# QUANTITATIVE CYTOCHEMICAL DETERMINATION OF DESOXYRIBONUCLEIC ACID WITH THE FEULGEN NUCLEAL REACTION

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Since the work of Feulgen and Rossenbeck (16) the Feulgen nucleal reaction has been used extensively to demonstrate and localize desoxyribonucleic acid (DNA) in histological preparations. From time to time both the specificity of the reaction and its ability to indicate the site of DNA in the cell have been challenged (5, 29–30). Although these challenges have been of value in pointing out the dangers of uncritical use (*cf.* reference 3), there can be little doubt today that the Feulgen reaction, if used with proper controls, is specific for DNA in histological preparations, and that it shows the site of localization of DNA in the cell (4, 9, 23, 33).

The quantitative use of the Feulgen nucleal reaction as a microchemical tool was investigated by Jorpes (19), Widström (39), Caspersson (6), and Brachet (3). Recently the Feulgen nucleal reaction has been used by several authors for a quantitative determination of DNA in histological preparations (14, 15, 24, 25, 32).

Quantitative cytochemical determinations cannot be relied upon until the methods used have been checked in some way against a standard chemical procedure. In the absence of such a comparison with accepted techniques, cytochemical methods based on light absorption may well be reproducible, but nothing can be stated with regard to their accuracy. In most instances it would be very difficult to devise such a comparison of cytochemical and chemical procedures, but in the case of desoxyribonucleic acid this has become possible with the discovery of the constancy of the DNA content in diploid nuclei of certain vertebrates (2, 22, 37, 38). This constancy permits a comparison of cytochemical determinations on single nuclei with the average value obtained by chemical methods on a large number of isolated nuclei. A series of such values for the DNA content of various vertebrate nuclei (Mirsky and Ris (22) and unpublished data) served as standards in the work to be described in this paper demonstrating that the Feulgen nucleal reaction can be used as a quantitative cytochemical procedure.

## *Apparatus and Procedure of Measurement*

The intensity of the Feulgen reaction in nuclei was measured by using the microscope as a colorimeter. The instrument used has been described and illustrated else-

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where (24). A mercury vapor arc ($AH_4$) served as light source. With a Wratten filter No. 74 the green line (546 m$\mu$) was isolated and used to illuminate the microscope. In the optic axis, 10 inches from the ocular, the phototube was mounted with a circular diaphragm that limited the area in the image plane. The phototube used was a RCA electron multiplier tube (1 P 28) in connection with the photovolt electronic photometer Model 512. The phototube was mounted interchangeably with a focusing magnifier. A cell structure to be measured was moved into the center of the field, then focussed upon in the image plane with the focussing magnifier, and moved into the center of the image plane-diaphragm. This diaphragm was closed to the size of the area to be measured. Then the focussing magnifier was replaced by the phototube and a reading was taken. The specimen was now moved until an empty area on the slide near it filled the image plane-diaphragm. From this and the first reading the transmission and extinction of the cell structure were calculated. The intensity of the light was adjusted with neutral density filters between light source and microscope.

The amount of absorbing substance can be calculated from the extinction and the dimensions of the structure if the specific extinction of the substance is known. According to the Beer-Lambert law the following equation holds:

$$C_x = \frac{E_x}{E_s D_x} \text{ mg./cc.} \tag{1}$$

where $C_x$ = the unknown concentration in the cell structure, $E_x$ = the extinction of the cell structure, $D_x$ = the thickness of the absorbing layer in centimeters, and $E_s$ the extinction of 1 mg./cc. of the substance with 1 cm. thickness of the absorbing layer as determined for instance in the Beckman spectrophotometer. The total amount of substance present is obtained by multiplying the concentration by the volume of the cell structure. In the simplest case, if the structure is cylindrical, we find:

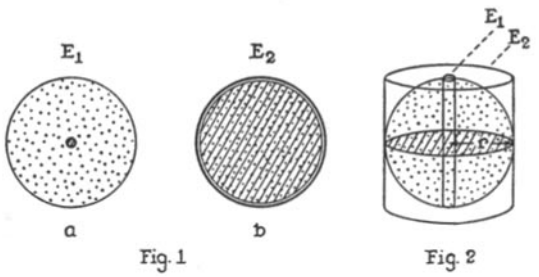
$$\text{Total amount} = \frac{E_x A D_x}{E_s D_x} \text{ mg./cc.;} \qquad \text{or} \tag{2}$$

$$\frac{E_x A}{E_s} \text{ mg./cc.} \tag{3}$$

where $A$ is the cross-sectional area of the cylinder measured in square centimeters. We see from equation (3) that the thickness of the cylinder measured does not have to be known. This is of great advantage, since accurate thickness measurements with the microscope are very difficult. The cross-sectional area is easily determined from the diameter of the image plane-diaphragm and the magnification used.

In the case of cell nuclei, however, we are not dealing with cylinders, but, in the simplest case, with spheres. The concentration in a sphere can be found by measuring the extinction of a small central cylinder and the diameter of the sphere (Fig. 1 *a*). But nuclei are rarely perfectly spherical, so that errors in the

measurements of diameters may lead to great errors in the determination of the volume. It would be of great advantage if it were feasible to use the extinction of the cylinder closely circumscribing the sphere (Fig. 1 *b* and Fig. 2). The determination of the total amount of absorbing substance would then become very simple by using equation (3). However, an error is introduced by this method, since the absorbing material is not evenly distributed in the cylinder measured, but concentrated in the sphere within the cylinder. It is shown below how the error thus introduced can be determined empirically. It was found to be within a few per cent and therefore negligible. We are, therefore, justified in the case of nuclei which are nearly spherical in shape to circumscribe with the image plane-diaphragm the maximal cross-sectional area of the nuclei and to apply equation (3) for calculating the total amount of absorbing substance contained in the nuclei.




FIGS. 1 and 2. For explanation see text.

For relative measurements it is not necessary to know $E_s$ in equation (3). $E_x A$ is then a convenient comparative value.

### *Checks of Apparatus and Procedure of Absorption Measurements*

Since so many factors influence absorption measurements with the microscope, some of which cannot be evaluated exactly by theoretical considerations alone, it is necessary to check the method against some independent determinations with a standard procedure. In the same way some of the factors influencing absorption measurements were investigated empirically. (For a theoretical discussion of the possibilities and limitations of absorption measurements consult the fundamental paper by Caspersson (7).)

1. A very simple check on the microscope colorimeter will be described first. Lightly colored cellophane (for instance bluish-red, similar in color to the Feulgren reaction) is cut into strips. Three such strips are mounted in clarite on a microscope slide so that they form steps of 1, 2, and 3 layers in thickness. The slide is fixed to a cardboard with a slit, so that it can be fitted into the Beckman spectrophotometer. The extinctions for the 1, 2, and 3 layers are determined using the spectrophotometer with minimal

slit width according to the instructions furnished by National Technical Laboratories. In this way a spectral band width of only 2 to 3 mμ is obtained at the setting for 546 mμ. The extinctions of the cellophane strips are then determined with the microscope colorimeter, at a wave length of 546 mμ ($AH_4$ mercury vapor arc and Wratten filter No. 74). Table I gives the values obtained with a 2 mm. and 4 mm. apochromat (Zeiss) and with the Beckman spectrophotometer. They show that for objects of uniform thickness the microscope colorimeter gives results which are in good agreement with those obtained with the Beckman spectrophotometer.

2. To simulate more closely the conditions encountered in measuring absorption of cell nuclei another check was used which had been described first by Caspersson (7). Sudan IV was extracted for 24 hours with hexane. A component of the dye went into solution. The insoluble part was centrifuged off and the extinction of the dye in hexane was measured in the Beckman spectrophotometer at 546 mμ. The hexane solution was then emulsified in 30 per cent sucrose by shaking in a test tube. The dye is insoluble in sucrose and, with the refractive index of hexane and 30 per cent sucrose

TABLE I

*Absorption of Cellophane as Determined in the Beckman Spectrophotometer and with the Microscope (546 mμ)*

| Cellophane layers | *E* Beckman | *E* microscope | |
|---|---|---|---|
| | | 4 mm. objective | 2 mm. objective |
| 1 | 0.130 | 0.131 | 0.125 |
| 2 | 0.265 | 0.268 | 0.244 |
| 3 | 0.385 | 0.377 | 0.367 |

almost identical, microscopic absorbing but non-refractile droplets were obtained. A drop of the emulsion was introduced into a cell about 100 μ deep, which was then placed on the microscope stage. Using a small central spot for absorption measurements the extinction at 546 mμ and the diameter of thirty droplets were measured with a 2 mm. and 4 mm. objective. In Fig. 3 the extinction of each droplet is plotted against the diameter. The solid line is calculated from the value obtained with the Beckman spectrophotometer. The average extinction for 1 cm. thickness was then calculated for the thirty measurements and compared with the extinction obtained with the Beckman spectrophotometer (Table II). The measurements on the droplets obtained with the microscope colorimeter are in close agreement with the measurements on the solution with the spectrophotometer.

3. These droplets of Sudan IV in hexane suspended in sucrose also made it possible to determine empirically the error introduced by calculating the amount of absorbing substance in a sphere from the extinction of a cylinder enclosing the sphere according to equation (3). On twenty-five droplets the following measurements were made: (*a*) the extinction ($E_1$) using a small central spot was determined at 546 mμ (Figs. 1*a* and 2); (*b*) the extinction ($E_2$) of the cylinder enclosing the same droplet was then measured (Figs. 1 *b* and 2). The image plane-diaphragm was closed on the maximal cross-

sectional area of the sphere (area = $\pi r^2$). $E_1$ is proportional to the concentration in the droplet, while $E_2$ is proportional to the average concentration in the cylinder around the sphere, neglecting the error due to the uneven distribution of absorbing material in the cylinder. Since the volume of the cylinder is $\frac{3}{2}$ of the volume of the

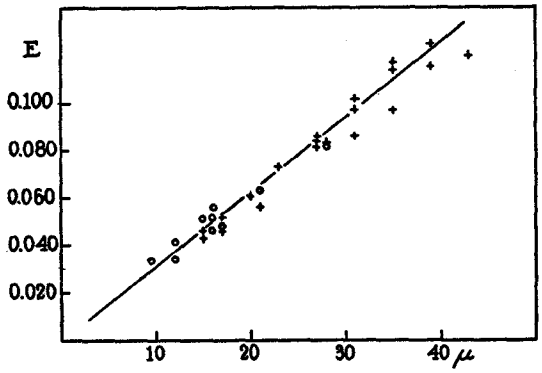



FIG. 3. Absorption of 30 droplets of Sudan IV in hexane, suspended in 30 per cent sucrose, measured with the microscope (+ with 4 mm. objective, O with 2 mm. objective). Extinction plotted against diameter of droplets. The solid line is calculated from the extinction of the same solution of Sudan IV in hexane as determined in the Beckman spectrophotometer.

TABLE II

*Absorption of Saturated Solution of Sudan IV in Hexane as Determined in the Beckman Spectrophotometer and on 30 Droplets with the Microscope*

| Sudan IV in hexane | Beckman $E$ for 1 cm. | Microscope Average $E$ calculated for 1 cm. | Difference |
|---|---|---|---|
| Diluted 50 × | 0.615 | — | |
| " 100 × | 0.325 | — | |
| Undiluted (calculated) | 31.6 | 30.5 ± 0.44 | 3.5 per cent |

sphere and the total amount of absorbing material is the same, the average concentration in the cylinder must be $\frac{2}{3}$ of the concentration in the sphere. Consequently

$$\frac{E_1}{E_2} = 1.5$$

The average ratio $\frac{E_1}{E_2}$ obtained from measurements on twenty-five nuclei was found to be 1.45. The error due to the uneven distribution of absorbing substance in the cylinder around the droplet is therefore very small and can be neglected. We are thus justified in using the area circumscribing the sphere for absorption measurements and calculating the total amount of absorbing substance according to equation (3).

4. The nuclei one encounters in tissues are rarely exactly spherical. It is therefore sometimes necessary to use an area larger than $\pi r^2$ to enclose all nuclear material. This, of course, increases the unevenness of distribution of absorbing material in the space measured. In the following series of measurements an attempt was made to estimate the error introduced by making the area of the image plane-diaphragm larger than the maximum cross-sectional area of the nucleus. The nuclei used were treated as described later in this paper to give an even distribution of DNA and consequently uniform Feulgen staining inside the nucleus. A large nucleus of low DNA concentration (turtle liver nucleus) and a small nucleus with high DNA concentration (turtle erythrocyte nucleus) were measured. First the image plane-diaphragm was closed exactly around the nuclear membrane, then the area measured was increased 1.2 times and 1.5 times. For each setting of the image plane-diaphragm ten determinations of $E$ times area were made on the same nucleus. From Table III one sees that with the large and lightly stained nucleus the increase in non-absorbing area around the nucleus did not affect the result. With such nuclei the image plane-diaphragm does

TABLE III

*Effect on Absorption Measurements of Uneven Distribution of Absorbing Substance in the Area Measured*

| Turtle liver nucleus lightly stained | | | | Turtle erythrocyte nucleus densely stained | | | |
|---|---|---|---|---|---|---|---|
| Area $\mu^2$ | | $E \times$ area | $n$* | Area $\mu^2$ | | $E \times$ Area | $n$ |
| 41 | 1 | 5.5 ± 0.03 | 10 | 20 | 1 | 5.2 ± 0.04 | 10 |
| 50 | 1.2 | 5.4 ± 0.08 | 10 | 23 | 1.2 | 4.6 ± 0.03 | 10 |
| 63 | 1.5 | 5.6 ± 0.04 | 10 | 31 | 1.6 | 4.4 ± 0.04 | 10 |

* $n$ = number of determinations on the same nucleus. Mean and standard deviation.

not have to fit closely around the nucleus. With the smaller and more densely stained erythrocyte nucleus, however, the increase in non-absorbing area around the nucleus caused a considerable decrease in the product of extinction times area, as is to be expected with the uneven distribution. In measuring small, densely stained nuclei, therefore, even distribution of the absorbing material is essential and the image plane-diaphragm has to fit closely around the outline of the nucleus.

5. The microscope colorimeter differs from the usual colorimeter or spectrophotometer in that the light rays are not parallel through the absorbing material, but they form a definite angle depending on the numerical aperture of the system. The effect of this on absorption measurements was discussed by Caspersson (7) and by Uber (36). The difference in absorption as compared with parallel light is almost impossible to evaluate, since a change in the aperture of the system causes a change in other factors affecting absorption. A decrease in the aperture of the light cone for instance increases the error due to light diffraction and a larger aperture of the light cone increases the error due to scattered light. Therefore, a series of measurements were made to determine empirically the effect of a change in the aperture of the light cone. Ten independent determinations of $E$ times area for a carp liver and erythrocyte nucleus stained with Feulgen were made, with the substage diaphragm set at a nu-

merical aperture of 0.25 and 0.85. The results are given in Table IV. The increase in numerical aperture from 0.25 to 0.85 caused only a negligible change in the measurement. In the following measurements the substage diaphragm was usually set between 0.3 and 0.6 numerical aperture.

6. Glare caused by stray light is an important factor causing errors in absorption measurements. To reduce such scattered light the field diaphragm on the lamp should be closed so that only a small area around the structure to be measured is illuminated. The same opening of the field diaphragm should be used for all measurements that are to be compared.

7. In the method described here the area illuminated on the phototube changes according to the size of the structure measured. Therefore, it must be made certain that the response of the phototube is uniform over the area used. This was checked

TABLE IV

*Effect of Numerical Aperture of System on Absorption Measurements with the Microscope*

| Numerical aperture, condenser | Carp liver nucleus | | Carp erythrocyte nucleus | |
|---|---|---|---|---|
| | $E \times$ area | $n$* | $E \times$ area | $n$ |
| 0.25 | 3.85 ± 0.05 | 10 | 3.5 ± 0.02 | 10 |
| 0.85 | 3.96 ± 0.02 | 10 | 3.68 ± 0.02 | 10 |

* $n$ = number of determinations on same nucleus.

TABLE V

*Uniformity of Response of Phototube for Various Areas Measured*

| | | | | | | |
|---|---|---|---|---|---|---|
| Diameter of image plane-diaphragm | 4 | 5 | 7 | 8 | 9 | 10 |
| Reading on galvanometer | 16 | 25 | 48 | 65 | 80 | 95 |

in the following way. The microscope was focussed on a slide which was then moved to an empty area so that the image plane was uniformly illuminated. The image plane-diaphragm was then set for various diameters from 4 to 10 mm. and the response on the galvanometer was read for each diaphragm setting. As we see from Table V the response of the phototube was proportional to the area up to 10 mm. diameter.

### *The Feulgen Nucleal Reaction*

The exact course of the Feulgen nucleal reaction is still not fully understood. But it is generally assumed that the reaction involves a liberation of aldehyde groups through hydrolysis and that these aldehyde groups react with the leuco-basic fuchsin, forming a colored product. This aldehyde-pararosaniline–$SO_2$ dye has a broad absorption maximum from 550 m$\mu$ to 570 m$\mu$ (6, 34). Caspersson found that in the test tube the intensity of the color developed is proportional to the amount of DNA within certain limits of concentration (0.02 per cent to 1.5 per cent), but only if the sample consisted of purified DNA and especially

in the absence of proteins. The intensity of color developed by a given sample of DNA varied greatly, depending especially on the pH, time and temperature of hydrolysis, the pH of the staining solution, and the length of time the reaction was allowed to proceed. The reaction was never found to be quantitative, only part of the DNA taking part. Since it was also impossible to completely standardize the reagents, the reaction could only be used in conjunction with a standard treated together with the unknown.

Some of the factors which affect the intensity of the reaction in histological preparations will be discussed now.

TABLE VI

*Time of Hydrolysis and Intensity of Feulgen Reaction after Acetic Acid–Alcohol Fixation (Calf Liver)*

| Time of hydrolysis | $E \times$ area $n^* = 5$ |
|---|---|
| *min.* | |
| 2 | 3.1 ± 0.08 |
| 3 | 3.7 ± 0.12 |
| 4 | 3.8 ± 0.07 |
| 6 | 5.2 ± 0.13 |
| 8 | 5.9 ± 0.14 |
| 10 | 5.9 ± 0.17 |
| 11 | 6.1 ± 0.10 |
| 12 | 6.0 ± 0.05 |
| 13 | 6.0 ± 0.06 |
| 15 | 6.1 ± 0.06 |
| 20 | 4.8 ± 0.10 |

* $n$ = number of nuclei measured.

*1. Time of Hydrolysis.*—The effect of time and temperature of hydrolysis on the intensity of the Feulgen reaction was first investigated by Bauer (1) who found that it depended on the type of fixative used. With certain fixatives, hydrolysis exceeding an optimum decreased the intensity of the color developed while with other fixatives prolonged hydrolysis did not change the maximal color intensity. This was confirmed by Hillary (17) on DNA in agar blocks and plant nuclei and by the absorption measurements of Di Stefano (14, 15).

In the present work two fixatives were used: (*a*) acetic acid–alcohol (1:3), and (*b*) formalin (10 per cent and 20 per cent). In the following some measurements on the relation of time of hydrolysis to the intensity of the color after these two fixatives will be reported.

(*a*) *Fixation with Acetic Acid–Alcohol.*—Calf liver nuclei isolated according to the citric acid method (*cf.* reference 21) were fixed with acetic acid–alcohol, smeared on a slide, and dried. They were then hydrolyzed for various lengths of time in 1 N HCl at 60°C. and stained in leucobasic fuchsin for 1 hour. After rinsing in $SO_2$ water three times for 5 minutes each, they were rinsed in tap water for 15 minutes, dehydrated, and mounted in clarite X. A slide treated in the same way except for the leucobasic

fuchsin was mounted in clarite X to determine the blank; *i.e.*, the absorption of unstained nuclei at 546 mμ. It was found that unstained nuclei are practically invisible in clarite and transmit over 95 per cent of light. The blank was therefore neglected for nuclei mounted in clarite in all the measurements reported below.

The absorption of spherical nuclei was measured as described above. Since we were only interested in relative measurements, $E_s$ in equation (3) does not have to be known and extinction times area was used as a measure of the dye content. The values for extinction times area after various periods of hydrolysis are given in Table VI. There is a broad maximum of color intensity from 8 minutes to 15 minutes of hydrolysis. Longer hydrolysis decreases the intensity of the reaction. In the following 12 minutes of hydrolysis were used whenever the material had been fixed in acetic acid–alcohol.

(*b*) *Fixation with Formalin.*—Rat liver perfused with 10 per cent formalin and frog liver fixed in 20 per cent formalin were sectioned 10μ and 15μ thick, hydrolyzed for

TABLE VII

*Time of Hydrolysis and Intensity of Feulgen Reaction after Fixation in Formalin*

| Time of hydrolysis | Rat liver nuclei (tetraploid) $E \times$ area ($n = 10$) | Frog liver nuclei $E \times$ area ($n = 10$) |
|---|---|---|
| *min.* | | |
| 4 | — | 8.8 ± 0.11 |
| 5 | 8.1 ± 0.14 | — |
| 8 | — | 12.0 ± 0.2 |
| 10 | 10.3 ± 0.04 | 12.3 ± 0.17 |
| 12 | — | 12.4 ± 0.14 |
| 13 | 10.9 ± 0.17 | 13.8 ± 0.17 |
| 15 | 12.1 ± 0.1 | 13.8 ± 0.16 |
| 20 | 11.8 ± 0.14 | 14.2 ± 0.20 |
| 25 | 12.2 ± 0.14 | 13.9 ± 0.14 |

various lengths of time, stained for 1 hour in leucobasic fuchsin, and mounted in clarite. The absorption of spherical nuclei (in medium size rat nuclei = tetraploid, see below) was measured and extinction times area calculated. Table VII shows the effect of time of hydrolysis on the intensity of color produced. A maximum intensity was reached after 13 minutes of hydrolysis and continuous hydrolysis up to 25 minutes did not decrease the intensity of the reaction. In the measurements reported below 15 minutes of hydrolysis was used after formalin fixation.

(*c*) *Comparison of Maximal Color Intensity after Acetic Acid–Alcohol and Formalin Fixation.*—Calf liver nuclei fixed in acetic acid–alcohol and with formalin were hydrolyzed for 15 minutes and stained together. As Table VIII shows the maximal intensity of the reaction is the same after the two fixatives.

2. *Time of Staining.*—Caspersson (6) found that in the test tube the color intensity varied with the time the reaction was allowed to proceed. A maximum was reached after 3 to 4 hours after which the intensity decreased rapidly. To determine the influence of time of staining in histological preparations, calf liver fixed in acetic acid–alcohol was sectioned 10μ thick, hydrolyzed for 12 minutes, and stained for

various periods of time. The results are shown in Table IX. The maximum color intensity of the nuclei is reached within one-half hour. Prolonged immersion in leucobasic fuchsin decreases the intensity of the reaction, probably due to continuous hydrolysis in the acid solution.

*3. Method of Preparation of the Leucobasic Fuchsin.*—Feulgen and Rossenbeck (16) described two methods for the preparation of the leucobasic fuchsin. It can be prepared by bubbling $SO_2$ gas through the basic fuchsin solution until it is decolorized or by adding sodium metabisulfite (or potassium metabisulfite (*cf.* reference 12)) and

TABLE VIII

*Maximal Intensity of Feulgen Reaction in Calf Liver Nuclei after Acetic Acid–Alcohol and Formalin Fixation*

| Fixation | $E \times$ area ($n = 5$) |
|---|---|
| Acetic acid–alcohol | 6.1 ± 0.06 |
| Formalin 20 per cent | 6.1 ± 0.1 |

TABLE IX

*Effect of Time of Staining on the Intensity of the Feulgen Reaction in Calf Liver Nuclei (Fixation Acetic Acid–Alcohol)*

| Time of staining in leucobasic fuchsin.... | 30 min. | 1 hr. | 1 hr., 30 min. | 24 hrs. |
|---|---|---|---|---|
| $E \times$ area ($n = 5$) | 6.8 ± 0.1 | 6.5 ± 0.1 | 6.9 ± 0.1 | 4.7 ± 0.1 |

TABLE X

*Method of Preparation of Leucobasic Fuchsin and Intensity of Feulgen Reaction. (Mouse Liver, Diploid Nuclei; Fixation Acetic Acid–Alcohol)*

| Preparation of leucobasic fuchsin | $E \times$ area $n = 5$ |
|---|---|
| Rafalko (1946) | 5.1 ± 0.17 |
| Coleman (1938) | 5.0 ± 0.17 |

N HCl to the basic fuchsin solution. Since the pH of the reagent influences the intensity of color produced and it is very difficult to control this pH with the first method, Feulgen and Rossenbeck (16) and later Caspersson (6) recommended the second method of preparation. Recently Rafalko (26) claimed that the reagent prepared by bubbling $SO_2$ gas through the basic fuchsin produced a more intense coloring of nuclear constituents. To compare the color intensity produced by the two types of reagents mouse liver sections fixed in acetic acid–alcohol were stained in the two reagents and the absorption of the smallest nuclei present was measured. The results are presented in Table X. The intensity of color produced was the same with both reagents. Actually, if the excess $SO_2$ in the reagent prepared according to Rafalko was not removed by letting it stand exposed to air for several hours, the intensity of color produced was less than with the reagent prepared with metabisulfite.

*4. Fading of the Feulgen Reaction.*—The nuclei were always measured within 24 hours after the treatment with leucobasic fuchsin. One slide was measured again after 4½ months and the same result was obtained as right after staining. The intensity of the Feulgen stain in microscopic preparations therefore does not change within several months at least (*cf.* reference 17).

### *Measurements on Various Nuclei of Known DNA Content*

*1. Liver Nuclei.*—In the course of a study on the DNA content of nuclei in the tissues of vertebrates, the DNA content of isolated liver nuclei of a number of vertebrates had been determined (Mirsky and Ris (22), and unpublished data). Some of these nuclei were fixed in acetic acid–alcohol, smeared on slides, and stained by the Feulgen procedure. The relative intensity of the reaction was then measured with the microscope colorimeter and compared with the DNA content as determined by chemical methods on a known number of nuclei. The results are shown in Table XI.

TABLE XI

*Intensity of Feulgen Reaction and DNA Content of Isolated Nuclei of Shad (Alosa sapidissima), Chicken, and Calf Liver, the Calf Liver Used as Standard. Fixation Acetic Acid–Alcohol*

| | Shad | Chicken | Calf |
|---|---|---|---|
| $E \times$ area | 1.20 ± 0.04 ($n$ = 5) | 1.57 ± 0.05 ($n$ = 10) | 4.3 ± 0.17 ($n$ = 5) |
| DNA per nucleus mg. $\times 10^{-9}$ chemical determination | 2.0 | 2.4 | 6.5 |
| DNA per nucleus calculated from Feulgen calf as standard | 1.8 | 2.4 | 6.5 |

In the first row the values for extinction times area calculated from the absorption measurements are given. In the second row the DNA content of these nuclei is shown, and in the third row the DNA content of the same nuclei calculated from the Feulgen reaction by using the calf liver nucleus as standard. These values agree closely with those obtained by standard chemical determinations. It is therefore possible to determine the DNA content of nuclei with the Feulgen reaction, if nuclei of known DNA content are used as standard, treated together with the unknown.

Another series of measurements were made on sections of livers of five different fish. The DNA content in nuclei of these fish had been determined chemically on erythrocytes. It was shown that in fish the DNA content of erythrocyte nuclei and liver nuclei is the same (Mirsky and Ris (22), and unpublished data). Small pieces of liver were fixed in acetic acid–alcohol, sectioned at 10μ, hydrolyzed 12 minutes, and stained in leucobasic fuchsin for 1 hour. Sections of shark liver were placed on every slide as standard. In making absorption measurements on nuclei in sections one must, of course, make certain that the entire nucleus is within the section. Table XII shows the results for these nuclei. In the third row again the DNA content of the nuclei calculated from the Feulgen reaction by using the shark liver nuclei as

standard is given. By comparison with the second row we see that the relative values calculated from the Feulgen reaction agree with the microchemical determinations within about 10 per cent.

Since we used both smears of isolated nuclei and nuclei in sections, measurements were made to find out whether any DNA was lost during isolation of the nuclei or whether the DNA content as determined by the Feulgen reaction was the same in

TABLE XII

*Intensity of Feulgen Reaction and DNA Content of Liver Nuclei of Yellow Tail (Ocyurus chrysurus), Red Hind (Epinephelus guttatus), Houndfish (Tylosurus acus), Yellow Grunt (Haemulon flavolineatum) and Dusky Shark (Carcharias obscurus), with the Shark Used as Standard. Fixation Acetic Acid–Alcohol*

| | Yellow tail | Red hind | Houndfish | Yellow grunt | Dusky shark |
|---|---|---|---|---|---|
| $E \times$ area ($n = 5$) | 0.95 ± 0.03 | 0.76 ± 0.01 | 1.05 ± 0.04 | 0.55 ± 0.01 | — |
| Shark | 2.20 ± 0.03 | 2.20 ± 0.1 | 2.24 ± 0.05 | 2.28 ± 0.03 | — |
| DNA per nucleus mg. $\times 10^{-9}$ chemical determination | 2.1 | 2.1 | 2.2 | 1.2 | 5.5 |
| DNA per nucleus calculated from Feulgen shark as standard | 2.4 | 1.9 | 2.5 | 1.3 | 5.5 |

TABLE XIII

*Comparison of the Intensity of Feulgen Reaction in Isolated Calf Liver Nuclei and Calf Liver Nuclei in Sections. Fixation Acetic Acid–Alcohol*

| | $E \times$ area $n = 10$ |
|---|---|
| Isolated citric nuclei | 6.38 ± 0.14 |
| Nuclei in sections (10$\mu$) | 6.44 ± 0.11 |

isolated nuclei and in nuclei fixed *in situ*. Calf liver fixed in acetic acid–alcohol was sectioned at 10$\mu$. On the same slide calf liver citric nuclei which had been fixed in acetic acid–alcohol, were smeared and fixed on the slide by drying. The slide was then stained by the Feulgen procedure and the absorption of the nuclei at 546 m$\mu$ determined. From Table XIII it can be seen that the intensity of the Feulgen reaction is the same in isolated nuclei and in nuclei fixed *in situ*. Accordingly no DNA is lost during the isolation of these nuclei in citric acid.

In many tissues of mammals the nuclei are not all the same size. In a number of investigations by many authors it was found that in general the volumes of these nuclei form ratios of 1:2:4 etc. By stimulating such nuclei to divide it was demonstrated that the larger nuclei are polyploid, that with each step of

increase in volume the chromosome number had been doubled (27, 35). This polyploidy is especially pronounced in the liver of rodents. Rat liver, fixed in 10 per cent formalin by perfusion and sectioned at 15$\mu$, was used to find out whether the intensity of the Feulgen reaction was proportional to the number of chromosome sets in these polyploid nuclei. In rat liver three sizes of nuclei are common, corresponding presumably to a diploid, tetraploid, and octoploid number of chromosomes. In Table XIV the values for extinction times area obtained by absorption measurements on these nuclei stained with Feulgen are given for the three sizes of nuclei. The ratios of intensity of the Feulgen reaction are very close to 1:2:4 and are therefore proportional to the ratios of chromosome number.

TABLE XIV

*Size of Nuclei (Polyploidy) and Intensity of Feulgen Reaction in Rat Liver. Fixation 10 Per Cent Formalin*

| Size of nuclei | $E \times$ area $n = 10$ | Ratio |
|---|---|---|
| Smallest nuclei | 5.5 ± 0.1 | 1 |
| Medium sized nuclei | 10.4 ± 0.1 | 1.9 |
| Largest nuclei | 19.9 ± 0.2 | 3.6 |

TABLE XV

*Intensity of Feulgen Reaction in Hepatic and Erythrocyte Nuclei of the Dusky Shark (Carcharias obscurus) and the Chicken. Fixation Acetic Acid-Alcohol*

| | Liver nuclei $E \times$ area $n = 10$ | Erythrocyte nuclei $E \times$ area $n = 10$ | Ratio |
|---|---|---|---|
| | | | *per cent* |
| Dusky shark | 3.96 ± 0.13 | 2.70 ± 0.17 | 68 |
| Chicken | 1.57 ± 0.05 | 1.04 ± 0.02 | 66 |

*2. Measurements on Nuclei of Different Tissues.*—So far we have compared only nuclei of one tissue and with similar structure, distribution of chromatin, and DNA concentration. Does the proportionality of the Feulgen reaction with DNA content hold also for nuclei with entirely different structure and DNA concentration?

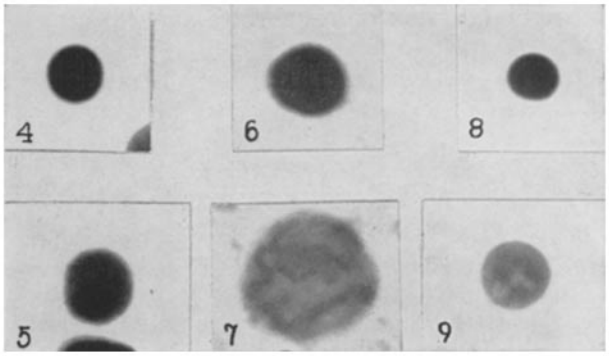
In the liver of lower vertebrates we find two types of nuclei which are convenient for the study of this question, namely the relatively lightly staining large hepatic nuclei and the much smaller and very densely staining nuclei of the erythrocytes. The intensity of the Feulgen reaction was measured for hepatic and erythrocyte nuclei in sections of shark liver and in smears of isolated nuclei from chicken liver, both after fixation in acetic acid-alcohol. The results are shown in Table XV. We find that in both shark and chicken the intensity of the Feulgen reaction as determined with the microscope colorimeter

is more than 30 per cent less in erythrocyte nuclei than in hepatic nuclei. However, it is known from chemical determinations that the DNA content of erythrocyte and hepatic nuclei is the same (Mirsky and Ris (22), and unpublished data). It seems, therefore, that it is not possible to determine the relative DNA content of nuclei from the intensity of the Feulgen reaction if these nuclei differ markedly in structure.

Considering a possible cause for this disproportionality one may think first of the great difference in the distribution of chromatin in these nuclei fixed with acetic acid–alcohol. Now it was found that the distribution of chromatin in nuclei depends very much on the state of the nucleus before fixation and on the type of fixative used (28). In the living nucleus and in the nucleus isolated in non-electrolytes (for instance sucrose) the chromatin is more or less uniformly distributed, since the chromosomes are greatly swollen and fill the entire nuclear space except for the nucleoli. Nuclei with the chromatin in this extended state can be fixed for instance with formalin without changing the distribution of the chromatin. After treatment with the Feulgen reagents the nuclei are then stained quite uniformly, only the nucleoli remaining unstained. In tissues removed from the animal the nuclei were always found to be in the condensed state no matter how quickly they were fixed. But the chromatin in these nuclei could be extended by isolating them in sucrose. No DNA was lost even if they remained in sucrose overnight in the cold (28).

The most uniformly stained nuclei were obtained in the following manner. The tissue was cut into small pieces in the cold and then homogenized in 30 per cent sucrose with an Elvehjem-Potter homogenizer. This was continued until microscopic examination showed most cells fragmented and the nuclei isolated. When there was much pigment present in the tissue, the pigment granules were removed by differential centrifugation according to the procedure of Hogeboom, Schneider, and Pallade (18). A drop of the homogenate was then smeared on a microscope slide and inverted on 20 per cent formalin in a Petri dish. After 10 minutes of fixation the slides were washed for 2 to 3 hours in running water to remove all traces of formaldehyde. After hydrolysis for 15 minutes in 1 N HCl at 60°C. the slides were stained with the leucobasic fuchsin for 1 hour, rinsed in $SO_2$ water in three changes for 5 minutes each, and mounted in clarite. The nuclei were now found to be stained uniformly by the Feulgen reaction. Figs. 4 to 9 show hepatic and erythrocyte nuclei prepared in this way from carp, chicken, and green turtle (*Chelonia mydas*).

Spherical nuclei were then selected and the intensity of the Feulgen reaction determined with the microscope colorimeter in both hepatic and erythrocyte nuclei. In measuring the absorption of erythrocyte nuclei it was made certain that the image plane-diaphragm fitted closely around the nuclei and no empty area was enclosed, since, as was discussed above, large errors are introduced by that. The results of these measurements are given in Table XVI. We see that with the much more even distribution of the chromatin in the nuclei the difference in the values extinction times area for the two types of nuclei has become negligible. The values obtained with this




FIGS. 4 to 9. Erythrocyte and hepatic nuclei isolated in sucrose, fixed with 20 per cent formalin, and stained with Feulgen. Note the uniform distribution of the DNA. Figs. 4 and 5, erythrocyte and hepatic nuclei of carp, Figs. 6 and 7 from green turtle, Figs. 8 and 9 from chicken. (2 mm. Zeiss apochromat, 15 x ocular. × 2400).

TABLE XVI

*Intensity of Feulgen Reaction in Hepatic and Erythrocyte Nuclei with Even Distribution of DNA. Nuclei Isolated in 30 Per cent Sucrose and Fixed in 20 Per cent Formalin*

| | Liver nuclei | | Erythrocyte nuclei | |
|---|---|---|---|---|
| | $E \times$ area | $n$ | $E \times$ area | $n$ |
| Carp | 3.8 ± 0.05 | 20 | 3.4 ± 0.04 | 20 |
| | 3.85 ± 0.05 | 10 | 3.5 ± 0.02 | 10 |
| Green turtle | 5.3 ± 0.1 | 16 | 5.3 ± 0.1 | 14 |
| (*Chelonia mydas*) | 5.5 ± 0.03 | 10 | 5.2 ± 0.04 | 10 |
| Chicken | 2.9 ± 0.04 | 20 | 2.6 ± 0.05 | 20 |

TABLE XVII

*Intensity of Feulgen Reaction and DNA Content in Liver Nuclei with Even Distribution of DNA. Nuclei Isolated in Sucrose and Fixed in 20 Per Cent Formalin*

| | Carp | Bull frog | Green turtle | Chicken |
|---|---|---|---|---|
| $E \times$ area $n = 20$ | 3.8 ± 0.05 | 18.3 ± 0.02 | 5.3 ± 0.1 | 2.9 ± 0.04 |
| DNA per nucleus mg. $\times 10^{-9}$ chemical determination | 3.3 | 15.7 | 5.1 | 2.4 |
| DNA calculated from Feulgen using carp as standard | 3.3 | 15.8 | 4.6 | 2.5 |
| DNA calculated according to Di Stefano (1948) mg. $\times 10^{-9}$ | 0.8 | 4.0 | 1.1 | 0.5 |

method from absorption measurements on Feulgen-stained nuclei are proportional to the DNA content even for nuclei with greatly different structure and DNA concentration.

Table XVII shows measurements on Feulgen-stained liver nuclei in the extended state from four different vertebrates. In the third row we find again the DNA content of these nuclei as calculated from the intensity of the Feulgen reaction, using the carp liver nucleus as standard. A comparison with the second row shows that the relative amounts of DNA for these nuclei as determined from the Feulgen reaction agree closely with the microchemical analysis of isolated citric nuclei.

### *Absolute Quantitative Determination of DNA from the Feulgen Nucleal Reaction*

Di Stefano (14, 15) and Pollister and Leuchtenberger (25) have calculated the absolute amounts of DNA per nucleus from absorption measurements on Feulgen-stained nuclei, by assuming with Wieland and Scheuing (40) that one molecule of basic fuchsin combines with two aldehyde groups liberated by the hydrolysis from two purine nucleotides, and that all the reactions involved proceed to completion. The specific extinction of the basic fuchsin -$SO_2$-aldehyde molecule was determined on leucobasic fuchsin colorized with formaldehyde, assuming that it is identical with the specific extinction of the basic fuchsin -$SO_2$-DNA-aldehyde.

The findings of Caspersson (6) on the Feulgen reaction *in vitro* and the recent work of Chong-Fu Li and Stacey (9) make it most unlikely that the Feulgen reaction on histological material is a quantitative process as assumed by those authors.

If we calculate the amount of DNA per nucleus from the intensity of the Feulgen reaction in the liver nuclei shown in Table XVII, we find that the result is indeed quite different from the values obtained by the standard microchemical analysis. The values calculated according to Di Stefano are about 25 per cent of the values obtained from microchemical analysis (Table XVII, fourth row).

It follows that the intensity of the Feulgen nucleal reaction can only be used for relative determinations of DNA in cellular structures. If absolute values are desired, standard nuclei of known DNA content must be treated together with the unknown material.

## DISCUSSION

If the Feulgen nucleal reaction is to be used for a quantitative cytochemical determination of desoxyribonucleic acid (DNA) it must be shown, (1) that the reaction is specific for DNA, (2) that the colored product is not diffusible but

remains at the site of DNA in the cell, and (3) that the intensity of color produced is proportional to the amount of DNA present.

There seems to be general agreement today that in fixed histological preparations there is no material other than DNA present that can give a true nucleal reaction. With true nucleal reaction is meant the development of color with leucobasic fuchsin after, but not before hydrolysis in N HCl.

Stedman and Stedman (30, 31) claimed that the fuchsin-$SO_2$–DNA product of the Feulgen reaction was diffusible and they therefore challenged the possibility of localizing the site of DNA in the cell with the Feulgen reaction. Furthermore, it was shown that chromosomes are stained by leucobasic fuchsin recolorized by hydrolyzed DNA (5, 10, 11, 13, 30). Danielli, however, made it clear that this staining with the developed nucleal stain was essentially different from the Feulgen reaction in that not only the chromosomes but also the cytoplasm was stained. It is possible that under certain conditions a diffusible fuchsin-$SO_2$–DNA product is formed that acts as a basic dye, but the recent work of Chong-Fu Li and Stacey (9) and Overend and Stacey (23) makes it most unlikely that this occurs under conditions of a properly executed Feulgen reaction on histological material. This conclusion is supported also by a comparison of the Feulgen reaction with the sites of absorption at 2600 Å (8). We can, therefore, safely assume that the Feulgen nucleal stain in histological preparations reveals the site of localization of DNA.

The work of Widström (39) and Caspersson (6) established that under certain conditions the intensity of the nucleal reaction was proportional to the concentration of DNA. This was true in concentrations from 0.02 per cent to 1.5 per cent and for purified DNA, but not in the presence of proteins. The reaction did not proceed quantitatively, only part of the DNA taking part.

In the present paper it was shown that in nuclei with a DNA concentration up to a few per cent the intensity of the color is proportional to the DNA content. This was demonstrated by comparing the absorption of nuclei at 546 mμ with the DNA per nucleus as determined by independent chemical determination on a known number of nuclei. This comparison was made possible by the remarkable fact that diploid nuclei within one species contain the same amount of DNA (2, 22, 37, 38). It was thus possible to compare a single nucleus with the average of a large number of nuclei (in the absence of polyploidy).

The possibility of a quantitative use of the Feulgen reaction was recently questioned by Lessler (20). He prepared various concentrations of DNA in gelatin, placed a drop on a slide, and applied the Feulgen reaction. The intensity of the color developed was judged by eye. In this way he found that concentrations above 0.15 per cent did not yield greater intensity of color. It is of course impossible to compare light absorption of densely stained preparations in this manner, and the observations of Lessler are therefore no valid criticism of a quantitative use of the Feulgen reaction.

In histological preparations as in the test tube the nucleal reaction does not proceed quantitatively. It is therefore not possible to calculate the absolute amounts of DNA from the amounts of basic fuchsin present as was done by Di Stefano (14, 15) and Pollister and Leuchtenberger (25). Only relative values can be obtained from the intensity of the nucleal reaction. For absolute determinations nuclei of known DNA content must be treated together with the unknown to serve as a standard.

The nucleal reaction executed according to a standard procedure yields reproducible results. But even so it is advisable to use nuclei of known DNA content as standard either on the same slide or on another slide treated together with the unknown material.

Determinations of the color intensity after various periods of hydrolysis on material fixed with acetic acid–alcohol and with formalin confirmed in general the observations of Bauer (1), Hillary (17), and Di Stefano (15), that the relation of hydrolysis to color intensity depends on the type of fixation. In the case of acetic acid–alcohol an optimum curve was found in agreement with the authors cited. However, the curve showed a broad maximum after hydrolysis for 8 to 15 minutes and not a narrow peak after 12 minutes as reported by Di Stefano (14, 15). After formalin fixation a maximum intensity of the Feulgen reaction was reached with a 13 minute hydrolysis in both rat and frog liver. Longer hydrolysis, up to 25 minutes, did not decrease the intensity of the reaction. This is in marked contrast to the result obtained by Hillary (17), who found that after formalin fixation the intensity of color decreased with hydrolysis beyond a narrow optimum just as after acetic acid–alcohol fixation. Only after chromic acid did he find persistent maximal intensity with prolonged hydrolysis. The data of Hillary were based primarily on experiments with DNA in agar blocks and it is likely that under these conditions DNA behaves differently than when combined with nuclear proteins.

The effect of time of staining on the intensity of the nucleal reaction has not been studied quantitatively before. Our measurements have shown that the intensity of color is the same after staining for one-half up to several hours. Prolonged sojourn in leucobasic fuchsin, however, decreases the intensity of the reaction.

The concentration of the fuchsin-$SO_2$–DNA product in the nuclei was determined by absorption measurements with the microscope at a wave length of 546 m$\mu$. The possibilities and limitations of absorption measurements on cellular structures with the microscope were first investigated by Caspersson (6), who discussed in detail the conditions under which such measurements are feasible as well as the major sources of error. A simplified apparatus and method of measurement were described by Pollister and Ris (24). The same apparatus and procedure were used in the present paper. The accuracy of the method and the magnitude of the errors caused by certain simplifying assumptions were

determined in a series of measurements. It was found that absorption measurements with the microscope on cellophane strips and on droplets of Sudan IV in hexane agreed within a few per cent with determinations on the same material with the Beckman spectrophotometer. Furthermore, it was shown that the errors due to the simplifying assumptions were so small that they could be neglected.

The non-homogeneity of distribution of absorbing material in cellular structures is one of the main sources of error, especially in absolute absorption measurements. The chromatin in nuclei for instance is distributed in fibers and clumps after the usual methods of preparation. Relative measurements on nuclei of similar structure are possible despite this uneven distribution with an error of about 10 per cent as was shown in this paper for various liver nuclei. However, if nuclei of different structure and DNA concentration were compared an error of over 30 per cent was found. Fortunately, this uneven distribution of DNA in the nucleus can be avoided. In the living nucleus DNA is distributed homogeneously and it can be fixed in this condition so that nuclei become uniformly stained by the Feulgen reagents (28). Absorption measurements on such nuclei were found to agree within 10 per cent even if the DNA concentration varied as much as in hepatic and erythrocyte nuclei of lower vertebrates.

In conclusion it can be said that with the apparatus described, absorption measurements on microscopic structures can be made with an acuracy of a few per cent and that relative determinations of the DNA content of nuclei with the Feulgen reaction yield results that agree within 10 per cent with the values obtained from determinations with standard microchemical methods.

## SUMMARY

The possibility of using the Feulgen nucleal reaction for a quantitative cytochemical estimation of desoxyribonucleic acid (DNA) was investigated. The intensity of the reaction in nuclei was determined by absorption measurements with the microscope. The accuracy of such measurements was tested by comparison with measurements on the same material with a Beckman spectrophotometer. The values obtained with the microscope agreed within a few per cent with those obtained with the Beckman spectrophotometer. Furthermore, the errors introduced by uneven distribution of absorbing material, by variations in the numerical aperture of the system, and by variation in the area used on the phototube were investigated empirically.

The following variables were studied with regard to their effect on the intensity of the Feulgen reaction: type of fixation, time of hydrolysis after acetic acid–alcohol and formalin fixation, time of staining in leucobasic fuchsin, method of preparation of leucobasic fuchsin.

The intensity of the Feulgen reaction in liver and erythrocyte nuclei of various vertebrates, fixed in acetic acid–alcohol, was then compared with the DNA

content of these nuclei as determined by chemical analysis on a known number of nuclei. The intensity of the reaction was found to be proportional to the DNA content of the nuclei, if nuclei of similar structure and DNA concentration were compared. In nuclei of different structure and DNA concentration (*i.e.* liver and erythrocyte nuclei), fixed in acetic acid–alcohol, the intensity of the Feulgen reaction was, however, not proportional to the DNA content. This difficulty was overcome by isolating nuclei in sucrose and by fixing them in formalin. Uniform distribution of DNA and therefore uniform coloring after the Feulgen reaction were thus obtained. In such nuclei with uniform distribution of absorbing material the Feulgen reaction was found to be proportional to the DNA content of nuclei, even if they differed greatly in their DNA concentration.

The Feulgen nucleal reaction is not quantitative in an absolute sense. For absolute determinations nuclei of known DNA content must be treated together with the unknown material to serve as standard.

From these data it therefore appears possible to determine cytochemically relative amounts of DNA in cellular structures by measuring their absorption after treatment with the Feulgen nucleal reaction.

*Note Added to Page Proof.*—In addition to the papers on quantitative cytochemical applications of the Feulgen nucleal reaction by Di Stefano (14) and by Pollister and Leuchtenberger (25) which have been referred to in this paper there has recently appeared a paper by Schrader and Leuchtenberger (*Proc. Nat. Acad. Sc.*, 1949, **35**, 464). In this case the authors have made cytochemical measurements by the procedure used previously (14, 25) on cells of different tissues of *Tradescantia* and consider that the "DNA values are relative and not absolute." From the experiments reported in the present paper it can be seen that when measurements on nuclei of certain different cell types are made by a procedure much the same as that used by Schrader and Leuchtenberger widely different values are obtained (Table XV), although when the measurements are made on nuclei in which the DNA has been more uniformly dispersed essentially the same values are found (Table XVI). It is, therefore, unlikely that the determinations of Schrader and Leuchtenberger have a relative quantitative validity. There has indeed been no cytochemical procedure for nucleic acid determination by the Feulgen nucleal reaction for which evidence has been given indicating either absolute or relative quantitative validity.

If the possibilities of error in the measurements of Schrader and Leuchtenberger are recognized, their measurements do not warrant their conclusion: "The unavoidable conclusion is that the amount of DNA carried in a given chromosome may vary in different tissues."

This very conclusion is, however, supported by determinations of DNA given in a paper (Mirsky and Ris, 22) to which Schrader and Leuchtenberger refer, but incorrectly. In referring to this paper Schrader and Leuchtenberger say, "Working on tissues in vertebrates, they arrived at the conclusion that all the diploid cells of an organism carry identical amounts of DNA," and, "Contrary to the findings of several

workers for the tissues of vertebrates, the nuclei in different tissues of the plant *Tradescantia* carry different amounts of DNA." Now what Mirsky and Ris (22) actually said with respect to diploid nuclei was that in certain vertebrates (in some fish and amphibia and in a reptile and a bird) the quantity of DNA in the nucleus of an erythrocyte is the same as in the nucleus of a liver cell of the same species; and it may be noted that these results are nicely confirmed in the present paper. In presenting their data on the DNA contents of the nuclei of cattle, Mirsky and Ris (22) said, "It will be seen that there are some variations from the simple relationships found in other vertebrates." This sentence appears under a table showing how the DNA content per nucleus varies in different diploid nuclei of cattle.

It should be said that Schrader and Leuchtenberger in the above quotations were referring to papers by Boivin and the Vendrelys as well as to the paper by Mirsky and Ris, and possibly this has made some difficulty in quoting the various authors correctly.

A. E. MIRSKY

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