OFF-PEAK ABSORPTION MEASUREMENTS IN FEULGEN CYTOPHOTOMETRY

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ABSTRACT

The use of off-peak measurements in Feulgen cytophotometry is reported, using intact diploid (x) and tetraploid (y) nuclei of the human anterior pituitary gland as the experimental model studied. Results indicate that the ratio y/x is similar at the 14 wavelengths examined over the range 450 mμ to 650 mμ. The value of this ratio was 1.95, falling slightly under the theoretical ratio of 2.0. It was concluded that off-peak absorption measurements are of value in Feulgen cytophotometry. A discussion of the possible justification of off-peak absorption measurements was presented for the case in which the Beer-Lambert relationship was experimentally determined to be followed at a peak wavelength, for all concentrations under discussion, and the curves preceding and/or following the peaks were straight lines coming from a common point. If the curve best fitting the data is a straight line, it follows that the rate of change of absorbance with respect to a linear wavelength scale is constant. This means that for a given increase, or decrease, in wavelength, the same change in absorbance is obtained. From this it follows that absorbance readings at any wavelength in such a region will be equally valid to those taken at the peak. While the finding of such a linear relationship at several concentrations does not guarantee that it will occur at all other concentrations, it is suggestive. The closer the spectra approximate straight lines, the more valid does the use of off-peak measurements become.

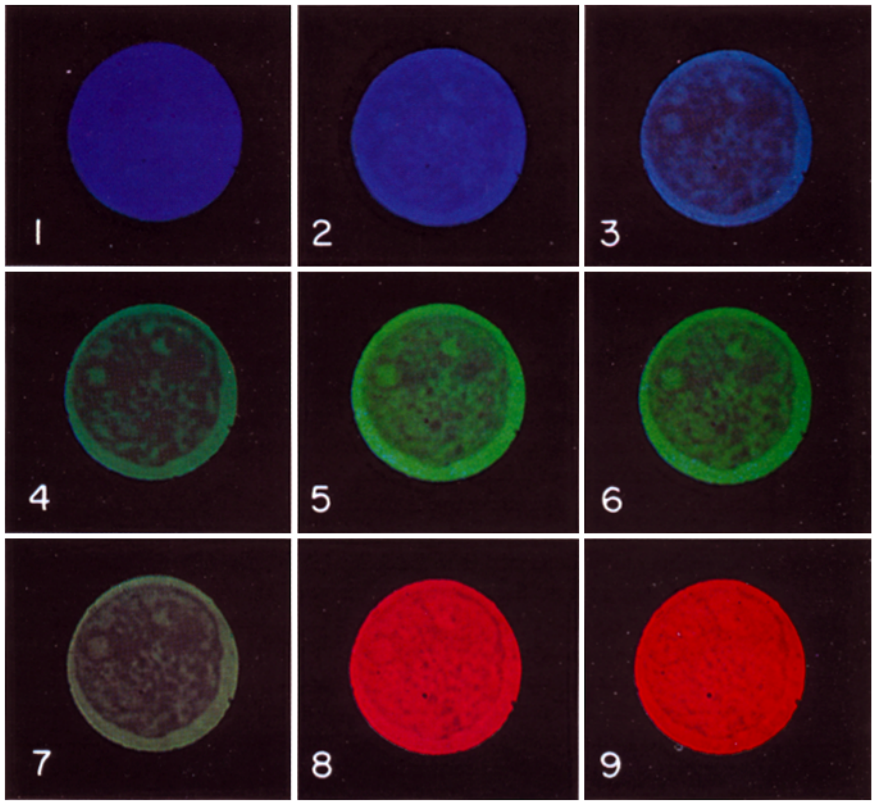
The literature records considerable discussion about the appropriateness of off-peak measurements, or relative extinctions, in cytophotometry (4, 6, 8, 12). It is the purpose of this report to present data on the consistency of the results obtained experimentally when using off-peak compared with maximal absorption measurements for Feulgen-stained human pituitary nuclei measured in the Barr and Stroud integrating microdensitometer (2), and a discussion of the possible justification of such measurements.

MATERIALS AND METHODS

TISSSUE PREPARATION: Imprints from the cut surface of fresh, human, anterior lobe were made directly after receiving pituitary glands from postmortem examination. The preparations, on long coverslips, were snap frozen in isopentane over dry ice and then freeze-substituted with ethanol (14). When the substitution was complete, they were transferred to alcoholic formalin for fixation prior to Feulgen staining. In our hands the optimal time of hydrochloric acid hydrolysis for these preparations was 20 minutes, after which the aldehydes formed from the deoxyribonucleic acid were demonstrated by Lillie's cold Schiff reagent (7) at pH 2. After staining, the imprints were covered with a mylar strip over immersion oil of refractive index 1.5150.

INSTRUMENTATION: For determining the absorption of the Feulgen-stained nuclei, we used the Barr and Stroud, Ltd. integrating microdensitometer,

originally developed and described by Deeley in 1955 (2). The light source is a tungsten lamp whose intensity is controlled by a neutral density filter. Wavelengths over the visible spectra can be selected by simple rotation of a series of filters which are incorporated into the optical chassis of the machine. The scanning system permits the recording of the



FIGURES 1 to 9 Photographs taken through narrowed field of integrating microdensitometer at varying wavelengths.

Fig. 1, 450 mμ	Fig. 2, 475 mμ	Fig. 3, 500 mμ
Fig. 4, 525 mμ	Fig. 5, 550 mμ	Fig. 6, 575 mμ
Fig. 7, 600 mμ	Fig. 8, 626 mμ	Fig. 9, 650 mμ

Each figure depicts the same human pituitary nucleus. × 1500.

sum of all absorption in the microscopic field, which can, itself, be narrowed so as to include only a single nucleus. Thus, whole nuclei rather than selected areas are measured. For those nuclei whose dense chromatin makes them difficult to measure, the crushing condenser permits the squeezing of the nucleus through the mylar strip in order to insure that the specimen has a sufficiently high transmission to permit accurate measurement.

EXPERIMENTAL DESIGN: Typically there are a number of difficulties relating to the sample characteristics in efforts to assess the validity of the Beer-Lambert law. These include problems in determining sample thickness, the presence of interfering constituents, and the non-random distribution of stained material. (For an excellent treatment of these considerations, see the papers by Pollister and Ornstein, reference 11, and by Shugar, reference 13.) The experimental studies to be reported solved these in the following way: All measurements were made on pituitary imprints in which the region studied was only one cell thick; only whole nuclei whose structure was typical in appearance were measured; the Integrating Microdensitometer, by virtue of summing the absorption over the whole nucleus, minimizes the problem of non-random distribution of stained elements.

Finally, our experimental model was the assessment of absorption values for diploid and tetraploid interphase nuclei in the same preparations. Our hypothesis was that for all wavelengths, the diploid

value, x, should predict the tetraploid value, y; i.e., $y = 2x$.

Four pituitary glands from adult males were studied. Readings of 5 diploid nuclei and 2 tetraploid nuclei were obtained from each of the glands at 14 wavelengths ranging from 450 to 650 mμ. Intervals of 25 λ were observed except over the range 525 to 575 mμ, where narrower intervals (5 to 10 λ) were assessed.

RESULTS

Figs. 1 to 9 are color reproductions of a single nucleus as seen in the appropriately narrowed field of the microdensitometer at each of 9 wavelengths. It will be noted that the staining density appears to be greatest at 550 to 575 mμ, while in the red (625 to 650 mμ) and blue (450 mμ) the stained boundary of the nucleus is difficult to clearly ascertain. These differences in density are depicted in the curves in Fig. 10. This figure also demonstrates the change in slope of the curves connecting both 475 to 450 mμ and 625 to 650 mμ. The deviation of the point at 450 mμ can be expected from the effect of a non-specific, light-scattering component in the histological structures; the deviation at 650 mμ is probably due to the use of an immersion oil whose refractive index is imperfectly matched with that of the specimen (11). We note in passing that the deviation at 650 mμ seems to influence both the diploid and tetraploid nuclei similarly; this is not the case for the deviation at 450 mμ.

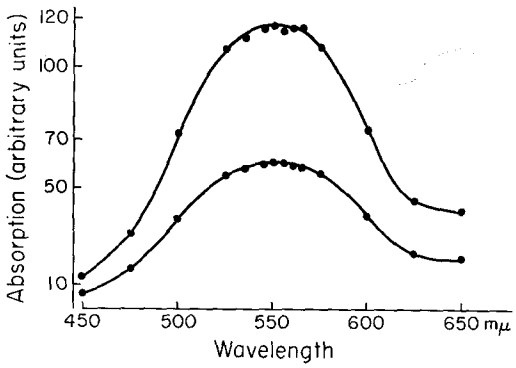


FIGURE 10 Absorption (in arbitary units) of Feulgen-stained tetraploid (upper curve) and diploid (lower curve) human pituitary nuclei over the visible spectrum.

Table I gives the measurements, in arbitrary units, for the four cases studied; readings for the diploid nuclei are in the x column, those for tetraploid nuclei, in the y column. These data can also be presented as the value of the ratio of tetraploid to diploid nuclei at each of the wavelengths recorded. They are charted in Table II and depicted graphically in Fig. 11.

It can be seen that there is very little variation in the tetraploid/diploid ratio over the range of wavelengths at which measurements were made. Our predicting equation, as derived statistically from these data, is $y = 1.95\,x$. The largest variance of this ratio, observed in Case 4, is 0.03. Whether

TABLE I

DNA (Arbitrary Units) in Diploid (x) and Tetraploid (y) Pituitary Cells as Measured at 14 Wavelengths

	Case 1		Case 2		Case 3		Case 4	
Wavelength	x	y	x	y	x	y	x	y
450	7.0	14.7	7.3	15.6	6.5	12.2	4.5	11.6
475	16.4	32.3	17.6	30.8	14.4	28.8	17.1	35.1
500	37.8	76.3	38.7	76.4	33.9	61.1	40.1	77.3
525	56.7	112.6	57.4	107.4	52.9	102.5	56.3	108.5
535	58.7	115.5	59.5	113.2	54.6	109.4	59.1	110.3
545	60.4	119.9	62.2	117.0	58.1	114.7	59.3	113.2
550	62.7	120.5	62.0	119.8	59.6	116.1	59.2	113.0
555	63.3	122.0	60.5	117.5	57.2	105.4	59.6	114.6
560	60.7	119.1	61.4	114.8	55.2	110.6	60.5	121.4
565	60.0	118.3	57.3	115.5	58.1	115.3	59.0	116.9
575	58.8	107.0	54.6	107.3	56.1	115.0	55.2	103.0
600	38.4	76.3	37.9	73.0	39.9	74.4	39.0	74.1
625	23.3	44.6	23.8	45.2	24.1	47.4	23.4	44.6
650	21.7	40.4	22.3	43.7	21.0	40.6	21.2	39.4

this represents a biologically significant departure from the theoretical ratio of 2.00 is difficult to assess, but it should be noted that if the ratios at 450 mμ were excluded, as could be justified on the grounds that readings at this wavelength were inaccurate because of the light-scattering effect, the variance in each series would be much smaller. Put another way, the data in Table II reveal that in the 56 ratios tallied, only 11 are 2.00 or above; were we to exclude the values at 450 mμ, we would also exclude more than ¼ of these values.

TABLE II

Ratio of Tetraploid/Diploid Absorption

Wavelength	Case 1	2	3	4
450	2.10	2.14	1.88	2.58
475	1.97	1.75	2.00	2.05
500	2.02	1.97	1.80	1.93
525	1.99	1.87	1.94	1.93
535	1.97	1.90	2.00	1.87
545	1.98	1.88	1.97	1.91
550	1.92	1.93	1.95	1.91
555	1.93	1.94	1.84	1.92
560	1.96	1.87	2.00	2.01
565	1.97	2.02	1.98	1.98
575	1.82	1.97	2.05	1.87
600	1.99	1.93	1.86	1.90
625	1.91	1.90	1.97	1.91
650	1.86	1.96	1.93	1.86

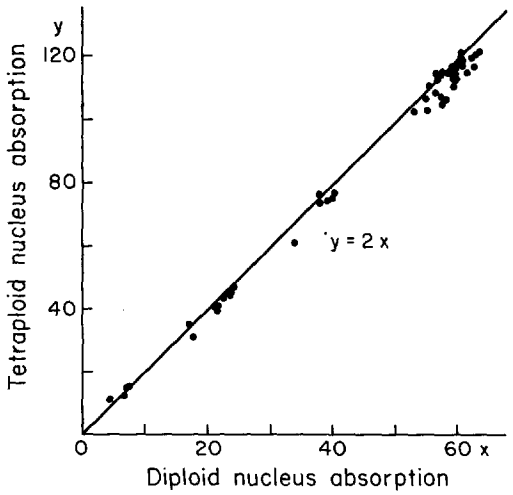


FIGURE 11 Tetraploid nucleus absorption of Feulgen-stained pituitary cells as a function of diploid nucleus absorption. Dark line depicts theoretical ratio of $y = 2x$.

DISCUSSION

Experimental justification of off-peak absorption measurements is provided by this study of Feulgen-stained anterior pituitary nuclei in which the preparations were made and measured so as to avoid the problems due to section thickness and non-random distribution of stained material. Choice of diploid and tetraploid interphase nuclei provided a 2 n–4 n system in which the theoretical predictor of the tetraploid nucleus would be exactly twice the diploid nucleus (15). The predicting equation developed in our studies is $y = 1.95\ x$.

Hale (5) has recently reported that the tetraploid/diploid ratio of liver and kidney cells measured under the same experimental conditions used herein is consistently, but not invariably, greater than 2. The values from microspectrophotometric studies of pituitary nuclei thus far reported (3, 9) suggest that there may be unusual variability of the cell population with respect to diploid DNA content. Should this be confirmed, it is possible that the tetraploid nuclei measured in this study represent a cell type different from that being sampled for diploid nuclei; in such a case the ratio might spuriously depart from the theoretical one. The high incidence of tetraploid nuclei in the adult pituitary observed in this study and our earlier one (3) does depart from the expected values for a gland in which mitosis is so rarely seen; further work is in progress to identify the cytoplasmic lineage of the tetraploid cells.

With regard to the Schiff stain (1), which had been known to follow the Beer-Lambert relationship at some wavelengths, our findings suggest that the measurement of absorption values at 2 wavelengths for 2 structures will yield valuable data on whether or not one is dealing with the same substance, thus corroborating the suggestion made in 1952 by Pollister (10).

The Beer-Lambert relationship, which was originally presented empirically, can, of course, be derived on the assumption that the decrease in the electromagnetic intensity with distance depends upon the original intensity, the wavelength, the concentration of absorbing substance, and an intrinsic constant characteristic of the material. If the Beer-Lambert relationship is experimentally

found to hold at one wavelength, can we state that it will hold at all other wavelengths? One could argue that in the derivation

$$\frac{dI}{dx} = -I \cdot c \cdot \epsilon$$

where I = intensity, x = distance, c = concentration, and ϵ is the extinction coefficient, we set $\epsilon = f(\lambda)$, $\epsilon \not\equiv f(c)$ in order to arrive at the final result of $I = I_0 e^{-x \cdot c \cdot \epsilon}$ (that is, we assumed ϵ was a function of the wavelength, but not of the concentration). Such an assumption is not a guarantee that extraneous phenomena will not occur at other wavelengths. At a constant concentration, the optical density as a function of the wavelength has the properties of being continuous, single valued (in the sense that each wavelength has associated with it only 1 OD) and positive or zero (therefore real in the region we are considering). These, however, are not stringent mathematical requirements, and do not preclude aberrations such as dimerization, and so on.

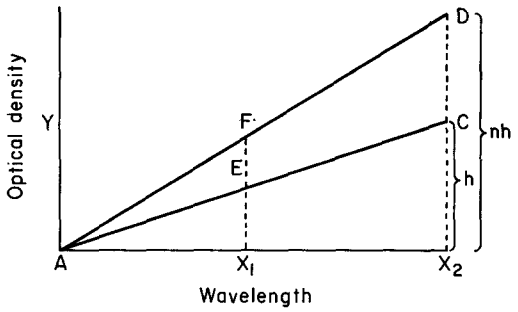


FIGURE 12 Schematic diagram showing optical density as a function of wavelength. X_1 is an arbitrary wavelength; X_2 represents an absorption maximum.

The fact that rigorous proof cannot be obtained that the Beer-Lambert relationship holds at all points, if it has been found to be valid at one wavelength, need not necessarily deter us from use of off-peak measurements. There is one case in which there is highly suggestive geometric reasoning as to the use of off-peak studies. Assume that the Beer-Lambert relationship has been experimentally found to hold at a particular wavelength. Also assume that two or more absorption curves, determined at different concentrations, proceed from a common zero point, to the wavelength under discussion. If on a linear wavelength scale these curves approximate straight lines, then the Beer-Lambert relationship must hold at all wavelengths along the experimentally determined lines. Consider the diagram in Fig. 12 showing the optical density as a function of λ. By the properties of similar triangles:

$$\frac{X_1}{X_2} = \frac{E}{C} \quad \text{and} \quad \frac{X_1}{X_2} = \frac{F}{D}$$

Therefore, $E/C = F/D$ and $D/C = F/E$. By inspection then, the optical density can readily be read off at other points along the straight line.

If the best fitting curve to the data is a straight line, it follows that the rate of change of absorbance with respect to wavelength is constant. This means that for a given increase, or decrease, in wavelength, we get the same change in absorbance. From this it follows that absorbance readings at any wavelength in such a region will be equally valid to those taken at the peak. It may here be noted that the limiting conditions imposed for this justification, *i.e.* the best fitting curve to the data being a straight line, can be met for almost all absorption spectra over a sufficiently narrow range. The illustration suggests that the more well behaved the absorption spectrum, the wider the wavelength range over which these measurements will be valid. It could be argued that finding linear relationships at two or more concentrations does not insure that this will hold for all other concentrations. This is, of course, true, but if the relationship holds at the concentration extremes to be encountered in the experiment, then it will likely hold at the intermediate values.

We wish to emphasize that we do not deny that there may be legitimate objections to off-peak measurements. Among the more important practical objections are two: (*a*) slight variations in wavelength cause more change when on the slope than when at the absorption plateau; (*b*) in most measuring systems there is a constant error as well as a percentage error, and off-peak measurements are, by definition, smaller and hence their accuracy more compromised. But the objections are exclusively ones of technique.

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