium looks much the same. Loss of dye into an inappropriate mounting medium may take a few days. The colour spreads outwards from the specimen, and the specific staining is lost.

Chapter 5

1. (a), (c) and (e) are coloured. (a) and (c) are cationic dyes.

2. The 3,4-benzpyrene molecule is hydrophobic (no atoms capable of hydrogen bonding), so it behaves like a solvent dye. It has been used as a fluorochrome for cytoplasmic lipid droplets (Pearse, 1985, p. 799), but is unpopular on account of its carcinogenicity. Nile red (Chapter 12) is a safer alternative.

3. Weigert's stain contains a large excess of Fe$^{3+}$ ions even after all the haematoxylin has been oxidized to haematein and formed soluble [iron–haematein] complex ions or molecules. This excess of FeCl$_3$ also makes the solution acid. Free Fe$^{3+}$ competes with the tissue for binding of the dye–iron complex. The high [H$^+$] breaks iron–tissue, iron–haematein and [iron–haematein]–tissue bonds, except at the sites of strongest binding, which are in nuclear chromatin.

With Heidenhain's method, all the iron-binding sites in the tissue are saturated and the bound iron is then complexed with haematein. Subsequent treatment with an Fe$^{3+}$ salt removes haematein as a soluble [Fe$^{3+}$–haematein] complex. The acidity of the solution breaks the [complex]–tissue and Fe(III)–tissue bonds. The weakest bonds to components of the tissue (those to extracellular and cytoplasmic proteins) are the first to be broken. Bonds to membrane-rich structures (mitochondria, myelin) follow, and the strong bonds to nuclear chromatin are the last to be broken.

4. (a), (b) Staining is due primarily to ionic bonding so urea in the solution is only a weak competitor. High concentrations of urea will inhibit staining.

(c) Binding of this large molecule requires non-ionic, non-covalent bonding. Urea competes effectively with these weak forces, and prevents staining of the tissue.

(d) Urea is very hydrophilic, so it does not interfere with the attachment of a hydrophobic dye to a hydrophobic substrate.

5. (a) and (d) have coloured cations, and binding to anionic substrates is competitively inhibited by inorganic cations in the solvent. See also Chapter 11.

(b) is a reactive dye, and a salt in the solution does not prevent its combination with −OH, −COOH and −NH$_2$. The salt may, however, promote the movement of unreacted dye into the interstices of the substrate.

(c) is anionic but is a direct cotton dye and as such it is bound mainly by non-ionic, non-covalent forces. An inorganic salt does not inhibit staining, and may even enhance the uptake of dye.

6. Dye–metal binding is explained in Sections 5.5.8 and 5.9.4.4.
7. An excess of OH\(^{-}\) ions decolourizes the dye by changing all the coloured cations into molecules of the leucobase.

8. (a) Colour changes to violet.
(b) Colour changes to pale yellow.
(c) Solution becomes purple. Darkens with addition of alkali; changes to red with addition of a mineral acid.
(d) Solution becomes grey-black. Changes to deep purple with addition of alkali; changes to red with addition of a mineral acid.

The colour changes are due to the acid-base indicator properties of free haematein and of its metal complexes.

9. (a) A dark precipitate is formed. It can be collected and dissolved in alcohol. This is a salt in which the anion and cation are both dyes. When the oppositely charged parts of the ions are close together, the exposed parts of the dye molecules are not hydrophilic enough to allow solution in water. (A staining method using a mixture of this type is included in Chapter 6.)
(b) There is no precipitation, because no reaction occurs between the two cationic dyes.
(c) As with (a), an alcohol-soluble precipitate forms. (Eosinates are discussed in Chapter 7.)
(d) The two anionic dyes mix without precipitation.

10. The compound has structural similarities to the Sudan dyes (few atoms able to form hydrogen bonds; not ionized). It can be used as a stain for fat, but its colour is not appealing.

Chapter 6

1. The Nissl substance of the neurons (cytoplasmic RNA) is strongly stained (blue) by azure A but is usually pink with H&E. Some blue colour can be seen in Nissl substance if the alum–haematein has not been adequately differentiated.

2. Weigert’s solution contains an excess of Fe(III) which oxidizes all the haematein to useless substances in a few days (Chapter 5, Section 5.9.12.3). Oxidation of the haematein in Mayer’s haemalum can be brought about only by atmospheric oxygen, which is not available in large amounts in a closed bottle.

3. (a), (c) and (f) are unsuitable because they are cationic dyes and they too would stain nuclei. (b) and (e) are suitable because their colours contrast with the blue of iron–eriochrome cyanine R and being anionic dyes, they stain cytoplasmic and extracellular proteins. (d) is unsuitable because although it is anionic, its colour does not differ appreciably from that of the stained nuclei.

4. The formaldehyde has reacted with all the amino groups of the tissue, so there are no cationic sites left to attract eosin anions.
5. Everything is stained, including structures that are not usually basophilic, because oxidative deamination of proteins has left an abundance of carboxylic acid groups in the tissue.

6. Alum–brazilin (Section 6.1.3.5), adequately differentiated by acid-alcohol, will give selective nuclear staining similar to that obtained with haemalum. Alum–nuclear fast red (Section 6.1.3.7) stains nuclei progressively. Fast green FCF is a suitable counterstain.

7. For (a), (b) and (c), see Sections 6.1.3, 6.1.1 and 6.1.2, respectively.

Chapter 7

1. The shapes of the nuclei are clearly shown, as are the sizes and shapes of the cells. The different types of granular leukocyte cannot be identified because their cytoplasms are all stained pink.

2. The methanol is needed to make a concentrated solution. Water is needed to solvate the dye anions and cations, which must be present in the staining solution.

3. Formaldehyde, by reacting with amino groups of protein, reduces the number of potentially cationic sites in the tissue. A more acid staining solution opposes this effect by promoting protonation of the remaining amino groups, thereby increasing the overall affinity of the tissue for eosin anions. Blood films are fixed in alcohol, which does not react with amino groups.

4. Unfixed cells flatten and spread as they settle. If the cells are fixed in suspension, they retain their spherical shapes and become physically stronger. When the fixed cells settle, they do not flatten very much. A smaller area is in contact with the glass slide, so fixed cells are more likely to be dislodged during staining than unfixed cells. Cells in smears and films are often distorted as they are dragged across the surface of the slide. When cells are centrifuged on to a slide, they flatten and spread more than cells that have settled slowly. Consequently, the apparent sizes of cells vary with the methods used to deposit them on the slide.

5. The metachromasia (see Chapter 11) of the basophil granules makes these cells (normally less than 1% of the leukocyte population) conspicuous. (Eosin alone, in alkaline solution, can be used for selective staining of eosinophils, whose granules contain much arginine. See Chapter 10 for rationale.)

Chapter 8

1. The chemistry of collagen and elastin is reviewed in Section 8.1. The staining properties of elastin are largely attributable to the abundance of hydrophobic amino acids, whereas collagen is stained by more hydrophilic dye anions. Silver staining of reticulin, which is due to the presence of oxidizable carbohydrate, involves chemical reactions between reagents and tissue (see Chapter 18).

2. The actions of the heteropolyacids are discussed in Section 8.2.4.

3. One such method is: Iron–haematoxylin for nuclei (black), followed by aldehyde–fuchsin for elastin (red-purple) and then picro–aniline blue for muscle and other cytoplasm (yellow) and collagen (blue). It is also possible to combine orcein (for brown elastin) with iron–eriochrome cyanine R (blue nuclei) and Gabe’s trichrome (red cytoplasm, greenish collagen). Verhoeff’s elastic stain is often followed by van Gieson’s method.

4. Aniline blue entered and stained nuclei that had been cut by the microtome knife, but orange G stained only those nuclei that were entirely contained within the section. It has been claimed, however, that blue staining occurs in nuclei with higher RNA content, because RNase treatment increases the proportion of orange-staining nuclei (Baccari et al., 1992).

5. The blue colour is strongest in collagen. Stannous ions reduce the bound phosphomolybdate to molybdenum blue (see Section 8.2.4.1).
Chapter 9

1. The sections must be treated with sodium borohydride (see Chapter 10) to remove aldehyde groups derived from the fixative. If this is not done, everything in the tissue will stain with Schiff’s reagent even without prior hydrolysis of nucleic acids.

2. Treat sections with the enzymes. Stain with a dilute solution of a cationic dye. After pure RNase, only the nuclei (excluding nucleoli) are stained. After pure DNase, staining is confined to nucleoli and Nissl substance (RNA in cytoplasm of neurons). A mixture of the two enzymes abolishes almost all basophilia in central nervous tissue. (The nucleases have no effect on the basophilia due to substances other than nucleic acids, such as that of cartilage matrix and mucus.)

3. Nucleoproteins are quite strongly basic, so they can be stained with neutral or slightly alkaline solutions of anionic dyes (see Chapter 10). Many more cationic sites are made available if the DNA is first extracted, chemically or enzymatically. Some of the [dye–iron] complex methods for nuclei probably demonstrate both nucleoprotein and DNA (see Chapter 6).

4. Cross-linking of proteins by aldehyde fixatives makes the tissue less permeable to the large enzyme molecules.

5. Extract the RNA either with RNase or chemically. Verify the extraction by showing that in some sections nucleoli are no longer stainable with basic dyes. Stain other sections to show that AgNORs are still demonstrable.

6. Obtain a cDNA probe that recognizes an mRNA for some part of the Fc segment of the type of immunoglobulin detected in the lesions. In situ hybridization will then reveal sites in which the gene for that type of immunoglobulin is being transcribed.

8. In a large nucleus, the DNA is diluted, so the intensity of staining in a section is less. A small nucleus contains a higher concentration of DNA, and is also more likely to be contained entirely in a section than a large nucleus.

Chapter 10

1. Acidophilia is due to protonated amino groups and/or guanidino (arginine) groups. The former can be removed by deamination or blocked by acetylation or benzoylation. The latter can be blocked by reaction with benzil or diacetyl. If staining can be obtained at high pH, it must be due to the strongly basic guanidino group. Paneth cell granules are rich in arginine.

2. Unless the incubation is brief, the section is destroyed. Carefully controlled incubation in trypsin can remove some proteins and make others accessible to histochemical reagents such as antibodies (see Chapter 19). With animal tissues, non-protein structures are lost when the proteins around them are digested, so trypsin has little or no value as a test for protein. In plant tissues digestion of cytoplasmic protein is likely to cause loss of such objects as lipid droplets and starch granules, even though the structural matrix (cellulose, lignin, etc.) is not attacked by proteolytic enzymes.

3. (a) At pH 1.0 only sulphate esters are stained: cartilage matrix and the granules of any mast cells in the section, but not nuclei, cytoplasm or collagen.

(b) Methylation blocks the basophilia of the cartilage matrix by removing sulphate (leaving −OH in its place) and esterifying carboxyl groups. The section therefore becomes unstainable.

(c) Ether-sulphuric acid introduces sulphate ester groups at sites of hydroxy groups, which are widespread. Thus, all components of the tissue (including desulphated cartilage matrix if sulphation follows methylation) will be stainable with a basic dye at pH 1.0.

4. (a) With Millon’s reagent or by coupling with a suitable diazonium salt (such as fast black K salt).
(b) In the hydroxynaphthaldehyde method.
(c) By reaction with hydroxynaphthoic acid hydrazide followed by coupling with a diazonium salt.

Chapter 11

1. (a) Glycoproteins; neutral polysaccharides (glycogen, starch, cellulose).
   (b) Glycoproteins that contain \( \Delta \)-glucose (i.e. collagen) or \( \Delta \)-mannose (most secretory glycoproteins); polysaccharides that contain these sugars in \( \alpha \)-linkage (starch, glycogen, not cellulose). Most PAS-positive substances stain with Con A; cell surfaces and basement membranes are more conspicuous with the latter. All Con A-positive substances are PAS-positive.

2. Nearly all components of the tissue become basophilic, and can be stained with strongly acidic (pH 1) solutions of cationic dyes.

3. (a) None.
   (b) and (d) All that were initially PAS-positive, and also sialic acid groups that initially were acylated at C8.
   (c) No effect on PAS reactivity, but the reagents can cause loss of some sialic acid residues, causing diminished staining of some types of mucus.

4. (a) and (b) No effect of acetylation on alcian blue staining.
   (c) Nothing stainable with alcian blue at pH 1.0 or 2.5.
   (d) Restoration of stainability at pH 2.5. Nothing stainable at pH 1.0.
   (e) Loss of some of the stainability at pH 2.5 (that due to sialic acids).

5. Hyaluronan, chondroitin-4- and -6-sulphates, dermatan sulphate, heparin, heparitin sulphate and glycoproteins containing sialic acids. (Not the keratan sulphates or glycoproteins that lack sialic acids.) Amylase has no effect on binding of fluorescent aprotinin. Hyaluronidase should diminish staining in extracellular materials (cartilage matrix, etc.). Neuraminidase suppresses staining of mucus and basement membranes.

6. Glycogen can be stained with PAS, Best’s carmine, and the lectins of Group 1 in Table 11.1. Instructions are given in this chapter. It is important to remember that most fixatives do not react chemically with glycogen and that this polysaccharide is soluble in water.

7. Chemical blocking procedures are of low specificity. Periodate oxidation blocks lectin-binding by (a) but not by (b). Before staining with the lectin it is necessary to block periodate-engendered aldehyde groups (see Chapter 10), which would combine non-specifically with lectins or any other proteinaceous reagents. Specific binding can be inhibited by adding an appropriate sugar to the solution containing the lectin.

8. Chitin accounts for only half the material in the exoskeleton. The other half includes PAS-positive substances.

9. NANA is identifiable with specialized PAS methods (Section 11.4.2.2), alcian blue at pH 2.5, or some of the lectins of Group 5 in Table 11.1, and it can be extracted enzymatically or chemically. After removal of NANA the glycoprotein would be PAS-positive only with the standard technique and it should show increased affinity for lectins in Group 3 of Table 11.1 (PNA, SBA, PHA, RCA). The Group 3 lectins with preference for \( \alpha \)-linked galactosides (DBA, MPA) would not stain this substance. Prior incubation of a section in \( \beta \)-galactosidase would prevent all staining of the glycoprotein, and no other glycosidase would have this effect.
Chapter 12

1. Triglycerides can be extracted with cold acetone before staining. Phosphoglycerides resist extraction by cold acetone.

2. (a) Not stained, because melting point of saturated fatty acid (stearic) is too high.
   (b) Stained at higher temperature, which melts tristearin.
   (c) Not stained, because no olefinic linkages.
   (d) Not stained, because not a plasmalogen.

3. (a) and (b) Stained, because cis-unsaturated oleic acid is liquid at room temperature.
   (c) Stained, because of the unsaturated linkage in each oleyl group.
   (d) Not stained, because not a plasmalogen. Oxidation of –C=C– by air could cause a pseudoplasmal reaction.

4. Positive Schiff reaction indicates that some of the unsaturated linkages in the fatty acid chains of the myelin lipids have been oxidized by air to –CHO.
   (a) Reduce pre-existing aldehydes to hydroxyls with borohydride before carrying out the plasmal reaction.
   (b) Alkaline hydrolysis prior to staining lipids by any method (Sudan black B is suitable) does not extract fatty acid amides.

5. In order to take up Nile red from the oxidized Nile blue solution, the lipid droplets must be in liquid state. Requirement of a higher temperature for staining indicates a higher proportion of saturated fatty acid chains, which impart higher melting temperatures. (These considerations do not apply when Nile red is detected with much greater sensitivity by fluorescence microscopy. Smaller amounts of the dye may then be seen also in hydrophobic domains other than those of lipid droplets).

6. The adrenal droplets can be stained by any method for hydrophobic lipids. Extraction by cold acetone excludes phospholipids. Alkaline hydrolysis removes esters (fats, cholesterol esters) but not steroid hormones (which are, however, extracted by acetone).

7. Sulphatide can be stained with cationic dyes at pH <2, because of the strongly acid sulphate-ester group.
   (a), (b) Any proteoglycans and glycoproteins that stain similarly will not be extracted by prior treatment of the sections with hot methanol-chloroform.
   (c) Cerebrosides are not basophilic (no ionizable acid group).
   (d) Gangliosides contain sialic acids, which are basophilic, but not stained at low pH.
   (e) Neutral fats are not basophilic (no ionizable acid group).
   (f) Although the acyl groups of sulphatide are largely saturated, tests of unsaturation (OsO₄, PdCl₂) are likely to be positive because of other lipids present in or near the deposits.

8. The paraffin inclusions are sudanophilic at temperatures approaching the melting point of wax (50–60°C). They are not extracted by alcohol or acetone (excluding fats, free fatty acids and free cholesterol), but they are soluble in chloroform-methanol (excluding lipoprotein and other insoluble hydrophobic materials). They do not reduce osmium tetroxide (excluding almost all lipids except cholesterol esters), are unaffected by alkaline hydrolysis (excluding cholesterol esters), and cannot be stained with basic dyes or by the PAS method (excluding glycolipids).
Chapter 13

1. Na⁺ occurs as soluble salts, mainly in extracellular fluids. The few reagents that precipitate sodium (e.g. potassium pyroantimonate, zinc uranyl acetate) penetrate and react too slowly to ensure accurate localization (Pearse, 1985, pp. 1006–7).

2. Immerse tiny fragments of tissue or sections of unfixed freeze-dried tissue in AgNO₃ solution. Wash in water. Expose to light. Accuracy of localization is poor for the reasons given for sodium above. Specificity is also poor, because of binding of Ag⁺ to proteins (see Chapter 18).

3. See Section 13.4. Test for masked iron if Perls reaction is negative.

4. Precipitation of insoluble, coloured salts; acid hydrolysis to release metal ions (‘unmasking’) from insoluble compounds in tissue; formation of insoluble, coloured chelates; use of chelating agents to ‘mask’ (extract) coloured chelates of metals one does not want to stain. When an ion is released for reaction with a precipitant, it diffuses away from its site of origin and may bind to other components of tissue.

5. It is the free base of a cationic azo dye. Metal binding involves the phenolic hydroxyl and the azo nitrogen attached to the pyridine ring, to form a six-membered chelate ring.

6. Phagocytosed material derived from blood contains iron, stainable by the Perls reaction. The other two pigments are Perls-negative. Lipofuscin is the only one of the three pigments that is fluorescent in unstained sections, and the only one stained in paraffin sections by Sudan black B. Melanin is bleachable with hydrogen peroxide; the other two pigments are not.

Chapter 14

1. Diffusion of the enzyme during fixation or staining; diffusion of intermediate or final reaction products; binding of intermediate or (more commonly) final reaction product to structures other than those containing the enzyme. Hydrophobic final products are particularly prone to migration.

2. See answer to Question 1 above for artifactual binding of final reaction product to ribosomes, secretory granules, etc. An enzyme uniformly distributed in the cytosol is likely to form a final product that consists of solid particles, and these may be confused with lysosomes or mitochondria in light microscopy.

3. The substrate is the physiological one. The products of enzymatic reaction are the oxidized substrate, electrons and hydrogen ions. The trapping agent is the tetrazolium salt, which is reduced by the released electrons to a coloured, insoluble formazan. This is an oversimplification. See Chapter 16 for more.

4. (a) 85 ions
   (b) 22,780 ions.

Chapter 15

1. General principles are explained in Chapter 14.
   (a) Often (as in the case of esterases), the hydrolysis of an artificial substrate is catalysed by more than one enzyme.
   (b) See answer to Question 1 of Chapter 14.

2. A reversible inhibitor competes with the substrate. It must be included in the incubation medium. An irreversible inhibitor binds firmly to the active site of the enzyme, so sections can be pre-incubated, and then incubated in substrate without added inhibitor.
3. (a) Arylesterase or acetyesterase (could be distinguished using PCMB after E600).
   (b) Butyrylcholinesterase (= pseudocholinesterase).
   (c) Butyrylcholinesterase. AChE would give stronger reaction with AThCh, and not be
   inhibited by ethopropazine or very low concentration of DFP.

Thus, the cells contain at least two esterases: BuChE, and also arylesterase and/or
acetyesterase.

4. The incubation medium would contain TPP (substrate), cysteine (to inhibit interfering
   alkaline phosphatase), CaCl₂ and TRIS or barbitone buffer at about pH 8 (sufficiently basic
   for precipitation of calcium phosphate). After incubation, the sections are treated with
   an aqueous solution of a Co²⁺ salt, washed and then placed in dilute aqueous ammonium
   sulphide, which converts the cobalt phosphate to the black sulphide. They can then be
   dehydrated, cleared and covered. For reasons given in Chapter 4, Section 4.3.1.1, Canada
   balsam is the mounting medium of first choice.

5. Stain the substrate film by the Feulgen method, or with a cationic dye. Sites of hydrolysis of
   DNA appear as clear areas. Controls should be stained with an anionic dye, to make sure that
   the loss of DNA is not due to proteolytic enzymes making holes in the gelatin film.

Chapter 16

1. (a) Sodium ions are reduced to the metal at the cathode.
   (b) Cupric ions are reduced to metallic copper; iron is oxidized to ferrous ions.
   (c) Hydrogen gas is oxidized; the carbon atoms of the olefin are reduced (oxidation number
   changes from −1 to −2).

2. The long conjugated chains of nitro-BT, which include chromophoric azo (N=N) and imine
   (C=N) linkages and two −NO₂ groups, ensure absorption of visible light. The formazan is
   insoluble and uncharged; it is a pigment, not a dye.

3. One of the molecules of hydrogen peroxide is the oxidizing agent; the other is the reducing
   agent.

4. A compound with an $E'$ of +1.0 V would be a much stronger oxidizing agent than FAD
   (see Table 16.1), so it would be expected to accept electrons from FADH₂ ($E' = -0.22$ V),
   the reduced prosthetic group of SDH. However, such a strong oxidizing agent would also
   accept electrons from all the cytochromes ($E' = +0.22$ to $+0.82$ V) and from non-enzymatic
   reducing groups (such as −SH) in the tissue. Although the oxidation–reduction potentials
   are only a crude indicator of the properties of a tetrazolium salt (see Section 16.4.1), an $E'$
   of +1.0 is far too high (see Table 16.2), and such a compound could not interrupt the flow of
   electrons from FADH₂ to oxygen.

5. For (a) and (b), the medium must contain the natural substrate of the enzyme and a
tetrazolium salt. For (a), it is also necessary to include NAD⁺. For other ingredients, see
Sections 16.4.4 and 16.4.5. Controls include omission of the substrate and, for NAD-
linked dehydrogenases, omission of the coenzyme to exclude an NADP-linked enzyme or
a flavoprotein that catalyses oxidation of the same substrate. If a specific inhibitor of the
enzyme is available, this should also be used. If an intermediate electron carrier such as PMS
is not included in the incubation medium for (a), it is necessary to ensure that the sections
contain NADH diaphorase.

6. (i) XH₂:NAD⁺ oxidoreductase in mitochondria of type B cells.
   (ii) NADH diaphorase in mitochondria of both cell-types.
   (iii) An enzyme that does not require NAD⁺ or NADP⁺, presumably a flavoprotein
   XH₂:NAD⁺ oxidoreductase, has been detected in the type A cells.
7. Citrate ions form a complex with cupric ions, so that \([\text{Cu}^{2+}]\) is too low to allow precipitation of the sparingly soluble copper ferricyanide. The reduced coenzyme or flavin nucleotide formed by the action of the enzyme reduces \(\text{Fe(CN)}_6^{3–}\) to \(\text{Fe(CN)}_6^{4–}\). Copper ferrocyanide (Hatchett’s brown) is extremely insoluble, so it is precipitated even in the presence of citrate, and it is the electron-dense final reaction product.

8. This is the classical NADI reaction. It does not occur in the presence of azide or cyanide ions, which inhibit cytochrome oxidase.

9. In mammals, enzymatic activity is seen in melanocytes, at the junction of dermis with epidermis and commonly also within the dermis. These cells secrete their pigment granules, which are immediately taken up by the basal cells of the epidermis (pigment donation). Consequently, most of the melanin in the skin is in the epidermis. In amphibian skin, the melanophores synthesize and store melanin, so the pigment and the monophenol oxygenase are found in the same cells.

**Chapter 17**

1. The fluorescence method can detect approximately 1 781 000 molecules of NA, which are contained in 81 chromaffin granules.

2. Proteins containing tyrosine, histidine and tryptophan (see Chapter 10).

3. Histamine can be demonstrated with o-phthaldialdehyde (low specificity) or immunohistochemically. The sulphated proteoglycan can be stained by alcian blue, pH 1.0 or other methods described in Chapter 11. Serotonin in rat mast cells can be demonstrated by formaldehyde-induced fluorescence or by an azo-coupling method.

**Chapter 18**

1. (a) Golgi methods.
   (b) A silver method for axons, counterstained with a basic dye.
   (c) Cajal’s gold-sublimate method.

2. OsO4 blackens all lipids. Cell membranes and membrane-bound intracellular vesicles and organelles are present at high density in grey matter. Consequently, both grey matter and white matter (myelin) are blackened. This is an advantage in electron microscopy because the individual membranes within myelin sheaths and elsewhere are clearly resolved.

3. The \(\text{SO}_3^{2–}\) ions withdraw unreduced silver from the sections, as a soluble complex. This extracted silver makes the solution a physical developer. Sulphite also reacts with the oxidation product of hydroquinone to form a colourless, soluble product.

4. Immerse the film in a physical developer. (If the fixer used is a simple aqueous solution of \(\text{Na}_2\text{S}_2\text{O}_3\) it is possible to do this. An acidic fixing and hardening solution, which is usual for black and white films, can dissolve the latent image, and the negative is then irretrievable.)

5. Silver methods stain axons of all types but can be unreliable, especially for fibres of small diameter. Iodide-osmium methods are preferable for some tissues. Histochemical methods for choline esterases (Chapter 15) and biogenic amines (Chapter 17), and immunohistochemical methods for various enzymes and peptides are valuable for axons that contain these substances.

6. Macromolecular carbohydrates of the cell wall.

7. A known positive section of identically fixed and processed tissue should be stained alongside the unknown, to show that the technique is working properly. A false negative result could also be obtained if that particular section did not contain enough spirochaetes for one to be recognizable. This would be an error of sampling, not of technique.
Chapter 19

1. The eye can detect smaller quantities of a fluorescent substance (see Chapter 1).

2. Coagulant fixatives distort the shapes of protein molecules, usually exposing more antigenic sequences of amino acids (see Chapter 2). Picric acid also partly suppresses autofluorescence. By cross-linking proteins, aldehyde fixation can mask epitopes. Aldehyde fixatives, especially glutaraldehyde, also induce fluorescence non-specifically in some tissues.

3. Treat sections with rabbit anti-HRP, followed by fluorescently labelled goat anti-rabbit immunoglobulin. For negative controls:
   (i) omit the primary antiserum.
   (ii) substitute non-immune rabbit serum for the primary antiserum.
   (iii) mix the primary antiserum with purified HRP to absorb out the antibodies.

   The enzyme is also easily demonstrated by direct histochemical staining for peroxidase activity.

4. Sensitivity is much less than with indirect methods. EPOS antibodies greatly increase sensitivity but are available only for staining only the antigens most commonly studied in histopathology labs.

5. The reasons are explained in Sections 19.5.5 and 19.6.

6. Apply (a), (b) and (c) sequentially to the section, with washes between treatments. This forms the sandwich:

   \[(\text{Antigen A})+(\text{Anti-A})+(\text{Fluorescent Antigen A})\]

   With a monoclonal IgG antibody, only one fluorescent antigen molecule is bound at each antigenic site. If AntiA is a polyclonal antiserum, several molecules can bind to each molecule of Antigen A by attaching to different epitopes. The method is then more sensitive.

7. When glutaraldehyde cross-links proteins, many free aldehyde groups are bound to all parts of the tissue (see Chapter 2). These free aldehydes will bind any soluble proteins, including immunohistochemical reagents, that are subsequently applied to the section. Non-immune goat serum binds to the aldehyde groups first, and thereby prevents the non-specific binding of primary and secondary antisera and of PAP.

8. It is important to provide secondary antibody molecules in numbers large enough to bind monovalently to the Fc segments of all the attached molecules of the primary antibody. This provides an excess of free Fab segments (of the secondary antibody molecules) to bind the subsequently applied PAP.

9. The rabbit PAP could not attach to goat anti-mouse immunoglobulin. It would be necessary to use mouse PAP if the primary antibody had been raised in a mouse. An alternative procedure is to use a bacterial Ig-binding protein (Section 19.10.3) instead of a secondary antiserum in stage (b) of the procedure. Protein A binds to the Fc segments of most IgG molecules, thus building the sandwich:

   \[(\text{Mouse anti-hormone})-(\text{Protein A})-(\text{PAP})\]