Histochemical Journal **12**, 153–168 (1980)

A rapid cytofluorometric method for quantitative DNA determination on fixed smears

G. MAZZINI[1], P. GIORDANO[1], C. M. MONTECUCCO[2] and A. RICCARDI[2]

[1]*Centro di Studio per l'Istochimica del C.N.R. and Istituto di Anatomia Comparata dell'Università di Pavia, Italy*
[2]*Istituto di Patologia Medica I dell'Università di Pavia, Italy*

Received 27 June 1979

Summary

A study has been made of the possibility of using propidium iodide (PI) for the cytofluorometric determination of DNA in fixed smears. A preliminary spectrofluorometric study made it possible to define the optimal conditions for the excitation wavelength and the intensity of the emitted fluorescence. The stoichiometry and specificity of the DNA–PI bond were studied in different materials and for different fixation procedures. In order to check whether RNA might interfere, it was extracted enzymatically from test preparations selectively. The data from such treated sections were not significantly different from those obtained by means of the fluorescent Feulgen reaction (Pararosaniline-SO_2) on the same material. On the other hand, some of the advantages of the PI method are important: (a) the time required for making ready and staining the preparations is very short, and in any case is considerably shorter than the Feulgen method; (b) the high quantum yield of the DNA–PI complex induces very high fluorescence intensities, which can, therefore, be easily measured, even with low sensitivity instruments; (c) the spectral conditions are particularly favourable for excluding the inner filter effect from the measurement; (d) the photo-decomposition is considerably lower compared to that found in preparations stained by the conventional Feulgen method. The wide possibility of excitation (from the u.v. to the green), together with the limited extent of the emission band (which is mainly in the red) are also conditions that are particularly favourable for obtaining multi-parametric determinations simultaneously from the same cell.

Introduction

The quantitative determination of DNA in single isolated cells in smears is still an important problem in histochemistry, and also for many fields of application in biology. The Feulgen reaction is the method most widely used. Its fluorescence form (employing both conventional and Schiff-type reagents) (Ruch, 1966; Böhm &

0018-2214/80/020153-16$03.60/0

Sprenger, 1968; Prenna *et al.*, 1974a) has also introduced a considerable improvement compared with conventional light absorption methods. On the other hand, microdensitometric scanning techniques, while they may offer results analogous to the cytofluorometric ones, call for longer measuring times and more complex instruments.

The development and the application of flow microfluorometry, which makes possible very short measuring times on cell populations in suspension, although it is particularly valid in certain applications, has not taken the place of conventional cytofluorometry. The first flow methods were derived from conventional cytofluorometric methods (Van Dilla *et al.*, 1969; Trujillo & Van Dilla, 1972; Prenna *et al.*, 1976), and subsequently intercalating dyes made a decisive contribution to the amplification of staining methods of flow microfluorometry.

Following the studies of Le Pecq & Paoletti (1967) some authors used ethidium bromide for the quantitative determination of DNA (Dittrich & Göhde, 1969; Crissman & Steinkamp, 1976; Zante *et al.*, 1976; Anderson & Skagen, 1977) and frequently the results obtained were compared with those resulting from the use of 'non-intercalating' methods (Crissman *et al.*, 1975; Noeske, 1975; Severin, 1975; Crissman & Steinkamp, 1976). However, the phenanthridinic derivatives, ethidium bromide and propidium iodide (Crissman & Steinkamp, 1973; Krishan, 1975; Fried *et al.*, 1976), and mithramycin (Crissman & Tobey, 1974; Barlogie *et al.*, 1976) have come into normal use. Mithramycin is also used in conventional cytofluorometry (Johannisson & Thorell, 1977).

Ethidium bromide and PI are bound to DNA by means of two types of bond: primary bonds (intercalation), which induce an approximately 20-fold increase in the quantum yield of the fluorochrome; and secondary bonds, which are formed without any appreciable increase in the quantum yield (Le Pecq & Paoletti, 1967; Olmsted & Kearns, 1977). A similar behaviour in this respect has been established between DNA and chromatin by Angerer & Moudrianakis (1972) or to be more precise, at the chromatin level, two different affinities for ethidium bromide have been observed, this being probably evidence of the presence of many different primary bonds (Lawrence & Louis, 1974; Lawrence & Daune, 1976).

By exploiting the fluorescence characteristics of PI (especially the high quantum yield of the intercalated molecule), we studied the possibility of devising a quantitative cytofluorometric method by using it as a fluorochrome for determining DNA in fixed smears. The intrinsic ability of this fluorochrome to increase its quantum yield considerably only in the intercalated form, makes it particularly suitable for quantitative purposes.

In this paper, ways of using PI are defined through a series of experimental observations: (a) by determining the spectral excitation and emission characteristics for the selection of the most suitable spectral conditions for fluorescence excitation and measurement; (b) by trying out various fixation conditions and evaluating the

influence of RNA on the results of the measurements; (c) by evaluating the trend of the fluorescence intensity (FI) as a function of the concentration of dye, as well as the FI as a function of time, also at different concentrations; and lastly, (d) by determining whether photo-decomposition has any effect on the measurements.

The results were compared with those obtained from the same preparations subject to the conventional Feulgen fluorescence reaction. In addition, consideration was given to the possibility of conserving the preparations in different mounting media, in order to keep them for a different length of time. With an eye to further developments, a description is given of the versatility of the method for the simultaneous cytofluorometric determination of several components from the same cell.

Materials and methods

All the experiments were carried out on human leukocytes; smears of peripheral blood were prepared, both with and without enrichment. Peripheral blood and marrow from a patient suffering from leukemic lymphosarcoma was also used.

Fixation

Three different fixatives were employed: (a) 70% ethanol; (b) 4% formalin; and (c) 3 : 1 v/v ethanol–glacial acetic acid. For fixatives (a) and (b), fixation times of 30, 60, 90 and 120 min were tried, and for fixative (c) 15 and 30 min. All the fixatives were used cold (about 4° C).

RNAse treatment

After fixation, some preparations were treated with RNAse for 10–60 min at 37° C in order to extract their RNA content. Ribonucleases A type 1-A (from bovine pancreas) were purchased from Sigma (St. Louis, U.S.A.), Boehringer (Mannheim, West Germany) and Worthington Biochemical (Freehold, U.S.A.). They were used in concentrations ranging from 10 units/ml to 50 units/ml in an 0.1 M pH 7.4 phosphate buffer.

Staining

Propidium iodide was obtained from Calbiochem. A stock solution of 100 μg/ml in distilled water was prepared and stored in the dark at 4° C. This solution can be kept without undergoing alteration for a few weeks (Fried *et al.*, 1976). Solutions of different concentrations were obtained from 1 to 50 μg/ml, both in a phosphate buffer (0.1 M, pH 7.4) and in distilled water. Smears were stained by either immersing them directly in the dye solutions or by smearing a layer of dye solution on the slide.

The following methods of mounting were investigated for examining the stained preparations in the fluorescence microscope: (a) preparations were mounted directly in the dye solution; (b) washed and mounted in the buffer; (c) washed in the buffer and mounted in various media (distilled water; 95 : 5 or 50 : 50 v/v ethanol–water, glycerol and Carbowax 400); (d) washed in distilled water and mounted in a rigid medium (Gurr u.v. inert aqueous mounting medium). Preparations mounted in the liquid media were sealed with Fluormount (Gurr) to prevent evaporation of the solvent.

Spectral analysis

Excitation and emission spectra were obtained by means of a Leitz microspectrograph, with an oscillating mirror and photon-counting system described elsewhere (Cova *et al.*, 1974; Prenna *et al.*, 1974b). For excitation spectra, the light source was a xenon lamp (Osram XBO 75 W), followed by the Leitz prism monochromator, a TK 580 dichromatic mirror, and a K 610 barrier filter. For emission spectra, the exciting source was the same lamp, coupled with the following filters; heat-protecting KG 1 (2 mm), BG 38 (5 mm), interference filter 480 mm (13 nm-hbw), TK 495 dichromatic mirror, and K 530 barrier filter. For both measurements, a Leitz Fl 54X, NA 0.96 objective was used. For this analysis, use was made of normal enriched peripheral blood smears, fixed in 3 : 1 v/v ethanol–glacial acetic acid for 15 min, washed in running water for 10 min, and stained for 10 min in 25 μg/ml PI in the buffer, as previously described. Preparations were mounted in this staining solution.

For purposes of comparison, fluorescence spectra from smears subjected to a Feulgen reaction (Pararosaniline-SO_2) were also recorded under the same instrumental conditions.

Determination of fluorescence intensity as a function of (a) staining time, and (b) dye concentration

(a) The microspectrofluorometer equipped with a single proton counting system already described elsewhere was employed (Cova *et al.*, 1974; Prenna *et al.*, 1974b). Smears of normal peripheral blood, fixed as for 'spectral analysis', were used. The cells were identified and centred in the measuring diaphragm by phase-contrast optics and with a water-immersion objective (Leitz PV WE 80X NA 1.00). After the beginning of the measurement, the water was replaced by the staining solution. Readings were taken for different concentrations of PI (from 1 to 50 μg/ml), both in water and in the buffer. So that photo-decomposition (albeit low) should not change the trend of the curves, a low excitation intensity was used: an XBO 75 W lamp, a 480 nm (13 nm-hbw) interference filter combined with a Zeiss attenuation filter, with a transmission of $T = 0.12$

(b) Using a Leitz MPV 1 microphotometer, equipped with the same optical accessories as for experiment (a), cytofluorometric analyses were made on a series of slides stained with different concentrations of PI in an 0.1 M pH 7.4 phosphate buffer. The analyses were performed directly in the dye after 30 min of staining. This time ensures that the fluorescence value measured is the maximum attainable one, even at lower concentrations (1 μg/ml).

Photodecomposition

By recording the fluorescence intensity emitted by the cell (Prenna *et al.*, 1974b) the photodecomposition trend was evaluated as a function of the mounting medium. Green light selected with a 546 nm (12 nm-hbw) interference filter was used as continuous excitation.

Cytofluorometric measurements

Various preparations were employed for arriving at the best conditions for staining and mounting, taking into account, *inter alia*, different fixation methods and the need, or otherwise, of extracting RNA beforehand. In order to compare the results of the measurements, some smears (from both normal and pathological blood) were subjected to a standard Feulgen fluorescence reaction as described elsewhere (Prenna *et al.*, 1974a). The measurements were performed with a Leitz MPV 1 equipped with a stabilized XBO 150 W xenon lamp and a vertical illuminator for fluorescence excitation with incident light according to Ploem (1967). A 546 nm (12 nm-hbw) interference filter was coupled to the lamp, and a TK 580 dichromatic

mirror was set in the epilluminator. The barrier filters used were: K 610 for smears stained with PI, and RG 630 for those stained by the Feulgen reaction.

The electronic equipment was as follows: the EMI 9558 QA photomultiplier, fitted with an electrostatic shield for noise reduction, was power-supplied by an NSHM BN 60 Knott Electronik and the output current was measured with a 150 B Keithley microvoltammeter.

Results

FIXATION

Of the various fixatives tried, the best results for fluorescence observations and cytofluorometric data were obtained using cold 3 : 1 v/v ethanol–glacial acetic acid for 15 min. The other two fixatives tried did not provide reliable results.

In bone-marrow smears, a high degree of background (that is, intercell) fluorescence is sometimes apparent. This not only varies from one preparation to another, but is greater after formalin fixation than after ethanol fixation, and is virtually negligible in smears fixed with ethanol–glacial acetic acid.

RNASE TREATMENT

Sufficient fluorescence was observed in preparations not subjected to RNAse digestion to demonstrate the need for extractions of RNA in order to obtain a correct quantitative determination of the DNA. The best results for extracting RNA were obtained with Worthington RNAse; the incubation time and optimum concentration varied between smears of marrow and smears of peripheral blood, and also differed with different pathological situations.

Good results were obtained on smears of normal peripheral blood with RNAse concentration of 25 μg/ml for 10 min. For marrow smears, it is necessary to increase the concentration and reaction time up to 50 μg/ml and 30 min respectively. Fig. 1 shows two preparations so treated. The first (a) is of enriched blood from a normal subject and the second (b) is from a patient with leukemic lymphosarcoma. Both were stained with 25 μg/ml PI in 0.1 M pH 7.4 phosphate buffer for 10 min, washed rapidly in distilled water, and mounted in Gurr's u.v. inert mounting medium.

In Figs. 1c and d it is possible to see, in some of the cellular elements, the difference with and without enzymatic extraction of the RNA.

STAINING

There are no substantial differences in the results of staining with PI concentrations of 20 μg to over 50 μg/ml. At these concentrations and with long staining times, the polymorphonuclear cells (unlike mononuclear cells) tend to lose their morphology and to break up when they have been in the dye for more than an hour. This fact is particularly accentuated by alcoholic fixative, being more evident in passing from distilled water to the 0.1 M buffer, and even more in 0.2 M buffer. At lower concentrations (10 μg/ml), this phenomenon was only observed occasionally and after very long staining times.

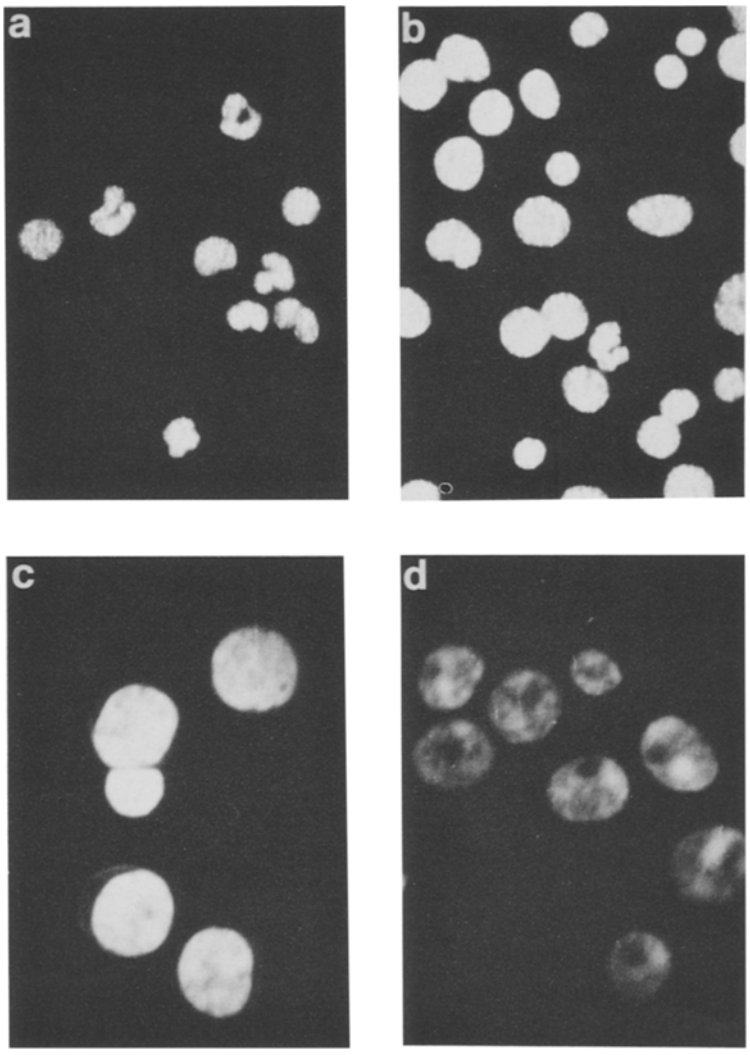

Fig. 1. Leukocytes from (a) enriched normal and (b) leukemic lymphosarcoma peripheral blood. Smears were stained, after RNAse treatment, with 25 μg/ml PI in 0.1 M pH 7.4 phosphate buffer, washed in distilled water and mounted in u.v. inert aqueous mounting medium. For both (a) and (b), an Fl 54 × NA 0.96 objective was used. (c) and (d) are at higher magnifications (Fl 95 × NA 1.10–1.30), (c) is the same preparation as in (b) but without RNAse treatment. The disappearance of the nuclear RNA in some cells in (d) is evident.

At high dye concentrations, there is a clear tendency, even though quantitatively slight, for the fluorescence of the mononuclear cells to increase, as compared with the polymorphonuclear cells. No substantial differences in fluorescence staining were observed when the pH of the dye solution was changed from 6.5 through to 8.0. Changing the dye vehicle from distilled water to 0.2 M buffer (with staining times of

up to 30 min) also had no effect. Smears washed briefly in distilled water and mounted in Gurr's medium (method d) gave the best results.

Washing in distilled water has to be short (a few seconds), since removing the free dye tends to reduce the concentration of intercalated dye. The results of the cytofluorometric measurements showed that the decrease in FI following the washing is in any case stoichiometric to the quantity of DNA present in the cell. On the other hand, it is not possible to mount preparations in glycerol or in a rigid medium without first eliminating the salts from the buffer solution; the latter tend subsequently to become crystallized, thus ruining the cells and considerably increasing the background.

SPECTRAL ANALYSIS

Fig. 2 shows the excitation and emission spectra obtained by staining with PI and with the conventional Feulgen fluorescence reaction. With PI, no significant

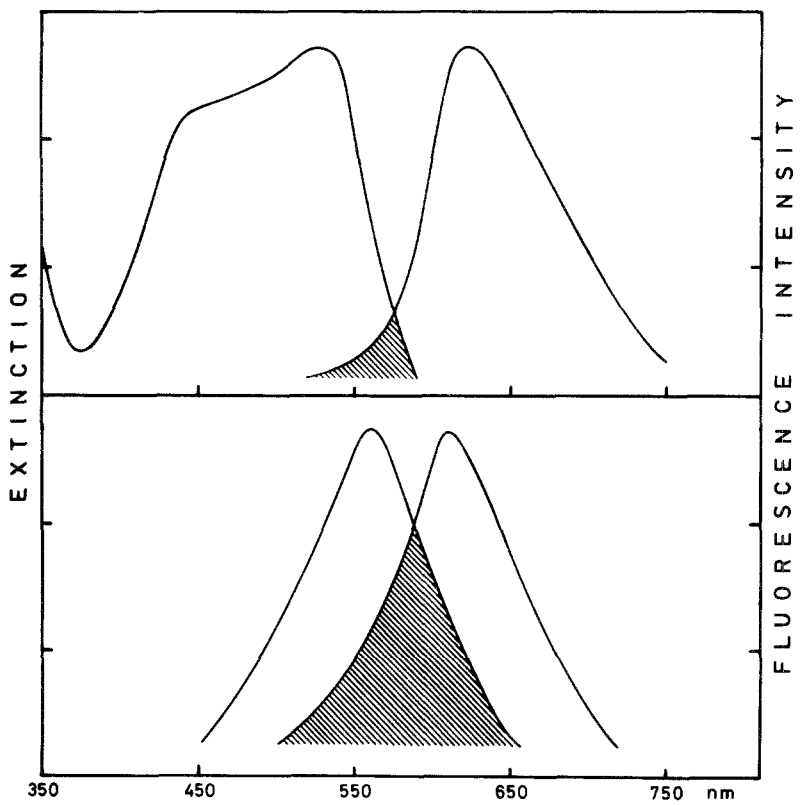

Fig. 2. Schematic representation of excitation (left) and emission (right) spectra of propidium iodide (above), and conventional Feulgen stained cell (below). The hatched area represents the overlapping zone in both cases.

variations in the spectral shape were detected, even at the highest dye concentrations. The area of overlap between excitation and emission spectra is very small; consequently, the inner filter effect in this case will be negligible. This fact makes it possible to perform cytofluorometric measurements on a fairly large area of the emission spectrum; in fact, with a K 610 barrier filter, it is already possible to exclude most of the inner filter effect incidence from the measurements.

The excitation range extends from blue to green, with a considerable excitation band in the u.v. around 330 nm (not shown in the figure). Due to the high quantum yield of the DNA–PI intercalation complex, high fluorescence intensities are obtained, even with low dye concentrations. On the other hand, in the visible range, high extinction values are never obtained, so that excitation in the green becomes optimal.

The fluorescence emission is orange-red, with an extension that prevails beyond 600 nm.

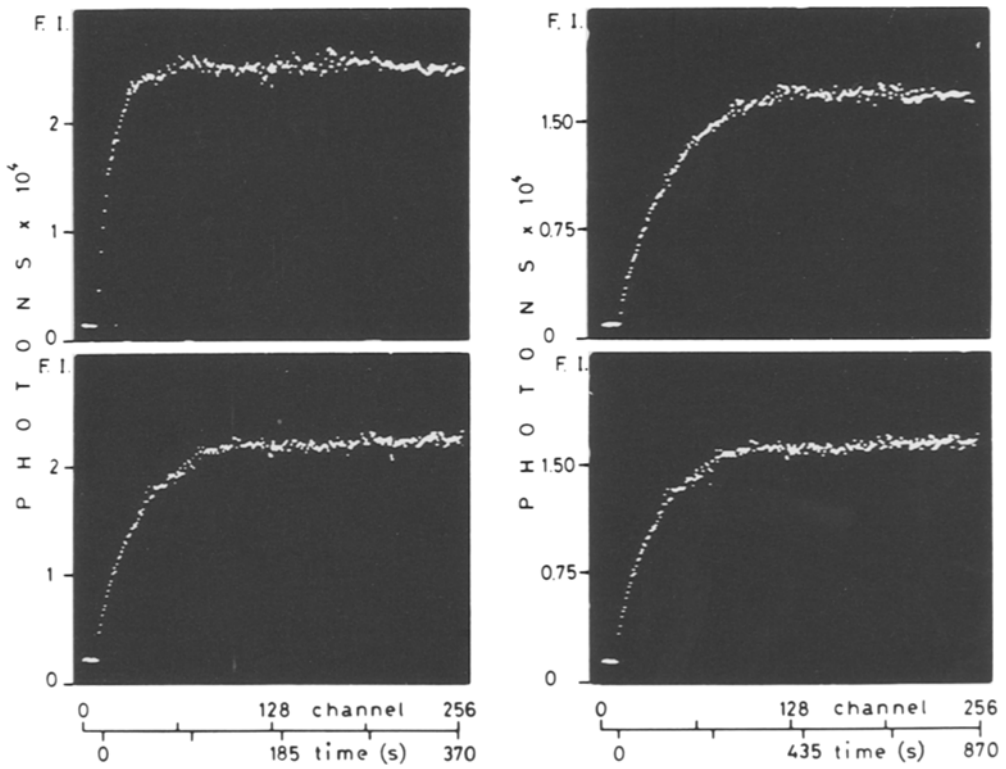

Fig. 3. Relation of fluorescence intensity to time. Fluorescence intensity was automatically recorded by a multi-channel analyser. Normal peripheral leukocytes were used. Dye concentration was: (a) 50 μg/ml (above), 10 μg/ml (below); (b) 2.5 μg/ml (above), and 1 μg/ml (below). The different time scales for (a) and (b) should be noted. After 10 min, maximum fluorescence intensity was reached for all concentrations.

RELATION OF FLUORESCENCE INTENSITY TO TIME AND DYE CONCENTRATION

(a) Time

Fig. 3 shows graphs representing fluorescence intensity (FI) as a function of staining time. The speed with which the dye is intercalated in the double helix is extremely rapid at the beginning, saturation afterwards being reached more and more slowly. This trend varies, and depends on dye concentration. It should be noted that in Fig. 3 there is a difference in the time scale between the two left-hand curves (50 and 10 μg/ml) and the right-hand curves (2.5 and 1 μg/ml). These curves indicate that a staining time of 10 min is sufficient to reach saturation, even with a dye concentration equal to, or less than, 10 μg/ml. The incidence of photodecomposition in the trend of these curves was kept to a minimum by reducing the intensity of the incident beam, operating with discontinuous excitation and with sufficiently low measuring times (0.1 s/channel), while taking into account the statistics of the measurements.

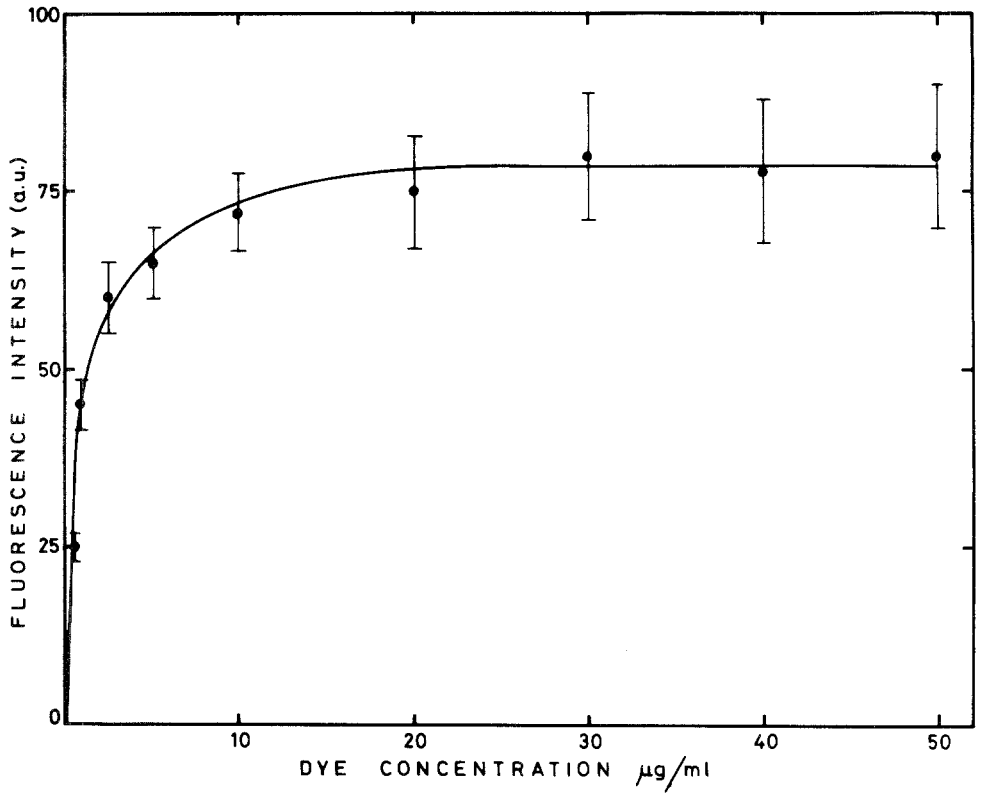

Fig. 4. Relation of fluorescence intensity (in arbitary units) to dye concentration. Cytofluorometric measurements were performed, using a PV WE 80 × NA 1.00 objective, directly in the dye solution. Each point represents the average of 50 determinations on human leukocytes. The SD of each measuring group is also shown.

(b) Dye concentration

Cytofluorometric analyses were performed on smears of normal peripheral blood without RNAse treatment. The results are given in Fig. 4. Each point represents an average of 50 determinations of different leukocytes (both mono- and polymorphonuclear cells). The standard deviation of each group of measurements is also shown.

As has already been noted earlier, there are no substantial FI variations for dye concentrations between 20 and 50 μg/ml and over; variations are, however, clearly noticeable at very low dye concentrations. The best results (in terms of data dispersion) were obtained with a dye concentration of 10 μg/ml; at this concentration the lowest SD (%) was recorded. Since this concentration also yielded good results on mounted preparations (see below), this seems the best one to use.

PHOTODECOMPOSITION

Fig. 5 shows the photodecomposition curves recorded in two preparations stained with PI. The upper curve was obtained from a slide mounted in a rigid mounting medium while the one in the centre refers to measurements performed directly in the staining solution. Below, for purposes of comparison, is a curve obtained from a preparation subjected to fluorescent Feulgen reaction and measured with the same instrumental conditions. Smears mounted in a rigid medium were found to be more stable towards radiation compared with those mounted in the staining solution.

CYTOFLUOROMETRIC MEASUREMENTS

Consistent results were obtained from preparations stained and mounted in the same dye solution. This can only be done when the measurements can be carried out in a short time.

Stained smears, washed and mounted in 95% ethanol were not measured, because they had a marked background fluorescence. For the same reason, measurements performed on preparations mounted in 50% v/v ethanol–water were found to be unacceptable. In these preparations there is a considerable increase in nuclear fluorescence (up to 20% more, compared to mounting in a buffer or in water) though this is accompanied by a comparable fluorescence enhancement in the free dye and in the dye that, in any case, is not intercalated.

Cytofluorometric analyses performed on preparations mounted in glycerol gave satisfactory results; however, the preparations required a period of stabilization (24 h) before the measurements could be performed. In the bone marrow smears, some background fluorescence was sometimes noted, but this never compromised the measurement of the cells. Preparations mounted in Carbowax 400 were found to be unsuitable not only because the background was high, but more importantly because the mountant induced considerable swelling in the cellular elements, especially in the polymorphonuclear cells.

Even though certain mounting media increase to a greater (ethanol) or lesser

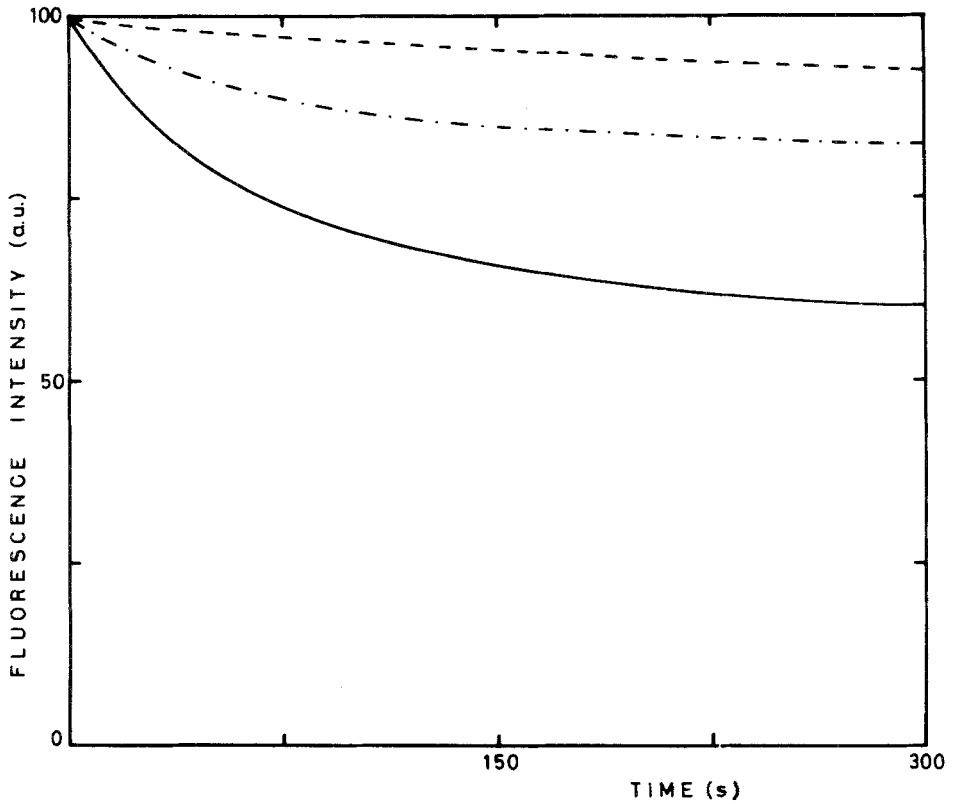

Fig. 5. Photodecomposition curve. The rate of photodecomposition (fluorescence intensity in arbitrary units) in leukocytes stained with PI and recorded directly in staining solution (- · · · -) is lower compared to that recorded in the same cells stained with a conventional Feulgen fluorescence reaction (Pararosaniline-SO_2) (——). The photodecomposition is still lower if, after staining with PI, the cells are mounted in u.v. inert aqueous mounting medium (- - - -).

(glycerol and Carbowax) extent than the emission of the intercalated dye, the highest fluorescence intensities are obtained from preparation measured directly in the dye. This is due to the fact that a subsequent wash, however short, by varying the equilibrium of the dye, diminishes the quantity of intercalated dye. In terms of quantitative analysis, it is of fundamental importance to check whether the stoichiometry is maintained even after washing in water. Figs. 6 and 7 show DNA distribution histograms of both normal and leukemic peripheral blood stained with PI and the Feulgen reaction. In the case, the leukemic leukocytes were mostly represented both by small (2c) and by large (4c) immature cells. As can be seen, the results obtained with the two methods can clearly be made to overlap. The preparations stained with PI were washed in distilled water before being mounted.

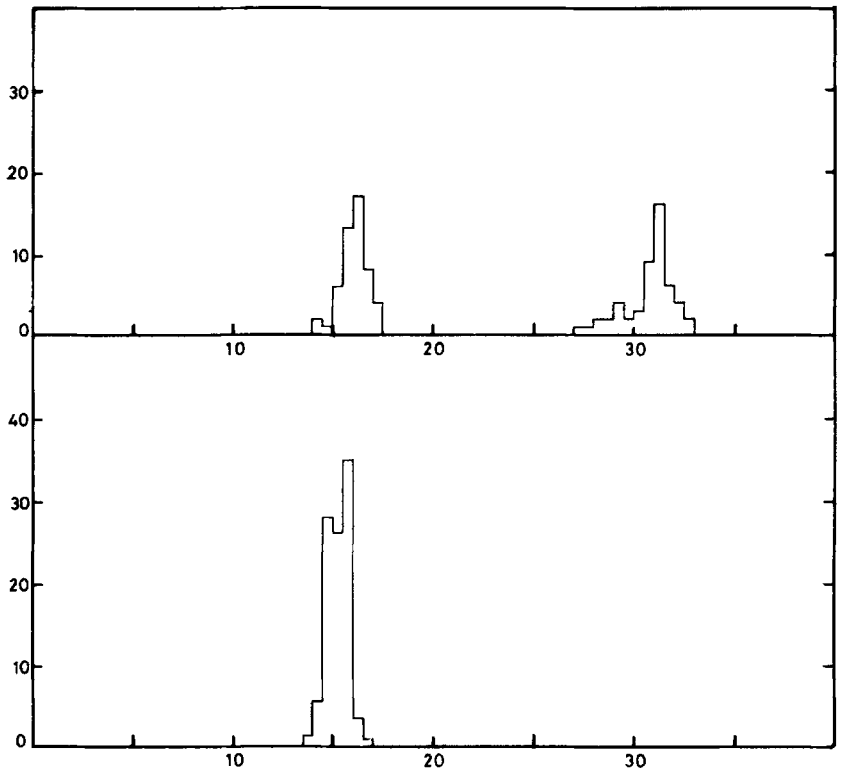

Fig. 6. DNA distribution obtained by cytofluorometric measurement of normal peripheral blood (below) and, in one case of leukemic lymphosarcoma (above), stained with 10 μg/ml PI in an 0.1 M pH 7.4 phosphate buffer for 10 min. The enriched smears were fixed in 3 : 1 v/v ethanol–acetic acid and subjected to enzymatic extraction with RNAse. After staining, preparations were washed in distilled water and mounted in u.v. inert aqueous mounting medium. Mean fluorescence values and SD are also given for 2c and 4c peaks: mean 15.9, SD 4.15% (2c) and mean 30.86 SD 3.4% (4c) above; mean 15.3, SD 3.7% (2c) below.

Discussion

The precision of a cytofluorometric method depends upon the stoichiometry with which the fluorochrome is bound to the macromolecular structure to be determined. In its turn, the fluorescence intensity at each point of the element to be measured is proportional to the fluorochrome concentration or, depending on the wavelength of the excitation used, to the extinction.

Theoretically, therefore, it is advisable to saturate the primary bond in order to be able to effect a correct stoichiometric determination. Experimentally, on the other hand, we found that the best results are obtained with lower concentrations of dye. By using a 10 μg/ml staining solution, we obtained the best SD (%) on a heterogenous population of normal leucocytes (Fig. 4), and a satisfactory 2c/4c ratio

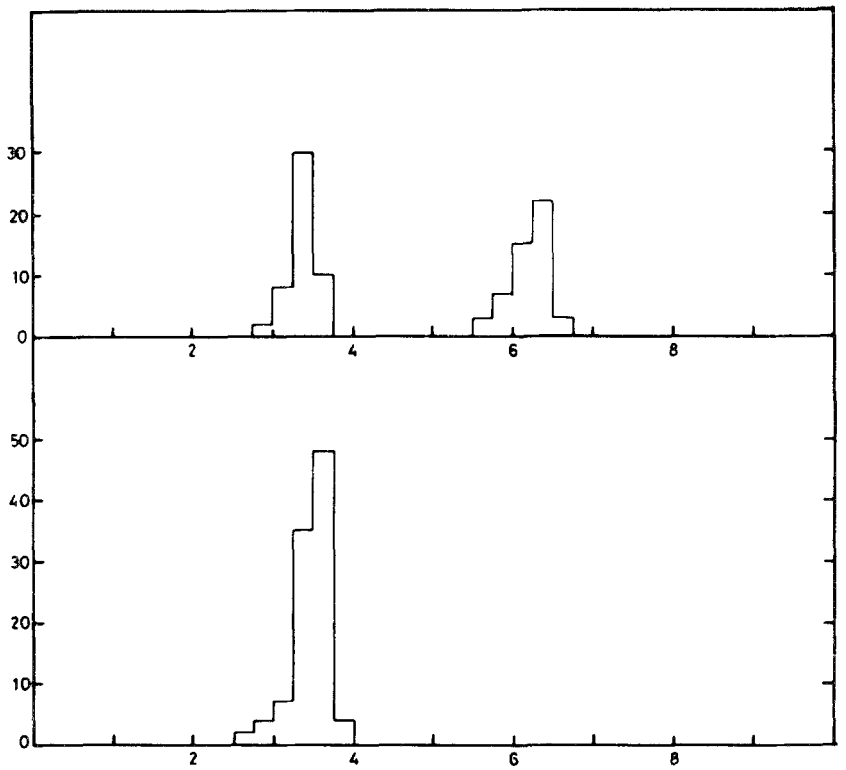

Fig. 7. DNA distributions obtained by cytofluometric measurements of normal peripheral blood (below), and, in one case of leukemic lymphosarcoma (above), stained with conventional Feulgen reaction (Pararosaniline-SO_2). Mean fluorescence values and SD are also given for 2c and 4c peaks: mean 3.36, SD 4.76% (2c) and mean 6.2, SD 3.7% (4c) above; mean 3.45, SD 6.4% (2c) below.

in the case considered in Fig. 6. With a high dye concentration (20–50 μg/ml), there is an obvious even though slight (10–15%) difference in FI between mononuclear and polymorphonuclear cells, the latter having a consistently lower value. Even if this discrepancy does not disappear completely, it is nevertheless insignificant at a concentration of 10 μg/ml.

These results agree with the findings of Vindeløw (1977) for the same experimental conditions (albeit in FMF). This author assumes the existence of a constant number of high-affinity bonds and of a variable number of low-affinity bonds in the same cell cycle phase. Extending the concept to morphologically different elements explains the anomaly of the results for high dye concentrations described above.

At all the concentrations tested, the extinction of the nuclei of the smaller cells (small lymphocytes) was very low. Operating at 546 nm and at the optimal

concentration already mentioned, all the cells have an absorbance lower than 0.1. Unlike what happens with the Feulgen method (Prenna *et al.*, 1974a) there are not, therefore, any particular problems associated with the FI-extinction relationship.

As regards the interference of the RNA, the only component able to interfere is the double-helix one (Bittman, 1969). Operating on fixed cell smears – and not on bare nuclei, as in most of the flow microfluorometry methods – we found extraction with RNAse to be necessary. This is above all called for in smears of neoplastic material, in which the presence of double-helix RNA is more probable (Patnaik & Taylor, 1973; Torelli *et al.*, 1975; Mansson & Pero, 1978). On the contrary, some authors consider that enzymatic extraction is not necessary, inasmuch as the interfering RNA may be very slight, or even resist enzymatic treatment, or else be in a quantity proportional to the cellular DNA (Fried *et al.*, 1976).

Conclusions

Since the results of the cytofluorometric measurements agree with those obtained by the Feulgen method on the same materials, consideration may be given to some advantages.

The time required for setting up the preparations is very short: by mounting the slides in the dye, the measurements can be begun in less than an hour. A few more minutes suffice to mount the preparations in a rigid medium so that they can be preserved for a long time before measuring. We encountered no significant variations in the cellular FI of preparations mounted in a rigid medium for three months.

The fluorescence intensity of the individual cells is always high and, therefore, it can be measured easily with sufficient precision, without particularly sophisticated instruments. On the other hand, since the inner filter effect is very slight, most of the emission can be sent to the photomultiplier without adversely affecting the precision of the results. Small cells with highly compacted chromatin also show low extinction at peak absorption (in the visible); the cytofluorometric measurements can, therefore, be performed under conditions of the maximum fluorescence obtainable.

The photodecomposition of the DNA–PI complex is slight, and in preparations mounted in a rigid medium is certainly negligible, even with a high excitation intensity and measuring times of a few seconds.

The specific PI spectral situation offers the prospect of being able to perform multiple fluorochromization on the same cell. In flow microfluorometry, a number of multi-parametric methods have already been devised. These make it possible to measure several components of the same cell simultaneously (Crissman & Steinkamp, 1973, 1976; Göhde *et al.*, 1976). Similarly, we are looking into the possibility of combining this method with that for determining acid phosphatase activity in fluorescence (Prenna *et al.*, 1977) on the same preparation. It will thus be possible to correlate two cellular components by measuring them one after the other on the same cell, without having to resort to laborious mapping methods.

Acknowledgements

This work was supported by C.N.R. (the Italian National Research Council). We thank also Dr G. Starace and Dr G. Badaracco for helpful discussion and Mr G. Michelazzo for aid to the photographic section.

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