The use of methyl green as a histochemical reagent

By JOHN R. BAKER and ELIZABETH G. M. WILLIAMS

(From the Cytological Laboratory, Department of Zoology, University Museum, Oxford)

Summary

The cation of methyl green carries two positive charges, that of malachite green only one; but the two dyes behave towards tissue-constituents in almost exactly the same way. These dyes are not specific for chromatin. They colour certain objects that are devoid of DNA, even when they are used in very dilute solution. The granules of cells called *Körnchenzellen* in the connective tissue of the common snail, *Helix aspersa*, are strongly coloured by both dyes from very dilute solutions, and thus provide a striking instance of the unspecificity of these dyes.

Malachite green, which is stable and free from contamination by metachromatic impurities, can advantageously replace the methyl green commonly used in mixtures with pyronine.

It is suggested that pyronine may have a greater capacity for penetrating into close-textured objects, such as nucleoli and ribosomes, than methyl and malachite greens.

Introduction

METHYL green was introduced into microtechnique by Calberla in 1877. It is evident from what he says that the dye (which had been synthesized for the first time only a few years before) was seriously contaminated with the mixture of dyes known nowadays as 'methyl violet'. He made no claim that methyl green has a specific affinity for the nucleus or any part of it.

Carnoy (1884) drew attention to the affinity of methyl green for nuclein. He remarks, 'Nous connaissons déjà le vert de methyl comme étant la pierre de touche de la nucléine du noyau', but in the same paragraph he allows that it is not specific for this constituent of the nucleus, and he notes in particular its strong affinity for silk.

Matthew pointed out long ago (1898) that methyl green will stain hyaline cartilage, certain mucins, and egg albumen. It also stains the colloid substance of the thyroid gland, the granules of mast-cells, and lignin (Pollister and Leuchtenberger, 1949; Kurnick, 1950*a*, 1955; Taft, 1951*a*).

In general, the reactions of dyes with their substrates are of a very unspecific kind. The bonds they form are commonly salt-linkages—in which case they merely give evidence of basiphilia or acidophilia—or else hydrogen bonds, which are even less specific. Nevertheless, the opinion has persisted that methyl green has an altogether special affinity for chromatin.

The belief that methyl green has special properties appears to be due to four main causes, as follows.

1. Methyl green is peculiar among cationic (basic) dyes in having two positive charges on the cation. The structural formula is shown here alongside

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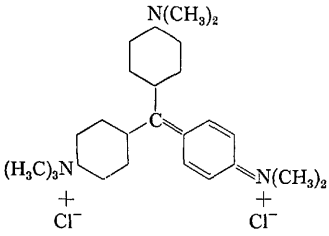
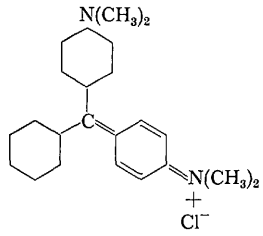
that of the related dye, malachite green, which is an ordinary cationic dye of almost exactly the same colour, carrying a single positive charge.



methyl green



malachite green

2. In reactions carried out in glass vessels with pure substances, highly-polymerized DNA takes up methyl green in a definite proportion by weight (Kurnick and Mirsky, 1950).

It may be remarked that in a reaction between DNA and methyl green, in the absence of any other substance that might react or interfere, a stoichiometric proportion might be expected, for the basic groups of the dye would presumably be bound to the phosphoric groups of the DNA; but this would throw no light on the question whether methyl green will or will not also react with other substances—and that must obviously be the test of specificity.

3. Stress has been laid on the fact that methyl green will colour chromatin from extremely dilute solution. Thus, if a single drop of a methyl green solution of ordinary strength (say 0·5%) be added to a slide-jar of distilled water, a section left overnight in this very dilute solution will show a green coloration of the chromatin. Some experiments bearing on this fact are recorded in the present paper.

4. The familiarity of methyl green as the dye for chromatin in mixtures based on those of Pappenheim (1899) and Unna (1902) has probably encouraged belief in the special properties of this dye. In fact, Pappenheim himself (1899) made some experiments with malachite green as a substitute for methyl green in his mixture with pyronine, but he does not appear to have carried them very far. Hitchcock and Ehrich (1930) substituted malachite green and acridine red for methyl green and pyronine. Sandiford (1938) substituted malachite green for methyl green in a complicated variant of the Pappenheim–Unna mixture, elaborated for a special purpose in bacteriological technique. Kurnick (1950*a*, 1955) claimed that malachite green coloured DNA more faintly than methyl green.

It is well known that methyl green is often contaminated with the highly metachromatic mixture of dyes called 'methyl violet', so that it requires to be extracted repeatedly with chloroform or some other suitable solvent. Methyl green is unstable in solution except between pH 3·5 and 5·0 (Kurnick, 1950*a*).

The present investigation was undertaken partly to discover whether the

possession of two positively charged groups confers special properties on methyl green, and partly to find out whether malachite green—a stable dye, not liable to contamination by metachromatic impurities—might replace it in practical use, especially in mixtures based on those of Pappenheim and Unna.

Materials

The experiments described below were carried out on the following tissues:

common snail (*Helix aspersa*): skin, cerebral ganglia with surrounding connective tissue, oesophagus, salivary glands, mucous gland of the reproductive system;

house-cricket (*Acheta domesticus*): mid-gut, fat-body, testis;

common frog (*Rana temporaria*): hyoid apparatus;

domestic mouse (*Mus musculus*): small intestine, pancreas, liver, submandibular and sublingual glands, kidney, spleen, anterior mesenteric ganglion, trachea;

rabbit (*Oryctolagus cuniculus*): trachea, thyroid gland.

The chief fixatives used were Zenker's (1894) and Clarke's (1851). The latter (usually attributed to Carnoy (1886)) consists of absolute ethanol with one-third its volume of glacial acetic acid. It has been objected to Zenker's fluid that DNA is depolymerized, with change of staining reaction (Kurnick, 1950*a*); but in the present investigation the period of fixation was limited to 3 h, and this change was scarcely noticeable. A disadvantage of Zenker's fluid is that the most external cells of a piece of tissue show a different staining reaction from all the rest; but these external cells are easily disregarded, and the brilliancy of the colours produced by dyes after the use of this fixative more than outweighs the defect. It follows that for work of the kind described in this paper, Zenker's fixative is inapplicable to very small pieces of tissue. The ideal size is about one-third of a mouse's testis.

Materials fixed in Zenker's fluid were washed overnight in running water, treated with iodine solution during dehydration, and embedded in paraffin. Sections were usually cut at 8 μ. Zenker material was treated a second time with iodine solution after sectioning, and then with sodium thiosulphate solution. The sections were thoroughly washed with running water and finally with distilled water.

Materials fixed in Clarke's fluid were washed in absolute ethanol and embedded in paraffin. Sections were usually cut at 8 μ (sometimes 10 μ). They were brought down to distilled water before dyeing.

The chief dyes used in this investigation were these:

methyl green (G. T. Gurr, no. 13852), repeatedly extracted with chloroform; malachite green (British Drug Houses, no. 2200880);

pyronine Y (Edward Gurr, no. 339).

It is important to insist on the very great differences between the various pyronines on the market. A prolonged study is necessary to obtain the right concentration of the solution, if one wishes to substitute a pyronine of one manufacturer for a similarly named dye made by someone else.

In the description of the experiments, reference will be made to the use of a few other dyes and of certain familiar histochemical reagents.

Great care was taken throughout the investigation to ensure that the ethanols used for dehydrating sections were free from contamination (especially by acid). The dyed sections were usually mounted in Canada balsam or DPX.

Experiments

The affinities of methyl green and malachite green in very dilute solutions

The dyes were dissolved separately at 0·5% in distilled water. A single drop of the dye solution was delivered by a pipette to 70 ml of distilled water. The pipette used for the purpose delivered 1 ml in 33 drops. Thus the dye solutions were used at a concentration of about 0·00022%. Sections (8 μ or 10 μ) of tissues fixed in Zenker's or Clarke's fluid were left overnight (16 h) in these solutions. They were then dipped in 95% ethanol, left for 1 min altogether in two lots of absolute ethanol, and passed through xylene into Canada balsam or DPX.

Tissues fixed in Zenker's and Clarke's fluids reacted so similarly to very dilute dyes that it is not considered necessary to make separate reports. Most of the work was done with tissues fixed in Zenker's fluid.

Methyl green. In the testis of *Acheta* and the colon of the mouse, nothing was coloured by methyl green except chromatin. It is especially to be remarked that although the abundant mucus of the colon is basiphil (and chromotropic) by conventional techniques, no trace of colour could be detected in it. Methyl green, used in this way with these organs, is indeed a *pierre de touche* for chromatin.

Nearly the same result was obtained with the testis of the mouse, though the nuclei of the mature spermatozoa were very pale green or unstained. This was the only exception noted to the general rule that methyl green colours chromatin even when it is used in very dilute solution. Some of the stainable granules (*die tingirbaren Körnchen*) of von Ebner (1888) in the later spermatids were just tinged with green, though they contain no DNA.

The colloid substance in the thyroid gland of the rabbit was unstained in the great majority of follicles, but just detectably coloured in a few.

In the pancreas of the mouse, the ergastoplasm of the exocrine cells was rather strongly dyed by methyl green. Both here and in von Ebner's granules, the dye was presumably reacting with RNA.

In the trachea of the rabbit and the hyoid apparatus of the frog, the matrix of cartilage was coloured fairly strongly by methyl green. It will be understood that the matrix was of the full thickness of the sections, and thus had a better opportunity of being stained than most other tissue-constituents.

Certain cells of the connective tissue of the snail contain sub-spherical granules, up to about 4 μ in diameter, that take up methyl green strongly from very dilute solution. The cells are usually ovoid, but sometimes irregular in shape. Their cytoplasm is crowded throughout with the granules that show

this remarkable affinity for methyl green. These granules provide such a striking example of the unspecificity of the dye that it is necessary to consider them in some detail.

The cells in question occur in the tissue below the superficial epithelium of the skin, but we have studied them chiefly in the sheath that surrounds the cerebral ganglia. They were first seen and figured by Semper (1856) in *Limnaea stagnalis*. He called them *Bindesubstanzzellen mit Fett*. Brock (1883) saw them in *Helix nemoralis* and named them *Körnchenzellen*: he denied that the granules are fatty. These cells were studied in greater detail by Cuénot (1892) in *Limnaea*, *Planorbis*, and *Helix pomatia*. Cuénot compared them with the *Mastzellen* of vertebrates and called them *cellules mucoïdes*. He noted that their granules were colourable by methyl green, safranine, and dahlia. It appears that the significance of these cells is still uncertain. In the present paper they will be called by Brock's name of *Körnchenzellen*.

We thought it necessary to undertake a small histochemical investigation of the granules in the *Körnchenzellen*, to gain some information about the composition of the substrate with which very dilute methyl green reacts. The results may be briefly summarized as follows.

The granules are negative to Feulgen and Rossenbeck's test (1924) for DNA. They colour with cationic dyes and (rather feebly) with aluminium-haematein, but scarcely or not at all with the typical anionic dyes, Biebrich scarlet and light green. Their reaction with toluidine blue is orthochromatic, and they do not become chromotropic when treated with 10% chromium trioxide. They are negative to the Sakaguchi test for arginine (Baker, 1947), and they appear to be negative also to the DAB test for tryptophane (Adams, 1957); but this test gives a strong reaction with the ground cytoplasm of the *Körnchenzellen*, and it is difficult to be sure that the granules are quite negative. They are negative to Driessen's test (1905) for glycogen, and they persist and remain colourable by cationic dyes when treated with saliva. They are strongly positive to the PAS test (McManus, 1946, 1948).

It must be considered probable, from the results of these tests, that the granules consist wholly or largely of a polysaccharide possessing acidic properties, but, unlike the typica lacid mucopolysaccharides, the substance responsible for the reactions is not chromotropic.

The *Körnchenzellen* may possibly be the same as those called 'mucin-staining cells' by Pearse (1961), who found that the granules respond positively to the germanic acid test for polyvalent alcohols.

Malachite green. In the great majority of cases this dye, in very dilute solution, behaves so similarly to methyl green that one would not be able to guess which had been used. A few differences were, however, noted, and these may be briefly summarized as follows. Malachite green colours the chromatin of certain cells rather less strongly than methyl green, but on the contrary it stains the nuclei of mature spermatozoa of the mouse more strongly. It also stains the granules of von Ebner in the spermatids more strongly. It gives a just-detectable tinge to the contractile substance of

striated mammalian muscle (sternohyoid, attached to trachea of mouse), when present in the full thickness of an 8 μ or 10 μ section. The only substance in the tissues studied by us that reacts with very dilute malachite and methyl greens in a markedly different way is the colloid substance of the thyroid gland, for this is rather deeply coloured by the former dye, but scarcely at all by the latter.

It follows from this study with very dilute dyes that methyl green has scarcely any more specificity for chromatin than malachite green has. This finding is not in accord with the opinion of Vercauteren (1950), who attributed the affinity of methyl green for DNA to the presence of two neighbouring negatively-charged phosphoric acid groups of the nucleic acid at a distance apart corresponding to the separation of the two positive charges on the dye.

The substitution of malachite green for methyl green in the Pappenheim–Unna technique

The similarity between methyl green and malachite green is exhibited not only in the experiments with very dilute dyes, but also when malachite green is substituted for methyl green in the pyronine/methyl green (PMG) techniques of Pappenheim (1899) and Unna (1902). We have devised a technique called the pyronine/malachite green or PMaG technique, which gives essentially the same results as PMG, with the advantage that an uncontaminated green dye, stable in solution, takes the place of an unstable one that is often contaminated with metachromatic by-products and requires to be purified before use. We have followed Taft (1951*b*) in buffering the dye solution with acetic acid/sodium acetate.

Details of the pyronine/malachite green technique. The following solutions are required:

acetate buffer at pH 4·8

acetic acid, 0·2 N	81 ml
sodium acetate, 0·2 M	119 ml

pyronine Y, 4% aq. (It is essential to use the pyronine Y No. 339 of Edward Gurr, 42 Upper Richmond Road West, London, S.W. 14.)

malachite green, 0·3% aq. (We have used the British Drug Houses dye, no. 2200880.)

pyronine/malachite green solution (PMaG)

acetate buffer at pH 4·8	50 ml
pyronine Y solution	40 ml
malachite green solution	10 ml

Note. Different specimens of the dyes, obtained from the same manufacturers, do not appear to be quite identical in their effects, and it may be found desirable to change the proportions of the pyronine Y and malachite green solutions from 40:10 to 40·5: 9·5 or 41:9.

sodium bicarbonate, 0·025% aq. The technique is carried out as follows:

1. Fix small pieces of tissue in Zenker's fluid, 3 h.
2. Wash in running water overnight.
3. Dehydrate, with iodine treatment; embed in paraffin.
4. Cut sections. 8 μ is the best thickness for general use, but when ribonucleic acid is extremely abundant in the cytoplasm (as in the mammalian pancreas) it is necessary to cut them thinner (e.g. 4 μ).
5. Bring sections to water (with the usual treatment with iodine solution, followed by sodium thiosulphate solution and thorough washing in tap water followed by distilled water).
6. Dye in the PMaG solution for 20 min.
7. Blot with filter paper.
8. Dip momentarily in the sodium bicarbonate solution.
9. Dip momentarily in 95% ethanol.
10. Absolute ethanol, two lots, 2 min exactly altogether.
11. Xylene; DPX or Canada balsam.

Note. It is important that the 95% and absolute ethanols used for dehydration should be perfectly free from contamination, especially by acid.

Chromatin is usually coloured blue, sometimes with a reddish or greenish tinge, according to the proportion of RNA contained in it. The nuclei of the late spermatids and spermatozoa of the mouse are coloured green. The granules in the *Körnchenzellen* of the connective tissue of the snail are coloured blue (or sometimes green).

It has already been mentioned (p. 5) that the coloration of the most external cells of a piece of tissue must be disregarded.

The chief substance coloured red or pink by this technique is RNA, but this must be confirmed by the use of ribonuclease. Tissues fixed for only 3 h in Zenker's fluid allow rapid removal of RNA by ribonuclease. A 0·1% solution of the enzyme in 0·01 M phosphate buffer at pH 6·0, used at 37° C, usually removes all RNA in 1¼ h, and often in a much shorter time. A curious exception is provided by the nucleoli in the epithelial cells of the tubular part of the submandibular gland of the male mouse. The RNA is removed from these nucleoli remarkably slowly, presumably because they are close-textured and the enzyme has difficulty in entering. (For details of the histology of this gland, see Tupa, 1926; for the remarkable sex differences in it, see Lacassagne, 1940.)

The testis of the mouse (cut into two or three pieces) is a suitable tissue on which to learn the PMaG technique, on account of the bright red colour given to von Ebner's granules in the late spermatids and residual cytoplasm.

Experiments on the mechanism of the PMaG technique. A simple experiment showed us that malachite green attaches itself to tissue-constituents in a very different manner from pyronine. Pieces of the intestine and pancreas of the mouse were subjected to the PMaG technique with this variation, that the

dyed sections were washed for half an hour in repeated changes of either distilled water or 95% ethanol. The results were very different, according to whether water or ethanol was used for washing. If water was used, chromatin was everywhere green, not blue; there was scarcely a trace of red or pink in any part of the intestine; the ergastoplasm of the pancreas was bluish purple instead of bright red. If, on the contrary, ethanol was used, the chromatin was pale pink, not blue; no green was seen anywhere; nucleoli, as usual, were pink, and ergastoplasm red. It follows that pyronine is readily extracted by water, and malachite green by ethanol.

During the war, when the pyronines were difficult to obtain, it was found that rhodamine S could be used instead in the PMG technique (Baker, 1942). We have now made some further experiments with rhodamine. We find that rhodamine can be substituted, though experiment is necessary to find the best proportion in which to mix this dye with malachite green, since the rhodamines of different manufacturers are probably not identical. We have used the rhodamine 6G of the British Drug Houses. The result is very similar to that given by PMaG, but not identical. Rhodamine does not impart such a bright colour as pyronine, and it dyes nucleoli less intensely; it has a slight tendency to give a tinge to the contractile substance of smooth muscle. There is no practical advantage in replacing pyronine by rhodamine, but the essential similarity in the action of the two related dyes throws light on the mechanism of the PMG and PMaG techniques (see p. 12).

Discussion

The PMG technique has been very fully discussed by Gerola and Vannini (1948, 1949), Pollister and Leuchtenberger (1949), Kurnick and Mirsky (1950), Kurnick (1950 *a*, *b*, 1955), Panijel (1951), Taft (1951*a*), and others. One cannot regard either this or the PMaG technique as histochemical tests, unless controlled by the use of ribonuclease. By varying the proportions of pyronine and methyl (or malachite) green, or altering the pH of the solution, one can favour one dye or the other and strike a balance at which the red will colour chiefly RNA, and the green, or green and red together, will colour chiefly chromatin. (When a microscopical object is coloured by both methyl (or malachite) green and pyronine, the colour produced is blue or bluish.)

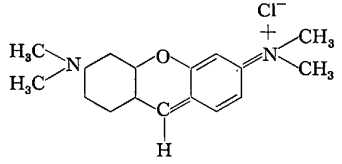
Malachite green seems preferable to methyl green for this and perhaps for all other purposes, except those in which contamination by a metachromatic by-product is desirable. The fact that the cation of methyl green carries two positive charges does not confer any useful properties that outweigh the disadvantages of contamination and instability.

Although neither methyl nor malachite green is specific for chromatin, yet obviously a difference between the affinities for various tissue-constituents is shown by the two green dyes on one hand, and by pyronine and rhodamine on the other. Full weight must be given to the conclusions reached by the authors mentioned in the first paragraph of this discussion. In particular, the degree of polymerization of the nucleic acids has been shown to play a part

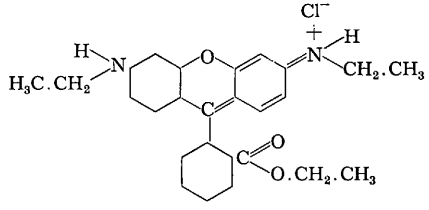
(Pollister and Leuchtenberger, 1949; Kurnick, 1950*a*, 1955). Nevertheless, it seems likely that other factors may also be involved.

The chief objects coloured by pyronine (or rhodamine, when used instead of pyronine) in the PMG and PMaG techniques are nucleoli and the basiphil components of the ergastoplasm, that is to say, the ribosomes. In both these sites the acidic component that attracts the cation of the dye is RNA. The differences in the behaviour of the green and red dyes may perhaps be due in part to the more rapid penetration of close-textured objects by pyronine and rhodamine than by methyl and malachite greens (though these can also enter when not in competition with the red dyes). The green dyes are more powerful, and to a greater or less extent they predominate over the red dyes in the loose-textured objects that can readily be entered by both classes of dyes.

It will be remembered that methyl and malachite green are triarylmethane dyes, and that anionic (acid) members of this group are chosen when a powerful dye, with limited capacity to enter close-textured acidophil objects, is needed. Thus methyl blue, aniline blue WS, and light green are used to colour collagen (which is loose-textured), while more close-textured acidophil objects are coloured by a more penetrating dye of contrasting colour, such as xylidine red.



pyronine Y



rhodamine 6G

The fact that methyl and malachite greens, methyl and aniline blues, and light green penetrate close-textured objects with greater difficulty than many other dyes is probably attributable rather to the awkward shape of their coloured ions, or to their greater tendency to ionic aggregation, than to their high molecular or ionic weights. Gurr (1962) has put forward the hypothesis that the differential action of pyronine and methyl green may be due to the

differences in their molecular or cationic weights, but the results with rhodamine and malachite green, recorded in the present paper, do not support this hypothesis.

Like the pyronines, the rhodamines are xanthene dyes. The formula for rhodamine 6G is here compared with that for pyronine Y.

The molecular and cationic weights of pyronine Y, rhodamine 6G, methyl green, and malachite green are shown in table 1.

TABLE 1

The molecular and cationic weights of certain dyes (to the nearest unit in each case)

	Molecular weight	*Cationic weight*
Pyronine Y	303	267
Methyl green	458	388
Rhodamine 6G	451	415
Malachite green	365	329

It will be noticed that whereas the molecule and cation of pyronine Y are much lighter than those of methyl green, the molecule and cation of rhodamine 6G are much heavier than those of malachite green. Nevertheless, as we have seen (p. 10), the two pairs of dyes—pyronine/methyl green and rhodamine/malachite green—give essentially the same results. In each case, when the dyes are mixed in suitable proportions, the xanthene dye colours the RNA.

Malachite green and pyronine differ not only in their ability to enter close-textured objects, but also in the readiness with which they are extracted from objects by water or by ethanol (see p. 10). It must be presumed that their mode of attachment to the objects they colour is different, and that this difference may be connected with such measure of differential dyeing capacity as they possess.

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