APPARENT ANOMALIES IN NUCLEAR FEULGEN-DNA CONTENTS

Role of Systematic Microdensitometric Errors

K. S. BEDI and D. J. GOLDSTEIN

From the Department of Human Biology and Anatomy, University of Sheffield, Sheffield S10 2TN, England

ABSTRACT

The Feulgen-DNA contents of human leukocytes, sperm, and oral squames were investigated by scanning and integrating microdensitometry, both with and without correction for residual distribution error and glare.

Maximally stained sperm had absorbances which at λ_{max} exceeded the measuring range of the Vickers M86 microdensitometer; this potential source of error could be avoided either by using shorter hydrolysis times or by measuring at an off-peak wavelength.

Small but statistically significant apparent differences between leukocyte types were found in uncorrected but not fully corrected measurements, and some apparent differences disappeared when only one of the residual instrumental errors was eliminated. In uncorrected measurements, the apparent Feulgen-DNA content of maximally stained polymorphs measured at λ_{max} was significantly lower than that of squames, while in all experimental series uncorrected measurements showed apparent diploid:haploid ratios significantly greater than two. In fully corrected measurements no significant differences were found between leukocytes and squames, and in four independent estimations the lowest diploid:haploid ratio found was 1.99 ± 0.05, and the highest 2.03 ± 0.05.

Discrepancies found in uncorrected measurements could be correlated with the morphology of the nuclei concerned. Glare particularly affected measurements of relatively compact nuclei such as those of sperm, polymorphs and lymphocytes, while residual distribution error was especially marked with nuclei having a high perimeter:area ratio (e.g. sperm and polymorphs). Uncorrected instrumental errors, especially residual distribution error and glare, probably account for at least some of the previously reported apparent differences between the Feulgen-DNA contents of different cell types.

On the basis of our experimental evidence, and a consideration of the published work of others, it appears that within the rather narrow limits of random experimental error there seems little or no reason to postulate either genuine differences in the amounts of DNA present in the cells studied, or nonstoichiometry of a correctly performed Feulgen reaction.

According to a hypothesis formulated by Boivin et al. (10) and independently by Mirsky and Ris (51), nonreplicating nuclei of a given higher organism contain a constant amount of DNA per haploid set of chromosomes. This "constancy hypothesis," together with the independent but complementary concept that nuclear DNA is on the whole metabolically stable, has been accepted as valid by most workers, at any rate as far as mammals are concerned. Thus, tissue-specific amplification of genes has not been demonstrated in mammals (see e.g. reference 34), and claims that a proportion of the DNA in certain organs is labile or "metabolic" (60–62, 70, 81) have been contested (e.g. references 11, 16, 43).

In nonmammalian species, on the other hand, several exceptions to nuclear DNA constancy are well documented. Examples include the shedding of nuclear DNA in *Parascaris* (66), the variable B chromosomes of plants and certain insects (33, 67), selective changes in the DNA content of protozoan macronuclei (63), extra formation of DNA in certain puffs and under-replication of heterochromatin in dipteran polytene chromosomes (39, 71), specific amplification of certain chromosome regions in sciarid insects (58), under-replication of specific fractions of the genome in some locust tissues (18), and the amplification of ribosomal genes in the oocytes of fish, mollusks, worms, insects, and amphibians (19, 34, 44, 45). "Magnification" of ribosomal DNA, occurring in both somatic and germ cells of male *Drosophila*, may be a mechanism for regulating the extent of ribosomal gene redundancy (69, 79), and findings somewhat similar to those in *Drosophila* have been reported for the toad *Xenopus* (2).

It may be that these nonmammalian exceptions to nuclear DNA constancy are in some sense special cases, of only limited biological significance. Nevertheless, in view of the theoretical importance of the concept of DNA constancy, and the fact that it features prominently in current views regarding the control of metazoan cell differentiation and cell activity, it may be premature to regard the controversy as closed. Periodical reappraisal of the relevant evidence therefore appears prudent. In fact, not all the evidence adduced in support of the constancy hypothesis appears on close scrutiny to be altogether convincing. Thus, although Mirsky and Ris (51) stated that in a number of nonmammalian vertebrates the quantity of DNA "... per sperm cell is one half that found in a somatic cell ...," their biochemical data showed discrepancies of up to 8% between observed and expected values for the DNA content per somatic nucleus. Their original paper also reported a number of cattle somatic tissues to contain between 2.2 and 3.0 times as much DNA per nucleus as do sperm.

Variations within a cell population are not readily demonstrated by in vitro biochemical methods. Microdensitometric measurement of individual nuclei, such as the studies of Swift (77) on Feulgen-stained material, are perhaps better evidence for or against DNA constancy. Swift's careful work, the results of which were strongly in favour of DNA constancy, were performed using "plug" (nonscanning) microdensitometry, which is known to be liable to systematic errors. Although more sophisticated microdensitometric procedures have been used by some subsequent workers, the extensive literature contains a number of disturbing discrepancies. A particularly controversial topic concerns the Feulgen-DNA content of different mammalian leukocyte types, which are convenient and popular test objects for microdensitometry. Using a scanning microdensitometer, Atkin and Richards (3) found the apparent Feulgen-DNA content of human polymorphonuclear leukocytes, lymphocytes, plasma cells, and fibroblasts to be about 10% lower than that of normal uterine and cervical epithelial cells. Differences in the apparent Feulgen-DNA contents between different leukocyte types, or between leukocytes on the one hand and other cell types on the other, have also been reported by other workers (12, 22, 23, 31, 32, 36, 47, 49) but have not been confirmed by all investigators (see Table I). The ratio of the Feulgen-DNA contents of diploid and haploid cells has also been investigated microdensitometrically by several groups of workers, with somewhat conflicting results (Table II).

Relatively small, but statistically highly significant apparent deviations from Feulgen-DNA constancy are thus fairly common in microdensitometric studies. Instrumental factors which might result in systematic microdensitometric errors have been considered in detail by a number of workers, but the majority attribute apparent Feulgen-DNA anomalies to nonstoichiometry of the Feulgen reaction. Some other investigators continue to maintain the existence of genuine biological variation in nuclear DNA content. However, only relatively recently, apparatus and procedures have become available which permit the correction of two important sources of error in scanning microdensi-

TABLE I

Differences in Apparent Feulgen-DNA Contents between Different Leukocyte Types

Author(s)	Measuring technique	Preparation (smears unless stated)	Cells	Differences in Feulgen-DNA content and comments
Atkin and Richards (3)	Scanning	Methanol freeze substitution	Human leukocytes, epithelial cells from cervix and uterus	Epithelial cells have 10% more than leukocytes
Stich, Florian, and Emson (74)	Two-wavelength method	Formalin; sections	Normal human lymphocytes, polymorphs and epithelial cells	No differences found from expected values
Hale (32)	Scanning	Freeze-substitution in butanol or ethanol	Human leukocytes, cells from liver, kidney and stomach epithelia	10% less in leukocytes than other cell types
Garcia (20, 21)	Two-wavelength method	Methanol-formalin-acetic acid (85:10:5)	Human and rabbit leukocytes	Same mean values for different types of leukocyte
Garcia (22)	Two-wavelength method	Methanol-formalin-acetic acid (85:10:5)	Rabbit granulocytes, mononuclears and metamyelocytes	Metamyelocytes about 10% more than granulocytes, which in turn about 10% more than mononuclears
Garcia and Iorio (26)	Scanning One-wavelength 2-area Two-wavelength method	Methanol-formalin (9:1)	Rat leukocytes	Monocytes ~ 16% > small lymphocytes Monocytes ~ 32% > small lymphocytes Monocytes ~ 33% > small lymphocytes
Garcia (23)	Scanning	Methanol-formalin (9:1)	Rat leukocytes	Monocytes ~ 23% > small lymphocytes in the "mean of means"
den Tonkelaar and van Duijn (13–15)	Photographic elution	Methanol-formaldehyde	Rat leukocytes, liver and kidney cells	No differences found; condenser NA 0.3–0.4; Schott 546 nm filter
Deitch et al. (12)	Two-wavelength and plug method	Bovin's; sections	Human lymphocytes and endometrial cells	Small lymphocytes contained 10% less than other diploid cells; extraction of acid-soluble proteins failed to increase the amount
Böhm et al. (9)	Scanning	Methanol-formalin-acetic acid	Guinea pig lymphocytes and granulocytes	No significant differences found (cut off at 0.75 OD)
Noeske (55)	Multiple plug readings per nucleus	Ethanol	Granulocytopoietic and erythrocytopoietic cells	Mature cells contained 10–21% less than other diploid cells; extraction of acid-soluble proteins eliminated differences
Mayall (47) Mayall and Mendelsohn (49)	Scanning	Ethanol at −70°C	Human leukocytes	Monocytes contained 4% more than lymphocytes and 6% more than polymorphs
Gottlieb-Rosenkrantz and O'Brien (31)	Two-wavelength method	Neutral buffered formalin	Human lymphocytes and granulocytes	Significant differences between granulocytes and lymphocytes regardless of hydrolysis time
Fontaine and Swartz (17)	Scanning	Methanol-colloidin	Human leukocytes	Significant fluctuations in amounts in all leukocytes at various times of the day
James (36)	Scanning	Methanol-formalin-acetic acid	Human lymphocytes	Feulgen-DNA content inverse function of nuclear area

tometry, namely glare (28) and residual distribution error (29). It seemed desirable to apply these improved microdensitometric methods in a re-investigation of the question of Feulgen-DNA constancy. The present article reports work on leukocytes, sperm, and oral squames which suggests that systematic instrumental errors may have been underestimated by previous workers, and probably account for at least some of the reported apparent exceptions to Feulgen-DNA constancy. Preliminary reports of some of this work have appeared (4-6).

MATERIALS AND METHODS

Smears of peripheral blood from two healthy men were air-dried, fixed for 1 h at room temperature in methanol-formalin-acetic acid 85:10:5 (9), washed in running tap water for 60 min, hydrolyzed in 5 N HCl at 25°C in an agitated water-bath for up to 200 min, rinsed in distilled water, stained for 40 min in Schiff's reagent, rinsed in distilled water, dehydrated in a series of ethyl alcohols, and mounted via xylene in Polymount (Stayne Laboratories Ltd.). This mounting medium was excellently matched in refractive index to unstained cytoplasm and nuclei of the cells studied, as demonstrated by the almost complete invisibility of the mounted cells when examined by bright field, phase-contrast or reflectance microscopy. In a given experiment, slides were put into the hydrolysis bath in a sequence permitting all slides to be removed and stained simultaneously, irrespective of the duration of hydrolysis. The Schiff reagent was made by the de Tomasi method (59) with Basic Fuchsin supplied by G. T. Gurr Ltd., and was used 1 wk after preparation. Stained slides were stored in the dark to minimize fading (37, 54, 76), and measurements were in general carried out within a few days of staining.

To compare the Feulgen-DNA contents of human leukocytes, oral squames and sperm, cells from a given donor were smeared and air-dried on different parts of a single microscope slide. Before smearing, freshly ejaculated semen was diluted with Eagle's tissue culture medium (Wellcome Reagents Ltd.). Fixation and staining were carried out as described above. A similar experiment was performed using rabbit blood and a suspension of epididymal sperm from the same animal.

In Feulgen-stained preparations, all lobed nuclei were classified as "polymorphs," and no attempt was made to distinguish nuclei of neutrophils from those of eosinophils or basophils. Small, darkly stained, approximately round or slightly indented nuclei were deemed to belong to lymphocytes, and larger, more indented nuclei to monocytes, while nuclei of intermediate appearance and uncertain classification were not measured.

A Vickers M86 scanning and integrating microdensitometer equipped with a ×100, NA 1.3 planapochromatic objective was used to measure the integrated absorbance of Feulgen-stained nuclei at either λ_{max} (ca. 565 nm) or an off-peak wavelength (ca. 490 nm). The off-peak wavelength was necessary for the measurement of sperm nuclei hydrolyzed to give maximum intensity of staining, since at λ_{max} the absorbance of such nuclei exceeded the maximum absorbance of 1.3 measurable with the Vickers M86. The field illuminated had a diameter of 40 μm in some experiments, and 60 μm in others.

Glare was measured as the apparent transmission of an opaque piece of activated charcoal of about the same size as the Feulgen-stained nuclei to be measured, and examined under identical optical conditions. Glare estimated in this way can be misleadingly high with opaque but reflecting particles, due to reflections from the objective being re-reflected by the top surface of the specimen. Direct measurement of reflectance with a Leitz incident-light microscope equipped with a photomultiplier tube showed, however, that the reflectance of the carbon particles used was never greater than 1% and was usually much less than this. Even assuming that 10% of the light reaching the objective was reflected onto the specimen, the final contribution of reflection to the apparent glare would be negligible, about 0.1%. Apparent glare values obtained were usually about 4%. In some experimental series, this amount of glare was offset electronically by adjusting the apparent transmission of such a carbon particle to zero, using a control provided originally for the compensation of the photomultiplier tube dark-current. The rationale and the detailed practical instructions for this procedure are given by Goldstein (28).

Although distribution error is minimized by scanning microdensitometry, a certain amount of residual error from this source remains, due to the necessarily finite size of the measuring spot. The magnitude of this residual distribution error has been shown to be approximately proportional to the diameter of the measuring spot. This enabled the error to be corrected by measuring the object with spots of different diameters and extrapolating the apparent integrated absorbances obtained to find the value corresponding to zero spot size (29). Integrated absorbances reported as "uncorrected for residual distribution error" were those obtained using the smaller measuring spot. In all cases, the empty background was set to ca. 90% apparent transmission, and the apparent integrated absorbance of an area of background was subtracted as a "blank" from the apparent integrated absorbance of the specimen. The number of replicate measurements made of each nucleus varied in different experiments, and is specified under Results.

In the Vickers M86, the hole which is imaged into the object plane as the measuring spot is situated at the exit aperture of the monochromator, so that increasing the diameter of the measuring spot increases the effective spectral bandwidth of the system. For example, by using a 0.48-μm diameter measuring spot with the entrance slit of the monochromator fully open, at wavelength 565

TABLE II

Ratio of Feulgen-DNA Contents of Diploid and Haploid Cells

Author(s)	Measuring technique	Preparation (smears unless stated)	Cells	Diploid:haploid ratio found (±SE)*
Boivin et al. (10)	Biochemical	–	Bull sperm vs. liver, kidney, pancreas, and thymus cells	1.91
Mirsky and Ris (51)	Biochemical	–	Bull sperm vs. liver, kidney, and lymphoid cells	Between 2.20 and 3.00
Swift (77)	Plug	Formalin	Frog spermatids vs. lymphocytes	2.00 ± 0.05
			Grasshopper spermatids vs. malphigian tubule nuclei	1.83 ± 0.05
			Mouse spermatids vs. spermatocytes II	1.99 ± 0.03
Pasteels and Lison (57)	Plug	90% alcohol-formalin (9:1)	*Sabellaria aveolata* (annelid), sperm vs. 1st polar body	4.35 ± 0.14
Moore (53)	Plug	Carnoy's acetic alcohol (sections)	Haploid and diploid embryo tissues from *Rana pipiens*	Varied between 1.33 ± 0.18 to 2.27 ± 0.21
Leuchtenberger et al. (40)	Plug	Carnoy's solution	Human sperm vs. skin cells	2.18 ± 0.04
Alfert and Swift (1)	Plug	Formalin	*Sabellaria aveolata* sperm vs. 1st polar body	2.08 ± 0.06
Thomson and Frazer (80)	Plug densitometry on photomicrographs	Ethanol-acetic acid (3:1)	Rat sperm vs. kidney cells	1.73
Ito and Leuchtenberger (35)	Plug	Carnoy's solution	Clam sperm vs. testis epithelial cells	1.88 ± 0.09
Leuchtenberger et al. (41)	Plug	Bouin's, Carnoy's, or 10% Formalin	Normal male spermatids vs. spermatocytes II	
			Case no. Le 937	1.96 ± 0.05
			Case no. Le 1315	1.80 ± 0.07
			Case no. Le 1314	1.99 ± 0.06
			Case no. Le 1313	1.90 ± 0.04
Leuchtenberger et al. (42)	Plug	10% Formalin or alcohol:formalin:acetic acid:water (50:10:2:40)	Normal bull spermatids vs. spermatocytes II	1.71 ± 0.05
			Normal bull spermatids vs. liver (gp 1 cells)	1.60 ± 0.04
den Tonkelaar and van Duijn (13–15)	Photographic elution	Methanol-formaldehyde	Bull sperm vs. thymus cells	2.30 ± 0.07
Böhm et al. (9)	Scanning	Methanol:formalin:acetic acid	Guinea pig sperm vs. diploid liver cells	1.99 ± 0.25
			" vs. lymphocytes	1.87 ± 0.24
			" vs. granulocytes	1.97 ± 0.24

TABLE II (CONTINUED)

Author(s)	Measuring technique	Preparation (smears unless stated)	Cells	Diploid:haploid ratio found (±SE)*
Garcia (23)	Scanning	Methanol:formalin	Rat sperm vs. granulocytes	2.25
			" vs. Small lymphocytes	1.98
			" vs. Medium lymphocytes	2.22
			" vs. Large lymphocytes and	2.44
			" vs. Monocytes	
Billings and Swartz (7)	Scanning	Ethanol:acetic acid (3:1) (sections)	*Xenopus* sperm vs. spermatocytes II	1.94 ± 0.11
Rasch et al. (64)	Scanning	Formalin	*Drosophila melanogaster* hemocytes vs. sperm	
			Case No. DM-V 2A	1.99 ± 0.16
			Case No. DM-V1 4A	1.92 ± 0.07
			Case No. DM-V 3B	1.91 ± 0.05

nm and 490 nm, the respective spectral bands passed, as measured with an eyepiece spectroscope, were approximately 50 nm and 20 nm. With a 0.95-μm diameter measuring spot, values of 55 nm and 25 nm were obtained. It may be noted that these values are the widths of the spectral band at total cutoff, and not half-band widths; the exit-slit functions at these settings of the entrance and exit slits of the monochromator were unknown (30).

In preliminary experiments, the adherence of the microdensitometer to Lambert's (Bouguer's) law was tested, with all the wavelength and spot size combinations used in the main experiment. In these preliminary experiments, a variable-pathlength spectrophotometer cell (Precision Cells, Inc., New York) was used with a variety of solutions, which included aqueous and alcoholic Basic Fuchsin, and Schiff's reagent recolorized with either formaldehyde or glutaraldehyde. The solution which proved to have an absorbance spectrum most closely resembling that of Feulgen-stained nuclei was formaldehyde-recolored Schiff's reagent (see under Results). This was prepared by adding 1 ml of 40% formaldehyde and 500 ml of water to 2 ml of Schiff's reagent. Absorbance spectra of solutions were plotted using the Vickers M86 itself, and also with a Bausch & Lomb Spectronic 505 double-beam recording spectrophotometer (Bausch & Lomb Inc., Rochester, N. Y.). Single absorbance measurements at selected wavelengths were made with a Unicam SP500 single-beam spectrophotometer. Procedures for investigating and, if necessary, correcting microdensitometric errors arising in the monochromator system of the instrument are described in detail elsewhere (30).

Projected areas of stained nuclei were measured by planimetry on photomicrographs of known magnification. Values for Student's t test, the regression coefficient (r), and the slope (b) were calculated by standard statistical methods. Ratios of two experimental values, each with its standard error, were estimated by use of the formula

$$\frac{x \pm \sigma x}{y \pm \sigma y} = \frac{x}{y}\left(1 + \frac{\sigma y^2}{y^2}\right) \pm \left(\frac{x^2\sigma y^2}{y^4} + \frac{\sigma x^2}{y^2}\right)^{1/2}$$

(38; Walker, personal communication, 1971).

RESULTS

Possible Errors Due To Imperfect Monochromaticity of the Light

As measured with the Vickers M86, Schiff's reagent recolorized with formaldehyde had an absorbance spectrum with a peak absorbance wavelength (λ_{max}) indistinguishable from, and a shape similar to, that of the integrated absorbance spectrum of Feulgen-stained nuclei (Fig. 1, Table III). The adherence of the microdensitometer to Bouguer's law, as tested by plotting the pathlength through recolorized Schiff's solution against the apparent absorbance, was therefore considered to be a reasonable test for the linearity of the instrument when used for measuring the integrated absorbance of stained nuclei.

The apparent absorbance at λ_{max} (ca. 565 nm) of a given thickness of recolorized Schiff's reagent was slightly lower with the Vickers M86 than with the Unicam SP500 (Fig. 2), but with both instruments the apparent absorbance was directly proportional to the pathlength within the limits of experimental error. At λ_{max} the Vickers M86 gave the same results with both spot sizes. At the offpeak wavelength (ca. 490 nm) used for the measurement of densely stained nuclei, a given pathlength of solution had a slightly higher apparent absorbance when the microdensitometer was used with the larger of the two measuring spots, but with a given spot size the pathlength was directly proportional to the apparent absorbance. The conformance of the system to Bouguer's law implies that, with a given spot size and wavelength, no systematic error due to imperfect monochromaticity of the light would occur in the comparison of heavily and lightly absorbing specimens.

Relationship of Hydrolysis Time to (Uncorrected) Apparent Integrated Absorbance

Smears of human blood were hydrolyzed for between 20 and 200 min before staining, and in each slide the integrated absorbance was measured of 10 lymphocyte, 10 monocyte, and 10 polymorph nuclei. The smaller (0.48 μm) diameter measuring spot was used at λ_{max} and no correction was made for glare (measured at 4%). Each cell was measured twice, with two blanks. In another hydrolysis series, slides were used on which had been smeared blood, sperm, and oral squames. Replicate measurements were again employed, and, at each hydrolysis time, 10 leukocytes, 10 sperm, and 10 oral squames were measured, but in this series the wavelength used was 490 nm.

The hydrolysis curves were similar for all cell types studied (Figs. 3 and 4). A plateau of maximum staining intensity was seen between about 60 and 100 min; in subsequent work, a "peak" hydrolysis time of 80 min was chosen. At all hydrolysis times, the mean apparent integrated absorb-

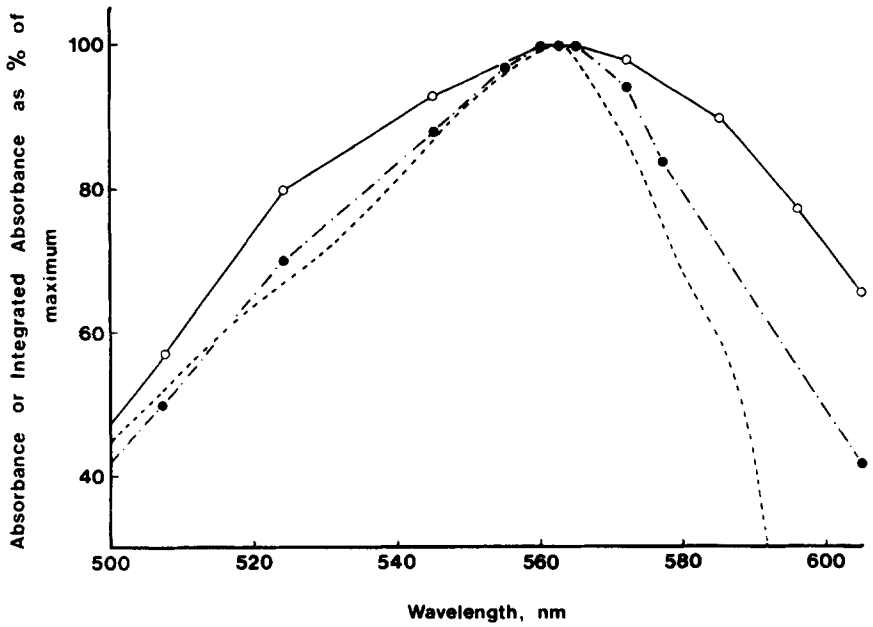

FIGURE 1 Absorbance spectra of de Tomasi Schiff reagent recolorized with formaldehyde measured on: (----) the Bausch & Lomb dual beam recording spectrophotometer, and (●-·-·-●) the Vickers M85 microdensitometer fitted with a variable pathlength cell. Measurements were carried out with a stationary spot (0.48 μm diam). The (○—○) is the absorbance spectrum of a Feulgen-stained nucleus as measured by the Vickers M85 in the scanning and integrating mode of operation with a 0.48-μm diam spot. Six measurements with six blank readings were taken at each point, and the mean value was used. For the sake of comparison, measurements are expressed as a percentage of the maximum absorbance or integrated absorbance.

TABLE III

λ_{max} as Measured on the Vickers M86 Microdensitometer

	Feulgen-stained nuclei	Aqueous basic fuchsin	Ethanolic basic fuchsin	Formaldehyde-Schiff	Glutaraldehyde-Schiff
λ_{max} (nm)	565	545	550	565	610

Scanning and integrating measurements were used for nuclei, and stationary spot measurements for solutions.

ance at λ_{max} of monocyte nuclei was higher than that of the other leukocyte types (Fig. 3).

When measurements were carried out at 490 nm, the hydrolysis curves (Fig. 4) showed no significant differences between leukocytes and squames, or between the observed mean values for sperm and those expected from the measurements of somatic cells (assuming a 1:2 haploid:diploid ratio). In these series, each nucleus was measured only twice, with two blanks. In subsequent series (Table VII, discussed below), uncorrected measurements at 490 nm showed small but statistically significant apparent differences between certain of the cell types; the discrepancy between the series can be attributed to the fact that in the later series the results were more precise, each nucleus being measured 10 times.

Effect of Residual Distribution Error and Glare on Apparent Integrated Absorbance

By using blood smears hydrolyzed for 40, 60, 80, or 200 min, the projected area and apparent integrated absorbance (uncorrected for residual distribution error or glare) were measured on nuclei of all three leukocyte types (Table IV, Fig. 5). Each nucleus was measured twice with two blanks and the mean taken. In all preparations, a highly

significant positive correlation was found between nuclear area and apparent integrated absorbance. When measurements were corrected for glare (but not residual distribution error), the projected nuclear area was still positively correlated with the apparent integrated absorbance, but both the slope of the plots and the correlation coefficients were less than with uncorrected measurements (Table IV, Fig. 6).

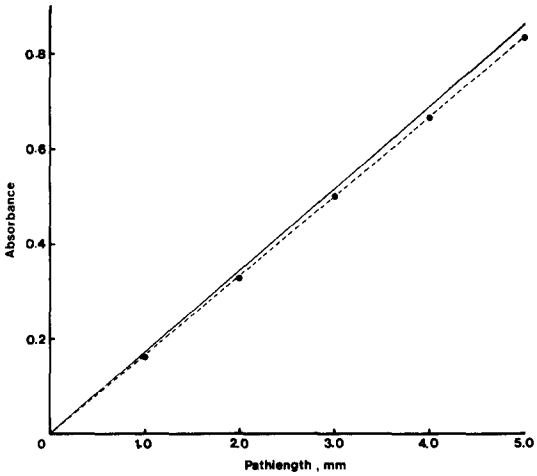

FIGURE 2 Plots of the absorbance against the pathlength of a solution consisting of de Tomasi Schiff reagent recolorized with formaldehyde contained within a variable pathlength cell. Measurements were carried out at λ_{max} (ca. 565 nm). The continuous line was that obtained on a Unicam SP500 single beam spectrophotometer. The broken line was that obtained using the Vickers M85 microdensitometer with a stationary spot. The diameter of the measuring spot (either 0.48 μm or 0.95 μm) did not significantly affect this line.

By using blood smears hydrolyzed to give maximum staining (80 min), the apparent integrated absorbances of nuclei of the three leukocyte types were measured under four sets of conditions, i.e. with two sizes of measuring spot, in each case both with and without electronic offsetting of residual glare. Each nucleus was measured 10 times (with 10 blank measurements of empty background) under each set of conditions; measurements wholly uncorrected or corrected for glare only were obtained using the smaller spot, while measurements corrected for residual distribution error (with or without additional correction for glare) were calculated from measurements made with both spot sizes. In one experimental series (Table V), the mean uncorrected integrated absorbance of monocyte nuclei was significantly higher than that of lymphocyte nuclei (by about 5%), which was in turn significantly higher than that of polymorph nuclei (by about 4%). The difference between monocytes and lymphocytes disappeared when measurements were corrected for residual glare, and no significant difference was seen be-

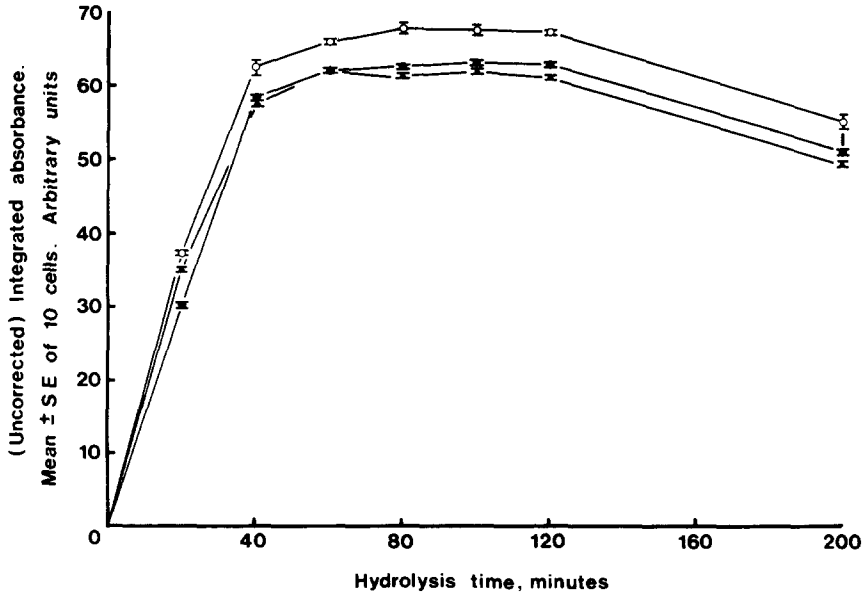

FIGURE 3 Hydrolysis time vs. integrated absorbance curves for different leukocyte types: ○, monocytes; ●, lymphocytes; and ▲, polymorphs. Hydrolysis was carried out in 5 N HCl at 25°C. Measurements at λ_{max}, uncorrected for residual glare or distribution error.

tween lymphocytes and polymorphs when residual distribution error was corrected. Fully corrected measurements (i.e. corrected for both glare and residual distribution error) showed no significant differences between the mean apparent integrated absorbances (Feulgen-DNA values) of the three cell types. When the entire experiment was repeated with a smear from a different donor, the results obtained were broadly similar in that no significant differences between the cell types were found when measurements of apparent integrated absorbance were corrected for both glare and residual distribution error. In this series of measurements, however, the apparent Feulgen-DNA contents of polymorphs and of lymphocytes were similar even with uncorrected measurements, while the apparent difference between monocytes and lymphocytes seen in wholly uncorrected measurements was not completely eliminated by correcting glare alone. These minor differences between the experimental series may, as discussed later, have been due to small variations in the preparation of the stained smears.

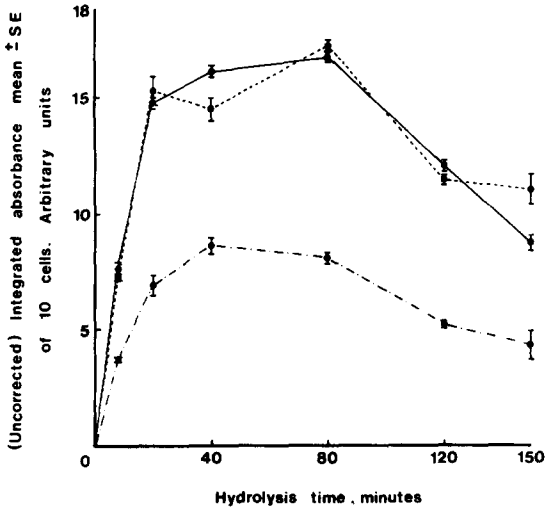

FIGURE 4 Hydrolysis time vs. integrated absorbance curves for (●—●), leukocytes; (●----●), oral squames; and (●-·-·-●), sperm, all from a single donor. Hydrolysis was carried out in 5 N HCl at 25°C. Measurements at λ 490 nm, uncorrected for residual glare or distribution error.

In further experiments, the apparent Feulgen-DNA content of leukocyte nuclei was compared with that of oral squames. No significant differences between the cell types were found with either corrected or uncorrected measurements made at λ_{max} after subpeak hydrolysis (Table VI), or with either corrected or uncorrected measurements made at an off-peak wavelength after peak hydrolysis (Table VII). However, squames had a significantly higher mean apparent Feulgen-DNA content than polymorphs when uncorrected measurements were made at λ_{max} after peak hydrolysis; this difference disappeared when measurements were corrected (Table VIII).

Sperm nuclei could not be measured at λ_{max} after peak hydrolysis, as under these conditions their absorbance was too great (at least with glare offset). At 490-nm wavelength the absorbance of leukocyte nuclei was about one-quarter of that at λ_{max}, while the highest point absorbance of maximally stained sperm nuclei at 490 nm was slightly above unity. It therefore appears that the true (glare-corrected) absorbance of sperm at λ_{max}

TABLE IV

Relationship between Projected Nuclear Area and Integrated Absorbances (at λ_{max}), (Both Corrected and Uncorrected for Residual Glare) of Pooled Human Leukocytes

	Hydrolysis time	No. of cells measured	r	P of r	b
	min				
Uncorrected integrated absorbances	40	32	0.80	<0.001	0.09
	60	36	0.58	<0.001	0.06
	80	35	0.81	<0.001	0.15
	200	34	0.69	<0.001	0.10
Integrated absorbances corrected for residual glare	40	35	0.28	<0.1	0.01
	60	35	0.45	<0.01	0.01
	80	30	0.44	<0.05	0.03
	200	38	0.56	<0.001	0.04

Hydrolysis was carried out in 5N HCl at 25°C. Each nucleus was measured two times with two blanks. r, correlation coefficient; b, slope of regression line.

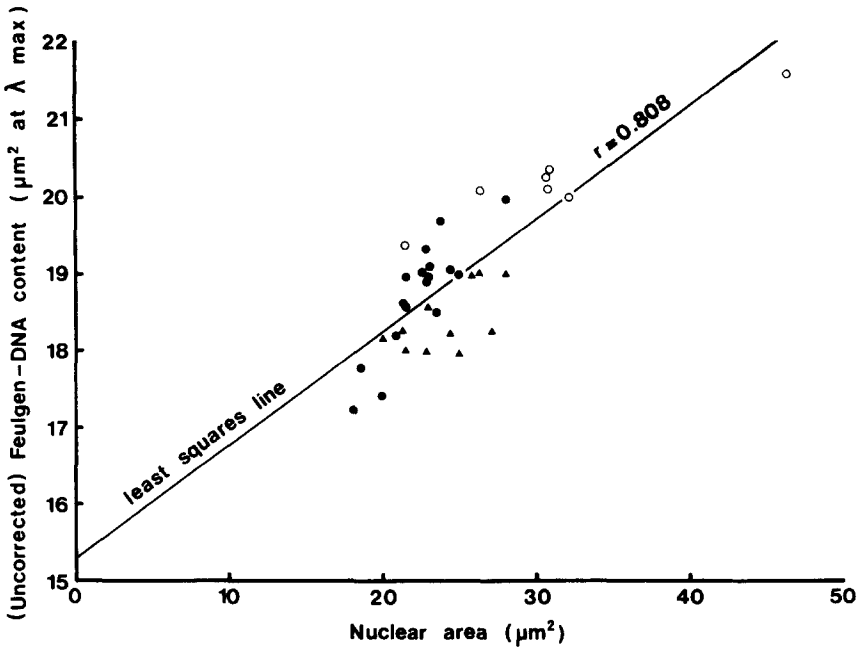

FIGURE 5 Relationship between the Feulgen-DNA content (uncorrected for residual glare or distribution error) and the projected nuclear area of leukocytes: ○, monocytes; ●, lymphocytes; and ▲, polymorphs hydrolyzed for 80 min in 5 N HCl at 25°C. Each nucleus was measured twice, each measurement being accompanied by a blank reading, and the mean taken. r is the correlation coefficient, (P of $r < 0.001$).

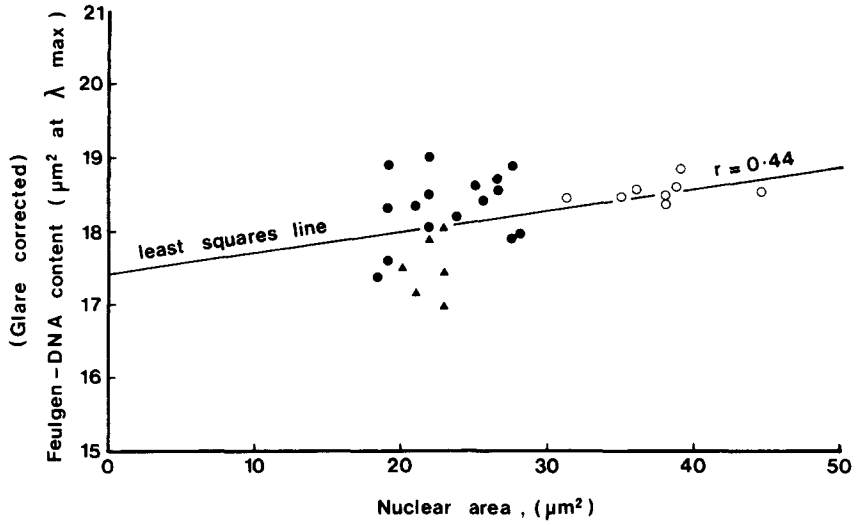

FIGURE 6 Relationship between the Feulgen-DNA content (corrected for residual glare) and the projected nuclear area of leukocytes: ○, monocytes; ●, lymphocytes; and ▲, polymorphs hydrolyzed for 80 min in 5 N HCl at 25°C. Each nucleus was measured twice, each measurement being accompanied by a blank reading, and the mean taken. r is the correlation coefficient, (P of $r < 0.05$).

would be of the order of 4.0, which is far beyond the 1.3 unit measuring range of the Vickers M86. If glare is uncorrected, the apparent absorbance would of course be lower. For example, no apparent value higher than 1.3 is possible in the presence of 5% glare. When uncorrected measure-

TABLE V

Feulgen-DNA Content at λ_{max} (ca 565 nm) of Different Types of Human Leukocyte Hydrolyzed to Give Maximal Staining (80 min)

	No. of cells measured	Uncorrected		Corrected for residual glare		Corrected for residual distribution error		Corrected for both residual glare and distribution error	
Experimental series I	(L) 9	19.24 ± 0.25		21.67 ± 0.15		19.46 ± 0.28		21.89 ± 0.21	
	(P) 9	18.54 ± 0.19		20.98 ± 0.11		18.84 ± 0.21		21.62 ± 0.10	
	(M) 8	20.18 ± 0.23		22.03 ± 0.17		20.18 ± 0.21		22.06 ± 0.23	
t	L < M	2.76*	L < M	1.59*	L < M	2.06§	L < M	0.55‡	
	L > P	2.23*	L > P	3.71‖	L > P	1.78‡	L > P	1.16‡	
	P < M	5.50‖	P < M	5.19‖	P < M	4.51‖	P < M	1.75‡	
Experimental series II	(L) 12	16.59 ± 0.14		17.79 ± 0.13		16.58 ± 0.18		17.85 ± 0.12	
	(P) 12	16.82 ± 0.09		17.90 ± 0.08		16.88 ± 0.13		17.96 ± 0.09	
	(M) 11	17.63 ± 0.07		18.35 ± 0.10		17.49 ± 0.09		18.12 ± 0.13	
t	L < M	6.64‖	L < M	3.41‖	L < M	4.52‖	L < M	1.53‡	
	L < P	1.38‡	L < P	0.72‡	L < P	1.35‡	L < P	0.73‡	
	P < M	7.10‖	P < M	3.51‖	P < M	3.86‖	P < M	1.01‡	

Results given as mean integrated absorbance (μm^2 units ± SE). Each nucleus was measured 10 times with 10 blanks under each set of conditions. Hydrolysis was carried out in 5N HCl at 25°C. Absolute values can only be compared within an experimental series as the staining of the slides in the two experimental series was carried out on different days, and using different batches of Schiff reagent. *L*, lymphocytes; *P*, polymorphs; *M*, monocytes.

* $P < 0.05$.

‡ Not significant.

§ $P < 0.06$.

‖ $P < 0.01$.

TABLE VI

Feulgen-DNA Content at λ_{max} (ca 565 nm) of Different Human Cell Types Hydrolyzed to Give Nonmaximal Staining (7 min)

	No. of cells measured	Uncorrected	Corrected for residual glare	Corrected for residual distribution error	Corrected for both residual glare and distribution error
Leukocytes	9	10.26 ± 0.08	10.74 ± 0.08	10.16 ± 0.11	10.85 ± 0.06
Oral squames	10	10.29 ± 0.09	10.59 ± 0.05	10.20 ± 0.12	10.69 ± 0.11
Sperm	9	4.73 ± 0.06	5.12 ± 0.09	4.90 ± 0.07	5.34 ± 0.12
Ratio: Leukocytes/sperm		2.17 ± 0.03	2.10 ± 0.04	2.07 ± 0.04	2.03 ± 0.05
t		5.25*	2.45‡	1.99§	0.70§
Ratio: Oral squames/sperm		2.18 ± 0.03	2.07 ± 0.04	2.08 ± 0.04	2.00 ± 0.05
t		5.25*	1.83§	2.13§	0.06§
Ratio: Oral squames/leukocytes		1.00 ± 0.01	0.99 ± 0.01	1.00 ± 0.02	0.99 ± 0.01
t		0.25§	1.60§	0.26§	1.28§

Results given as mean integrated absorbance (in μm^2 units ± SE). Each nucleus was measured 10 times with 10 blanks under each set of conditions. Hydrolysis was carried out in 5N HCl at 25°C.

* $P < 0.01$.

‡ $P < 0.05$.

§ Not significant.

ments of integrated absorbance were made at λ_{max} after subpeak hydrolysis, or at 490 nm after maximal staining, the apparent Feulgen-DNA content of human sperm was slightly less than half that of oral squames or leukocytes (Tables VI, VII). These discrepancies, however, disappeared when measurements were corrected for residual distribution error and glare. In these corrected measurements the diploid:haploid ratios were extremely close to two; of four independent estimations, the lowest value was 1.99 ± 0.05 and the highest 2.03 ± 0.05 (Tables VI, VII). Similar results were obtained using rabbit sperm and leukocytes (Table IX).

DISCUSSION

In our experiments, technically refined scanning and integrating microdensitometric measurements

TABLE VII

Feulgen-DNA contents at Wavelength 490 nm of Different Cell Types Hydrolyzed to Give Maximal Staining (80 min)

	No. of cells measured	Uncorrected	Corrected for residual glare	Corrected for residual distribution error	Corrected for both residual glare and distribution error
Lymphocytes	8	5.71 ± 0.05	5.97 ± 0.09	4.99 ± 0.17	5.62 ± 0.16
Oral squames	8	5.86 ± 0.09	5.96 ± 0.11	5.42 ± 0.10	5.58 ± 0.12
Sperm	8	2.73 ± 0.05	2.91 ± 0.05	2.56 ± 0.07	2.80 ± 0.04
Ratio: Lymphocytes/sperm		2.09 ± 0.04	2.05 ± 0.05	1.95 ± 0.09	2.01 ± 0.06
t		2.17*	1.11‡	0.58‡	0.12‡
Ratio: Oral squames/sperm		2.15 ± 0.05	2.05 ± 0.05	2.12 ± 0.07	1.99 ± 0.05
t		2.87§	0.94‡	1.70‡	0.13‡
Ratio: Oral squames/lymphocytes		1.03 ± 0.02	1.00 ± 0.02	1.09 ± 0.04	0.99 ± 0.04
t		1.46‡	0.06‡	2.07‡	0.18‡

Results given as mean integrated absorbance (in μm^2 units ± SE). Each nucleus was measured 10 times with 10 blanks under each set of conditions. Hydrolysis was carried out in 5N HCl at 25°C.

* $P < 0.05$.

‡ Not significant.

§ $P < 0.02$.

TABLE VIII

Feulgen-DNA contents at λ_{max} (ca 565 nm) of Human Polymorphs and Oral Squames Hydrolyzed to Give Maximal Staining (80 min)

	No. of cells measured	Uncorrected	Corrected for residual glare	Corrected for residual distribution error	Corrected for both residual glare and distribution error
Polymorphs (P)	8	19.33 ± 0.13	20.62 ± 0.15	20.16 ± 0.27	21.62 ± 0.22
Oral squames (S)	8	20.99 ± 0.26	21.80 ± 0.25	20.98 ± 0.25	21.67 ± 0.24
t		S > P, 5.71*	S > P, 4.05*	S > P, 2.23‡	S > P, 0.15§

Results given as mean integrated absorbance (in μm^2 units ± SE). Each nucleus was measured 10 times with 10 blanks under each set of conditions. Hydrolysis was carried out in 5N HCl at 25°C.

* $P < 0.01$.

‡ $P < 0.05$.

§ Not significant.

of Feulgen-DNA contents of oral squames and different types of blood leukocyte did not differ significantly from each other, and were almost precisely twice that of sperm. Since the constancy or otherwise of the amount of DNA per haploid set of chromosomes is the primary question at issue, it appears logically inadmissible to take our results as evidence both for the constancy hypothesis and for the validity of the technical procedures adopted. The circularity of this argument has been pointed out, e.g. by Swift (78) and Walker and Richards (83). Nevertheless, possible alternative interpretations of our data involve somewhat implausible assumptions. If, for example, we suppose that the DNA contents of the cells studied were *not* identical, the differences were fortuitously compensated for by systematic errors in the staining and/or microdensitometry. It seems equally improbable that the DNA contents were actually constant, and that nonstoichiometry of the Feulgen procedure was precisely neutralized by undetected errors of opposite sign in the measurement. The most logically economical interpretation of our data is, therefore, that the staining and (corrected) measuring procedures used gave quantitatively valid results, and that in the cells studied the amount of DNA per haploid chromosome complement was constant within the (rather narrow) limits of random experimental error.

When scanning and integrating microdensito-

TABLE IX

Feulgen-DNA Content at λ_{max} (ca 565 nm) of Rabbit Lymphocytes and Sperm Hydrolyzed to give Nonmaximal Staining (7 min)

	No. of cells measured	Corrected for residual glare	Corrected for both residual glare and distribution error
Lymphocytes	13	7.13 ± 0.07	7.15 ± 0.07
Sperm	13	3.38 ± 0.06	3.45 ± 0.06
Ratio: Lymphocytes/sperm		2.11 ± 0.04 2.57*	2.07 ± 0.04 1.77‡

Results given as mean integrated absorbance (in μm^2 units ± SE). Each nucleus was measured 10 times with 10 blanks under each set of conditions. Hydrolysis was carried out in 5N HCl at 25°C.

* $P < 0.02$.

‡ Not significant.

metric measurements were carefully made using standard procedures, but without compensation for residual distribution error or glare, several statistically significant apparent deviations from Feulgen-DNA constancy were found. These discrepancies could often be attributed to particular sources of error. For example, glare appeared to be mainly responsible for apparent differences between monocytes and lymphocytes, residual distribution error accounted for the apparent differences (found in one experimental series but not in another) between lymphocytes and polymorphs, and both glare and residual distribution error appeared to contribute to the apparent differences between monocytes and polymorphs. These results correlate nicely with the morphology of the three types of nucleus. On theoretical grounds (28), it would be expected that measurements of darkly stained polymorph and lymphocyte nuclei would be more affected by glare than those of relatively pale monocyte nuclei. Similarly, it is not surprising that residual distribution error, which is a function of the extent of the perimeter of the scanned specimen (29, 49), affected measurements of lobed polymorph nuclei more than measurements of the relatively round and compact nuclei of lymphocytes. Trivial differences between the (uncorrected) results of different experimental series may perhaps have been caused by minor or local variations in the extent of spreading or intensity of staining of the cells. The crucial observation is that significant differences between the three leukocyte types were not found in either experimental series, when measurements were fully corrected for both glare and distribution error.

A highly significant correlation found between the nuclear area and the apparent integrated absorbance, as seen in our uncorrected measurements, has also been described by previous workers (e.g. references 31, 75). However, the fact that correcting for glare reduced both the slope of the plots and the correlation coefficients suggests that the Feulgen-DNA content of nonreplicating nuclei is independent of the size of the nucleus, and that the apparent correlation between these two parameters in uncorrected measurements (Table IV, Fig. 5) was due to instrumental errors.

Entirely consistent results were obtained with sperm and oral squames, no deviations from the expectations of the constancy hypothesis being observed when fully corrected measuring techniques were employed. Precision measurements of the Feulgen-DNA content of oral squames do not appear to have been published previously, and from the point of view of the constancy hypothesis it seems significant that the apparent Feulgen-DNA content of these specialized ectodermal epithelial cells, with relatively flattened and extended nuclei, is identical with that of the much more compact nuclei of mesodermal leukocytes.

Sibatani (72) showed that in vitro, under conditions rather different from those employed in the staining of smears or histological sections, the presence of histones markedly affected the apparent stoichiometry of the Feulgen reaction. He subsequently (73) reported that the amount of protein present in rat liver nuclei, which differed markedly in starved and fed animals, had little or no effect on the Feulgen reaction when carried out on cover-glass preparations of fixed material. In our own work, the nature of the proteins associated with the nuclear DNA seemed relatively unimportant, since the apparent Feulgen-DNA content of sperm was precisely half that of somatic cells, despite the fact that protamines are present in the former and histones in the latter.

Statistically significant apparent anomalies in the Feulgen-DNA contents of both sperms and oral squames were sometimes observed when measurements were made in the presence of residual distribution error or glare. The discrepancies found can be correlated with the morphology of the nuclei concerned just as in the case of the leukocyte nuclei, discussed above. In the comparison of the Feulgen-DNA contents of oral squames and leukocytes, even uncorrected measurements showed no anomalies provided the absorbances of the measured nuclei were reduced by either sub-

peak hydrolysis or the use of an off-peak wavelength. This is readily explicable, since both residual distribution error and glare are relatively more important with strongly absorbing objects.

It may be noted at this stage that, in the case of the Vickers M86, the extrapolation procedure for the correction of residual distribution error simultaneously corrects or diminishes "white light error" resulting from the use of a finite spectral band-width. This is because, as mentioned earlier, changing the diameter of the measuring spot of this instrument alters the exit aperture of the monochromator (30). Reduction of the diameter of the measuring spot lessens residual distribution error and hence tends to increase the apparent integrated absorbance of the specimen at any wavelength setting. The effect of employing a narrower monochromator bandwidth, however, depends on the spectral characteristics of the specimen and on the wavelength of light used. At λ_{max}, higher apparent absorbances and integrated absorbances are obtained with a smaller bandwidth, but the opposite effect may be seen at off-peak wavelengths. At a given off-peak wavelength, the use of a smaller measuring spot may therefore exert effects of opposite sign on the residual distribution and chromatic errors. Which effect predominates depends on the specimen. In the case of Feulgen-stained nuclei measured at 490 nm, the monochromator effect proved to be the larger, higher apparent integrated absorbances being obtained using the larger of the two measuring spots. Measurements corrected for residual distribution error (and for chromatic error) were therefore lower than uncorrected measurements.

Our use of off-peak hydrolysis times in the Feulgen procedure requires justification. According to Rasch and Rasch (65), cell nuclei contain both acid-labile and relatively acid-resistant fractions of DNA, and the apparent cellular Feulgen-DNA content of a given cell, if measured after any period of hydrolysis other than a specific "cross-over time," depends on the ratio of the amounts of the two types of DNA present. Rasch and Rasch recommend that in the comparison of cell types ". . . ratios of Feulgen dye amounts be examined at several different hydrolysis times. If the ratios remain constant, then the Feulgen values obtained at curve peaks can be directly compared . . .", otherwise the cross-over time must be ascertained and employed. In our experiments, the hydrolysis curves of the various cell types studied appeared similar, and the ratios of the apparent Feulgen-DNA contents of two given cell types were not significantly different after different periods of hydrolysis. Our use of both maximal and submaximal hydrolysis times, therefore, appears to be in order, even assuming the validity of the model proposed by Rasch and Rasch. It should, however, be said that the experimental evidence presented by Rasch and Rasch in support of the supposed existence of two distinct varieties of DNA does not seem convincing. Indeed, some of their own data appear inconsistent with their postulates. For example, after peak hydrolysis (120 min), they found the apparent Feulgen-DNA content of avian lymphocytes, as measured by the two-area method, to be significantly lower than that of erythrocytes. They estimated the cross-over hydrolysis time to be 420 min, and after longer periods of hydrolysis found no statistically significant differences between mean Feulgen-DNA values for the two cell types. Since after relatively short periods of hydrolysis lymphocytes appeared to take up less Feulgen stain than erythrocytes, according to their theory lymphocytes contain a relatively high proportion of acid-resistant DNA, and should therefore stain *more* intensely than erythrocytes, after periods of hydrolysis longer than the cross-over time. This was not, however, the case. After no time of hydrolysis studied by Rasch and Rasch was the apparent Feulgen-DNA content of avian lymphocytes higher than that of erythrocytes, i.e. no cross-over point was apparent. This seems to throw considerable doubt on their theoretical edifice. The apparent differences between the Feulgen-DNA contents of the two cell types, which they found after relatively short periods of hydrolysis, are not good evidence for the postulated differences in the types of DNA present, because such apparent differences may well have been due to microdensitometric errors analogous to those in our uncorrected measurements. The fact that after long periods of hydrolysis Rasch and Rasch found statistically insignificant differences between the cell types is consistent with this interpretation, since (as already pointed out) the effects of systematic measuring errors in general become less marked with lower absolute intensity of staining.

We have presented evidence that in our experiments apparent deviations from Feulgen-DNA constancy were instrumental artifacts. The question remains whether a similar explanation holds for anomalous findings of other workers. That this may be the case is suggested by the similarity

between our uncorrected results and previously published data, and by the fact that close scrutiny of the methods employed by other workers indicates that in at least some instances systematic instrumental errors were probably present.

Regarding the comparison of our results with those of others, our uncorrected measurements showed a statistically highly significant positive correlation between the projected area and the apparent Feulgen-DNA content of leukocyte nuclei. Similar findings have been reported, e.g. by Garcia (23), Sullivan and Garcia (75), Gottlieb-Rosenkrantz and O'Brien (31) and James (36) for Feulgen-stained leukocytes, by Urasinski and Habicht (82) for leukocytes stained with gallocyanin-chrome alum, and by Mayall and Mendelsohn (48) for stained chromosomes. Our (uncorrected) results showing significant apparent differences between the mean Feulgen-DNA values of monocytes, lymphocytes and polymorphs, and discrepancies between observed and expected ratios of the Feulgen-DNA contents of haploid and diploid cells, are also very similar to published observations (see Tables I and II).

Invidious though it may be, discussion of possible instrumental errors in the work of other investigators appears unavoidable. Assessment of the significance of published data, or comparison with our own results, is not, however, always possible. In some instances, mean Feulgen-DNA values have been given without sufficient information to enable the calculation of a standard error (see e.g. references 23, 26, 74, 80). In certain other cases, the variability of the published results is so great that no detailed discussion appears worthwhile. For example, Böhm et al. (9) present data on the Feulgen-DNA contents of guinea pig sperm, liver cells, lymphocytes, and granulocytes, from which it is possible to calculate diploid/haploid ratios. These ratios do not differ significantly from 2.0, just as expected on the basis of the constancy hypothesis, but the actual ratios presented may have been anywhere between about 1.5 and 2.5 (best estimate ± two standard errors, see Table II). Similarly, from data presented by Billings and Swartz (7), the ratio of the Feulgen-DNA contents of *Xenopus* spermatocytes and sperm can be calculated to be 1.940 ± 0.111, i.e. the actual value could have been as low as 1.72 or as high as 2.16.

Most of the earlier, pioneering work on Feulgen-DNA constancy was carried out using the plug method of microdensitometry (Tables I and II). Some of this work, notably that of Ris and Mirsky (68), Mirsky and Ris (52) and Swift (77), exhibited considerable technical insight and awareness of possible errors, but the distributional error unavoidable with the plug method probably renders it inherently unsuitable for the demonstration of Feulgen-DNA differences of the magnitude (ca. 10%) under discussion. In addition, other sources of error (such as glare) were doubtless present in at least some series. The plug data in Tables I and II are therefore mainly of historical interest.

The two-wavelength and two-area methods of microdensitometry basically assume the projected area of the specimen to be of arbitrary shape and size, but of uniform internal absorbance (24, 25, 50, 56). These methods reduce but do not completely eliminate glare and distribution error. Systematic errors arising from failure of the specimen to conform to the basic requirement of internal homogeneity can be minimized by close attention to such factors as the correct choice of wavelengths, but it is not always clear that such precautions have been taken. Because few published articles give essential technical data, such as the amount of glare present and the bandwidth of the monochromator, it is seldom possible to assess the probable magnitude of systematic errors, but glare, residual distribution error and perhaps other errors plausibly account for at least some of the Feulgen-DNA anomalies which have been found using the two-wavelength and two-area methods (Tables I and II).

Photographic methods of microdensitometry can be affected by glare, chromatic and other errors, but can (e.g. with methods involving the assay of silver or dyes eluted from the photographic material) be more or less free from distribution error. They have not been widely used, perhaps because they tend to be laborious and are complicated by problems connected with the linearity of the response of the photographic emulsion. They have, however, been applied to Feulgen-DNA studies e.g. by den Tonkelaar and van Duijn (13–15). These workers found no discrepancies between the apparent Feulgen-DNA contents of various somatic cells, but reported slightly lower relative values than expected in sperm. It is possible that some glare was present in their system despite careful control of the specimen area illuminated.

Of the systematic errors which plague scanning and integrating microdensitometry, glare is probably the most important. A number of workers (e.g. references 36, 47, 49) minimize glare by use

of a field stop, but in our experience an appreciable amount of glare (e.g. 3%) usually remains after as much reduction of the illuminated specimen area as is practicable with a flying spot or image-scanning microdensitometer (considerably smaller illuminated fields are possible with an object-scanning instrument such as the Zeiss UMSP). Of the workers quoted (Tables I and II), only Mayall and Mendelsohn give a figure (1%) for glare measured in their system. Systematic errors from 1% glare would be negligible with many types of objects, but it may be noted that if a sperm head and a somatic cell nucleus have true absorbances of (say) 2.0 and 0.5, respectively, in the presence of 1% glare the apparent integrated absorbance of the sperm head would be about 3.5% less than would be expected from the actual dye contents of the two specimens. The amount of glare present in a given instrument may also vary appreciably from time to time. For example, we once found that with a standard setting of the field stop (ca. 60 μm diameter in the specimen plane), the measured glare of our Vickers M86 was about 8%. Removal of dust which had accumulated on the scanning mirrors immediately reduced this figure to a more usual value, i.e. 3%. It is therefore advisable to check the glare figure frequently, and certainly before any important series of measurements. If significant glare remains despite attention to the cleanliness of the optics and the size of the field stops, electronic off-setting can be resorted to (28), but this has not been employed by previous workers.

Residual distribution error, resulting from the necessarily finite size of the measuring spot employed in scanning microdensitometry, varies with objects of different geometry. The error can be compensated for by the extrapolation procedure we have employed, or minimized by the use of a measuring spot with a diameter approaching the diffraction-limited resolving power of the system. A relatively small spot, 0.375 μm diameter in the specimen plane, was used by Mayall and Mendelsohn (49), and the Barr & Stroud GN2 microdensitometer employed by a number of workers has a measuring spot which can be as small as 0.25 μm diameter. Some users of the Barr & Stroud instrument (e.g. references 9, 36) have, however, employed an effective measuring spot diameter of about 0.7 μm, and other workers have not specified their instrumental settings in sufficient detail for the spot diameter used to be assessed. A spot diameter of 0.7 μm would, according to our findings, result in an appreciable error in the measurement of small, dense objects such as stained lymphocyte or sperm nuclei. A proportionately greater error would be expected from the use of a 1.0-μm diameter measuring spot, as in the Zeiss UMSP measurements of James (36) and the multiple spot measurements of Noeske (55). It thus appears that residual distribution error was probably present in at least some previously published work.

In scanning and integrating, as in other forms of microdensitometry, systematic error in the comparison of lightly and darkly stained objects can result from the use of insufficiently monochromatic light (30). Mayall and Mendelsohn (49) calculated that, under their experimental conditions, error due to this cause was negligible, and in the present article we have presented empirical evidence that appreciable chromatic error did not affect our measurements. However, not all workers appear to have taken this possible source of error into account. Significant systematic errors can, for example, result from the use of a graded-spectrum interference filter of the type routinely supplied with the Barr & Stroud GN2 microdensitometer (30), and chromatic errors are likely to be especially marked when measurements are made at a wavelength on the "shoulder" of an absorption peak (as in the work of Garcia and Iorio [26]).

In scanning microdensitometry, the absorbance of the specimen should at no point exceed the measuring range of the instrument. The range of the Vickers M86 microdensitometer is from zero to an absorbance of 1.3, true values greater than this being recorded as 1.3. We have found maximally Feulgen-stained human and rabbit sperm to have true absorbances at λ_{max} considerably in excess of this value. Error from this cause is therefore a real possibility. Erroneous measurements can be avoided with the Vickers M86 by setting the area-measurement facility to record only specimen areas having an absorbance greater than (say) 1.25; a finite area measurement during a scan then indicates that some part of the specimen has an absorbance near and possibly exceeding the permissible maximum. Errors resulting from excessive specimen absorbance are also possible with other instruments. The Barr & Stroud GN2 microdensitometer, for example, records absorbances greater than a set "cut-off" value (usually 0.75 or 1.0) as zero. Böhm (8) found formalin-fixed, Feulgen-stained sperm to have absorbance

values greater than the maximum measurable with his Barr & Stroud microdensitometer, and it seems possible that other measurements of densely stained sperm heads using this instrument (e.g. 9) may also have been incorrectly low. A similar factor could conceivably have been responsible for the finding by Gledhill et al. (27), using a microdensitometer developed by Lomakka, of a deficit in the apparent Feulgen-DNA content of ejaculated bull sperm when compared with spermatids. Lomakka (46) does not explicitly state the measuring range of his instrument, but mentions that he normally measures only specimens having an absorbance less than 1.3. Microdensitometric errors, resulting from the theoretical measuring range of the instrument being exceeded by the apparent absorbance of the specimen, are of course only likely in systems where glare is slight or negligible, since (as already discussed) the maximum possible apparent absorbance of any specimen is limited by glare.

Turning from purely instrumental aspects of microdensitometry, there is good evidence that serious systematic errors in Feulgen-DNA measurements can result from incorrect specimen preparation. In particular, the fixation employed appears critical. Formal fixation has been used by many workers from Ris and Mirsky (68) onwards, including ourselves, and has been shown by Böhm et al. (9) to eliminate certain differences between the hydrolysis curves of different cell types. Some recent authors, notably Hale (32), Mayall (47), Billings and Swartz (7), Noeske (55) have, however, used fixatives or fixative mixtures without formalin, and this could account for some of the discrepancies in the literature.

In conclusion, some workers (in recent times notably Mayall and Mendelsohn, and Garcia) have made considerable efforts to assess, eliminate, or control instrumental microdensitometric errors. For example, Garcia and Iorio (26) carried out scanning measurements of Feulgen-stained nuclei at an off-peak wavelength (490 nm) to diminish distributional errors. Garcia (23) found that cell crushing, which would be expected to diminish most types of systematic error, had little or no effect on his measurements, and Mayall and Mendelsohn (49) were able to estimate quantitatively the maximum errors to be expected from various sources. We do not, therefore, claim that all apparent deviations from Feulgen-DNA constancy are due to instrumental errors, or that such errors were present in any particular piece of research. We nevertheless believe that systematic microdensitometric errors have been seriously underestimated by many previous workers, were probably present in much of the published research on the Feulgen-DNA contents of different cell types, and could account for many reported apparent deviations from the predictions of the constancy hypothesis. There therefore appears little reason to suppose either that the Feulgen procedure (when correctly performed) is nonstoichiometric, or that substantial biological variations in the amount of DNA present per haploid chromosome complement are present in the mammalian cell types thus far studied. Our measurements do not of course preclude the possible existence of variation in DNA content which, although less than the current limits of microdensitometric error (1–2%), could nevertheless be of considerable biological significance.

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