METHYL GREEN-PYRONIN

I. Basis of Selective Staining of Nucleic Acids

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PLATE 1

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Largely as the result of Brachet's investigations (1), it has become appreciated that the differential staining of tissues with the methyl green–pyronin mixture of Unna and Pappenheim is determined by their nucleic acid components. Thus, the green staining of nuclei was demonstrated to be due to their content of desoxyribonucleic acid, and the red staining of the cytoplasm to be determined by the ribonucleic acid content. It is the purpose of this paper to report some observations on the nature of this phenomenon of differential staining of similar acid substrates by two basic dyes.

In tissues, desoxyribonucleic acid apparently occurs as a much higher polymer than ribonucleic acid (2). It appeared to us that this difference in the degree of polymerization might be the determining factor in the differential staining with methyl green–pyronin. It is the purpose of this paper to present observations on the differences in staining between highly polymerized and depolymerized desoxyribonucleic acids. As will be seen, these tend to confirm the hypothesis that the affinity of nucleic acids for methyl green and pyronin is a function of their state of polymerization: methyl green stains preferentially highly polymerized nucleic acid, while pyronin has a special affinity for low polymers (depolymerized desoxyribonucleic acid and ribonucleic acid).

EXPERIMENTAL

Nucleic Acids and Nucleoproteins

1. Desoxyribonucleohistone (DNH) was prepared from calf thymus chromosomes by extraction with 1 M NaCl (3). The viscous solution was centrifuged at high speed to remove "residual chromosomes" and nucleoli. The slightly opalescent viscous supernate was stored in the cold with toluene as preservative. The phosphorus analysis of this solution was 0.27 mg./cc.

2. Desoxyribonucleic acid (DNA)[1] was prepared from the DNH solution by repeated mixing in a Waring blendor with chloroform-octyl alcohol (4) to remove

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[1] Prepared by Dr. A. E. Mirsky of The Rockefeller Institute for Medical Research.

the histone, followed by precipitation of the DNA from the clear viscous aqueous solution with alcohol, resolution in water, and reprecipitation in alcohol. The final white, fibrous precipitate was wound about a glass rod, squeezed dry, and dried in a desiccator at room temperature. This material swells and then dissolves in water to form clear, colorless, viscous solutions which give a pure nucleic acid absorption spectrum. The criteria for high polymerization of this material are based on ultracentrifugation, high viscosity of solutions, and fibrous character of the precipitates. A 1 mg./cc. solution was found to have a phosphorus content of 0.085 mg./cc.

3. Ribonucleic acid (RNA) was purchased from Schwarz Laboratories, Inc. The white granular material dissolved in water on the addition of a little NaOH to bring the pH to between 6 and 7. The solutions were colorless and not viscous. They gave a pure nucleic acid absorption spectrum. A 1 mg./cc. solution contained 0.076 mg. P/cc.

4. Depolymerized DNA was prepared from a solution of DNA by the addition of desoxyribonucleo-depolymerase (5). To 15 cc. of a 1 mg./cc. solution of DNA, 0.8 cc. 0.15 M $NaHCO_3$ was added to adjust the pH to 7.5, then 3 cc. 0.1 mg./cc. gelatin, 5 cc. 0.015 M $MgSO_4$, and 2 cc. 4 mg./cc. depolymerase. The solution was allowed to stand overnight at room temperature, then stored in the refrigerator with toluene as preservative. The resultant solution is clear, colorless, not at all viscous. The addition of lanthanum salts, alcohol, or dilute acid results in a granular precipitate as for RNA instead of the fibrous precipitate obtained with DNH and DNA.

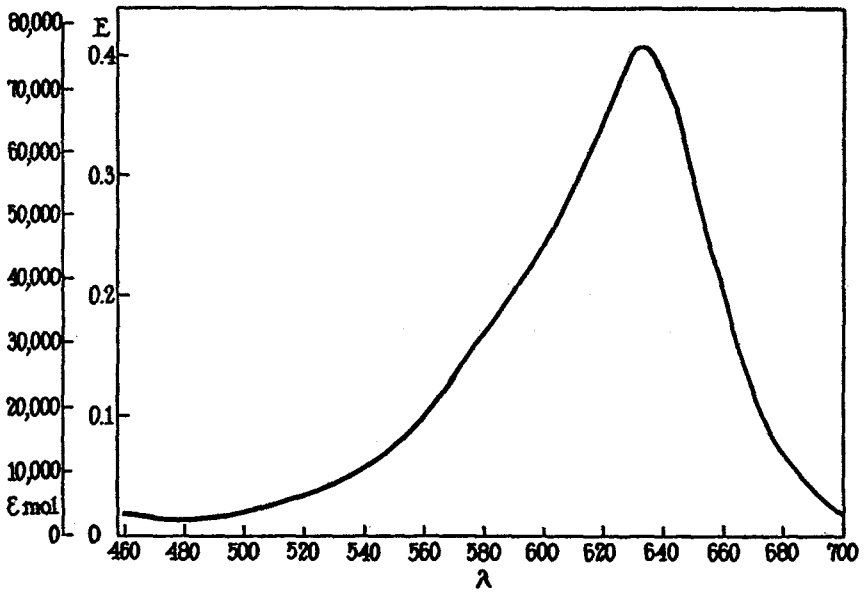
5. Ribonucleoprotein (RNP) was prepared from calf thymus as described by Mirsky and Pollister (6). The solution was stored in the cold with toluene as preservative. Its P content was 0.10 mg./cc.

6. Tobacco mosaic virus was provided through the courtesy of Dr. G. Oster of The Rockefeller Institute for Medical Research, Princeton, New Jersey. The solution contained 0.014 mg. P/cc.

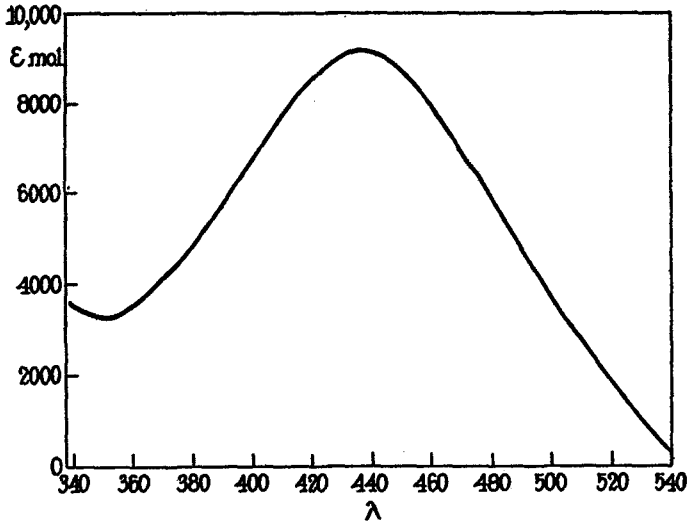
Stains

A brief description of the source and characteristics of the stains used follows:

1. The methyl green used in these experiments was labelled "National Aniline Corp., C.I. No. 684, certified for general histological staining, certification No. NC3." The commercial dye was made up to 0.25 per cent in 0.2 M pH 4.1 acetate buffer. In order to remove the considerable amount of crystal violet present in the commercial dye, the solution was extracted with successive portions of chloroform until the chloroform extracts were colorless. The absorption spectrum of this solution, suitably diluted with buffer, was determined in the Beckman spectrophotometer (Text-fig. 1). By comparison with the extinction coefficient at the maximum (635 mμ) of a known solution of the perchlorate (prepared by the precipitation of methyl green perchlorate from the purified solution of the dye described above by the addition of a few drops of a dilute aqueous solution of sodium perchlorate, washed with cold water, dried at 106°C., theoretical N 7.16 per cent, found 6.99 ± 0.2 per cent (Dumas)), this solution was found to contain 51.4 per cent of the labelled concentration of dye (ϵ_{635} (molar extinction coefficient at 635 mμ) = 74,400). This was consistent with the nitrogen content of the solution (theoretical N for 0.25 per cent solution 0.220 mg./cc., found 0.128 mg./cc., corresponding to a dye content of 55.7 per cent).



TEXT-FIG. 1. Absorption spectrum of methyl green perchlorate, 0.000323 per cent in pH 4.1 acetate buffer.

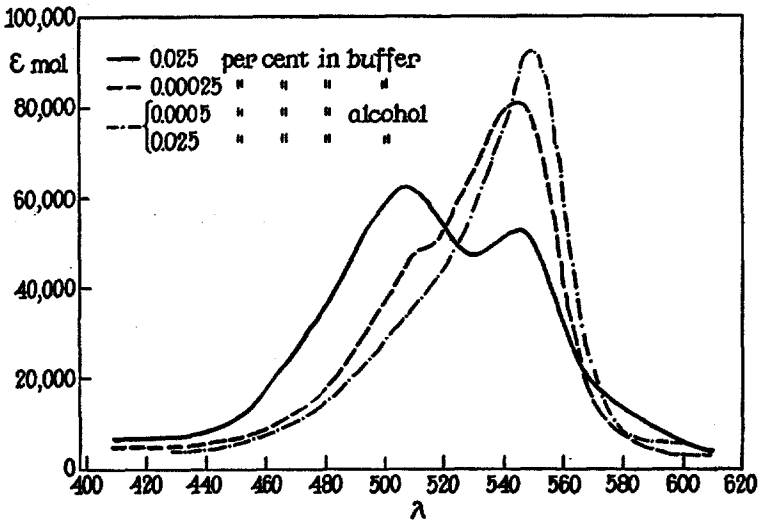


TEXT-FIG. 2. Absorption spectrum of methyl green in concentrated hydrochloric acid (0.00025 per cent and 0.0025 per cent).

Solutions of methyl green in concentrated HCl are brown with an absorption maximum at 440 mμ (Text-fig. 2). This brown solution follows Beer's and Lambert's laws, and has an ϵ_{440} of 8920.

In staining experiments with this dye, the use of a buffer at about pH 4.1 is essential. It was found that above pH 5.0 and below pH 3.5, solutions of the dye are very unstable to light. At pH 4.1, solutions stored in brown bottles for several months showed a reduction in extinction coefficients of less than 10 per cent, and no loss of absorption could be detected during the course of the experiments. It should be noted also that alcoholic solutions of the dye fade exceedingly rapidly. Solutions of the dye in the buffer follow Beer's and Lambert's laws, and the absorption spectrum is independent of the concentration.

2. Several samples of pyronin were investigated. All gave stable solutions which, unlike methyl green, showed no tendency to fade over the pH range 2.9 to 7.5. How-



TEXT-FIG. 3. Absorption spectrum of Grübler's pyronin in pH 4.1 acetate buffer and in alcohol.

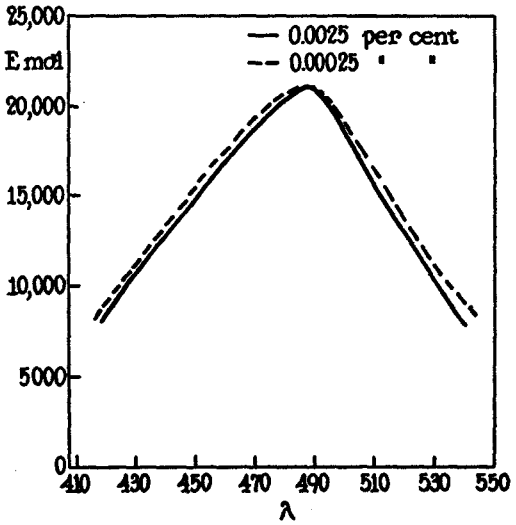
ever, most experiments were carried out in 0.2 M pH 4.1 acetate buffer as with methyl green. Phenol and glycerin, as prescribed in Unna's mixture, had no influence on the height or shape of the absorption spectrum. In dilute aqueous solutions, the absorption spectrum shows a slight irregularity at 510 mμ and a maximum at 550 mμ. In concentrated solutions, the slight hump at 510 mμ emerges as a definite maximum (Text-fig. 3). Beer's law is followed only in very dilute solutions. In alcohol a smooth curve with maximum at 550 mμ, uninfluenced by concentration, and with a molar extinction coefficient of 91,000, as compared to 79,000 in dilute aqueous solution, is obtained for pyronin Y. Alcoholic solutions obey Beer's law over a wide range of concentrations (Text-fig. 3). In concentrated HCl, a yellow-brown dye with a maximum at 490 mμ and $\epsilon_{490} = 21{,}100$ results (Text-fig. 4). This follows Beer's law over a wide range also (0.0025 per cent to 0.00025 per cent were compared). The dye content of the various dye preparations was determined by nitrogen determinations on aqueous solutions made up to 0.25 per cent by weight and by comparison of the absorption at

550 mμ of alcoholic solutions with an alcoholic solution of the corresponding perchlorate (prepared as for methyl green perchlorate from 0.25 per cent solution of pyronin by weight). The several pyronins used and their dye contents were:

Grübler pyronin, 10 per cent of theoretical nitrogen, 12 per cent of theoretical absorption (assumed to be pyronin B).

A. H. Metz pyronin, 13 per cent of theoretical nitrogen, 12 per cent of theoretical absorption (assumed to be pyronin B).

Pyronin Y, National Aniline Corp., certification No. NP 11, "38 per cent dye content," 24.5 per cent of theoretical absorption, 48.5 per cent of theoretical nitrogen (possibly contains contaminating nitrogenous salts).



TEXT-FIG. 4. Absorption spectrum of Grübler's pyronin in concentrated hydrochloric acid.

Pyronin B, Coleman and Bell, certification No. CP6, "41 per cent dye content," 53 per cent of theoretical absorption (ϵ_{550} = 104,700 in alcohol as determined for the perchlorate).

3. Ethyl green, C.I. No. 685, National Aniline Corp., certification No. NG-3, dye content 61.9 per cent by absorption, 56.2 per cent by nitrogen content (after $CHCl_3$ extraction of the solution). This dye was indistinguishable from methyl green in its properties.

4. Malachite green, C.I. No. 657, National Aniline Corp., certification No. NMg 13. This dye gives a slight β hump in concentrated solutions, but insufficient to cause significant deviation from Beer's law. (*Cf.* Text-figs. 7A and 7B.)

5. Crystal violet, National Aniline Corp.

6. Victoria Blue, National Aniline Corp.

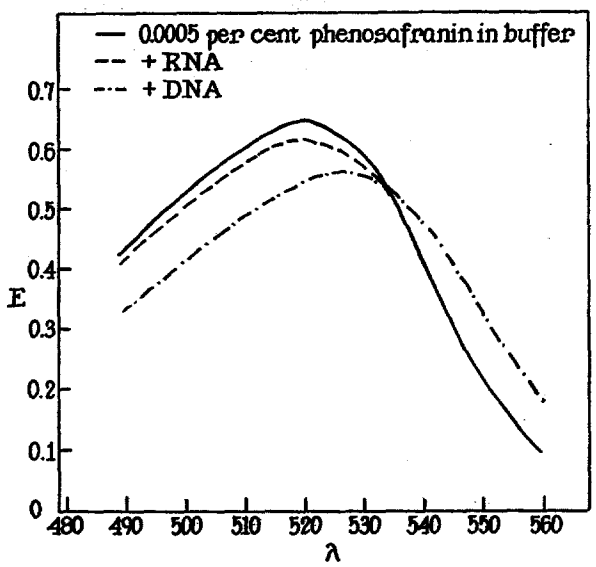
7. Phenosafranin, C.I. No. 840, National Aniline Corp. lot No. 6016. Whereas, unlike pyronin this dye reveals no β hump in its absorption spectrum, alcoholic solu-

tions and aqueous solutions containing DNA reveal maxima at longer wave lengths than simple aqueous solutions (Text-fig. 5). High concentrations in aqueous solutions result in a shift of the maximum to shorter wave lengths (Text-fig. 6).

8. Bismarck brown Y, C. I. No. 331, National Aniline Corp.

Staining with the Unna-Pappenheim Mixture

A simple qualitative test of the relationship between states of polymerization and selective staining by methyl green–pyronin was performed by staining

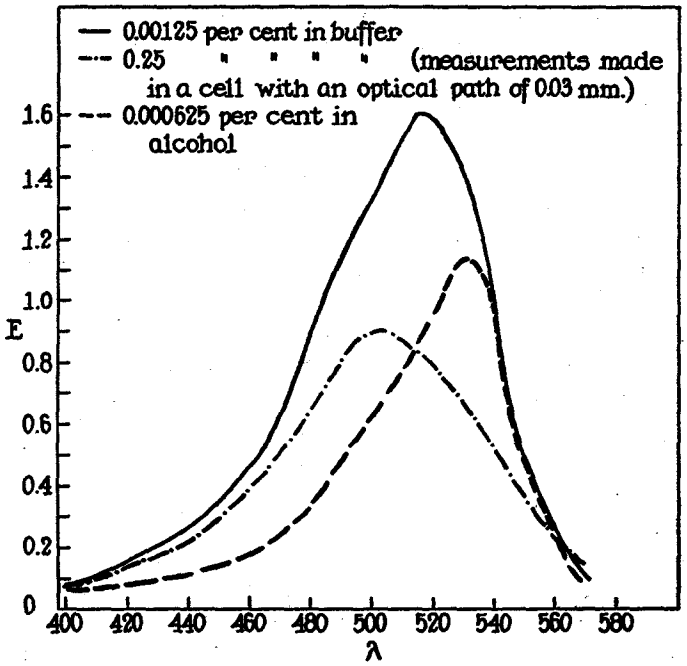


TEXT-FIG. 5. (*a*) Absorption spectrum of 0.0005 per cent phenosafranin in pH 4.1 acetate buffer. (*b*) Same with 4 mg. per cent RNA (P = 0.328 mg. per 100 cc.). (*c*) Same with 4 mg. per cent DNA (P = 0.32 mg./100 cc.).

nucleic acids and nucleoproteins deposited on glass slides. Measured drops of the solutions investigated were pipetted onto albumin-coated glass slides, allowed to dry at 45°C., stained for 30 minutes in the methyl green–pyronin mixture of Unna, rinsed in water, differentiated in 95 per cent alcohol, dehydrated in 100 per cent alcohol and xylene, and mounted in clarite. The solutions used were: (*a*) 0.010 cc. DNH (P = 0.27 mg./cc.), (*b*) 0.025 cc. DNA (P = 0.085 mg./cc.), (*c*) 0.042 cc. depolymerized DNA (P = 0.049), mg./cc.),(*d*) 0.015 cc. RNA (P = 0.152 mg./cc.), (*e*) 0.025 cc. RNP (P = 0.10 mg./cc.) (*f*) 0.2 cc.tobacco mosaic virus (P = 0.014 mg./cc.). After rinsing in water, all the spots appeared purplish brown, but after a few minutes in alcohol, differential staining became apparent. The DNH and DNA spots were bright green, while the depolymerized DNA, RNA, and RNP spots were pink (Fig. 1).

Staining with Dyes Separately

For quantitative and semiquantitative study of the degree of selectivity, it is necessary to work with each dye separately rather than with mixtures. That this was feasible, and that the peculiar selectivity was not a property of the mixture only, could be demonstrated by staining tissue sections with aqueous solution of each dye alone. Cells stained in 0.25 per cent (by weight of



TEXT-FIG. 6. Absorption spectrum of phenosafranin in pH 4.1 acetate buffer and in alcohol.

commercial dye) aqueous methyl green (chloroform-extracted) display green chromatin, while nucleoli and cytoplasm remain unstained. On the other hand, cells stained in 0.25 per cent aqueous pyronin reveal pink cytoplasm and nucleoli, and only very pale pink chromatin (which is consistent with the ribonucleic acid content of chromosomes (3)).

1. Methyl Green Alone.—The addition of DNA to dilute solutions of methyl green results in an increase in the absorption at the maximum, which is shifted from 635 to 645 mμ. It was observed that this change, although apparent at once to the eye as a change in color from blue-green to emerald green, progressed slightly at room temperature for 1 to 2 hours (about 15 per cent increase in E_{645} (optical density at 645 mμ) from zero time) and was stationary thereafter.

For this reason, in all precipitation and dialysis experiments, the mixtures were allowed to stand at room temperature in the dark for at least 2 hours before the precipitant was added or the dialysis begun. This color change was not observed with RNA and depolymerized DNA in the concentrations used in our experiments (0.1 to 0.3 mg. nucleic acid phosphorus in 5 cc. of 0.0025 to 0.005 per cent methyl green). However, ten to twenty times this amount of RNA shifted the absorption maximum to 640 mμ without increasing the extinction coefficient. On precipitation of the nucleic acid with lanthanum acetate, the dye which remains in the supernate is found to have the color and absorption spectrum of pure dye solutions.

A simple qualitative demonstration of the effect of polymerization is based upon this color change in the presence of DNA. To 0.5 cc. 0.028 per cent methyl green (corrected dye content) in 0.2 M pH 4.1 acetate buffer, were added 3.5 cc. buffer and 1 cc. 2 mg./cc. DNA (P = 0.158 mg.). Such mixtures, with controls consisting of 0.5 cc. 0.028 per cent methyl green and 4.5 cc. buffer, were incubated at 0°C., 22°C., 40°C., 60°C., and 100°C. Within 10 minutes, the DNA mixtures kept at 60°C. and 100°C. had changed from emerald green back to the blue-green of the controls, while the mixtures kept at the lower temperatures remained emerald green. Upon the addition of 0.5 cc. 15 per cent lanthanum acetate the solutions maintained for 2 hours at 0°C., 22°C., and 40°C. gave green fibrous precipitates, while those kept at 60°C and 100°C. gave almost colorless granular precipitates. This change in the character of the precipitates indicates that depolymerization of the DNA had occurred at the higher temperatures, with the consequent loss in affinity for methyl green. Reducing the temperature after exposure to 60–100°C. did not reverse the color change in the solutions nor the character of the $LaAc_3$ precipitates.

This observation appeared inconsistent with the reported lack of influence of hydrolysis, as performed in the Feulgen reaction, on methyl green staining (7). We therefore performed the following quantitative experiments.

(*a*) To 1 cc. 4 mg./cc. DNA (P = 0.320 mg./cc.), 0.5 cc. 3 N HCl was added so as to make a 1 N HCl solution as used in Feulgen hydrolysis; (*b*) to 1 cc. 4 mg./cc. DNA, 0.5 cc. 0.2 M pH 4.1 acetate buffer was added. These tubes were heated for 12 minutes in a 60°C. water bath and cooled. To (*a*), 0.4 cc. 5 N NaOH was added to approximately neutralize the solution, 1.9 cc. buffer to bring the pH to 4.1, 1.0 cc. 0.128 per cent methyl green. To (*b*), 2.3 cc. buffer + 1 cc. 0.128 per cent methyl green was added. After 1 hour at room temperature, 0.2 cc. 1.5 per cent $LaAc_3$ was added to each. Controls consisted of (*c*) a mixture prepared as in (*b*) but not heated and (*d*) a mixture consisting of 1 cc. H_2O + 2.8 cc. buffer + 1 cc. methyl green + 0.2 cc. $LaAc_3$. (*a*) and (*b*) each gave granular precipitates while the unheated DNA mixture (*c*) gave a fibrous precipitate. The precipitates were removed by centrifugation, the supernates diluted 1:100 with buffer, and their absorptions compared at 635 mμ. Aliquots of the supernates were used for phosphorus determinations and found to contain none. The absorptions at 635 mμ are given in Table I.

Thus heating DNA at 60°C. for 12 minutes results in considerable loss of staining with methyl green.

A similar experiment was performed with isolated calf liver nuclei (8):

(*a*) 1 cc. calf liver nuclei in 0.2 per cent citric acid (P_{DNA} = 0.228 mg./cc.) + 0.5 cc. 3 N HCl, heated at 60°C. for 12 minutes, cooled, + 0.4 cc. 5 N NaOH (to approximately neutralize), + 2.1 cc. 0.2 M pH 4.1 buffer (to adjust pH to 4.1) + 1 cc. 0.128 per cent methyl green. (*b*) 1 cc. nuclei + 3.0 cc. buffer, heated at 60°C. for 12 minutes, cooled, + 1 cc. methyl green. (*c*) 1 cc. nuclei + 3.0 cc. buffer, let stand at room temperature 12 minutes, + 1 cc. methyl green.(*d*) 1 cc. nuclei + 1 cc. 0.2 N HCl, let stand 12 minutes, at room temperature, + 2 cc. buffer + 1 cc. methyl green. (*e*) 4.0 cc. buffer + 1 cc. methyl green.

TABLE I

Mixture	E_{635}	$\frac{\mu M \text{ dye in supernate}}{\frac{E_{635} \times 100 \times 10^6 \times 5}{74{,}400 \times 10^3}}$	μM dye in precipitate	μM dye/μM P_{DNA}
(*a*)	0.314	2.11	0.35	0.034
(*b*)	0.286	1.92	0.54	0.052
(*c*)	0.239	1.61	0.85	0.083
(*d*)	0.366	2.46	0	

TABLE II

Mixture	E_{635}	μM dye in supernate	μM dye in precipitate	μM dye/μM P_{DNA}
(*a*)	0.341	2.29	0.17	0.0231
(*b*)	0.314	2.11	0.35	0.0476
(*c*)	0.315	2.12	0.34	0.0463
(*d*)	0.256	1.72	0.74	0.10
(*e*)	0.366	2.46	0	

After 2 hours, the nuclei were centrifuged off, the supernates diluted 1:100 with buffer, and the extinctions at 635 mμ were compared (Table II).

Thus, heating nuclei in 1 N HCl caused considerable loss of staining with methyl green. Whereas the heating of DNA to 60°C., even in a pH 4.1 buffer caused loss of staining with methyl green, nuclei were not affected by such treatment. The greater staining after treatment with cold dilute HCl is attributed to the displacement of competing histone (cf. similar competition between histone and crystal violet (9)).

Finally, nuclei smeared on glass slides and hydrolyzed for 12 minutes at 60°C. in 1 N HCl, then stained in buffered 0.128 per cent methyl green, failed to take any visible stain, whereas smears placed in 0.1 N HCl for 12 minutes at room temperature stained readily.

We were thus unable to confirm Di Stefano's results (7) and are of the opinion that acid hydrolysis for 12 minutes partially depolymerizes the DNA so as to interfere with methyl green staining. It may be that Di Stefano's findings resulted from the use of unpurified commercial samples of methyl green (personal communication), which contain considerable amounts of crystal violet. Since crystal violet staining is not dependent upon the state of polymerization of the nucleic acids, the loss of methyl green staining may have been obscured.

The effect of the state of polymerization on staining with methyl green was studied also by comparing the amounts of stain bound by nucleic acids precipitated from mixtures of the stain and nucleic acid solutions.

To 5 cc. of a 0.128 per cent solution of methyl green in acetate buffer, solutions of DNA, depolymerized DNA, or RNA containing similar amounts of phosphorus (P = 0.20 to 0.24 mg.), were added and made up to 10 cc. with buffer. The nucleic

TABLE III

	P in precipitate	μM P in precipitate	E_{440} of HCl solution calculated for 10 cc.	μM dye	μM dye/μM P
	mg.				
DNA	0.16	5.17	0.561	0.628	0.12
Depolymerized DNA	0.12	3.87	0.082	0.092	0.02
RNA	0.19	6.13	0.247	0.277	0.045

acid–stain complex was then precipitated with an equal volume of alcohol. The precipitate was washed several times with alcohol, until the washes were colorless. The polymerized DNA precipitate was now a deep green, whereas the depolymerized DNA and RNA were practically colorless. The washed precipitates were dried at room temperature, and dissolved in a known volume of concentrated HCl (4 cc. for RNA and depolymerized DNA, 10 cc. for DNA). The optical density of the brown HCl solution was measured against a concentrated HCl blank at 440 mμ. The phosphorus content of an aliquot of the HCl solution was determined. The results are given in Table III.

As will be noted, the polymerized DNA stains several times as intensely as the depolymerized DNA and RNA. This experiment is to be regarded only as a semiquantitative demonstration of the hypothesis, however, since unstained solutions of nucleic acids dissolved in concentrated HCl in the above concentrations gave E_{440} of 0.032 to 0.057. The effect of this correction on the μM dye/μM P_{DNA} would be to reduce it by less than 10 per cent, but in the case of depolymerized DNA and RNA, significant corrections result. As will be seen in the subsequent paper (10), the stoichiometry for DNA-methyl green found here is reproduced by other methods, whereas the intensity of RNA and depolymerized DNA staining indicated by these results is too great.

The effect of the degree of polymerization of the nucleic acids on staining with methyl green was also investigated by precipitating the stained compound from solution with lanthanum acetate. The amount of dye precipitated was determined by the loss of light absorption in the supernate as compared with a control (containing dye without nucleic acid) and the nucleic acid precipitated was determined by difference (nucleic acid phosphorus added minus phosphorus found in supernate). Since, as will be seen in the subsequent paper on the stoichiometry of the DNA-methyl green reaction (10), lanthanum competes with the dye for the nucleic acid, the results are only of relative value.

TABLE IV

	μM P in precipitate	E_{635} supernate	μM dye in 5 cc. supernate	μM dye in precipitate	μM dye/μM P in precipitate
DNA	5.0	0.530	0.356	0.199	0.04
Depolymerized DNA	5.68	0.810	0.545	0.010	0.0018
RNA	5.3	0.820	0.552	0.003	0.0006
Control	0	0.825	0.555	0	

TABLE V

	μM P in precipitate	E_{635} supernate	μM dye in 5 cc. supernate	μM dye in precipitate	μM dye/μM P in precipitate
DNA	5.1	0.121	0.081	0.223	0.044
Depolymerized DNA	5.62	0.445	0.295	0.009	0.0016
Control	0	0.452	0.304	0	

To 1 cc. 0.025 per cent dye in buffer + 2.5 cc. buffer, was added 1 cc. of DNA (P = 0.155 mg./cc.), depolymerized DNA (P = 0.176 mg./cc.), or RNA (P = 0.164 mg./cc.). After 2 hours, 0.5 cc. 15 per cent $LaAc_3$ was added, the precipitates removed by centrifugation, and the absorptions of the supernates at 635 mμ compared after diluting 1:10. The control contained 1 cc. H_2O instead of nucleic acid solution. The results are tabulated in Table IV.

The same experiment, using one-half the dye concentration and 0.5 cc. 0.6 per cent $LaAc_3$ as the precipitant instead of 0.5 cc. 15 per cent $LaAc_3$, gave the results tabulated in Table V.

Thus we note that depolymerization of the DNA has resulted in a 25-fold decrease in staining with methyl green.

As an indication of the specificity of staining by methyl green, and of the rôle of polymerization of carbohydrates in staining with this dye, 1 cc. of a solution of Pneumococcus Type III polysaccharide[2] (2 mg./cc.) was mixed

[2] The bacterial polysaccharides were provided by Dr. Goebel of The Rockefeller Institute for Medical Research.

with 4 cc. of buffered 0.003 per cent methyl green. No change in the absorption spectrum of the solution, such as has been described above with DNA, occurred. To 5.5 cc. of this mixture, 0.5 cc. 15 per cent lanthanum acetate was added. The flocculent precipitate which resulted was practically colorless, and the supernate revealed no loss of dye as measured by its absorption at 635 mμ. The very faint blue-green color in the precipitate is rapidly removed with alcohol. On the other hand, crystal violet applied in place of methyl green yields a deep purple precipitate which retains most of its color despite repeated washing with alcohol (crystal violet stains both RNA and DNA without selectivity).

Smears of capsulated Friedländer's bacillus did not stain at all with methyl green. When stained in the Pappenheim mixture (methyl green–pyronin), the organisms from young broth cultures appeared as pink outlines containing red polar bodies. A similar picture of a dark outline with two dense polar bodies is seen in the phase contrast microscope.

2. Pyronin Alone.—It was found that in sufficiently high concentration, pyronin caused the precipitation of nucleic acids and nucleoproteins from solution. It was thus possible to study the effect of the state of polymerization of nucleic acids on pyronin staining by precipitation with pyronin alone or, when dilute pyronin solutions were used, with the aid of alcohol or $LaAc_3$.

To 5 cc. (*a*) 1 mg./cc. DNA (P = 0.08 mg./cc.), (*b*) depolymerized DNA (P = 0.05 mg./cc.), (*c*) 1 mg./cc. RNA (P = 0.09 mg./cc.), (*d*) RNP (P = 0.1 mg./cc.), and (*e*) DNH (P = 0.11 mg./cc.), 5 cc. of 0.0625 per cent aqueous solution of pyronin Y (National Aniline) was added. In each case a precipitate resulted (fibrous in the case of DNA and DNH, granular in the others). Upon removal of the precipitates by centrifugation (the fibrous precipitates could usually be removed with a glass rod without centrifugation), the supernates were analyzed (*a*) spectrophotometrically for dye by comparison of the absorption at 550 mμ to a control dye solution and (*b*) by the method of Allen (11) for phosphorus content. The results are given in Table VI.

A similar experiment was performed with Grübler's pyronin as follows:—

Control: 2 cc. 0.025 per cent pyronin in pH 4.1 acetate buffer + 3 cc. H_2O.
RNA : 2 cc. 0.025 per cent pyronin + 2 cc. H_2O + 1 cc. 20 mg./cc. RNA (P = 1.58 mg./cc.).[3]
DNA : 2 cc. 0.025 per cent pyronin + 2 cc. H_2O + 1 cc. 2 mg./cc. DNA (P = 0.160 mg./cc.).

RNA yielded a granular precipitate and DNA a swollen fibrous precipitate. The precipitates were removed by centrifugation and the supernates analyzed for dye and phosphorus content as above. The results are presented in Table VII.

We can see from the two following tables that depolymerized DNA, RNA, and RNP stain similarly, while DNA and DNH reveal less affinity for pyronin.

[3] The large amount of RNA is required to obtain a significant precipitate.

With pyronin, therefore, as with methyl green, the state of polymerization of nucleic acid appears to influence the degree of staining.

Samples of nucleic acids and nucleoproteins containing 0.07 to 0.15 mg. P were precipitated from solution by the addition of alcohol and a little salt. These precipitates were stained with 0.025 per cent Grübler's pyronin. Such precipitates, when washed with alcohol, continued to lose stain for many days

TABLE VI

	DNA	Depolymerized DNA	RNA	RNP	DNH	Control
P content of precipitate, *mg*	0.4	0.2	0.3	0.44	0.53	0
μM	12.9	6.5	9.7	14.2	17.1	
Dye content of supernate, expressed as E_{550} diluted 1:100	0.671	0.614	0.609	0.404	0.761	0.875
Dye content of supernate, *μM**	8.5	7.77	7.7	5.11	9.6	10.9
Dye content of precipitate, *μM*	2.4	3.1	3.2	5.8	1.3	0
Dye/P, molar	0.19	0.48	0.33	0.41	0.076	

* $= \frac{E_{550} \times 1000.}{79}$

TABLE VII

	Control	RNA	DNA
P content of original mixture, *mg*	0	1.58	0.16
P content of supernate, *mg*	0	1.54	0.034
P content of precipitate, *mg*	0	0.04	0.13
μM		1.29	4.1
E_{625} of supernate, diluted 1:30	0.895	0.359	0.094
Pyronin in supernate, *μM*	1.70	0.682	0.179
Pyronin in precipitate, *μM*	0	1.02	1.52
Dye/P, molar		0.79	0.37

but quickly reached an end point with acid alcohol. Unlike pyronin Y, which is completely removed from nucleic acid precipitates by acid alcohol, a small amount of Grübler's pyronin remains attached. Such precipitates, washed with acid alcohol, were completely dissolved in a small volume of concentrated HCl and compared spectrophotometrically. The results were reproducible within 15 per cent (as contrasted with the variable results obtained with washed pyronin Y precipitates). The results of four experiments are averaged in Table VIII.

In another effort to compare the staining of nucleic acid solutions with pyronin, dilute solutions of pyronin and nucleic acids were mixed and the nucleic acid–stain complex precipitated with lanthanum acetate, as was done with

methyl green. Experiments were set up in acetate buffer so that the final concentration of dye was 0.0025 per cent (except pyronin B, where 0.004 per cent

TABLE VIII

	DNA	Depolymerized DNA	RNA	RNP	DNH
Dye/P, molar	0.0035	0.02	0.02	0.02	0.0023

TABLE IX

	Control	RNA	DNA	Depolymerized DNA
Pyronin Y				
Nucleic acid P in mixture, *mg.*		0.162	0.155	0.174
in supernate, *mg.*		0.086	0	0.125
in precipitate, *mg.*		0.076	0.155	0.049
P in precipitate, μM		2.44	5.0	1.61
E_{550} supernate, diluted 1:10	0.608	0.419	0.482	0.515
Pyronin Y in precipitate, μM (ϵ_{550} = 79,000)		0.138	0.087	0.062
Dye/P molar		0.057	0.017	0.039
Grübler's pyronin				
Nucleic acid P in mixture, *mg.*	0	0.131	0.128	
in supernate, *mg.*		0.036	0.003	
in precipitate, *mg.*		0.095	0.125	
P in precipitate, μM		3.06	4.03	
E_{547} supernate, diluted 1:10	0.619	0.281	0.380	
Pyronin Grübler in supernate, μM (ϵ_{547} = 79,000)	0.353	0.160	0.217	
Pyronin Grübler in precipitate, μM		0.193	0.136	
Dye/P, molar		0.063	0.033	
Pyronin B				
Nucleic acid P in mixture, *mg.*		0.162	0.155	
in supernate, *mg.*		0.047	0	
in precipitate, *mg.*		0.115	0.155	
P in precipitate, μM		3.7	5.0	
E_{550} supernate diluted 1:30	0.432	0.344	0.390	
Pyronin B in precipitate, μM (ϵ_{550} = 87,900)		0.15	0.072	
Dye/P, molar		0.04	0.014	

was used) and of nucleic acid phosphorus about 0.0032 per cent. To 4.5 cc. of these mixtures, 0.5 cc. 1.5 per cent lanthanum acetate was added, the precipitates were removed, and the supernates analyzed for dye content and phosphorus. The results are given in Table IX.

These experiments thus demonstrate a two- to threefold greater staining by pyronin of low polymers of nucleic acid than of high polymers.

It should be noted that in the mixtures described in these experiments, using dilute dye solutions, DNA changes the color of the solution from orange-red to rose-red with a shift in the absorption maximum from 550 mμ to 565 mμ. This change was much less marked with similar concentrations of RNA or depolymerized DNA, but did occur when the concentration of RNA was increased tenfold.

Staining with Related Dyes

The staining of nucleic acids by several other dyes was investigated in the hope that some light might be shed upon the structural determinants of dye selectivity. The experiments were set up as follows:—

Control: 0.5 cc. 0.05 per cent dye (by weight of commercial powder) in 0.2 M pH 4.1 acetate buffer + 4.3 cc. buffer + 0.2 cc. 1.5 per cent $LaAc_3$.

RNA: 0.5 cc. dye + 3.3 cc. buffer + 1 cc. 2 mg./cc. RNA (P = 0.164 mg.) + 0.2 cc. $LaAc_3$.

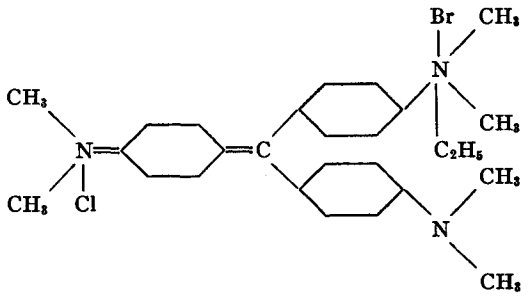
DNA: 0.5 cc. dye + 3.3 cc. buffer + 1 cc. 2 mg./cc. DNA (P = 0.160 mg./cc.) + 0.2 cc. $LaAc_3$.

Depolymerized DNA: 0.5 cc. dye + 2.3 cc. buffer + 2 cc. depolymerized DNA (P = 0.087 mg./cc.) + 0.2 cc. $LaAc_3$.

The composition of the precipitates was calculated from the change in the extinction coefficient (at the maximum absorption of the dye) of the supernate upon precipitation of the nucleic acid, and from the residual phosphorus content of the supernate.

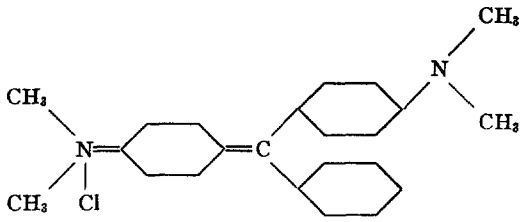
The following triphenylmethane dyes were examined in order to compare their selectivity to methyl green:

(*a*) Ethyl green:



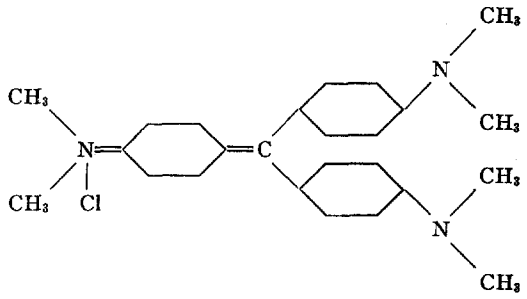
National Aniline Corp., certification No. NG-3, dye content 61.9 per cent by absorption, 65.2 by nitrogen content (after $CHCl_3$ extraction of solution).

(*b*) Malachite green:



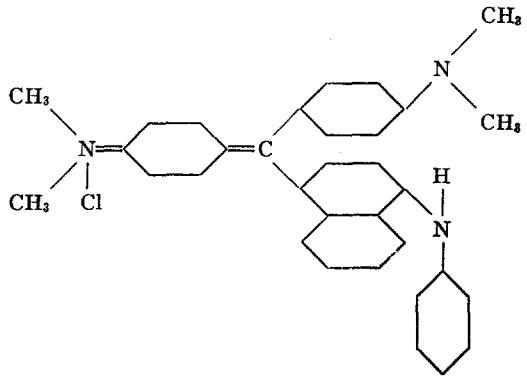
C. I. Number 657, National Aniline Corp., certification No. NMg 13

(*c*) Crystal violet:



National Aniline Corp., C. I. Number 681.

(*d*) Victoria blue:



National Aniline Corp., C. I. Number 729.

The results are tabulated (Table X). The values must be compared only for each dye, since no correction was made for dye content except for methyl green and ethyl green (based upon $\epsilon_{635} = 74{,}400$).

Even then, the values are of relative significance only, since the use of dilute dye solutions and lanthanum is not optimum for stoichiometric study. (See above and following paper (10).)

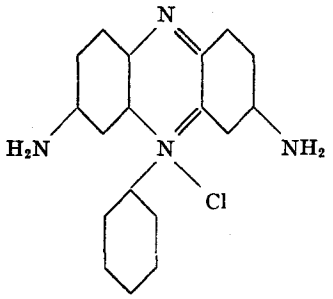
Ethyl green showed the same color change with DNA as did methyl green, and the same order of selectivity. Malachite green also demonstrated a shift

in the position of the absorption maximum from 620 mμ to 630 mμ with DNA but not with RNA or depolymerized DNA. It also revealed selectivity for the higher polymer, but to a lesser degree than did methyl green. Crystal violet and Victoria blue failed to demonstrate any selectivity.

TABLE X

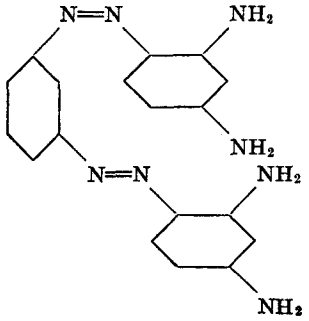
Dye	Dye/P, Molar		
	RNA	DNA	Depolymerized DNA
(1) Methyl green	0.004	0.05	0.004
(2) Ethyl green	0.007	0.04	0
(3) Malachite green	0.006	0.017	0.007
(4) Victoria blue	0.13	0.1	0.12
(5) Crystal violet	++	++	++

In the same manner we examined phenosafranin,



C. I. Number 840, National Aniline Corp., lot Number 6016

and Bismarck brown Y,



C. I. Number 331, National Aniline Corp., certification No. NN9.

Phenosafranin was selected because it has been recommended as a nuclear stain, and, like methyl green, has been used as a stain for lignin. Bismarck brown was chosen because it has been recommended as a stain for mucin in con-

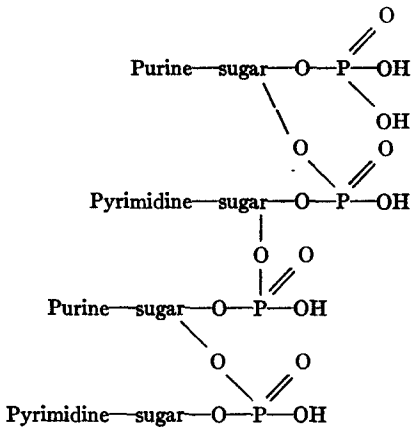
trast to methyl green (12). Both of these dyes failed to distinguish between the nucleic acids. Bismarck brown Y, even in dilute solutions, caused the precipitation of nucleic acids. It therefore provided an opportunity to compare the reaction with and without lanthanum. It was found that here, as with methyl green (10), lanthanum competed with the dye for the nucleic acid in that the addition of 1.5 per cent lanthanum acetate reduced the amount of stain bound by the nucleic acids by 55 per cent.

DISCUSSION

Whereas, we have observed that staining of DNA and presumably also RNA by methyl green and pyronin is a function of the state of polymerization of the nucleic acid substrates, we can only guess at the mechanism of the selection. The fact that mixtures of methyl green and pyronin are selectively adsorbed by strips of filter paper or permutit columns, suggests that the staining of nucleic acids may be, at least in part, a physical phenomenon, with selective adsorption determined by the physical state of the substrate molecules. However, the constant stoichiometry of methyl green staining of polymerized DNA, and the competition of lanthanum and of histone (10) point to chemical union with the phosphoric groups.

We have seen that triphenylmethane dyes with two methyl-amino groups, such as methyl green, ethyl green, and malachite green are selective for polymerized nucleic acids, while those with three amino groups, such as crystal violet and Victoria blue show no such selectivity. This appears particularly significant when one contrasts methyl green and crystal violet, in which the only difference lies in the methylation of one amino group of crystal violet, thus converting it into the quaternary ammonium ion of methyl green. Crystal violet stains both DNA and RNA with one dye molecule combining with two phosphoric groups (9), while methyl green stains polymerized DNA with one dye molecule per 10 phosphoric groups (10), and practically fails to stain RNA or depolymerized DNA. This suggests that the triphenylmethane dyes are bound to nucleic acid molecules by two amino groups. This requires that the spacing between pairs of phosphoric groups match that between a pair of amino groups in the dye molecule. In the case of crystal violet, three such spacings are possible, while in the methyl green molecule, only one exists. It may be that in highly polymerized DNA, every fifth pair of phosphoric groups presents the appropriate spacing, while in depolymerized DNA and in RNA only rarely does the correct spacing occur. The much less strict requirements of crystal violet permit all pairs of phosphoric groups to participate.

The selectivity of pyronin is even more puzzling. However, it is possible that this molecule combines preferentially with the dibasic phosphoric acid groups. If nucleotides are indeed linked as indicated by Levene's structure

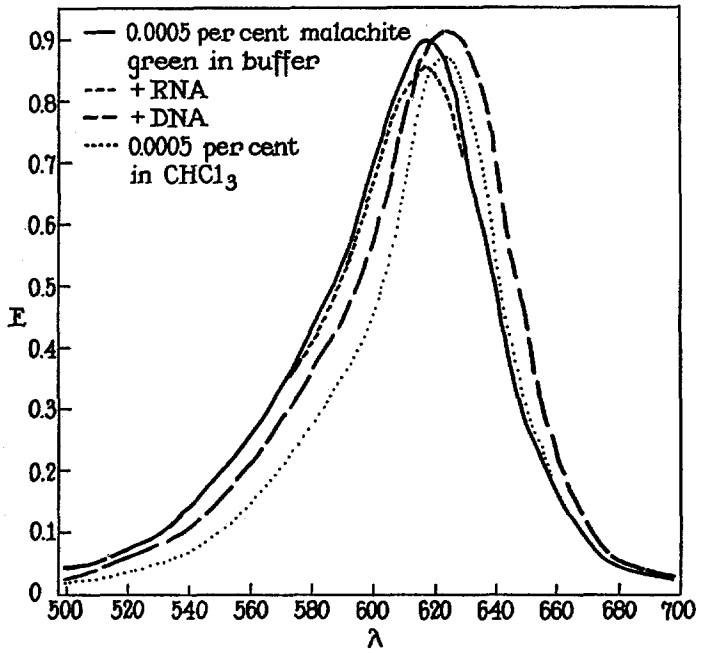


it follows that a high polymer will offer fewer dibasic groups than a low polymer. Thus, a tetranucleotide would contain one dibasic phosphoric acid and a 40-nucleotide would likewise contain only one dibasic group.

Similar considerations may explain our failure to observe staining of highly polymerized bacterial polysaccharides with methyl green. Nevertheless, it is possible that still higher degrees of polymerization would afford steric opportunities for staining with this dye. This is suggested by the observation that lignin, but not cellulose and mucin, is stained by methyl green (13). Whereas these polysaccharides have different chemical compositions, on maceration with strong nitric acid and potassium chlorate, lignin gives the color reactions of cellulose with iodine and sulfuric acid, and with zinc chloroiodide. This treatment very likely causes partial depolymerization of the lignin. Methyl green staining is lost following maceration (13). Basic dyes such as Bismarck brown and crystal violet, which lack the selectivity of methyl green, stain mucin and plant cellulose as well as lignin.

We have observed that dilute solutions of DNA shift the absorption maxima of methyl green, ethyl green, malachite green, phenosafranin, and pyronin to longer wave lengths, while RNA does so only in much more concentrated solutions. We believe that the shift in the absorption spectrum is a manifestation of the orientation of the dye by its attachment to an oriented structure (the fibrous DNA polymer in this case). Such orientation may also be construed as preventing polymerization of the dye, as suggested by Michaelis (14), but then we must assume polymerization, at least to a slight degree for methyl green, malachite green, and phenosafranin, which Michaelis considered always to be monomers. Indeed, such a possibility is suggested by the slight irregularity in the ascending limb of the absorption spectrum found in concentrated solutions of malachite green which disappears on dilution, suggesting a β curve (Text-fig. 7B), on the shift in the absorption spectrum of malachite green when dis-

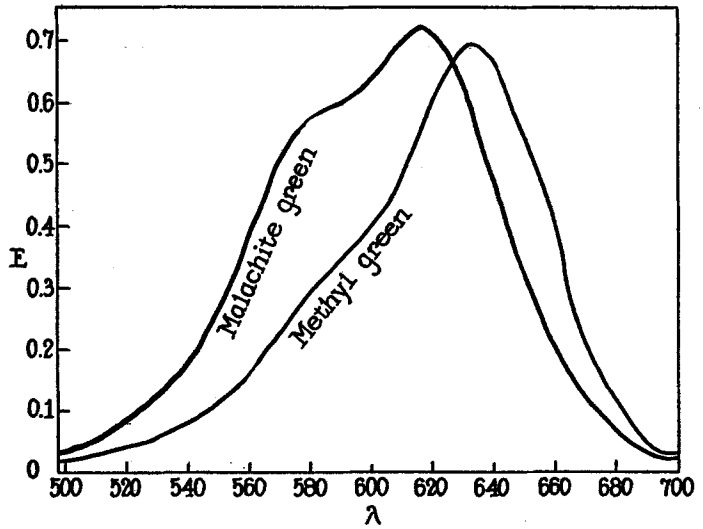
solved in chloroform (resembling that in DNA) (Text-fig. 7A), and by the shift in the phenosafranin spectrum on dilution of aqueous solutions or in alcohol (resembling that in DNA) (Text-figs. 5 and 6). Since neither methyl green nor phenosafranin ever exhibit a β maximum to indicate the presence of dimers, we must assume that the polymeric forms have maxima so close to that of the monomer as to result in smooth curves (note that mixtures of excesses of methyl green with DNA, which give absorption maxima between 635 mμ and 645 mμ



TEXT-FIG. 7A (*a*) Absorption spectrum of malachite green in pH 4.1 acetate buffer. (*b*) Same with 4 mg. per cent RNA (P = 0.328 mg./100 cc.). (*c*) Same with 4 mg. per cent DNA (P = 0.32 mg./100 cc.). (*d*) Malachite green in chloroform.

(indicating a mixture of the uncombined dye and the dye-DNA complex) still give smooth curves (10)). That such orientation of the dye by the nucleic acid may be the factor which determines the shift in the absorption spectrum, is indicated by the failure of dilute RNA solutions to cause the shift, even when the dye is not in excess (particularly with such dyes as phenosafranin and pyronin which stain RNA readily). The smaller RNA fails to provide the backbone for the orientation of neighboring dye molecules. However, when the concentration of RNA is increased manyfold, the shift is noted, suggesting that the "crowding" of the short linear (?) RNA molecules has imposed some orientation upon them. To state the hypothesis in another way, we can think of a dilute solution of methyl green as containing molecules of the dye moving

freely and at random and free to interact with each other. The addition of suitably polymerized nucleic acid molecules, containing chains of 20 or more nucleotides will bind two or more dye molecules to a single straight chain, thus restricting the motion of the dye molecules and preventing their interaction. Chains of less than 20 nucleotides will bind 0 to one molecule of dye and thus not significantly influence the opportunity for interaction. The shift of the absorption maxima by DNA toward longer wave lengths is that usually seen with dyes which are known to polymerize (14).



TEXT-FIG. 7B. Absorption spectrum of malachite green and methyl green in pH 4.1 acetate buffer, dye concentration 0.25 per cent, 0.03 mm. thickness.

The effect of polymerization on staining with methyl green–pyronin accounts for the occasional failure to obtain methyl green staining of nuclei in tissue sections. This is most apt to occur after aqueous acid fixation, such as Zenker's, where prolonged contact with the acid permits depolymerization of the DNA. Special care in the handling of tissues to avoid such depolymerization is essential if methyl green–pyronin is to be applied as a histochemical technique.

SUMMARY

1. Methyl green stains selectively highly polymerized desoxyribonucleic acid, and fails to stain, to any significant extent, depolymerized desoxyribonucleic acid and ribonucleic acid.

2. Pyronin stains preferentially low polymers of nucleic acid.

3. Triphenylmethane dyes with two amino groups appear to share the selectivity of methyl green. Those with three amino groups are not selective.

4. A stereochemical hypothesis is offered to account for these observations.

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EXPLANATION OF PLATE 1

FIG. 1. Unna-Pappenheim stain of nucleic acids, showing effect of depolymerization.

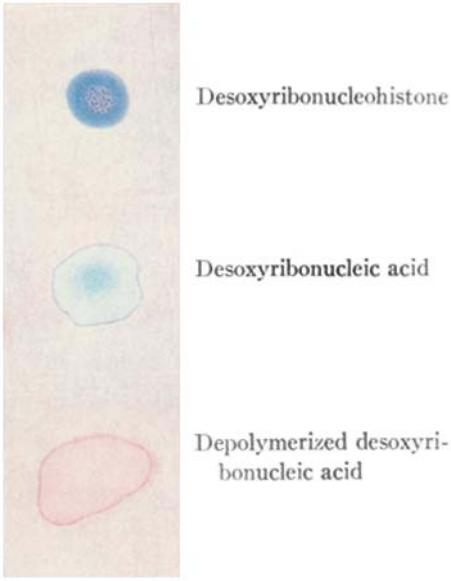

FIG. 1

(Kurnick: Methyl green–pyronin. I)