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Standardization of the Feulgen-Schiff technique

Staining characteristics of pure fuchsin dyes; a cytophotometric investigation

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Summary. Four fuchsin analogues (Pararosaniline, Rosaniline, Magenta II and New Fuchsin) usually found in Basic Fuchsin have been applied as chemically pure dyes to the Feulgen-technique. Total nuclear absorption and wavelength of the absorption maximum were measured by microspectrophotometry in Feulgen stained cytological and plastic embedded histological liver samples, and in lymphocyte nuclei in human peripheral blood smears; absorption spectra of Feulgen stained DNA-polyacrylamide films were determined by spectrophotometry. The grey value distribution of tetraploid liver cell nuclei was calculated with an image analyzer. The staining characteristics of the pure dyes were compared to commercial fuchsin samples from various suppliers. Reverse phase thin layer chromatography was used for characterization and qualitative separation of commercial batches.

Pure fuchsin analogues were all equally suitable for Feulgen staining: with respect of staining intensity all pure fuchsin dyes gave nearly identical results with a bathochromic shift of the absorption maximum from Pararosaniline to New Fuchsin of about 8 μm.

Differences in staining results observed among the commercial dyes were due to varying dye content, contamination with an acridine-like fluorescent compound or simply mislabelling of samples. Pure Pararosaniline is recommended for a standard Feulgen technique.

Introduction

Since its introduction by Feulgen and Rossenbeck in 1924 the Feulgen reaction (FR) has become one of the most widespread methods in quantitative cyto- and histochemistry. Although the physico-chemical background of the FR has not been fully elucidated it is considered to be specific and stoichiometric for DNA (Kasten 1960; Hardonk and van Duijn 1964a, b; Harms 1965; Romeis 1968; Duijndam and van Duijn 1975b; Horobin 1982; Oud et al. 1984; Pearse 1985).

When cytophotometric measurements are carried out on Feulgen stained cell nuclei it is found that staining intensity as an indicator of nuclear DNA content to a great extent depends on a number of preparatory factors among which the method of fixation, the mode of acid hydrolysis and the staining kinetics are particularly important; they have been investigated in detail (Sandritter et al. 1965; Böhm and Seibert 1966; Böhm 1968; Deitch et al. 1968; Greenwood and Berlyn 1968; Fand 1970; Rasch and Rasch 1973; Duijndam et al. 1973; Duijndam and van Duijn 1975a, b; Kjellstrand and Lamm 1976; Gill and Jotz 1974, 1976). However, there are still gaps in our knowledge of those dye-substrate relationships on which the typical staining result is based. So we do not know if all fuchsin analogues are equally suitable for obtaining the colour "purple" or whether there might be preferences for one or another analogue. An answer to this question has to rely on the employment of all fuchsin analogues in a highly purified state. The pure dyes were now available in sufficient quantity for the present study.

Schiff's reagent normally used in the FR is prepared from basic fuchsin, a variable mixture of four triaminotriphenylmethane analogues: Pararosaniline (=Magenta 0), Rosaniline (=Magenta I), Magenta II (="Basic Fuchsin") and New Fuchsin (=Magenta III). Pararosaniline is unmethylated, Rosaniline, Magenta II and New Fuchsin have one, two or three methyl groups, respectively (Fig. 1).

It was – among others – observed by Kasten (1960), Müller (1966) and Lillie (1977) that Schiff reagents prepared from different batches of basic fuchsin stained cell nuclei with different intensities, and Lodin et al. (1963) found significant differences in nuclear extinction for several commercial batches in their cytophotometric studies. However, they did not determine the purity of the dyes used. The same proves right for the paper of Dutt (1976) who found

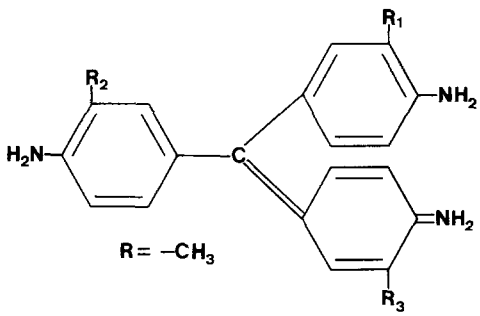

Fig. 1. Molecular structure of the triarylmethane dyes. Pararosaniline is unmethylated (i.e. R_1, R_2 and R_3 are hydrogen atoms); with increasing methylation of the dye molecule R_1, R_2 and R_3 are successively methylated

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no differences in staining performance between Pararosaniline and its three methylated analogues.

Teichman et al. (1980) extensively studied the staining properties of Pararosaniline, Magenta II and New Fuchsin. They applied their staining technique to a solution of hydrolyzed DNA and determined the spectral absorption of the dye-DNA-complex. However, such studies in solution have only limited value for the situation in the matrix of the fixed cell. Finally, they also did not use pure dyes, and they omitted Rosaniline.

In the present study we try to determine whether Feulgen stains prepared from different pure fuchsin analogues show different characteristics in spectral absorption and in dye-substrate interaction in situ.

Materials and methods

Cytological and histological material

Liver. The surface of a freshly cut human liver was smoothly pressed against a glass slide; after air drying for 1 h one half of the slides was fixed for 1 h in a mixture of methanol/formaline/glacial acetic acid 85/10/5 v/v (=*MFA*, Böhm 1968), the second half was fixed for 15 min in methanol p.a. After fixation the MFA-fixed slides were rinsed in distilled water for 10 min. Hydrolysis was carried out for 45 min in 5 *M* HCl at 22° C (temperature controlled water bath), followed by two 2-min rinses in distilled water. The slides were stained for varying times (5–120 min) in the Feulgen reagent (at 22° C), followed by two 5-min rinses in sulphite water, washed twice in distilled water for 5 min, dehydrated in ethanol (50%, 60%, 70%, 80%, 90%, 99% one min each) and mounted – after toluene for 3 min – in Flo-Texx (Lerner Lab., Stamford, USA, cat. no. 770-1).

Small pieces of liver tissue (3 × 3 × 3 mm) were fixed in 4% neutral buffered formaldehyde for 24 h at 22° C, washed in running tap water for 12 h, dehydrated in 50%–99% ethanol (each step 30 min) and embedded in glycolmethacrylate (JB_4, Polysciences, St. Goar, FRG, cat. no. 0226A). 4 micron sections were cut from these tissue blocks with "Ralph" knives (Bennett et al. 1976). Staining was performed as described above.

Blood. Heparinized peripheral blood smears from three human donors were prepared on an Abbott ADC-500 centrifugal blood spinner. After air drying for 1 h the smears were fixed in methanol p.a. for 10 min or in MFA for 1 h and stained as described above.

Unhydrolyzed control slides were used to test the specificity of the FR for both pure and commercial dyes. All slides were stored in the dark for 2 days before measurements were carried out.

Staining solution

Schiff's reagent was prepared from Pararosaniline (Colour Index 42500), Rosaniline (C.I. 42510), Magenta II (no C.I. number) and New Fuchsin (C.I. 42520) according to Graumann (1953): 0.5 g Pararosaniline-chloride (or equimolar amounts of the higher methylated analogues) are solved in 15 ml 1 *M* HCl. 85 ml of a 0.5% $K_2S_2O_5$-solution are added. After 24 h the solution is shaken with 0.3 g charcoal for 2 min and filtered. The colourless filtrate is ready for use and can be stored in the refrigerator for some months. For some experiments staining solutions with a dye content from 0.01% to 0.5% (the "normal" dye concentration) were prepared. The pure fuchsin dyes (Table 1) were obtained from SERVA GmbH, Heidelberg, FRG (charge-no.: Pararosaniline G-8501; Rosaniline G-8502; Magenta II G-8503; New Fuchsin G-8504). The commercial dyes are listed in Table 2. The pH of the staining solution and of the sulphite rinsing agent is about 1.1.

The pure dyes were used as chloride salts. The "standard" solution was prepared with Pararosaniline-chloride: When higher methylated fuchsin dyes or – in the case of commercial samples – other than chloride compounds were investigated *equimolar amounts* of the respective dye were used.

Table 1. Pure fuchsin dyes from Serva GmbH, Heidelberg, FRG

No.	Dye	Colour Index No.
A	Pararosaniline	42500
B	Rosaniline	–
C	Magenta II	42510
D	New Fuchsin	42520

Table 2. Commercial fuchsin dyes from various suppliers

No.	Dye (label)	Supplier	Batch
1	Pararosaniline acetate	Aldrich	–
2	Parafuchsin reinst	Serva	31627
3	Pararosaniline chloride	Michrome	722
4	Pararosaniline sulphate	Michrome	783
5	Pararosaniline	Chroma	1B533
6	Rosaniline	Merck	7604
7	Fuchsin basisch	Chroma	10757
8	Fuchsin basisch	Chroma	11308
9	Fuchsin basic	Difco	3928
10	Fuchsin basic	Michrome	DC568
11	Fuchsin basisch	Serva	21915
12	Magenta Basic	Michrome	DEC68
13	Neufuchsin	Chroma	6911
14	Neufuchsin	Bayer	61356
15	Neufuchsin	Chroma	181

DNA-polyacrylamide (PAA) films

The preparation of DNA-PAA films is described elsewhere (Wittekind and Gehring 1985). A DNA content of 5 mg calf thymus DNA (Sigma, München, FRG, no. 1501) in 1 ml PAA solution was used. The thickness of the films amounted to 200 micron (±15 micron). After polymerization the films were rinsed in distilled water for 15 min, hydrolyzed in 5 *M* HCl for 60 min at 22° C, rinsed in distilled water for another 15 min and stained with the Feulgen reagent for 1 h at 22° C. The stained films were washed in three changes of sulphite water (5 min each), and after another rinse in distilled water were measured in a spectrophotometer. Specificity of the staining reaction was controlled by immersion of unhydrolyzed films in Schiff's reagent.

Thin layer chromatography

The composition of the commercial fuchsin samples was tested by reverse phase thin layer chromatography (RPTLC) on Nano-SIL C_{18}-100 plates (Macherey-NAgel, Düren, FRG, no. 811052). The solvent system consisted of methanol 30 ml, water 20 ml and 25% NH_3 in water 10 ml; the solution was applied with a Pt-Ir capillary in form of a line 1 cm in length and 1 cm from the bottom of the RPTLC-plate. RPTLC-plates were positioned vertically in a glass container (25 cm × 30 cm × 8 cm) with a solvent phase 0.5 cm thick on the ground. Chromatography was stopped when the solvent front reached a line 1 cm from the top of the RPTLC-plate (which occurred after about 50 min).

Spectrophotometry

For calculation of the molar extinction coefficient 5×10^{-7} *M* of the pure fuchsin dye was solved in ethanol 95%. The absorption

was measured with a DMR 21 spectrophotometer (see below) with a 5 cm glass cuvette. A corresponding cuvette with dye free solvent was positioned in the reference beam.

The dye content of commercial samples also was assessed by spectrophotometry as described above. The absorption of the pure dyes was set 100%, the dye content of the commercial samples was calculated in per cent of the pure dye.

Nuclear magnetic resonance (^{1}H-NMR) spectrometry for the characterization of the dyes was performed by Dr. J. Wörth and T. Gehring, Department of Chemistry, University of Freiburg, FRG. Dyes were solved in dimethylsulfoxide-d_6 and measured at a frequency of 400 MC.

Instruments

Spectral absorption of DNA-PAA films was determined in a Zeiss DMR 21 spectrophotometer (Zeiss, Oberkochen, FRG). For all fuchsin dyes extinction was plotted against wavelength. A hydrolyzed unstained film was positioned in the reference beam.

Microdensitometry was carried out with a Vickers M 85a scanning microdensitometer (Vickers, York, UK). Objective 100X oil immersion (aperture 1.3); diameter of measuring spot: 0.5 micron. Monochromator bandwidth 20 nm; scanning wavelength: *Pararosaniline* 540 nm; *Rosaniline* 542 nm; *Magenta* II 546 nm; *New Fuchsin* 550 nm; light source: 100 W tungsten lamp.

Integrated nuclear optical density (INOD) as an indicator of the amount of dye bound was calculated in arbitrary units (au). 150 diploid, tetraploid and octoploid liver cell nuclei respectively and 100 randomly chosen lymphocyte nuclei in human blood smears were measured and the mean values and standard deviation calculated. All measurements were compensated for glare and distributional error (Goldstein 1970, 1971).

Nuclear grey value distribution was measured with the image analyzer IBAS 2000 (Kontron, Eching, FRG). The image was scanned with a Siemens K-30 tv-camera (vidikon-tube) through a 100X oil immersion objective (aperture 1.3). The light source (100 W tungsten lamp) was filtered through a 545 nm filter (Schott, FRG). After appropriate correction for shading and noise the image was stored in the computer for further processing.

In a second experiment INOD of cell nuclei were measured with the IBAS to compare the results with microdensitometric investigation.

Microspectrophotometry was performed with a modified UMSP 100 microspectrophotometer (Zeiss, Oberkochen, FRG). This instrument has been described in detail by Zipfel et al. (1984).

Data were investigated for statistical significance with Mann-Whitney *U*-test (two-sided). Differences were considered to be significant if $p < 0.01$.

Results

Careful microscopical examination of the slides showed the following results:

1) With regard to staining intensity and staining pattern there were no differences among the pure dyes; in contrast, commercial samples showed major variations of hue and intensity of nuclear staining.

2) Unspecific staining was never observed when the Feulgen reaction was performed "lege artis"; when the final sulphite rinse was omitted there was some unspecific cytoplasmic staining both with pure and commercial dyes.

3) Unhydrolyzed "control" slides and films were unstained.

4) After hydrolysis in 1 *M* HCl for 1 h at 60° C the Feulgen reaction was negative in lymphocyte cell nuclei.

5) Feulgen staining was also negative after 24 h DNase treatment of the same cells.

Figures 2 and 3 show cytological and histological slides, respectively, after staining with pure Pararosaniline. Staining with the higher methylated fuchsin analogues gave completely identical results.

Microdensitometry

The values of INOD are listed for both pure and commercial dyes in Table 3. Compared to the Vickers microdensitometer INOD values measured with IBAS were about 5% lower. Remarkable variation of staining intensity was found for the commercial samples, whereas no statistically significant differences were seen among pure dyes. Total nuclear extinction of MFA-fixed lymphocytes was about 7%–10% lower than the corresponding value of diploid hepatocyte nuclei. After methanol fixation we found the same nuclear extinction for hepatocyte nuclei as compared to MFA-fixation, whereas absorption of lymphocyte nuclei was about 15% lower after methanol fixation. This was true for all fuchsin dyes.

Table 4 gives the dye content of commercial samples which varied from 17% to 79%! Emphasis is given on the fact that the *total dye content* of commercial samples was tested; thus the extinction is due not only to the fuchsin dyes but also to any present coloured components which absorb at 540 nm. Note that with 5 among 15 commercial batches (33%!) staining intensity is significantly lower than with pure dyes.

Spectrophotometry of DNA-PAA-films

The absorption curve of a Feulgen-stained DNA-PAA model film is shown in Fig. 4. The curve is the mean of 20 measurements after staining with Pararosaniline. The absorption curves for the higher methylated analogues are nearly identical. Table 5A gives the mean values of the absorption and of lambda$_{max}$ for Pararosaniline and its analogues.

The following molar extinction coefficients were calculated from the pure fuchsin dyes in ethanol 95%: Pararosaniline 85700 ($\pm$990); Rosaniline 85300 ($\pm$850); Magenta II 86200 ($\pm$720); New Fuchsin 86800 ($\pm$1010). The extinction coefficient of Pararosaniline coincides well with the value reported by van Duijn and Riddersma (1973).

Microspectrophotometry

The absorption curve of a Feulgen stained lymphocyte nucleus is shown in Fig. 5. The curve is again the mean of 20 single measurements after staining with Pararosaniline. The absorption curves for the higher methylated analogues are nearly identical. In Table 5B the mean values of nuclear absorption and of lambda$_{max}$ are given.

Tables 3, and 5A, B, Figs. 4 and 5 show that pure fuchsin dyes gave nearly identical total nuclear extinction. A slight bathochromic shift of lambda$_{max}$ from Pararosaniline to New Fuchsin was observed for both DNA-PAA films and lymphocyte nuclei.

Figure 6 shows the influence of staining time on staining intensity for pure dyes; nearly identical staining kinetics were found for all fuchsin dyes; no statistically significant differences were seen. After a rapid increase of staining intensity a plateau phase is reached after about 50–60 min.

In Fig. 7 staining intensity of lymphocyte nuclei was

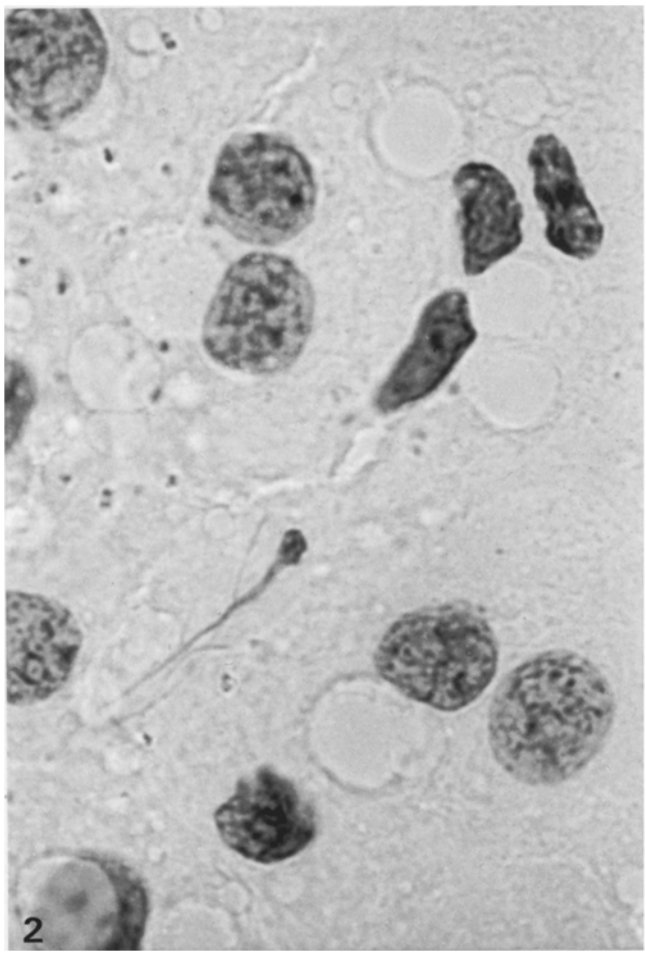

Fig. 2. Liver tissue imprint; air-dried, fixation in MFA for 1 h. Hydrolysis in 5 *M* HCl for 45 min at 22° C. Feulgen staining with Pararosaniline. 1:1800

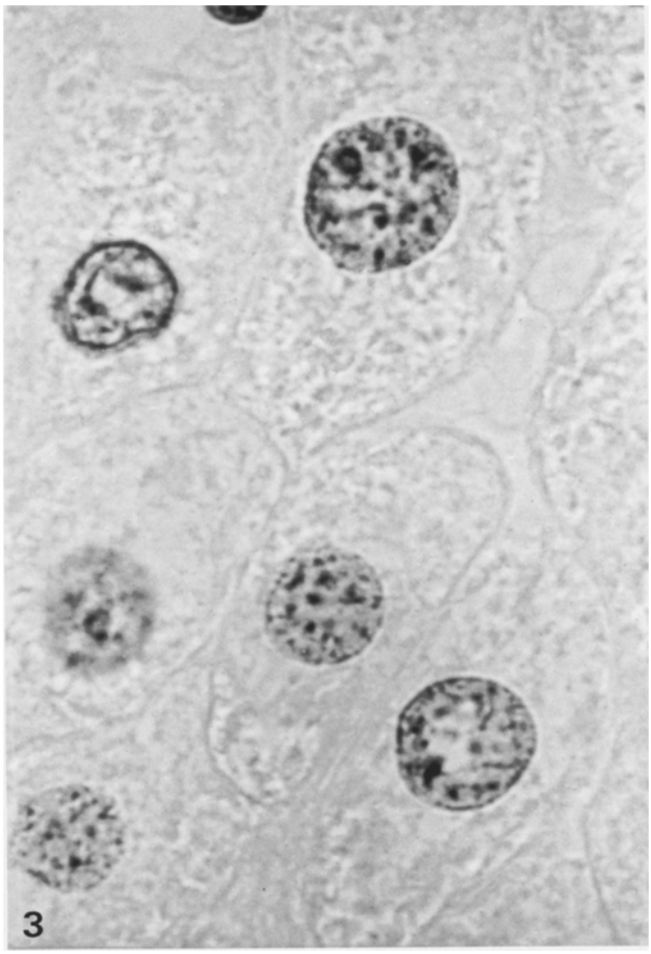

Fig. 3. Liver tissue section; fixation in neutral buffered formalin for 24 h; embedding in GMA. Hydrolysis in 5 *M* HCl for 45 min at 22° C. Feulgen staining with Pararosaniline. 1:1800

plotted against dye concentration. Over a wide range (0.1%–0.5%) there is nearly no change of total nuclear extinction; very low dye content (0.05% and less with proportional decrease of sulphite content) resulted in a decrease of staining intensity; this was found for all fuchsin dyes.

Thin layer chromatography

The results of RPTLC of pure and commercial fuchsin dyes are shown in Fig. 8A–D. The following Rf-values were calculated for the pure dyes: *Pararosaniline* 0.66; *Rosaniline* 0.53; *Magenta II* 0.37; *New Fuchsin* 0.23. These values differ slightly from those reported by Nettleton and Martin (1979), which is certainly due to differences in the solvent system used. Neither coloured nor fluorescent impurities were found in the pure samples, whereas most commercial samples contained impurities which showed a bright green-yellow fluorescence when excitation light of 366 and 254 nm was used. RPTLC proves that some batches (no. 4, 8, 12) simply were mislabelled. Batches no. 7 and 8 were obtained from the same supplier; the two samples are labelled identically ("Fuchsin basisch"), but RPTLC shows that they differ in both dye content and dye composition. This batch-to-batch variation is indeed a major problem in standardization of dyes and stains.

Figure 9A–D shows NMR-spectrometry of the pure fuchsin analogues which could be clearly identified.

Figure 10 shows the nuclear grey value histogram of tetraploid liver cell nuclei from cytological samples after staining with pure Pararosaniline. The histograms after staining with higher methylated analogues were nearly identical. The grey value histogram describes the numerical distribution of condensed and decondensed chromatin particles; it is thus a rough parameter for nuclear texture.

Discussion

The Feulgen-Schiff reagent has been applied now since almost sixty years in cyto- and histochemistry but – as far as the authors know – a fully standardized staining procedure has not been developed until today. "*Standardization*" of the Feulgen reaction means that a well-defined staining protocol including all preparatory steps such as fixation, hydrolysis, composition of the staining solution and the final rinsing procedure has to be developed to give a highly reproducible staining pattern; the need for standardization of the Feulgen reaction especially for automated cell pattern recognition (ACPR) has been discussed by Wittekind (1985) and Wittekind and Schulte (1987).

It has been mentioned above that some steps of the

Table 3. Integrated Nuclear Optical Density (INOD) of Feulgen stained lymphocyte and hepatocyte cell nuclei. Dye no. A–D and 1–15 see Tables 1 and 2. Hydrolysis in 5 *M* HCl for 45 min at 22° C. Vickers M 85a

Dye	INOD [mean values and standard deviation]			
	Lymphocytes	Hepatocytes		
		2c	4c	8c
A	11.6 (1.2)	12.7 (1.2)	24.6 (1.9)	50.3 (4.4)
B	11.3 (1.2)	12.4 (1.4)	25.4 (2.3)	51.2 (4.7)
C	11.9 (1.4)	13.1 (1.5)	26.8 (2.2)	54.0 (4.9)
D	11.8 (1.5)	13.2 (1.6)	25.6 (2.3)	52.4 (4.5)
1	12.1 (1.7)	13.5 (1.7)	24.9 (2.7)	52.2 (4.8)
2	11.8 (1.8)	13.3 (1.6)	25.2 (2.9)	50.4 (4.6)
3	10.9 (2.6)	11.4 (2.0)	21.5 (3.4)	45.4 (6.7)
4	9.2 (2.4)	9.7 (2.9)	18.1 (4.7)	39.4 (5.7)
5	12.2 (2.2)	13.4 (3.7)	24.8 (5.3)	49.7 (6.4)
6	11.2 (1.9)	12.0 (2.1)	23.7 (3.1)	49.5 (5.8)
7	10.2 (1.9)	11.8 (1.8)	24.0 (3.6)	50.3 (6.9)
8	9.5 (2.1)	10.3 (2.3)	19.3 (2.9)	41.7 (4.9)
9	8.3 (1.4)	9.6 (1.5)	17.9 (2.4)	36.9 (5.0)
10	10.1 (1.7)	11.4 (1.6)	24.3 (2.9)	50.5 (6.0)
11	9.1 (2.2)	9.9 (2.4)	18.5 (3.2)	38.3 (5.2)
12	11.5 (1.3)	13.1 (1.2)	25.3 (1.9)	51.5 (4.1)
13	11.0 (2.1)	11.6 (2.7)	24.5 (3.6)	47.3 (5.6)
14	9.1 (2.0)	10.2 (2.6)	22.5 (3.6)	47.6 (5.2)
15	11.0 (2.5)	12.0 (3.4)	25.1 (4.1)	51.3 (5.9)

Table 4. Dye content of commercial fuchsin analogues (for numbers see Tables 1 and 2). Dye content is calculated in per cent of the pure sample. All measurements with DMR 21

Batch number	1	2	3	4	5
Dye content [%]	47	41	65	33	58
Batch number	6	7	8	9	10
Dye content [%]	39	69	23	56	49
Batch number	11	12	13	14	15
Dye content [%]	32	79	72	17	51

Feulgen procedure – amongst them fixation, acid hydrolysis and the influence of the rinsing agent (Demalsy and Callebaut 1967; Elleder and Lojda 1971; Duijndam and van Duijn 1973) – have been investigated in the past, and these preparatory steps have been standardized to a certain extent. However, the question is still unanswered whether all fuchsin dyes are equally suitable for Feulgen staining and – if they are not – which dye should be recommended for a standardized staining protocol. A lot of staining recipes have been described where Pararosaniline, one of its methylated analogues or simply a variable mixture of these dyes, Basic Fuchsin, were recommended for Feulgen staining (for references see: Stowell 1945; Romeis 1968; Lane and Tripp 1971; Duijndam and van Duijn 1973; Kovacs and Longley 1975; Pearse 1985; Culling et al. 1985; Schulte 1986). Besides Feulgen staining with fluorochromes a palet of thiazine, phenazine and oxazine dyes have been investigated as a substitute for Pararosaniline; Thionin-SO_2, which was established by Van Duijn (1956), has been applied in combination with Congo Red to ACPR by Oud and his coworkers (1981).

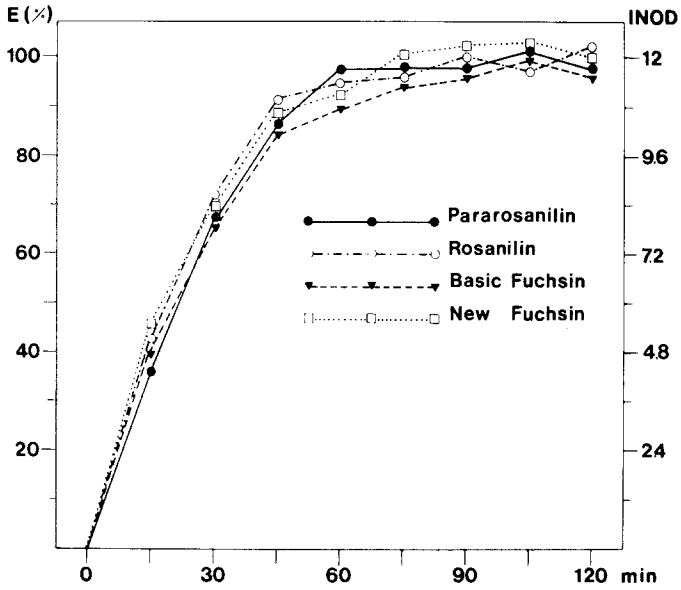

Fig. 4. Influence of staining time on nuclear absorption of Feulgen stained lymphocyte nuclei in human peripheral blood smear. Fixation for 1 h in MFA; hydrolysis in 5 *M* HCl for 45 min at 22° C. Staining was carried out at 22° C. Dye concentration: 0.5 g/100 ml. IBAS 2000. *Abscissa:* staining time (min); *ordinate:* absolute (INOD) and relative (E %) extinction. Each *points* represents the mean value of 100 randomly chosen cell nuclei

Table 5A. Absorption and wavelength of absorption maximum ($lambda_{max}$) of Feulgen stained DNA model films. Mean values (standard deviation) from 20 films. Measurements with DMR 21

Dye	DNA model films	
	$Lambda_{max}$	Absorption
Pararosaniline	541 (1.7)	0.91 (0.083)
Rosaniline	544 (1.3)	0.88 (0.079)
Magenta II	547 (1.1)	0.92 (0.084)
New Fuchsin	549 (1.4)	0.90 (0.081)

Table 5B. Absorption and wavelength of absorption maximum ($lambda_{max}$) of Feulgen stained lymphocyte nuclei. Mean values (standard deviation) from 20 cell nuclei. Measurements with IBAS 2000

Dye	Lymphocyte nuclei	
	$Lambda_{max}$	Absorption
Pararosaniline	542 (1.6)	0.62 (0.055)
Rosaniline	544 (1.4)	0.59 (0.063)
Magenta II	546 (1.4)	0.61 (0.048)
New Fuchsin	549 (1.6)	0.60 (0.053)

Standardization of a staining procedure – and even more the comparison of the "standard" with different staining variants – requires objective descriptors of the staining pattern. For nuclear staining with the Feulgen technique these features can be defined as: *1)* the chromaticity coordinates (hue, saturation and intensity of nuclear colour), and *2)* the chromatin texture. Both features are influenced by the dye-substrate affinity, and they can be investigated by means of cytophotometry. Thus the evalua-

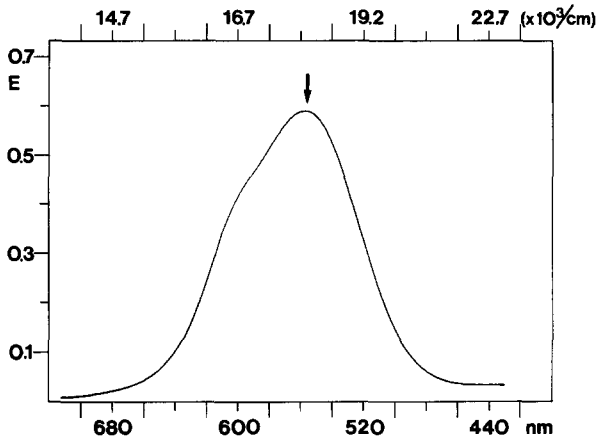

Fig. 5. Spectral absorption curve of a Feulgen stained lymphocyte nucleus in human peripheral blood smear. Fixation for 1 h in MFA; hydrolysis in 5 *M* HCl for 45 min at 22° C. Staining with Pararosaniline. The curve is the mean value of 20 measurements (Zeiss UMSP 100). The *arrow* points to lambda max. *Abscissa:* wavelength (wavenumber); *ordinate:* extinction

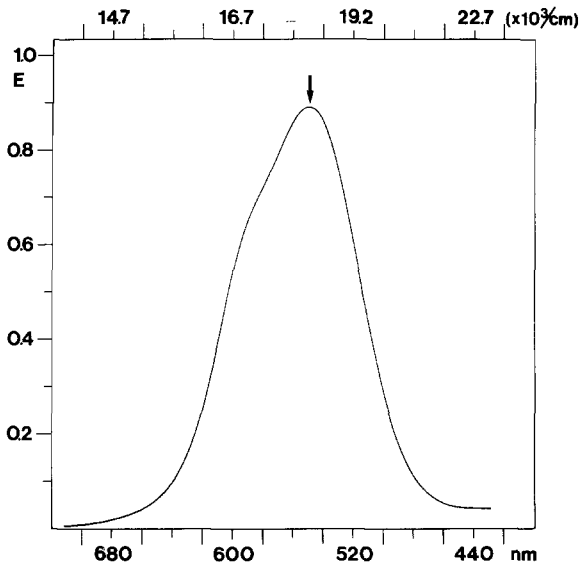

Fig. 6. Spectral absorption curve of a Feulgen stained DNA-polyacrylamide film. Hydrolysis in 5 *M* HCl for 45 min at 22° C. Staining with Pararosaniline. The curve is the mean value of 20 measurements (Zeiss DMR 21 spectrophotometer). The *arrow* points to lambda$_{max}$. *Abscissa:* wavelength (wavenumber); *ordinate:* extinction

tion of a standardized staining protocol has to rely on the assessment of these two features.

As shown by spectrophotometry the hue of nuclear colour differs slightly for all fuchsin analogues corresponding to the bathochromic shift of the absorption maximum with increasing methylation of the dye molecule. The human eye is not very sensitive for light around 550 nm; thus the bathochromic change of colour may easily be undetected.

The reproducibility of staining intensity is one of the most important features of a standardized dye (Wittekind 1985), especially when it is used for quantitative determination of DNA. In this respect there were no statistically significant differences among the fuchsin dyes. Furthermore

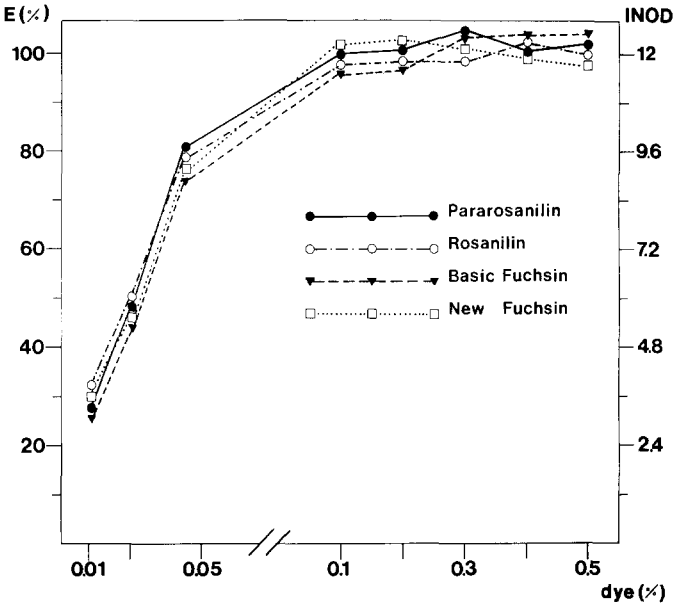

Fig. 7. Influence of dye concentration on nuclear absorption of lymphocyte nuclei in human peripheral blood smear. Fixation in 5 *M* HCl for 45 min at 22° C. Staining time: 1 h at 22° C. IBAS 2000. *Abscissa:* dye concentration (%); *ordinate:* absolute (INOD) and relative (E %) extinction. Each *point* represents the mean value of 100 randomly chosen cell nuclei

the staining kinetics (Figs. 6 and 7) were very similar; this finally means that the affinity of the dye towards its substrate was nearly identical for all fuchsin analogues.

Tables 3 and 5A, B give an answer to point *1)*: all fuchsin dyes gave nearly identical values of spectral absorption both on cell nuclei and on DNA model films. Small differences among the dyes were statistically not significant ($p > 0.05$). After methanol fixation absorption of lymphocyte cell nuclei was about 15%–20% lower than in diploid hepatocyte cell nuclei. This coincides well with findings of Atkin and Richard (1956), Mayall (1967), Garcia and Iorio (1968) and Garcia (1969) and is probably due to differences of chromatin compactness (Böhm et al. 1968; Duijndam and van Duijn 1975a; Mello 1983). Emphasis is given to the fact that this "proportionality error" was found for all fuchsin dyes to the same extent. A complete "restoration" of the diploid DNA value in lymphocyte nuclei after MFA-fixation as reported by Böhm and his coworkers (1968) was not observed: total nuclear extinction of lymphocyte nuclei was about 7%–10% lower than the IOD of diploid hepatocyte nuclei. Off-peak measurements of lymphocyte nuclei showed that this remaining proportionality error was not due to errors of measurement. A similar proportionality error after MFA-fixation was found by Deitch et al. (1968).

As mentioned before the Feulgen-Schiff reagent usually is a mixture of four fuchsin analogues. In general the proportion of each analogue in a certain batch is unknown and can only be roughly estimated by RPTLC (Nettleton and Martin 1979); a quantitative separation of fuchsin dyes using high performance liquid chromatography is possible (Sehlinger and Nettleton 1987) but requires special expensive equipment. Thus, in routine work the composition of the staining solution probably remains more or less unclear. Therefore the comparison of the single dye versus the dye

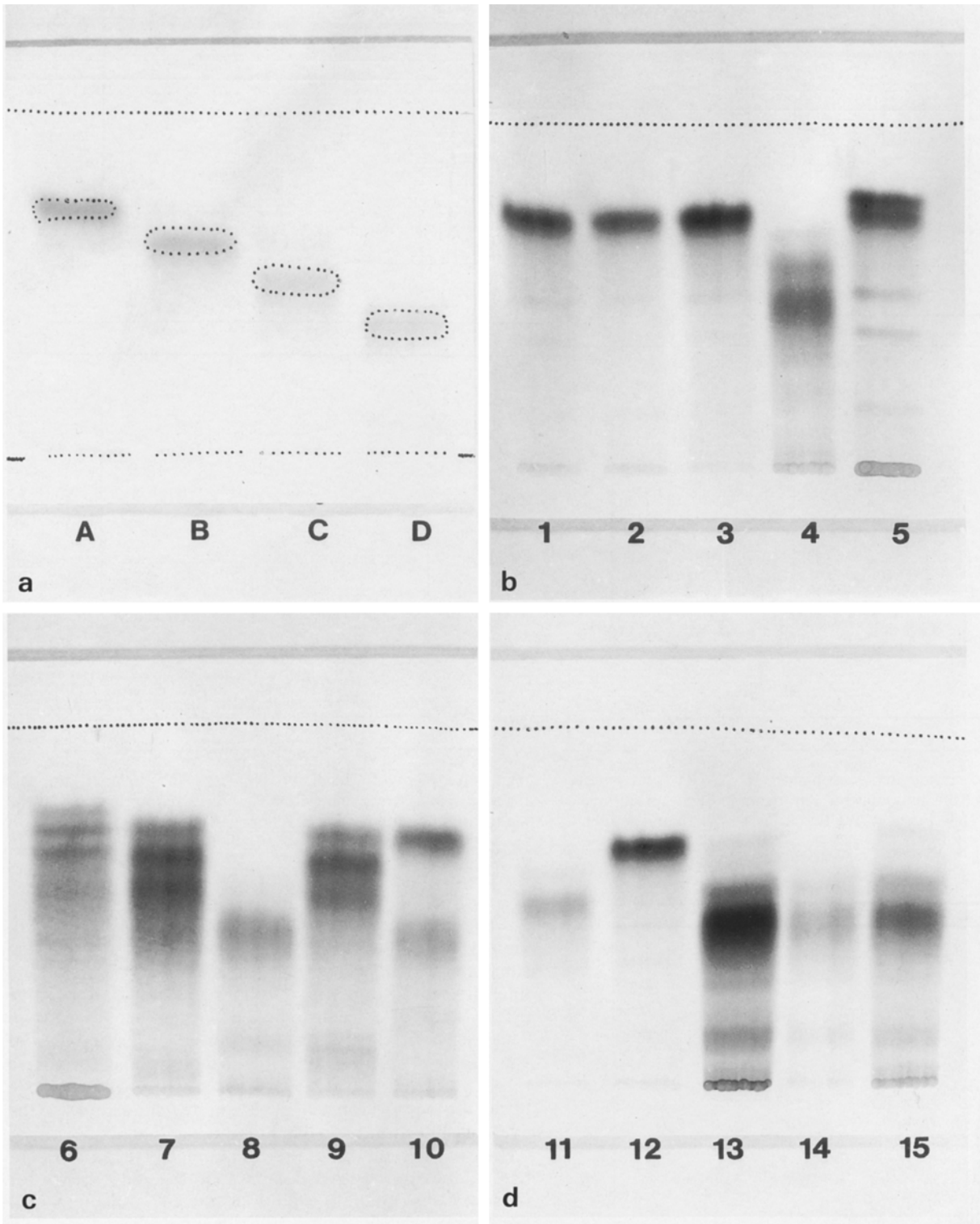

Fig. 8a–d. Reverse phase thin layer chromatography (RPTLC) of commercial and pure fuchsin dyes on Nano-SIL C_{18}-100 plates. Solvent phase: water/methanol/NH_4OH 25% = 30/20/10 ml. Dye no. 1–15 and *A–D* see Tables 1 and 2

mixture seems to be of utmost importance because we do not know how far the components of the dye mixture may contribute to the staining pattern obtained. Duijndam et al. (1973) and Duijndam and van Duijn (1975b) have discussed four different formulas for the Pararosaniline-DNA compound. It cannot be excluded that the methylated fuchsin analogues form different dye-DNA compounds. Therefore it seems reasonable to investigate the staining properties of the single dye in a highly purified state which will elucidate the question if all fuchsin analogues give staining results which are sufficiently comparable for reproducible and standardized staining in practice.

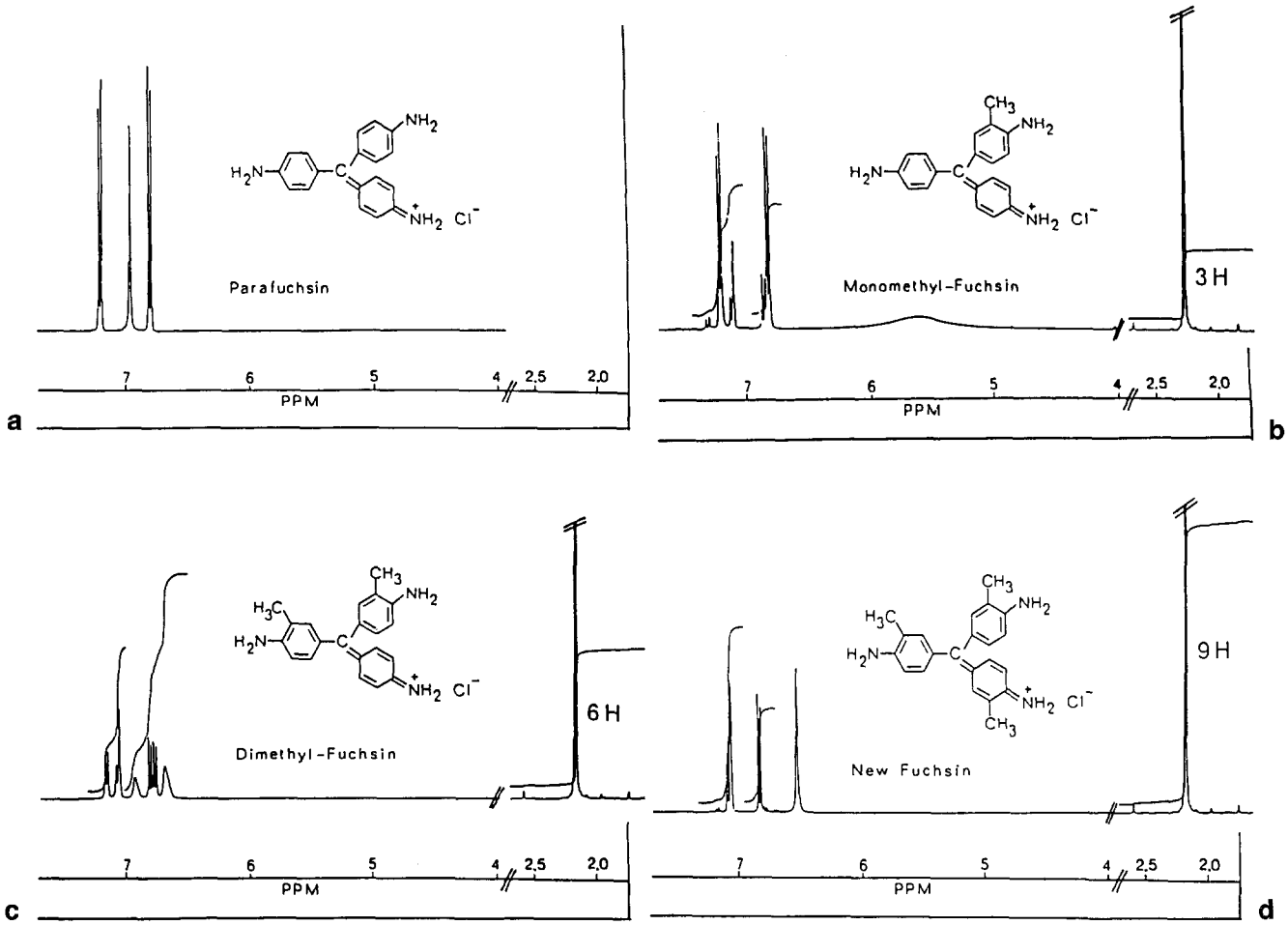

Fig. 9a–d. Nuclear magnetic resonance (^{1}H-NMR) spectrometry of the four fuchsin analogues. **a** Pararosaniline; **b** Rosaniline; **c** Magenta II; **d** New Fuchsin. We thank Dr. J. Wörth and T. Gehring, Department of Chemistry, University of Freiburg, for performing NMR-spectrometry. Dyes were solved in dimethylsulfoxide-d_6 and measured at a frequency of 400 MC

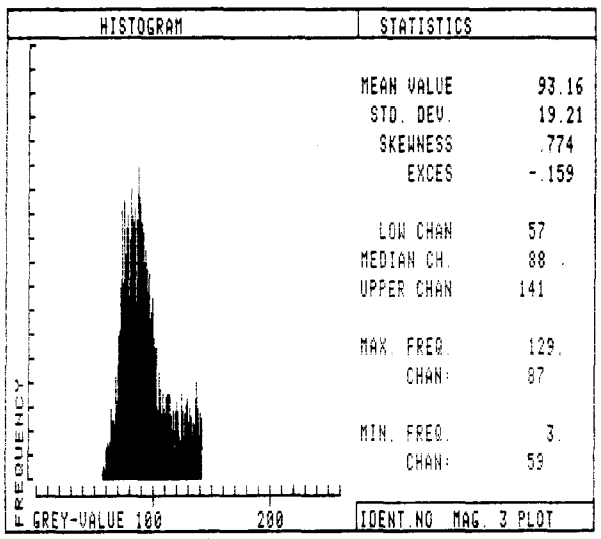

Fig. 10. Grey value histogram of tetraploid liver cell nucleus after Feulgen staining with Pararosaniline. Hydrolysis in 5 *M* HCl for 45 min at 22° C; dye concentration: 0.5 g/100 ml; staining time: 1 h at 22° C. IBAS 2000. Grey value "0" stands for "black", "255" represents "white"

The tables and figures show that pure fuchsin dyes obviously have more or less the same affinity towards the substrate. If the dye concentration is less than 0.1% staining intensity rapidly decreases – for all fuchsin dyes to the same extent. Thus 0.1% seems to be the "critical" concentration. This fairly agrees with results reported by Duijndam and van Duijn (1975b). Staining with commercial dyes gave rather variable absorption of model films and lymphocyte nuclei although – for nearly all commercial samples – the dye concentration probably was higher than the "critical 0.1%" in the staining solution. That means that a low content of the respective dye cannot be the only reason for the lower absorption of cell nuclei stained with commercial samples; other mechanisms must be involved.

We presume that contamination of the batch – e.g. with an acridine – leads to a competition between the fuchsin dye molecule and the contaminant for the DNA-binding sites. RPTLC revealed some colourless contaminants which showed a yellow fluorescence and the same Rf-value as 9-Aminoacridine.

Finally, the grey value histograms – as parameters of nuclear chromatin texture – were practically identical for the pure analogues.

From our own results we may draw the conclusion that the four dyes tested are equally suitable for the Feulgen reaction if they are used as pure dyes in a standard staining procedure.

However, that does not mean that differences among the four dyes do not exist. Ortmann et al. (1966), Mowry (1978), Mowry et al. (1980) and Lichtenstein and Nettleton (1980) reported that only Pararosaniline formed an aldehyde fuchsin whereas Rosaniline and Magenta II did not. This might be an indication that differences among the four dyes in their reactivity towards aldehyde groups do exist but are only found under special experimental conditions. Omission of charcoal treatment shows that different amounts of the four fuchsins are transformed to the leucoform: the four solutions differ in colour. The formation of the leucoform is not a prerequisite for the suitability of the dye in the Feulgen reaction (Kasten 1960; Böhm and Fukuda 1981), but we cannot exclude that differences in the reactivity towards sulphite imply differences in the staining characteristics. These results agree with Lodin et al. (1963) who found that different batches of basic fuchsin required different amounts of charcoal to decolorize them due to different dye content on the one hand and to different portions of the analogues on the other hand.

Longley (1952) reported that charcoal treatment of the Schiff reagent might diminish the amount of reactive leucoform with subsequent decrease of staining intensity; this certainly depends on the purity of the batches used: when commercial samples containing substantial amounts of acridines are treated with charcoal staining intensity usually increases because the acridine contaminant which competes for the aldehyde binding is removed (Yarbo et al. 1954; Gabler 1965; Müller 1966). In our own experiments charcoal treatment obviously left "enough" (more than the critical 0.1%) reactive fuchsin dye in the staining solution because there were no differences in IOD before and after charcoal treatment of pure dyes. Nevertheless charcoal treatment might be a critical step if commercial samples with a very low fuchsin content are used.

Conclusions

With respect of standardization of the Feulgen procedure we may draw the following conclusions:
Although all fuchsin analogues are obviously equally suitable for the FR under the experimental conditions chosen only one fuchsin analogue should be used in a standardized staining procedure for three reasons:
1) Mixtures of all four dyes differ in the position of the absorption maximum corresponding the varying portions of components; this is negligible for conventional visual microscopy but it might be the reason for photometric errors.
2) The analogues show different reactivity towards sulphite, and differences in dye-substrate affinity cannot be completely excluded under all experimental conditions.
3) The intensity of unspecific staining of unhydrolyzed cells is different for the fuchsins (and minimal for Pararosaniline) when the final sulphite rinse is incomplete or has been omitted.
4) Inconsistent results reported in the literature are probably due to the application of impure dye samples with unknown dye content and unknown dye composition.

A review of the literature describing Feulgen staining on DNA model films (Persijn and van Duijn 1961; Hardonk and van Duijn 1964a, b; van der Ploeg and Duijndam 1986) shows that our data sometimes differ from those found in other experimental studies; this is especially true for the staining kinetics and the dye-substrate affinity. To a certain extent this might be due to different chromatin compactness in cell nuclei or different DNA-concentrations in model films.

With regard to the staining results described above we prefer *Pararosaniline* for a standard staining procedure. Additionally from a chemist's point of view it seems much easier to synthesize a non-methylated dye in pure form than a dye with one or two methyl groups which is completely free from lower or higher methylated analogues. In this respect we do not agree with Teichmann et al. (1980) who recommended to use commercial batches containing a high proportion of Magenta II and to avoid Pararosaniline.

In a following study the use of pure fuchsin dyes in the PAS-reaction will be investigated.

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