ACADEMY OF SCIENCES; and from the Permanent Science Fund of the American Academy of Arts and Sciences.

[1] Both the Canadian and U. S. clones belong to Variety II. The former belongs to mating type J; the latter to type K.

[2] Chen, T. T., *J. Hered.*, **31**, 185–196 (1940); *J. Morph.*, **79**, 125–261 (1946). Wichterman, R., *Turtox News*, **26**, 2–10 (1948).

[3] Chen, T. T., *J. Morph.*, **78**, 353–395 (1946).

THE NATURE OF THE SPECIFICITY OF METHYL GREEN FOR CHROMATIN

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Of all substances which bulk large in the chemical composition of cellular structures, desoxypentose nucleic acid is perhaps the most clearly demonstrable by application of cytochemical methods to cells. For each one of the three components of nucleotide residues there is a specific method which enables the cytologist to visualize it on slides: the purines and pyrimidines have natural specific absorption in the ultra-violet spectrum; the Feulgen reaction reveals the desoxypentose; and the staining with basic dyes is an indication of the orthophosphoric acid component. It is this last specificity

TABLE 1

METHYL GREEN STAINING OF CYTOPLASM AND NUCLEI OF EPITHELIAL CELLS OF MOUSE LIVER

NO. OF EXPERIMENT	PART OF CELL MEASURED	STAIN	MEAN EXTINCTION
1	Cytoplasm	Methyl green	0.014 ± 0.001
1	Nucleus	Methyl green	0.294 ± 0.012
2	Nucleus	Methyl green	0.265 ± 0.014
3	Nucleus	Methyl green, Pyronin	0.274 ± 0.007

which many histologists might wish to question. It has been generally realized, since the chemical properties of nucleic acid became known, that for the most part the usual binding of basic dye in histological staining of the nucleus must be due to formation of a dye-nucleate salt (see especially Michaelis[1, 2]). However, to what extent the intensity of basic staining by any particular method can be strictly interpreted to indicate relative amounts of nucleic acid has not been so clear. It has been shown again and again that under the conditions of standard staining procedures the basic dyes color many structures that do not contain nucleic acid, and that the intensity of the color in chromatin is subject to many influences from both

within and without the tissue. In such a situation the only sure way has been that of checking the amount of dye-nucleate staining by use of specific nucleases, as Brachet[3] has done. In effect, this makes the problem one that can be attacked best by methods of quantitative chemical cytology. The present paper is concerned with demonstration, by photometric cytological analysis, that one basic dye, methyl green, can be used in such a manner that the staining is highly specific for desoxypentose nucleic acid, that it is quantitatively related to the amount of nucleic acid present, and that it is dependent upon the physical state of the nucleic acid.

Most common histological staining methods do not appear to be as highly specific for nucleic acid as has been suggested, for example, by Pollister and Ris.[4] With such dyes as Pyronin B, Pyronin Y or Basic Fuchsin we have found that there is a considerable residue of stainable substance in nuclei and cytoplasm after all nucleic acids and nucleotides have been removed chemically, table 2. These dyes also stain heavily

TABLE 2

METHYL GREEN AND PYRONIN STAIN BEFORE AND AFTER REMOVAL OF NUCLEIC ACIDS WITH HOT TRICHLORACETIC ACID

METHOD, MATERIAL, WAVE-LENGTH MEASURED	BEFORE TRICHLORACETIC ACID	AFTER TRICHLORACETIC ACID
Methyl green, mouse liver, E_{625}	0.296 ± 0.012	0.010 ± 0.002
Pyronin, mouse liver, E_{550}	0.480 ± 0.014	0.110 ± 0.004

such material as thyroid colloid, which, as Caspersson and Gersh[5] have shown photometrically, almost certainly contains no nucleic acid. However, as was originally pointed out by Unna,[6] methyl green seems to be an exception in that it stains nothing but chromatin.

The method of staining which we have used closely follows that of Unna and Pappenheim.[7] The dye is a National Aniline product, C.I. No. 685, Certification No. NG 25, Total Dye Content 93%. Under this Color Index Number Conn[8] discusses a dye called ethyl green (synonym, methyl green) which he says is the "zinc chloride double salt of ethylhexamethylpararosanilin chlorobromide," molecular weight 653. As received, the dye contains a small amount of a violet compound which must be removed, for it does not have the specific properties of the green dye. This purification may be carried out just before use by Unna's method of shaking the dye with chloroform, in which the purple component alone is soluble. The purification does not seem to be necessary with Grubler's Iodine Green, presumably a closely related dye, which has the same staining properties as methyl green. For use 75 mg. of the purified dye was dissolved in 50 cm.^{3} of the phenol-alcohol-glycerin solution of Unna-Pappenheim. The pH of this solution is about 6.3. The absorption maximum of the dye as de-

termined in the Beckmann Spectrophotometer is about 630 mμ. Paraffin sections of vertebrate tissues, fixed in Carnoy's acetic alcohol (1:3), were stained in freshly purified, freshly prepared dye solution for 45 minutes at 56°C., blotted, then decolorized overnight in tertiary butyl alcohol (or in 95% ethyl alcohol, when methyl green was used without pyronin). This approximates a procedure which Michaelis,[2] has suggested as most likely to leave in the tissue only chemically bound dye. The intensity of stain in the cells was estimated by the absorption methods described by Pollister and Ris,[4] measuring the transmission of red light isolated from a tungsten lamp by a Farrand Interference Filter with a peak transmission at 630 mμ. The lower extinction values are from partial liver nuclei in 5 μ sections, the higher from whole nuclei in 8 μ sections. The whole nuclear diameter was projected onto the photosensory surface. The extinction values are means of a number of measurements, each mean being from a series of nuclei of approximately equal diameter. Unstained slides show an absorption equivalent to an extinction of 0.010 to 0.030; consequently values in this range indicate that there has been no staining reaction.

When slides have been stained as described above it is apparent at a glance that the methyl green stains only chromatin; the amount of color in either nucleolus or cytoplasm (table 1) being negligible. For any one type of nucleus the amount of dye bound is accurately reproducible, tables 1 and 2. The mean values for 5 μ sections of nuclei in four different experiments ranged from 0.265 to 0.296, the differences not being statistically significant. The amount of methyl green staining of chromatin is independent of the presence of another dye such as pyronin B (table 1) or basic fuchsin.

A series of experiments demonstrates what chemical component of the chromatin is responsible for this specific binding of methyl green. The methyl green is staining the nucleic acid of chromatin, not the protein, for after nucleic acid has been removed from the sections by 15 minutes' treatment with hot 5% trichloracetic acid (Schneider,[9] Pollister and Ris[4]) the chromatin no longer stains to any appreciable extent with methyl green, table 2. The methyl green is bound to desoxypentose nucleic acid, not to the pentose type; for two hours' action of ribonuclease on the sections has no effect upon the methyl green staining. The mean extinction of ten untreated whole nuclei was 0.374 ± 0.020 that of ten after enzyme action was 0.395 ± 0.020, which is not statistically different. In short, the series of experiments demonstrates conclusively that, in material which has been fixed in acetic alcohol, *purified methyl green is highly specific for desoxypentose nucleic acid.*

In reproducibility and specificity the methyl green staining is similar to a properly controlled Feulgen nucleal reaction (Stowell[10], Di Stefano[11]), which suggests that the methyl green binding likewise is quantitatively related to the amount of desoxypentose nucleic acid in a nucleus. This is

further supported by the fact that in diverse normal nuclei, with varying amounts of chromatin, the ratio of nucleal-regenerated fuchsin to methyl green staining is approximately the same, the ratio of extinctions being not far from 1.0. It seems likely that in the case of methyl green we are dealing with a chromatin staining which is solely chemical combination of dye and nucleic acid, presumably to form a methyl green nucleate salt.

Among other technical discoveries Unna noted that the methyl green staining of chromatin could be markedly reduced by pretreatment of sections with boiling water. Photometric measurements demonstrate this effect in a striking manner. When sections which have been treated with hot water are stained, the methyl green binding does not occur to any appreciable extent, table 3. Indeed the methyl green extinction after hot water is as weak as on sections from which the nucleic acid has been entirely removed, table 3; but the explanation of the loss of staining after hot water must be quite different, since it can be demonstrated that the treatment has not removed any of the desoxypentose nucleic acid from the chromatin (table 3) for: (*a*) the nucleal reaction is equally intense before and after hot water, showing that the desoxypentose content is unchanged; (*b*) there is no loss of purine or pyrimidine bases from the chromatin, for the 2537 Å. absorption is not reduced; and (*c*) gross analyses of liver before and after hot water, which Dr. Gerhardt Schmidt was kind

TABLE 3

METHOD, MATERIAL, WAVE-LENGTH MEASURED	BEFORE HOT WATER, MEAN EXTINCTION	AFTER HOT WATER, MEAN EXTINCTION
Methyl green, mouse liver, nuclei, E_{625}	0.340 ± 0.010	0.032 ± 0.004
Feulgen reaction, mouse liver, nuclei, E_{550}	0.427 ± 0.014	0.419 ± 0.015
Gross analysis (Schmidt), mouse liver, DNA phosphorus per 100 mg. of nitrogen	1.43 mg.	1.44 mg.
U. V. absorption, cartilage, *Amblystoma*, E_{253},	0.460	0.522

enough to make for us, show conclusively that the treatment causes no reduction of the phosphoric acid, the third component of the desoxypentose nucleic acid. This last is most interesting for it shows that the part of the molecule which is presumably most closely related to the binding of the basic dye to form a methyl green nucleate salt is still present after hot water in as great an amount as when the chromatin stained intensely with methyl green. The obvious conclusion from these data is that the loss of methyl green staining after hot water treatment is a consequence of a physical change in the nucleic acid molecule. From results reported by Kurnick,[11] we can infer that this change is in the nature of a depolymerization. In test-tube experiments Kurnick found that while methyl green combined readily with highly polymerized desoxypentose nucleic acid, the dye would no longer combine with nucleic acid which had been depolymerized by the enzyme. Pending the results of experiments with the

effect of purified thymonucleodepolymerase on tissue sections, it may be tentatively concluded that in cytological preparations also the methyl green staining of chromatin is dependent upon the presence of desoxypentose nucleic acid in a highly polymerized state, and that the hot water treatment causes depolymerization. If this is actually the effect of the hot water, it has not proceeded so far as to degrade the nucleic acid to lower acid-soluble polymers, for sections which have been treated first with hot water and then subjected to cold trichloracetic acid (after Schneider[9]) give as intense a Feulgen reaction as do untreated controls.

The more closely the methyl green reaction is examined the more conspicuous do its differences from other types of basic staining appear, and one feels impelled to look for special chemical properties of methyl green as an explanation. An obvious difference is that unlike all other common histological stains the methyl green colored ion carries two positive charges, which surely might be expected to give it unique properties as a stain. The divalent methyl green ion should have a much greater affinity for acidic groups like phosphoric acid than should the monovalent ions of other dyes and *a priori* one should therefore expect that methyl green, alone, would stain very strongly all acidic structures, and, when mixed with pyronin, it should be much the stronger staining component for acidic material. In the staining which has been described above this expected result is realized only to a limited extent. Where a structure is stainable with methyl green, as chromatin is, then the divalent cation completely predominates over the monovalent—that is, the pyronin exerts no measurable restriction on the methyl green reaction. Superimposed upon this readily understandable relationship between the two dyes, however, is another influence which appears as a sharp restriction of the methyl green reaction to one particular acidic component of the cell, the phosphoric acid of the desoxypentose nucleic acid. It seems possible that this also may be explained by the structure, a biologist is tempted to say the "morphology," of the molecule of methyl green. Michaelis[2] has reported that the spectroscopic characteristics of basic dye bound in tissue staining show conspicuous differences from those of the dye-nucleate in test tubes, which are best interpreted as evidence of a specific "fit" or structural relationship between the molecules of the dye and those of the nucleic acid. It is not difficult to imagine that the high specificity of methyl green for highly polymerized desoxypentose nucleic acid may be explained in somewhat the same way.

In the normal tissues the Feulgen-methyl green ratio is fairly constant; in other words, there appears to be a definite proportion of the total desoxypentose nucleic acid which is in the physical state suitable for combination with methyl green. Any decrease in relative amount of this presumably highly polymerized nucleic acid should lead to decrease in the methyl green reaction and should increase the Feulgen-methyl green ratio, above

the normal range about a mean value of 1.0. Thus, if it is combined with a parallel study of slides on which the Feulgen reaction has been performed, the methyl green staining is potentially an indicator of a physical alteration of the desoxypentose nucleic acid molecule. Such changes may be expected under many cytological conditions, especially, for example, where chromatin is degenerating. We have recently been examining with these techniques the pycnotic degeneration that occurs in cells of a tumor implanted into a new host (as originally described by Rössle[12]). It is interesting that the first major change in the chromatin is a decrease in the intensity of the methyl green reaction, leading to such Feulgen-methyl green ratios as 3:1 or eventually perhaps as high as 8:1.

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ERRATUM

In Section 4 of our paper "Some Properties of Rotational Flow of a Perfect Gas," March, 1948 (concerning Massotti Flow) the statement near the middle of the Section, that the vorticity equation

$$\operatorname{curl}\left[\frac{\vec{w} \times \operatorname{curl} \vec{w}}{1 - w^2}\right] = 0$$

is automatically satisfied, is incorrect and invalidates the remainder of the Section.

Actually, it is easily seen that this equation introduces the additional restriction that

$$1 - \left(\frac{\partial \phi}{\partial x}\right)^2 - \left(\frac{\partial \phi}{\partial y}\right)^2 = f(z)g(x, y)$$

where f and g are arbitrary functions. This restriction limits the solutions known at present to those for which $w^2 = \text{const.}$

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