ANALYTICAL METHODS IN HUMAN TOXICOLOGY

Part 1

Edited by
A. S. CURRY

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Introduction

Analytical toxicology encompasses a vast range of techniques. There will therefore be two types of readers who use these volumes: those who wish to find out the methods that are being used to solve a problem that has occurred in a particular area and those who need to see which technique is most pertinent to their area of research. The volumes reflect both approaches.

One theme is common, and this is the astonishingly small quantities of foreign materials that can be localised, identified and measured in the human body. One picogram is to one gram what one gram is to two hundred thousand five-ton elephants, and this analogy emphasises that the purity of all handling preparations in analytical toxicology must be ensured at all stages. So often a researcher may spend months, even years, tracking down a material that turns out to be a plasticiser, a wax or even a contaminant from the operator, possibly diurnal or sex-related. There is no easy way to overcome such problems except to read in depth the work of experts who have the greatest experience and it is the intention of these volumes to present such work.

Analytical toxicology does not stop when a particular compound has been traced and its metabolism followed in the body. The way in which it affects the normal action of the body can lead to dramatic consequences in medicine and some of the most modern advances concern toxicology at the cellular level. Indeed the entry of drugs into genetic pathways is being tracked by the analytical techniques that are described in these volumes.

Both the generalist and the specialist are concerned with the living and the dead, as are those who wish to produce better drugs to treat disease. Every life-saving or post-mortem analysis is a step towards understanding the very nature of life itself. The techniques now available cover the whole spectrum from the disintegration of molecules in mass spectroscopy to the immunological properties

of the body and the effect of light and radiation to reveal compounds within a cell.

The subject is fascinating in that there are so many ways to tackle a new problem. This, coupled with the high cost of 'black box' instruments, means that all of us need periodically to review the latest state of the art to help us decide which way to go.

It is the hope of the editor that the reader will find much of interest and of help in these volumes.

Reading, 1984 A. S. C.

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The detection of drugs by histochemical procedures

M. MORLEY

1.1 INTRODUCTION

Many methods of analysis are available to the toxicologist for determining the type and amount of drug within human tissues, but few have the potential of histochemistry for enabling the precise site of action of a drug to be defined. Histochemistry is essentially the examination of the chemical as opposed to the morphological structure of tissue preparations. Nondiffusible coloured or fluorescent reaction products which can be visualised under the microscope indicate the cellular localisation of the endogenous or exogenous substances under investigation.

Autoradiography has provided a useful histochemical tool in investigations with laboratory animals, but such procedures are obviously of limited use to the morbid anatomist. Few people obligingly imbibe radiolabelled drugs before their death! The application of other histochemical procedures to the problem of drug localisation within tissues would provide invaluable information about the toxic actions of a drug in man and would ultimately enable more precise determination of the cause of death. At present such conclusions have to be drawn from relatively gross toxicological data, as few poisons produce notable lesions within the body.

All of the published literature has concentrated on the development of fluorescence methods of detection but other types of histochemical reactions may prove equally suitable. In addition the use of microspectrofluorimeters and microdensitometers may allow some quantification of the data. The field is wide and needs to be exploited. I hope that the ensuing paragraphs will give some insight into the problems that will be encountered in developing such techniques and will excite the imagination of those interested in becoming active participants in this area of research.

1.2 PROCESSING TECHNIQUES

Before any tissue sample can be subjected to a histochemical staining procedure it must first be processed in such a manner that minimal disruption occurs in the morphological and chemical characteristics of the specimen. Procedures which preserve tissue morphology are not necessarily compatible with those which preserve tissue histochemistry and a compromise has to be reached between the two. Drugs may be soluble in the chemicals used for fixation, embedding and dehydration. Further problems arise if immunological methods are employed, for these chemicals may destroy the immunoreactive groups.

Although the precise method of tissue preparation is ultimately dependent upon the physico-chemical properties of the particular substance under investigation it should be noted that the characteristics of a pure drug may be different from those pertaining in the histochemical environment. For example, Δ-tetrahydrocannabinol (Δ-THC) could be considered as being suitable for processing by the aqueous technique of immunofluorescence because of its extreme degree of lipophilicity, but leaching from tissue section still occurs because it forms a water-soluble complex with its antibody (Morley and Gee, 1982). There are no precisely defined rules for the development of such procedures. Success is largely a matter of trial and error.

1.2.1 Fixation

The ultimate purpose of fixation in drug histochemistry is to prevent diffusion of the substances under investigation away from their sites of localisation at the time of tissue death. The type of fixative required will be determined both by the nature of the drug being investigated and the proposed histochemical staining procedure. For example, the presence of mercury salts in fixatives may be unsuitable for use with fluorescence techniques as they act as quenching agents. Similarly, fixatives which alter the reactive groups of a drug will be of little value when combined with methods involving chemical staining reactions.

No formal studies have been carried out on the suitability of different types of fixatives for use with drugs. The fixatives that have been used are those which are usually encountered in histochemical procedures for demonstrating biological molecules. Pertschuk and his colleagues have used ethanol as a fixative in the immunofluorescent detection of both phenobarbital (Pertschuk *et al.*, 1976) and methadone (Pertschuk and Sher, 1975; Pertschuk *et al.*, 1977). The high degree of water solubility of sodium phenobarbital was considered to be incompatible with drug retention during the aqueous processing procedures of immunofluorescence but this factor is much less critical with methadone. Methadone has been localised in both alcohol-fixed and unfixed human brain tissue. Very little is known about the chemistry of fixation by alcohol. It acts as a protein precipitant. The denaturation resulting from this action may alter the solubility charac-

teristics of the drug-receptor complex, so reducing the amount of leaching of the water-soluble drug from the tissue during processing.

Lipid-soluble compounds may not require fixation in tissues if aqueous processing techniques are to be employed. For example, Balkon and his colleagues (1980) have successfully localised morphine in rat brain by immunofluorescence. However, lipophilicity cannot always be taken as suitable property for using unfixed material with aqueous staining methods. As has been mentioned previously, Δ-THC cannot be detected by a simple indirect immunofluorescence technique because the drug forms a water-soluble complex with its antibody. 19% of the drug is leached from tissue sections after incubation with the primary antiserum and a further 7% is lost following incubation with the secondary antiserum (Figure 1.1). Intermediary fixation steps after each antiserum incubation may help to alleviate this problem but care should then be taken that a fixative is chosen which does not destroy the immunoreactive groups of the drug-antibody complexes. Much has been written about the effect of fixatives on protein immunoreactivity. Reviews published by Nairn (1976a), Pearse (1968a)

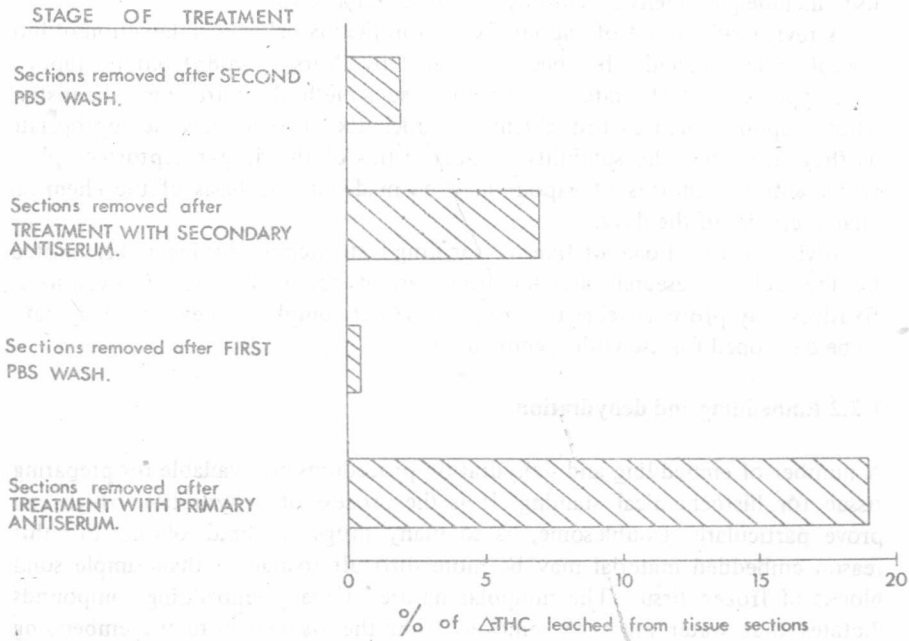

Figure 1.1 Estimation of the amount of Δ-THC leached from sections of spiked human liver during processing by the indirect immunofluorescence technique. Tissue sections spiked with 1 μg of radioactive Δ-THC were processed by the indirect immunofluorescence technique. Five sections were removed for scintillation counting at each stage of the procedure. The figure shows the amount of Δ-THC leached from the tissue sections at each stage of the procedure. (© ASTM, 1916 Race Street, Philadelphia, PA 19103, USA. Reprinted with permission.)

and Feteanu (1978a) are recommended for consultation. Other aspects of the effects of fixatives on immunofluorescent staining will be dealt with in the ensuing section on this technique.

Stora (1980a, b) has used both Carnoy's and Bouin's fixatives for the examination of the histological localisation of the chemical carcinogens aflatoxin B1, *N*-2-fluorenylacetamide and benzo(α)pyrene. Bouin's fixative is a mixture of picric acid, formaldehyde, glacial acetic acid and acetone, while Carnoy's fixative contains chloroform, glacial acetic acid and absolute ethanol. The intracellular pattern of distribution of the carcinogens was not affected by the choice of fixative. The efficiency of fixation proved to be limited, however, for subsequent deparaffinisation procedures removed all weakly bound molecules from the cells.

Some drugs may be totally unsuitable for use with liquid fixatives of any sort, as they may be leached from the tissue as soon as they come into contact with any aqueous or nonaqueous solvents. In these instances vapour fixation may provide a suitable alternative. Formaldehyde and osmium tetroxide vapours are most commonly encountered in histochemistry but others that have been used include glutaldehyde, chromyl chloride and alcohol.

A review of the use of vapour fixation in the histochemical detection of biological macromolecules has been published by Pearse (1968b) but no reports have appeared in the literature of the use of such methods in drug histochemistry. Those vapours, such as formaldehyde, which fix proteins may be appropriate as they may alter the solubility characteristics of the drug–receptor complex. Other suitable choices of vapour may be made on the basis of the chemical characteristics of the drug.

Advice on the choice of fixative for drug histochemistry is inevitably limited by the lack of research that has been carried out in this area. Conventional fixatives may prove suitable for this purpose but completely new ones may have to be developed for use with specific drugs.

1.2.2 Embedding and dehydration

A number of embedding and dehydration procedures are available for preparing tissue for histochemical staining. It is the process of dehydration which can prove particularly troublesome, as so many drugs are lipid-soluble. For this reason embedded material may be more difficult to handle than simple solid blocks of frozen tissue. The nonpolar nature of many embedding compounds dictates that water must be removed from the tissue before the embedding compound can penetrate into the tissue block. Similarly the embedding compound must be removed to allow penetration of the staining reagents. The use of organic solvents as dehydrating agents leaches many lipid-soluble drugs from the tissues.

The technique of freeze drying is a neat way of sidestepping this problem. Water is removed under vacuum from tissues which have been frozen rapidly at temperatures as low as −190 °C. This material can then be embedded directly

without recourse to dehydration by organic solvents. The technique has been used successfully in the detection of antibiotics such as terramycin (Helander and Böttiger, 1953), tetracycline (Böttiger, 1955a), chlortetracycline (Böttiger, 1955b) and doxycycline (Liss and Norman, 1975). Water-soluble waxes are available which may be used in conjunction with freeze-dried material should the drug under investigation be soluble in either nonpolar embedding agents or the organic solvents required for removal of these materials from the tissue before histological staining can begin. They are also useful for embedding materials which may be affected by the relatively high temperatures required for embedding in paraffin wax (56 to 60 °C), as the procedure can be carried out at 20 to 30 °C.

Unfortunately, freeze drying is a time-consuming process which requires the use of expensive apparatus that may not be available in all laboratories. Although simple unembedded frozen sections may provide a suitable alternative, some dehydration is still necessary in order to observe cytological detail. Sufficient dehydration may occur when such sections are thaw-mounted onto microscope slides. Egorin *et al.* (1974) describe the localisation of adriamycin and daunorubicin by virtue of their autofluorescent characteristics in thaw-mounted frozen sections of hamster kidney, heart and lung mounted in a phosphate-buffered saline-glycerol solution and similar dehydration procedures have been used in the immunofluorescent detection of phenobarbital (Pertschuk *et al.*, 1976), methadone (Pertschuk *et al.*, 1977) and morphine (Balkon *et al.*, 1980). This method of dehydration has not, however, proved satisfactory in our laboratory. The autofluorescence of both tetracycline and chlorpromazine was found to be masked by an opaque film overlying sections of simple thaw-mounted tissue. Pearse (1968c) notes that some kind of protein film forms on thaw-mounted preparations which interferes with the penetration of some staining reagents. This may be responsible for the quenching of fluorescence in our preparations.

The technique of section freeze-substitution (Chang and Hori, 1961) may provide a suitable alternative to either freeze drying or simple freeze-thaw procedures. Although in this process the ice crystals present in frozen sections of biological material are dissolved by floating the tissue sections in cold liquid dehydrating agents, the dehydration process is completed much more rapidly than it is with embedded material, thus reducing the amount of exposure of the drug to organic solvents. Obviously, a dehydrating agent has to be chosen in which the drug under investigation has little or no solubility. Both tetracycline and chlorpromazine have been localised satisfactorily by this method using acetone cooled to −20 °C as the dehydrating agent (Gelder and Gee, 1979). Even though chlorpromazine has a solubility of about 1 in 100 in acetone, sufficient quantities of this drug are retained in the sections to allow visualisation of its luminescence in the cortical region of the brain (Figure 1.2).

Section freeze-substitution would seem to be particularly suitable for use with water-soluble drugs. After dehydration the floating sections are sufficiently stretched for direct mounting on cover slips or microscope slides. Flotation and expansion of embedded materials in heated water baths can remove water-

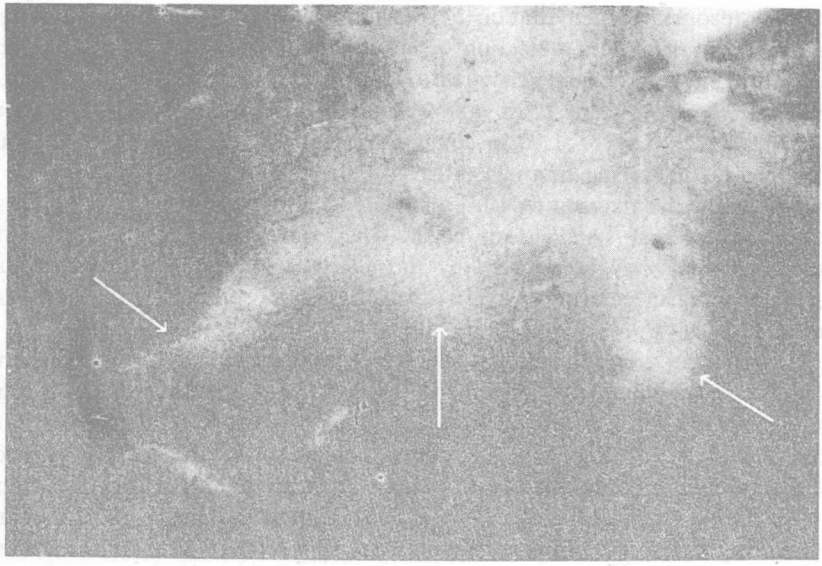

Figure 1.2 Distribution of chlorpromazine in the subcortical layer of mouse brain. Sections of mouse brain were prepared by the freeze-substitution technique. The specimen was maintained at −195 °C and excited with u.v. light. The areas of luminescence characteristic of the drug are arrowed (Gelder and Gee, 1979). (© *Journal of the Forensic Science Society*.)

soluble material. For example, Gelder and Gee (unpublished observations) have observed that such procedures leach tetracycline from soft paraffin-embedded tissues such as the liver and the kidney, although the drug is retained at sites of strong binding such as the areas of mineralisation in bone.

Ultimately the choice of embedding and dehydration procedures will be limited by the solubility characteristics of the drug in question and the amount of time available before the results are required. Although freeze-drying procedures would seem to offer an easy solution to the problem of dehydration, they can take up to a week to complete, whereas sections prepared by freeze substitution are ready for staining within a few minutes.

1.2.3 Mounting procedures

On completion of histochemical staining the tissue sections must be mounted in some material for subsequent observation on the microscope. It is important that a mountant should be chosen in which the drug is not soluble. Liquid paraffin has proved to be a suitable mountant for many of the antibiotics and for chlorpromazine. It has the added advantage that when it is used in conjunction with paraffin-embedded material it dissolves the paraffin wax surrounding the biological tissues. This obviates the need for exposure of the sections to organic

deparaffinisation agents which may leach drugs from the tissue. All but strongly bound molecules of the chemical carcinogens aflatoxin B1, *N*-2-fluorenylacelamide and benzo(α)pyrene, for example, are removed at this stage of the processing procedure (Stora, 1980a). Other properties of the mountant may also affect its suitability for use with a particular histochemical procedure. Fluorescent mountants will obviously be incompatible for use with fluorescent staining techniques. Many different mountants are available commercially and each should be considered carefully before the final choice is made.

1.3 FLUORESCENCE METHODS OF EXAMINATION

Spectrofluorimetric techniques for analysing drugs are well established in toxicology. Similarly, fluorescence microscopy is widely used for examining tissue structures. It seems logical that a combination of these two procedures should provide a means of localising drugs within tissue sections and successful histofluorescence techniques for a number of drugs have been described in the literature. Their position has been detected both by virtue of their autofluorescence characteristics (e.g. Gelder and Gee, 1979; Stora, 1980a,b) and by indirect procedures such as immunofluorescence (e.g. Pertschuk *et al.*, 1977; Balkon *et al.*, 1980). Other indirect procedures such as induced fluorescence may also prove to be of value, but these are entirely speculative at present.

Detailed theoretical descriptions of fluorescence will not be dealt with here. A comprehensive review of the subject has been published by Udenfriend (1962). It is simplest terms fluorescence can be defined as the radiation emitted by certain substances when they are irradiated or 'excited' with ultraviolet or short-wavelength visible light. A spectrum can be recorded of both excitation and emission radiations. The wavelengths at the peaks of these spectra are referred to as the excitation and emission maxima (Ex_{Δ} and Em_{Δ}). Use of the excitation maximum for irradation results in maximum fluorescence emission. The excitation and emission maxima are characteristic of each substance and consequently the conditions of examination can be controlled in such a way that only the substance in question is detected. The degree of control that can be exerted in fluorescence microscopy is more limited than it is in spectrofluorimetry because many naturally occurring cellular constituents autofluoresce. For this reason some drugs and flurochromes lend themselves more readily to this type of investigation than others. These problems are dealt with in the forthcoming section together with a brief discussion of the various types of equipment available to the microscopist.

1.3.1 Fluorescence microscopy

The ultimate aim of a histoflorescence technique is to produce an intense and specific image in the microscope of the fluorescent molecules under study. The

attainment of this goal will be aided if the contributions made by the components of the microscope are understood.

1.3.1.1 Factors affecting image intensity

1. Type of illumination Two types of illumination systems are available with fluorescence microscopes. They involve the use of either transmitted or incident light (Figure 1.3). For transmitted illumination the light source is positioned below the microscope stage and the section is excited by transmitting u.v. light through the tissue. For incident (epi-) illumination the light source is positioned above the microscope stage and the exciting radiations are directed onto the upper surface of the section.

Maximal fluorescence intensity is never observed with either of these systems because the biological tissues absorb light from both the excitation and the emission beams. With transmitted illumination the intensity of fluorescence from molecules in the upper cellular layers is reduced as a result of absorption of some of the excitation light during passage through the tissue. Similarly, fluorescence from molecules in the lower layers of cells is reduced as a result of tissue absorption of some of the emitted light. In epi-illuminated systems the intensity of fluorescence from these low-lying molecules may be reduced even further because wavelengths from both the excitation and the emission beams are absorbed by the tissues, but little attenuation of either of these radiations occurs in the upper cell layers. This results in a much brighter image than can be obtained with transmitted illumination, particularly when dealing with thick specimens.

Optimal visualisation of the fluorescence image is easier to achieve in epi-illuminated instruments because a single lens acts as both condenser (focusing the excitation light onto the section) and objective (collecting the light emitted by the section). Focusing of this lens onto the specimen results in the proper alignment of this part of the microscope. With transmitted illumination two separate lenses, each with an independent optical axis, are required for these purposes. These axes must be perfectly aligned before optimal visualisation can be attained and such alignment will not always be easy to maintain in routine use.

Other features of epi-illuminated instruments aid the ease with which a bright image can be obtained. For example, oil-immersion objectives are easier to use because a drop of oil on the specimen is sufficient for observation of fluorescence; in transmitted systems oil is also required on the surface of the condenser. In addition, since substage illumination is fully available in epi-illuminated instruments, fluorescence microscopy can be combined with transmitted brightfield microscopy. This can be extremely useful as it permits selection of the area of examination before irradiation of the specimen, and so reduces image fading.

The design features of epi-fluorescence microscopes thus allow a bright fluorescence image to be easily obtained. It is perhaps for this reason that the use of these instruments has become extremely popular in recent years.

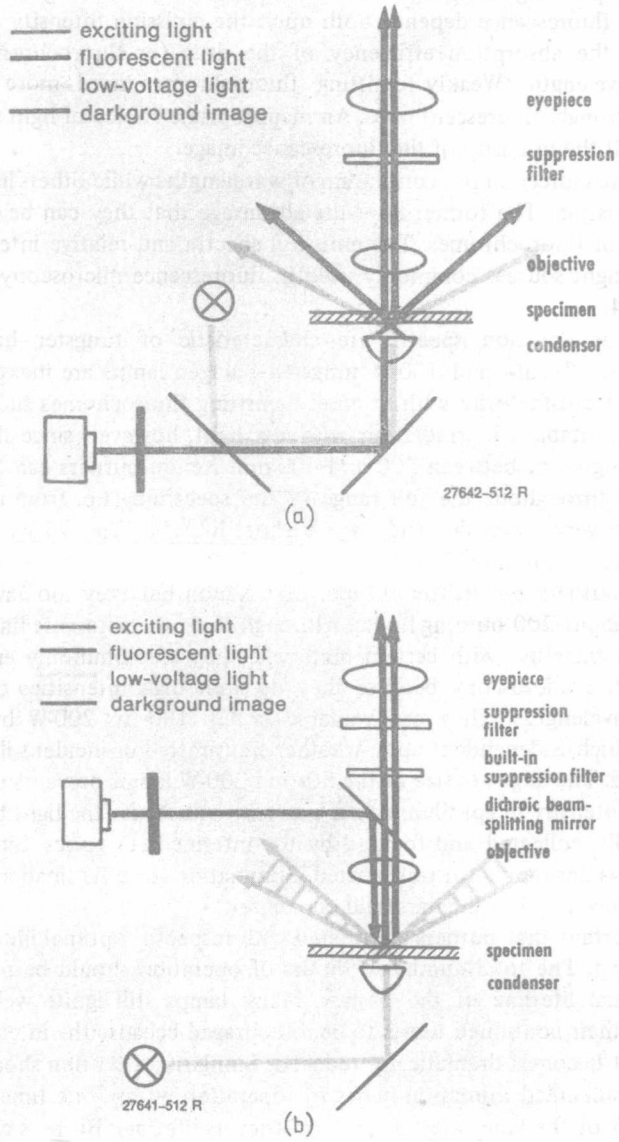

Figure 1.3 A comparison of transmitted and epi-illumination. In transmitted illumination (a) the specimen is excited by passing u.v. light through the tissue. The excitation beam is focused onto the section by means of a substage condenser. Emitted light is collected by the objective lens and observed through the eyepiece. In epi-illumination (b) the excitation beam is directed onto the upper surface of the section. The objective acts as both a condenser and a collecting lens. (*Courtesy E. Leitz (Instruments) Ltd.*)

2. Light sources The function of a light source is to provide light at a wavelength corresponding to the excitation maximum of the drug. The resulting intensity of fluorescence depends both upon the emission intensity of the light source and the absorption efficiency of the drug (or fluorochrome) for the emitted wavelength. Weakly emitting fluorochromes need more excitation light than strongly fluorescent ones. An inappropriate choice of light source may severely limit the intensity of the fluorescence image.

Some light sources emit a continuum of wavelengths while others have distinct peaks of emission. The former have the advantage that they can be used with a wide range of fluorochromes. The emission spectra and relative intensities of a number of light sources commonly used in fluorescence microscopy are shown in Figure 1.4.

Continuous emission spectra are characteristic of tungsten–halogen and xenon lamps. 12-, 50- and 100-W tungsten–halogen lamps are inexpensive and may be used satisfactorily with intensely emitting fluorochomes such as FITC. They are unsuitable for irradiation with u.v. light, however, since they do not emit wavelengths of between 300 and 400 nm. Xenon burners can be used for illumination throughout the full range of the spectrum (i.e. from u.v. to red) but they are very expensive and have a short lifetime. The 75-W, 110-W and 450-W bulbs have lifetimes of 400, 1200 and 2000 burning hours respectively.

Mercury burners are slightly cheaper than xenon but they too have a limited lifetime of about 200 burning hours. Although their characteristic line spectrum may restrict their use with certain materials, they are commonly encountered in fluorescence microscopy because they do emit high intensities over a wide range of wavelengths. They are available as 50-, 100- or 200-W burners, the choice of which is dependent upon whether transmitted or incident illumination is to be used. The large arc size of the 50- and 200-W lamps prevents optimal use of the light intensity in epi-illuminated instruments because the light bundle cannot be totally collected and focused by the intermediate optics. Similarly, the 100-W lamp is unsuitable for transmitted illumination since its small arc does not fill the entrance pupil of the darkfield condenser.

It is important that burners are treated with respect if optimal illumination is to be achieved. The total number of hours of operation should be restricted to the theoretical lifetime of the burner. Many lamps still ignite well past this period, but their continued use is to be discouraged because the intensity of the emitted light becomes dramatically reduced. Similarly, attention should be paid to the recommended minimum hours for operation at any one time. Frequent switching off of the lamp may severely restrict its lifetime. Burners can explode under these conditions and this is not only extremely noisy but it can cause extensive damage to the lamphouse!

3. Magnification and numerical aperture The intensity of the fluorescence image is affected by both the magnification and the numerical aperture of the lenses of the microscope. Maximal brightness is obtained by using minimal