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REVIEW

Standardization of reagents and methods used in cytological and histological practice with emphasis on dyes, stains and chromogenic reagents

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Summary

The need for the standardization of reagents and methods used in the histology laboratory is demonstrated. After definitions of dyes, stains, and chromogenic reagents, existing standards and standards organizations are discussed. This is followed by practical instructions on how to standardize dyes and stains through the preparation of reference materials and the development of chromatographic methods. An overview is presented of the problems concerned with standardization of the Romanowsky-Giemsa stain for cytological and histological application. Finally, the problem of how to convince routine dye and stain users of the need for standardization in their histology laboratories is discussed.

Introduction

In histology laboratories a not infrequent occurrence is that the staining results do not agree with the expected results. There may be a total failure or more often just a slight change. This can be in the hue of the stain or in its location. Perhaps the stain is just not as crisp as usual. What is the reason? Nearly always the phenomenon can be traced back to a change in reagents in the form of new batches or to slight, perhaps unrecognized, alterations of the applied method. What can be done about this state of affairs? The only answer is standardization of reagents and methods at a sufficiently high level. In the following, the problems are discussed more fully by some of the participants at the symposium on standardization held at the 9th International Congress of Histochemistry and Cytochemistry in Maastricht, The Netherlands in 1992. In addition to the introduction and conclusion, this review consists of a compilation of the following individual contributions:

1. Standardization of reagents for use in the histology department (Lyon)
2. Standardization and external quality control of dyes for microscopy by national and international institutions (Schulte)
3. Standardization of dyes and stains. A practical approach (De Leenheer, Lambert, van Liedekerke)
4. Differences in the staining pattern between the standard Romanowsky–Giemsa stain and non-standardized commercial versions (Wittekind)
5. Impure stains are troublesome, and such trouble is avoidable – but how do we convince the wo/man down the corridor? (Horobin)

In Part 1, Lyon discusses the quality of reagents, definitions of dyes, stains, and chromogenic reagents. This is followed by a brief summary of the data required for standardizing dyes and stains including methods for their use. In Part 2, Schulte elaborates on the quality problems of biological dyes and stains and how to solve them. This is followed by a description of commissions performing standardization of these reagents. In Part 3, De Leenheer, Lambert, and van Liedekerke give an overview of the general strategy for performing standardization of dyes

and stains. In Part 4, Wittekind gives a detailed example of how a specific stain (Romanowsky–Giemsa) has been standardized. This applies also to the application of the stain for both haematological (cytological) and histological work. Finally, in Part 5, Horobin gives several examples of problems caused by impure dye samples. A strategy for avoiding these problems is given. The difficulties in communicating this information to the users of biological dyes and stains are underlined. Ways for improving this situation are emphasized.

1. Standardization of reagents for use in the histology department

REAGENTS USED IN THE HISTOLOGY LABORATORY

Reagents used in cytology, histology, and histochemistry laboratories include inorganic and organic solvents, fixatives, decalcifying agents, embedding agents, buffers, dyes, stains, chromogenic reagents, enzymes, antibodies, nucleic acid probes, radioactive isotopes, and mounting media. To this impressive list must be added numerous acids, bases, and salts.

Quality of reagents

In the laboratory, one should as a general requirement use reagents that are pure (purum, rein, reagent quality) or of analytical grade (analytical reagent (AR), pro analysi (p.a.), zur Analyse) (Andersen *et al.*, 1991). This is normally easy to achieve when employing solvents, fixatives, and buffers. However, several proprietary brands of commercially available fixatives, decalcifying agents, clearing agents, and mounting media have insufficient, meaningless, or no declaration of their contents. More pronounced problems concerning purity arise when we turn our attention towards dyes, stains, chromogenic reagents, and enzymes. With antibodies, it is a question of ensuring specificity and avidity towards the antigenic epitope(s). For nucleic acid probes, specificity must be directed towards the target nucleic acid sequence. In both the latter cases, one must have information regarding the correct working conditions.

Definitions of dyes, stains, and chromogenic reagents

The following exposition will focus on dyes, stains, and chromogenic reagents but with occasional reference to the other types of reagents. To make clear what is meant in the following a few definitions might be in place (Lyon *et al.*, 1991; European Committee for Clinical Laboratory Standards, 1992a). By dyes, we mean coloured organic substances available as crystals or as powders which on solution in a suitable solvent may bind by physicochemical attraction to a substrate and impart colour to the latter. A stain is a solution of dye in a suitable solvent. A chromogenic reagent can react with suitable groups present or induced in the biological substrate with the formation of a dye *in situ*.

STANDARDIZATION

Standardization includes two aspects; these are: standardization of reagents and standardization of methods.

Standardization of dyes, stains, and chromogenic reagents

Standardization of dyes, stains, and chromogenic reagents includes specification of their physical and chemical characteristics. Minimum requirements include knowledge of the visible and ultraviolet absorption curve, molar extinction coefficient, and thin-layer chromatographic or high-performance liquid chromatographic data. However, only for a minority of these reagents do sufficiently precise data exist. This is due to the very few reagents, particularly dyes, so far produced in completely pure form. When a standard does exist, a candidate sample of the reagent must fulfil the specifications for the standard.

Standardization of methods

Standardization of staining methods calls for the whole procedure of preparation and staining to be standardized, in addition to the staining reagent. The introduction of model systems (Horobin, 1982, pp. 229–34) containing specified amounts of various molecules in a suitable matrix (van der Ploeg & van Duijn, 1964; van Duijn, 1976; van Duijn & van der Ploeg, 1980) has given much valuable information for achieving these goals. Examples of standardization involving both the dyes and their applications are the standard Romanowsky–Giemsa stain (see Part 4 of this review) and the Methyl Green–Pyronin Y method (Hoyer *et al.*, 1986). For further references see, for instance, Schulte *et al.* (1992).

FUTURE GOALS

To achieve maximum quality assurance of histological and cytological diagnosis, our goal might well be to circulate tissue and cell specimens between laboratories. This could be by post or by electronic video transmission. The need for national and international rules of standardization in this field is therefore urgent. We must find ways to assure drafting of such rules and adherence to these.

2. Improving biological dyes and stains: quality control and standardization for biomedical applications

QUALITY PROBLEMS OF BIOLOGICAL DYES AND STAINS: WHY ARE RULES FOR QUALITY CONTROL AND STANDARDIZATION NECESSARY?

Usually, commercial dyes and stains are not of uniform quality. Batch-to-batch variations of dye content, variable amounts of organic and inorganic impurities and even mislabelling of dye containers lead to corresponding variations of staining performance (Scott, 1972; Mowry & Kasten, 1975; Horobin, 1980; Mowry *et al.*, 1980). This is a major problem in research, and it may be detrimental when dyes and stains are used as diagnostic tools as for

instance in routine pathology. The need for quality control was recognized many years ago (Scott & French, 1924) and has since been repeatedly noted in the literature. A review of some relevant questions is given elsewhere (Wittekind, 1985; Schulte, 1991).

HOW CAN BIOLOGICAL DYES AND STAINS BE IMPROVED?

There are principally three ways to guarantee the good and uniform quality of dyes and stains. These are internal quality control, external quality control, and standardization.

Internal quality control

By definition, internal quality control is performed by the producer or vendor of the substance. The dye undergoes a specified testing procedure which is established by the firm. This means that the rules for the testing procedures are defined by the firm itself, and the user has finally no guarantee for obtaining the good and uniform quality expected. The user has to trust the firm that the quality testing procedures have been carried out with care. The Certistain (E. Merck, Darmstadt, Germany) and the Accustain projects (Sigma–Aldrich, USA) are such internal quality control procedures.

External quality control

By definition, external quality control is performed by independent organizations. A well-known example is the US Biological Stain Commission (US BSC) which was founded as an independent non-profit organization, originally under the name 'Commission on Standardization of Biological Stains' (Clark & Kasten, 1983; Conn, 1980a, b), to guarantee a high quality of dyes on the American market.

Representative samples of commercial dyes are tested by the US BSC according to a well-defined testing procedure and, when found to give satisfactory results, are labelled with a US BSC certificate which indicates that this sample proved to be satisfactory according to the US BSC protocol. The specifications for the test protocol include total dye content, absorption characteristics and a staining test according to reference staining procedures (Clark, 1974). Quality control of dyes and stains outside the USA has been discussed by Allison (1982, 1984).

Standardization

Creation of standards involves a unification of substances and methods, variations of which are in general use by those concerned: industrial consumers, organizations, or individuals (Wittekind, 1985). Standardization of a dye is based on physicochemical and technical specifications. The relevant dye is completely and exclusively characterized by these specifications as a chemical substance of high purity. Any commercial dye can be compared with the reference standard.

Standardization of staining procedures comprises more than just specifications for dyes. Indeed, the relevant stain, the staining protocol, and adjuvant techniques must be standardized to guarantee reproducible staining performance. Detailed specifications on the dye container label should inform the user about the quality of the actual batch. This type of standardization is performed by several independent and non-industrial organizations.

INSTITUTIONS FOR STANDARDIZATION OF BIOLOGICAL DYES AND STAINS

The German Bureau for Standardization (DIN, Berlin) has established standards for three dyes and for one staining procedure used in haematology (DIN, 1987). These standards have been adopted and extended by the European Committee for Clinical Laboratory Standards, Subcommittee on Reference Materials for Tissue Stains (ECCLS-SRMTS, 1992a–i). Recently, the above committee has changed its name (now: European Commission for Clinical Laboratory Standardization).

A standard staining procedure for blood and bone marrow smears was recommended by the International Committee for Standardization in Haematology (ICSH, 1984).

The Working Group 6 (WG 6) within the Comité Européen de Normalisation (CEN) will shortly establish a horizontal standard for dye specifications (Lyon, personal communication).

FUTURE ASPECTS

The need for standardization of dyes and stains will increase in the future. Despite the growing importance of immunocytochemical and immunohistochemical methods, stains are still important tools in cytological and histological practice.

Application of quantitative techniques such as high-resolution image analysis in cytology requires highly reproducible staining performance which – if at all – can be guaranteed only by standardized procedures (Wittekind, 1985).

3. Standardization of dyes and stains

INTRODUCTION

Variable chemical composition and the presence of impurities are two important factors underlying inconsistent staining of blood, bone marrow and tissue samples and contribute to potential diagnostic errors (Marshall & Lewis, 1974). Also, degradation of the constituting dyes by the presence of metal ions (Marshall & Lewis, 1975), by high temperatures (Dean *et al.*, 1977) or by oxidation (Marshall, 1978) drastically influences the quality of a staining solution. In addition, the concentration of the dye in solution as well as the molarity of the buffers affect the stability of dyes (Bins *et al.*, 1985). Therefore, to

prevent degradation, the addition of stabilizers (e.g. acids, alkylamines, and dimethylsulphoxide) has been thoroughly investigated.

To obtain reproducible and reliable results in staining performance it is of the utmost importance that chemically pure dyes and standardized procedures are used for the preparation of staining solutions as well as for the staining itself. In this way, standardized dyes and staining techniques will allow automated microscopy and automated cell pattern recognition (Green *et al.*, 1979; Wied *et al.*, 1980). Consequently, there is a need for analytical methods that can distinguish between the various homologues, isomers and degradation products of each individual dye to evaluate the quality of fresh commercial dyes as well as of aged staining solutions in the laboratory. A brief overview of the strategy and of the techniques involved in this process follows.

REQUIREMENTS FOR STANDARDIZATION

For standardization to be achieved, two requirements must be fulfilled. First, pure and structurally identified reference compounds should be available. Secondly, chromatographic techniques should be used to achieve the separation of the compound of interest from its impurities and degradation products.

Reference compounds

Reference compounds, especially of degradation products, are not always commercially available. However, they can be obtained by collection of the separated peaks of an impure or degraded dye from a (semi)-preparative high-performance liquid chromatographic (HPLC) system. After crystallization, these compounds have to be characterized and structurally identified by different spectroscopic approaches such as nuclear magnetic resonance (NMR), ultraviolet (UV) and infrared (IR) spectrometry and by mass spectrometry (MS) (Baeten *et al.*, 1989). After structure elucidation all these compounds can serve as reference materials in the second step of standardization of dyes and stains.

The availability of well characterized reference compounds now allows real quantitative measurements instead of expressions based on relative peak areas because exact molar absorption coefficients are now known. A representative example from the authors' laboratory of obtaining reference compounds is depicted in Fig. 1. It represents a semi-preparative HPLC separation of Azure B from a number of impurities and degradation products (van Liedekerke *et al.*, 1989; van Liedekerke & De Leenheer, 1990). Azure B is the well known cationic constituent (together with the anionic Eosin Y) of the Romanowsky–Giemsa staining solution applied in cytology and histology laboratories for colouration of blood and bone marrow smears. After elimination of other non-dye constituents that were present in the eluent, such crystallized compounds served as reference material for further quality control studies of these Romanowsky–Giemsa staining solutions.

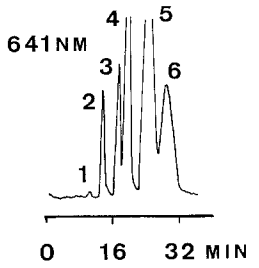

Fig. 1. Semi-preparative separation of Azure B and related compounds. Column: PRP-1, 25 × 2.15 cm i.d. Eluent: H_2O:CH_3CN (32:68 by vol) 0.124 M tetramethylammonium hydroxide (TMAH), 0.0027 M bis(2-ethylhexyl)-phosphoric acid (BEHP), pH: 2.5. Flow rate: 10 ml min^{-1}. Detection: 641 nm. Peak identification: (1) Thionin; (2) Azure C; (3) Azure A (asymmetrical dimethylthionin); (4) Symmetrical dimethylthionin; (5) Azure B; (6) Methylene Blue.

Chromatographic techniques

As already mentioned, the second step in the standardization procedure is always based on a chromatographic process. Appearance of extra peaks in the chromatogram unequivocally demonstrates degradation or contamination of the product. In the past, classical thin-layer chromatography (TLC), and, more recently, also high-performance thin-layer chromatography (HPTLC), have been applied for standardization purposes. The introduction of automatic application of the sample combined with computerized densitometry not only allowed quantitative work but also provided spectral information of the separated compounds. In addition, both techniques (TLC and HPTLC) offer the possibility of analysing a large number of samples in one single run with a much lower solvent consumption as compared to HPLC (Gupta, 1991; van Liedekerke *et al.*, 1991a). The application of liquid chromatography, however, undoubtedly offered substantial improvement and advantages as compared to TLC (Nelis & De Leenheer, 1986). Gradient elution permitted the separation of a large number of compounds from very different polarities in a reasonable run time, while ion-pair chromatography on bonded-phase packing materials allowed the chromatography of ionic substances (e.g. the constituents of Romanowsky–Giemsa staining solution, namely Azure B and Eosin Y) (van Liedekerke *et al.*, 1991b). In this way we investigated the stability of different salts of Azure B (e.g. thiocyanate, chloride) with or without addition of different Eosin Y forms (disodium salt or free acid) over a period of one year. It was shown that the Azure B chloride salt degraded more rapidly than the thiocyanate salt, but that its stability was increased by addition of Eosin Y acid (and not by Eosin Y disodium salt). These results are presented in Fig. 2, while a representative chromatogram of an impure commercial Azure B bromide standard is given in Fig. 3. In addition, photo-diode array detection offered on-line spectral

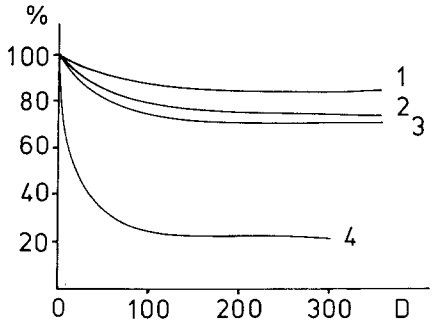

Fig. 2. Percentage of different Azure B salts left in solution in dimethylsulphoxide (DMSO) versus time (days). (1) Azure B SCN 0.75%/Eosin Y acid 0.12%; (2) Azure B SCN 0.75%; (3) Azure B Cl 0.75%/Eosin Y acid 0.12%; (4) Azure B Cl 0.75%.

information of the eluting peaks. An important advantage with regard to TLC and HPTLC concerns the easy recovery of separated compounds from collected fractions of the eluent. In this way, other spectroscopic techniques (e.g. IR, NMR and MS) can be applied on the same fraction (Baeten *et al.*, 1989).

The main purpose of these stability studies concerns the evaluation of the different parameters influencing the degradation of a constituent of a staining solution. This knowledge then leads to the optimal choice of the solvent, of the counter-ion or of the stabilizing agent, and will finally result in more reproducible colour development. In addition, the chromatographic techniques offer the possibility for national as well as international quality control studies in the future.

4. Differences in the composition and staining pattern between the standard Romanowsky–Giemsa stain and non-standardized commercial versions

INTRODUCTION

The Romanowsky–Giemsa stain is an efficient biological stain whose mechanism of action has been extensively studied. It may thus serve as a suitable example to illustrate the impact which this kind of work has on standardization and on the practical application of the stain. There is still a considerable gap between the creation of a stain standard and its employment in morphological diagnosis. This is evident for morphologists interested in the background of their diagnostic methods. The main characteristic of the use of the Romanowsky–Giemsa stain is the development of the colour purple in the stained specimen. According to an old assumption (Giemsa, 1902, 1904, 1923; Lillie, 1944), this is due to the cationic dye Azure B acting together with the anionic dye Eosin Y. This was proved correct by Wittekind *et al.* (1976).

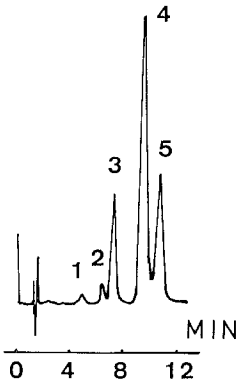

Fig. 3. Commercial Azure B bromide. Column: PRP-1, 15 × 0.4 cm i.d. Eluent: same as in Fig. 1. Flow rate: 1 ml min^{-1}. Detection: 641 nm. Peak identification: (1) Azure C; (2) Azure A (asymmetrical dimethylthionin); (3) Symmetrical dimethylthionin; (4) Azure B; (5) Methylene Blue.

The next logical step was to compare the stain performances of the Azure B–Eosin Y dye-pair with those of the commercial Giemsa, Leishman, and Wright dye mixtures (Wittekind & Gehring, 1985). These stains often varied widely in composition. In addition to Azure B, they contained closely related cationic thiazin derivatives Methylene Blue, Azure A, and sometimes even Thionin and the entirely ineffective Methylene Violet (Bernthsen). This comparative study demonstrated the superiority of the Azure B–Eosin Y combination over the commercial mixtures consisting of polychrome Methylene Blue and Eosin Y. In routine diagnostics, the Azure B–Eosin Y dye-pair demonstrated its superiority and its cost-effectiveness. The dye-pair Azure B–Eosin Y is simpler and gives staining results that are more reproducible and easier to evaluate. One might thus expect the dye-pair Azure B–Eosin Y to meet with widespread acceptance. This was, however, clearly not the case. It is pertinent to ask: what is the reason for this failure?

It is undeniable that the majority of Romanowsky, Leishman, and related type mixtures give the important colour purple of cell nuclei, mast cell and promyelocyte granules. Particularly the most recently produced commercial Giemsa brands can give staining results adequate for diagnostic purposes. Azure B can be obtained by direct synthesis (Green, 1980, personal communication) using the method of Holmes (1928). Very good results can be achieved when this directly synthesized Azure B replaces polychrome Methylene Blue in commercial Giemsa type stains.

CHARACTERISTICS OF THE AZURE B–EOSIN Y COMPLEX

The often satisfactory staining performance of non-standardized Giemsa type stains is not surprising considering the physico-chemical characteristics of the Azure B–Eosin Y molecular interaction. The non-standardized stains have been analysed in detail by model experiments, spectrophotometry, and microspectrophotometry (Zimmermann, 1983; Zipfel *et al.*, 1981, 1982, 1984; Hüglin *et al.*, 1986; Müller-Walz & Zimmermann, 1987; Friedrich *et al.*, 1990).

In the present context, there are two important physico-chemical features. The first is the location of the Romanowsky–Giemsa at wave number 18 100 cm^{-1}, corresponding to wave length 551 nm, close to the Eosin Y absorption maximum (514 nm). The second feature is the very intense absorption at this wave length (Zipfel *et al.*, 1984; Friedrich *et al.*, 1990). These properties are consistent with an assumption already proposed by Zanker (1981); this is that a charge transfer is involved in the formation of the Azure B–Eosin Y complex (Slifkin, 1971). One of the spectral characteristics of charge transfer is the high absorption of light caused by an electron moving from an electron donor (Azure B) to an electron acceptor (Eosin Y). The precise geometry of the aggregated Azure B–cations/Eosin Y–anions complex is still insufficiently understood. In commercial polychrome Methylene Blue–Eosin Y dye mixtures, no evidence supports the formation of mixed cationic dye aggregates, e.g., Azure B–Methylene Blue.

The Romanowsky–Giemsa-effect, i.e. the formation of the colour purple in the stained specimen, is due to the intense light absorption of the Azure B–Eosin Y complex. In older commercial Giemsa type stains, Azure B does not account for more than about 30–35% of the total of thiazin dyes present in polychrome Methylene Blue. The high absorption coefficient of the Azure B–Eosin Y complex ensures even in these cases that the colour purple becomes visible.

Another consequence of the Azure B–Eosin Y reaction is the high affinity of the dye-pair to biological and other substrate surfaces. This surpasses that of either dye alone. The affinity of Azure B–Eosin Y is greater than that of any other dye combination so far examined. The reason for this is so far insufficiently understood. Molecular interaction of dyes in aqueous surroundings is a state of affairs with which textile dyers and colourists are familiar (Daruwalla, 1974). However, there are differences in the chemical structures and the technical conditions of dye-application in the two fields.

DYE PURIFICATION AS A PREREQUISITE FOR THE STUDY OF THE STAINING MECHANISM AND FOR STAIN STANDARDIZATION

The elucidation of the physicochemistry and staining behaviour of the Romanowsky–Giemsa dyes, Azure B and Eosin Y, clearly required the availability of both dyes in pure form. Also, the other dyes forming part of Romanowsky, Leishman, and related dye mixtures had to be obtained in a sufficiently purified state to carry out the necessary control experiments. This meant that Methylene Blue, Azure A and Thionin had to be combined with Eosin Y. Similarly, in addition to Eosin Y, Azure B was combined with other representatives of the fluorescein group: tetrachlorofluorescein, Eosin B, Erythrosin B, Phloxine, Rose Bengal, and others (Wittekind & Grèzes, in preparation). A detailed description of these experiments is certainly beyond the scope of the present article. Suffice it to mention here that it proved impossible to imitate the Azure B–Eosin Y staining pattern with any other dye combination.

Components of the stain

The principal argument for using pure dyes for setting stain standards is thus established. The widely used Romanowsky dye mixtures may well perform satisfactorily in the laboratory routine. However, it is impossible to determine the contribution of the individual components of a dye mixture to the staining pattern. The general demands on high standards for analytical methods (to which biological staining techniques should belong) make it essential to classify stain components into three categories. These are: (a) components essential for the typical staining pattern, (b) indifferent additives, and (c) detrimental components. An elaboration follows below.

a. Indispensable components for the typical (i.e. the standardized) results are here Azure B and Eosin Y.
b. Indifferent additives are components that do not qualitatively interfere with the staining results. Examples are Methylene Blue, Azure A, or Tribromofluorescein. These components may, however, cause a quantitative deterioration of the staining result by decreasing the concentration of the nominal dye.
c. Components which are detrimental to the stain solution may act either by directly interfering with the staining pattern or by causing technical disturbances. A technical disturbance is, for instance, the reduction in solubility of the dyes in the solvent. Other examples are components leading to an alteration of pH or a reduction in dye stability. Ionic, alcoholic, and detergent additives might be placed in this category. On the other hand, deliberate addition of ions, for example the solution of Azure B in potassium acetate buffer, may increase stain performance (Horobin, 1982, p. 82).

The importance of pure dyes

The central role played by pure dyes in the heterogeneous and complex biological stains appears clearly on reviewing the factors effective in a staining prescription. It is, however, by no means the intention to recommend the 99 or 100% pure dye as the indispensable main constituent of a standard stain. Still, for the reasons stated below, the chemically pure dye should, at least once, have been prepared.

1. In principle, anyone advocating the application of dye mixtures should know the characteristics of the pure component dyes. This includes knowledge of the staining pattern obtained with the pure dyes. This makes it possible for producers and consumers of the dyes to determine tolerance limits for admissible concentrations of impurities.

2. The Beer–Lambert law can be put to practical use for computation of the concentration of the actual dye content in commercial dye samples of unknown composition. For this determination, the specific molar absorption coefficient (ϵ) is necessary. However, this can be obtained only from an extremely pure dye sample.
3. There are also technical reasons recommending familiarity with the physical and chemical properties of pure dyes. Their solubility in biologically important solvents need not be identical with those of the parent dye mixtures. The pH values obtained in one solvent are not the same after dissolving the pure dye and the variously contaminated dyes.

DIFFERENCES BETWEEN THE STANDARD ROMANOWSKY–GIEMSA STAIN AND NON-STANDARDIZED VARIANTS

Staining results

In Table 1 the general scheme of staining obtainable with the standard Romanowsky–Giemsa stain is listed. Further, comparison is made with deviating staining results frequently obtained with polychrome Methylene Blue-based variants of the Romanowsky–Giemsa stain. A few common technical faults seen with non-standard Romanowsky–Giemsa are also included in the table. The standard Romanowsky–Giemsa stain is described in detail elsewhere (Wittekind, 1986). Note that the results summarized in Table 1 refer to the staining of air-dried, alcohol-fixed cell films. The host of additional technical problems to be faced when transferring the application to histology are briefly summarized in the closing section.

Technical differences

The commercial polychrome Methylene Blue-based Giemsa, Leishman, Wright type stains have in common that methanol or preferably alcohol–glycerol mixtures are used as solvents for the dyes (Giemsa, 1904; Pappenheim, 1911; Lillie, 1944). It was decided (Wittekind, 1986) to replace alcohol/glycerol by dimethylsulphoxide (DMSO) for several reasons. DMSO is stable, non-volatile, and has a very high solvent capacity. It is non-toxic and inexpensive. Azure B is stable indefinitely in DMSO if combined with anions which will reduce, but of course not abolish, solubility in aqueous solutions. Suitable anions are, for instance, tetrafluoroborate and thiocyanate. The corresponding Azure B salts are easily obtained by slowly adding the ammonium salts of tetrafluoroboric acid or thiocyanic acid respectively, to aqueous solutions of Azure B in the chloride form. This matter will be dealt with in detail shortly (Wittekind, in preparation).

Table 1. General scheme of staining results on air-dried, alcohol-fixed cell films obtained with the Romanowsky–Giemsa stain and colour deviations due to common errors and deficiencies of commercial Giemsa, Wright, and Leishman type stains

Structure	*Standard Romanowsky–Giemsa stain*	*Commercial Giemsa, etc., stain*	*Probable cause of colour difference*
Nuclear chromatin	Purple	Blue	(a) Insufficient concentration of AB in polychrome MB; MB dominates (b) EY precipitated from staining solutions by the cationic dyes. Remainder of blue MB and AB suffices to stain nuclei blue (c) Faulty slide preparation
Cytoplasm (rich in RNA)	Blue	Greyish red, various shades	(a) Ratio of AB:EY too low (b) Concentration of MB too low to override the greyish-red cytoplasmic shades (c) Concentrations of the lower Azures (A, C, sometimes even Thionin) too high
Basophil granules	Dark purple	Invisible	No cationic dye in alcoholic fixative to precipitate strongly anionic heparin
Eosinophil granules	Dull brownish orange	Red-orange	Lower concentration of AB in polychrome MB provides for binding of EY (red-orange) alone. Possibly the only case where standard RG-colours are not superior to commercial dyes
Neutrophil granules	Clear purple	Weakly stained or unstained	(a) Concentration of AB too low for this specific substrate (b) Probably correlated to (a): gross divergence from optimum molar ratio AB:EY 8–10:1

AB = Azure B; MB = Methylene Blue; EY = Eosin Y; RG = Romanowsky–Giemsa.

THE STANDARD AZURE B–EOSIN Y STAIN, RELATED EOSIN Y-TYPE → AZURE B STAINS, AND GIEMSA-TYPE STAINS IN CYTOLOGY AND HISTOLOGY

Cytological material

In cytology, the exposure of cell films to the Romanowsky-type dyes is the main step in the staining process. The optimal pretreatment is air-drying followed by fixation in alcohol. The optimal post-treatment consists merely of rinsing in water and air-drying. These procedures are so widely used that it is admissible to consider the Romanowsky–type staining 'in this usual way' as approaching a status of standardization *per se*. For this reason, it has already proved possible to standardize the cytological Romanowsky–Giemsa stain (DIN, 1987; ICSH, 1984).

Histological material

In histology, the situation is completely different. In both pretreatment and post-treatment, many more factors are influential besides the solution of the two dyes in suitable solvents. The most important factor in pretreatment is *fixation*. Azure B, polychrome Methylene Blue, Eosin Y, and other fluorescein derivatives are far more sensitive to fixation-induced alterations of biopolymers than robust and simple stains such as Haematoxylin–Eosin and the anionic dye-based trichrome stains.

Thus, when staining with thiazin-cationic dyes (e.g. Azure B) and Eosin Y or other fluorescein derivatives, it makes a considerable difference whether the fixative contains aldehydes (formaldehyde, glutaraldehyde) or ethanol/acetic acid (for instance Carnoy). On the other hand, post-treatment is also of major importance. Thus, differentiation and dehydration are indispensable for obtaining multicoloured and reproducible staining patterns. The essential measure to achieve this goal, and also to avoid too much dye being extracted by differentiation and dehydration, is to prolong the staining time. This is particularly true for the Azure B staining. For the combined Azure B–Eosin Y stain, this will mean staining times of up to four hours to obtain the full Romanowsky–Giemsa staining pattern. However, such long times are impractical.

Extended model and histochemical experiments have shown that the only way to achieve acceptable 'Romanowsky–Giemsa-like' staining results in histology is to replace the simultaneous Azure B–Eosin Y application with a sequential Eosin Y → Azure B staining (not vice versa!). For specific histochemical applications, one might consider replacing Eosin Y by analogue fluorescein derivatives of higher molecular weight, for example tetraiodofluorescein (i.e. Erythrosin B). This dye binds more firmly to biopolymers due to increased van der Waals's forces aiding the electrostatic (Coulombic) forces. The intricate theoretical and technical problems involved will be dealt with in detail shortly (Wittekind, in preparation; Wittekind & Grèzes, in preparation).

5. Impure stains are troublesome, and such trouble is avoidable: but how do we convince the wo/man down the corridor?

IMPURE STAINS ARE TROUBLESOME

This warning is well documented, and needs no lengthy exposition. Here, for illustration, two entertaining examples are offered.

Some dyes discussed in the literature do not actually exist. For instance, Acetyl Sudan Black, advocated by certain histochemists and biochemists as a lipid and lipoprotein stain, cannot be synthesized by the methods given in the literature. Consequently, dye sold as Acetyl Sudan Black is merely Sudan Black with a different label (Lauder & Beynon, 1989).

As a second example note that other dyes described in the literature are in practice unobtainable. For instance, a dye called SITS has been used as an axonal tracer by neuroscientists. Unfortunately, chromatographic investigation of SITS samples showed that only samples containing a particular red impurity were effective in this role, and that the necessary red dye was present in only a single commercial sample (Horobin *et al.*, 1987). It should not be thought that such problems arise only with dyestuffs. The literature indicates that immunostains (Elias *et al.*, 1989), enzyme substrates (Jefree, 1970), and reagents for *in situ* hybridization (Koji & Nakane, 1990) are also on occasion impure.

SUCH TROUBLE IS OFTEN AVOIDABLE

This proposition, too, needs no extensive documentation. Analytical methods for stains have been developed, pure reagents produced, and standardized staining methods devised. The optimism of this sub-heading may be demonstrated by two comments. Thus, many dyes traditionally regarded as 'difficult to use' or 'giving erratic results' do not give problems if used in pure form (Wittekind *et al.*, 1982; Lyon *et al.*, 1983). Fortunately, an increasing number of reasonably pure dyes are currently available commercially, at reasonable prices: e.g. Azure B, for Romanowsky–Giemsa stains, and Pyronin Y for Methyl Green–Pyronin staining. However, even if reasonably pure dyes are not commercially available, there is a simple practical strategy for reducing problems due to impurities. This is to buy dye samples which carry US BSC certification. These meet minimum assay standards, and will reliably work for specified practical methods. Moreover, as browsing in catalogues demonstrates, US BSC certified dyes are no more expensive than non-certified samples; indeed they are sometimes cheaper. US BSC certified dyes have been quality checked by an independent organization, namely, the commission, as well as hopefully by the vendor. Consequently, they should not be confused with products which have only been subject to an in-house quality control scheme, however rigorous. Unfortunately, no comparable certification scheme currently exists for immunostains, for

enzyme substrates/visualizing agents, or for reagents used for *in situ* hybridization.

THE MESSAGE PROBLEM

The messages concerning impure dyes are not reaching routine users. Many benchworkers don't know, don't believe or don't bother about them. If this seems an extreme opinion, consider the following analysis of today's histochemical literature.

Look in the indices of current English-language histochemical books for terms such as 'purity' or 'purification' or 'analysis' or 'artifact'. Only a single book appears to have any reference to the effects of impurities (Lyon, 1991). Moreover, 'procedures' sections of staining manuals are little better. Indeed, some fail to mention dye impurity as a cause of problems in routine staining methods. One or two manuals, notably Chayen and Bitensky's *Practical Histochemistry* (1991), do contain such comments for many procedures. However, even in such a book, comments are not placed in any consistent position in the text, and so are easy to overlook.

SUGGESTIONS FOR IMPROVED COMMUNICATION

How do we bring the good news to the wo/man down the corridor? Possibilities include persuading editors in routine histopathology and biology journals to make recommendations to authors; persistently writing on this topic for technical magazines and proceedings; publishing low-cost booklets on stain im/purity; persuading authors and editors of staining manuals to add 'purity tips' to their practical recipes; and producing new kinds of information sources; e.g. databases and expert systems. Attention is focused on the last two options.

First consider adding purity tips to the 'procedures' sections of staining manuals. To be readily found, they should be consistently placed in each staining method: preferably immediately prior to the section describing the preparation of solutions. The purity tips should be written to some consistent format. As an example of how this might be done, consider the following draft entry for staining methods making use of the dye Alcian Blue in critical electrolyte concentration procedures.

'Alcian Blue samples not soluble in water to at least 5% are unsuitable – as are pH 5.7 acetate-buffered Alcian Blue solutions containing 2.0 M magnesium chloride, which rapidly precipitate dye. To minimize problems, use US BSC certified samples. Do not use samples labelled Alcian Blue 5G or 7G. For more complete criteria and suggestions, see Scott & Mowry (1970).'

Another approach to telling the world of dye purity problems, and of their solutions, is to exploit electronic information systems. These can be generated using hypertext or expert system shells, and could be run on the routine laboratory computers. Such electronic information systems do have certain advantages over printed media. Overloading benchworkers with unwanted information is more easily avoided than with books; and the systems can be more economically updated than can printed media. In this context, a prototype electronic advisory system has been produced by Ford, Horobin and Williams (Sheffield University, England), under the auspices of the US Biological Stain Commission. This system enables a benchworker to seek information in a wide variety of ways, for instance:

- To ask about common problems arising with named dyes (e.g. Alcian Blue, or Eosin), or about named staining systems (e.g. PAS or van Gieson)
- To ask for advice on recognizing problems due to impure dyes (e.g. pale staining, excessive background staining, non-selective staining), and then to troubleshoot the problems
- To obtain information on recommended methods of analysis and purification, both in general, and of named dyes
- To obtain other information, e.g. concerning dye names and nomenclature; the usefulness of the Biological Stain Commission and its stain certification programme; and why dyes are impure
- There is also a Bibliography, which can be browsed directly, in addition to its being cited in other segments of the system.

The present version of the system considers the routine dyes and many others in general use, some 48 in all. Following field testing, a report on the value of this advisory system will be prepared. If favourable, it is hoped that a version will become generally available.

Conclusion

Advanced quality assurance schemes for laboratories of histology and histopathology can be instituted only if full reproducibility of results is ensured. This makes standardization of reagents and methods indispensable. The implementation of existing and new internal quality control is under way. However, we must stress that external quality assurance schemes are also most important for achieving the goal: full reproducibility of staining results.

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