METHYL GREEN–PYRONIN

II. Stoichiometry of Reaction with Nucleic Acids

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We have observed (1) that the selective staining of nucleic acids by methyl green and pyronin is a function of the relative states of polymerization of the nucleic acids. The purpose of this paper is to present studies on the stoichiometry of the reactions, in order to elucidate the mechanism of the selectivity and to explore the possibility of quantitative histochemical application.

The studies, as applied to pyronin, met with only very limited success. Unlike methyl green, which appears to form a stable compound with polymerized DNA, pyronin-stained RNA or depolymerized DNA-precipitates lose stain continuously when washed with alcohol or aqueous buffers. The "end point" which is eventually reached, represents the retention of only a few per cent of the original dye bound (*cf.* Table VI and Table VIII of the preceding paper (1)). Consequently, the possibility of non-specifically adsorbed dye to the pyronin-nucleic acid complex which precipitates on the addition of pyronin to nucleic acid solutions (in high concentrations of dye) cannot be excluded, thus vitiating the significance of the stoichiometric data based upon precipitation experiments in which combined dye was determined by difference between that remaining in the supernate and the original concentration. Likewise, washing probably results in considerable dissociation of the complex so that the "end point" is also not reliable for stoichiometry. However, Table VI (preceding paper (1)) based on precipitation experiments, suggests that the low polymers of NA bind approximately one pyronin molecule for each pair of phosphoric acid groups, indicating that both amino groups are functional in the linkage.

Referring again to Table VI (1), a significant difference in staining is noted between DNA and DNH. We doubt that the difference between RNA and RNP is significant, since the preparations are obtained from different sources by unrelated methods. The possibility that the slightly greater staining of RNP is due to the staining of protein by pyronin is rendered unlikely, but by no means excluded, by the fact that heat-coagulated beef serum fails to stain with pyronin when treated in the same manner as the nucleic acid precipitates.

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The interference by histone with the staining of the nucleic acid by pyronin was confirmed by staining fibers of desoxyribonucleohistone prepared by precipitating the nucleohistone from 1 M NaCl solution by dilution with water. The fibrous precipitates (P = 0.27 mg. each) were wound about glass rods. One sample was immersed in 0.2 N HCl and then in pH 4.1 acetate buffer, another only in buffer. They were then transferred to 0.025 ger cent Grübler pyronin, washed in alcohol until the alcohol washes were colorless, and finally decolorized in 10 cc. acid alcohol at 60°C. overnight. Practically complete decolorization is achieved (*cf.* the low values for retained pyronin after acid alcohol washing of DNA and DNH in Table VIII (1)). The acid alcohol extracts were compared spectrophotometrically for dye content. The results indicated a molar dye/P ratio of 0.0117 for the nucleohistone which had been freed of practically all its histone by the acid treatment and 0.0072 for the intact nucleohistone (Table XI).[1]

TABLE XI

	E_{550} acid alcohol extract	μM dye in acid alcohol, based on $\epsilon_{550} = 91{,}000$	μM P	Dye/P, molar
DNA	0.925	0.102	8.7	0.0117
DNH	0.570	0.0627	8.7	0.0072

This competition of the histone with pyronin, but not of the more complex protein of ribonucleoprotein suggests that both the dye and histone are attached to the nucleic acid by phosphoric acid groups, while the protein of RNP is bound in a different manner.

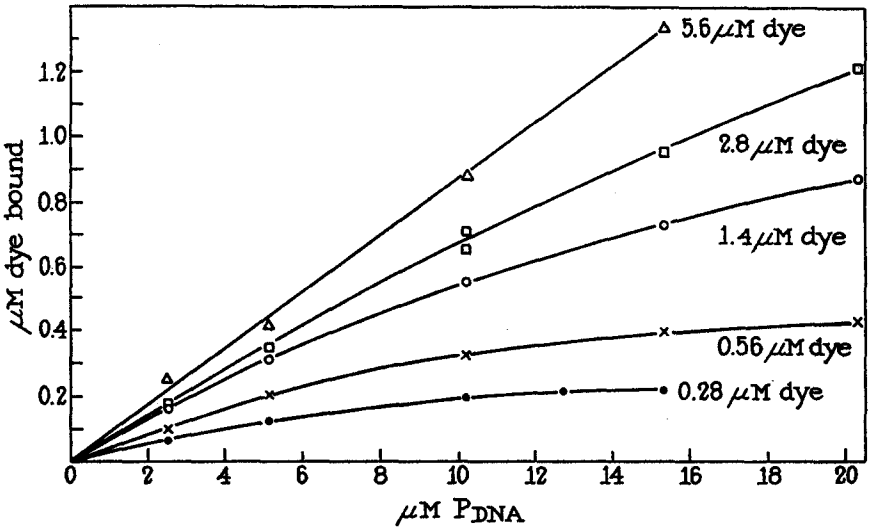
It should be noted that the problem of the stoichiometry of pyronin staining is further complicated by the dependence of the degree of staining on the dye concentration. Thus, when the pyronin concentration in the stain-nucleic acid mixture was 0.03 per cent, the dye/$P_{\text{depolymerized DNA}}$, molar = 0.48 (Table VI (1)), whereas in the lanthanum precipitation experiments with a dye concentration of 0.0025 per cent, the dye/$P_{\text{depolymerized DNA}}$, molar = 0.04 (Table IX (1)). Even relatively small differences in dye concentration influenced the degree of staining. Thus, the precipitates obtained in the following three experiments were compared:

(*a*) 5 cc. RNP (P = 0.1 mg./cc.) + 5 cc. 0.0625 per cent pyronin Y, (*b*) 3 cc. RNP + 2 cc. H_2O + 5 cc. pyronin Y, (*c*) 5 cc. RNP + 2 cc. H_2O + 3cc. pyronin Y. The resultant precipitates contained (*a*) 0.48 mg. P and 8.3 mg. dye, correspond-

[1] The tables in the present paper are numbered consecutively with those in the preceding paper (1).

ing to a molar ratio of dye: P of 0.45, (*b*) 0.3 mg. P and 6.2 mg. dye, corresponding to dye/P, molar = .54, (*c*) 0.42 mg. P and 5.7 mg. dye, with a dye/P, molar of 0.35.

Pyronin, therefore, appears to be unpromising as a quantitative histochemical stain, although as a qualitative test for the presence of RNA or depolymerized DNA as distinguished from polymerized DNA, after appropriate washing with alcohol, it appears to be of value. The specificity is enhanced by the use of ribonuclease (2).



TEXT-FIG. 1. Competition of methyl green and lanthanum acetate for DNA. Final volume 5 cc. in pH 4.1 acetate buffer. Content of dye (on curve) and DNA (on abscissa) varied as indicated. Ordinates indicate amount of dye bound in precipitate (DNA content of precipitate same as on abscissa). Lanthanum acetate final concentration 1.5 per cent.

Much more satisfactory results were obtained in the investigation of the stoichiometry of methyl green. From Table II of the preceding paper (1), we note that after treatment at room temperature with dilute HCl, calf thymus nuclei combine with methyl green in the ratio of 0.1 molecule of dye per phosphorus. The same value is found for the composition of the DNA-methyl green compound precipitated with alcohol (Table III (1)). Lower values were obtained in the experiments in which $LaAc_3$ was used as the precipitant (Tables I, IV, V (1)). This suggested that lanthanum competed with methyl green for the phosphoric acid groups of DNA. The experiment was set up so that the final volume of 4.5 cc. contained from 0.28 μM to 5.6 μM methyl green and 2.5 μM to 20.4 μM P_{DNA} in pH 4.1 acetate buffer. After these mixtures had been allowed to stand for two hours, 0.5 cc. 15 per cent $LaAc_3$ was added to each,

the fibrous precipitate removed with a glass rod, and the extinction coefficients of the supernates at 635 mμ determined. By comparison with controls containing no nucleic acid, the methyl green removed could be calculated. The precipitation of the DNA was quantitative under these conditions. As seen in Text-fig. 1, as the effective excess of dye is increased, the competition of the $LaAc_3$ becomes less effective, until finally it becomes insignificant and a straight line, indicating constant composition of the precipitated compound, regardless of the relative concentrations of the components in the original solution, results. This compound corresponds to a molar dye/P_{DNA} ratio of 0.09, which is consistent with the value of 0.1 obtained in the staining of nuclei and on alcohol precipitation of DNA-methyl green. RNA and depolymerized DNA, under the conditions which give this straight line curve for DNA, combine with only 0.004 molecule of dye per phosphorus.

TABLE XII

Methyl Green/P_{DNA}, Molar

Stain in bath	P in mixture	P in supernate	P in precipitate	P in precipitate	E_{635} mμ of supernates					
					Control *a* diluted 1:20	*a* diluted 1:20	Control *b* diluted 1:100	*b* diluted 1:100	Dye in precipitate (ϵ_{635} = 74,400)	Dye/P_{DNA}, molar
μM	mg.	mg.	mg.	μM					μM	
(*a*) 1.41	0.31	0.013	0.297	9.6	0.970	0.438			0.72	0.07
(*b*) 5.64	0.31	0	0.31	10.0			0.768	0.650	0.92	0.09

The competition with lanthanum could also be demonstrated by washing a sample of stained, precipitated DNA with alcohol until no further dye could be removed. This is accomplished quickly, since methyl green required no "differentiation"—it is only necessary to wash away the stain solution in contact with the sediment with alcohol. Washing with 0.2 M acetate buffer will now remove no dye from the green precipitate. Fifteen per cent $LaAc_3$, will, however, remove a considerable amount of stain as judged by the intensity of color in the wash fluid. Thirty per cent NaCl is practically ineffective in this regard (cf. reference 3). Similarly, staining with methyl green in the presence of 1 M NaCl reduces the amount of dye combined by only 10 per cent.

When the $LaAc_3$ concentration was reduced to the minimum which would precipitate the polymerized DNA quantitatively (0.06 per cent), even with relatively small excess of dye, the molar dye/P_{DNA} ratio was 0.07 to 0.09 (Table XII). These experiments were set up as follows:—

(*a*) 0.5 cc. 0.128 per cent methyl green + 2.3 cc. buffer + 2 cc. 2 mg./cc. DNA (P = 0.155 mg./cc.) + 0.2 cc. 1.5 per cent $LaAc_3$ (corresponding to 1.41 μM dye and 10 μM P_{DNA}).

(*b*) 2.0 cc. 0.128 per cent methyl green + 0.8 cc. buffer + 2 cc. DNA + 0.2 cc. 1.5 per cent $LaAc_3$ (corresponding to 5.64 μM dye + 10 μM P_{DNA}).

The controls were made up with water in place of DNA solution.

That histone also competes with methyl green staining is suggested by the observation (Table II of preceding paper (1)) that nuclei stain with only one-half as much methyl green before HCl treatment as after.

Whereas when a solution of methyl green in acetate buffer was dialyzed against acetate buffer, all the dye escaped from the cellophane sac, when DNA was first added to the dye, a considerable amount of dye failed to dialyze. While no absolutely steady state was reached, after prolonged dialysis with frequent

TABLE XIII

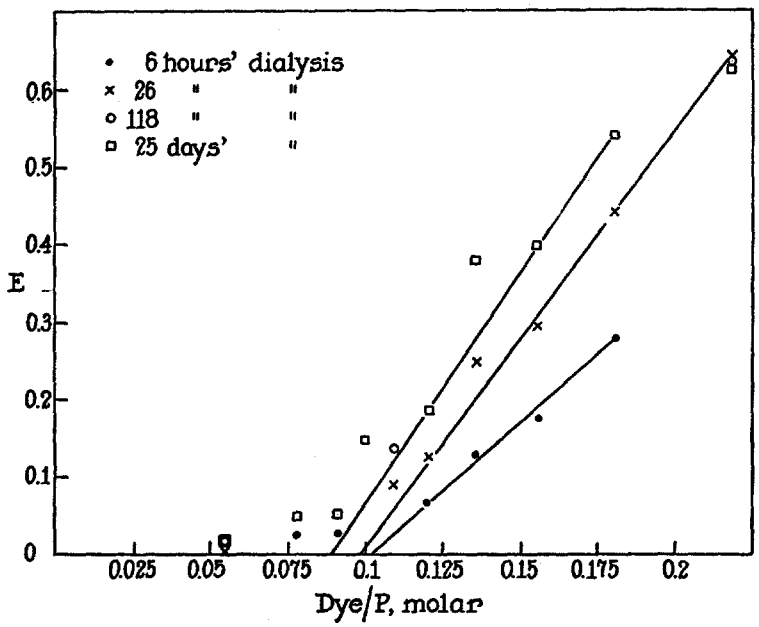
Time of dialysis	E_{645} dialyzed solution, diluted 1:20		μM dye per 10 cc. $\epsilon_{645} = 10^5$	μM P per 10 cc.	Dye/P, molar
	Control	Experimental			
hrs.					
26	0.221	0.531		10.3	
29	0.029	0.483	0.986	9.5	0.103
50		0.510	1.02		
74		0.488	0.976		
79		0.459	0.918	10.1	0.091
25	0.221	0.571		10.3	
27	0.062	0.534	1.068		0.103
32	0.015	0.520	1.04		
54		0.477	0.954		0.093
151		0.440	0.88		0.087
343		0.407	0.814	10.1	0.081

or constant changing of the dialysate, the rate of loss of dye from the mixture of DNA and dye became quite slow. This approximate end point corresponded to a composition of the DNA-methyl green complex retained in solution in the cellophane sac of 0.08 to 0.10 mols dye per P.

The experiment was performed by mixing 10 cc. 2 mg./cc. DNA (P = 0.160 mg./cc.) + 20 cc. 0.05 M acetate buffer (pH 4.1) + 20 cc. 0.128 per cent methyl green in 0.2 M acetate buffer. After standing overnight in the dark, the mixture was dialyzed in a cellophane bag against 0.05 M acetate buffer in a rocking, continuous flow dialyzer. Samples of the contents of the bag were withdrawn at intervals. The control contained water in place of DNA solution. The results are presented in Table XIII.

It was not possible to perform comparable experiments with RNA and depolymerized DNA because of the ease with which these nucleic acids passed through the membrane.

Mixtures of DNA and methyl green were prepared so that the ratio dye/P_{DNA} varied over the range 0.05 to 0.225, while the dye concentration was kept constant at 0.005 per cent. Four cc. of each mixture was dialyzed against 10 cc. of acetate buffer at 0°C. in the dark (without changing the dialyzing bath). At infrequent intervals varying from 6 hours to 1 month, the extinction coefficients of the dialysates were measured at 635 mμ. When the extinction values at a given time were plotted against the molar dye/P ratio of the original mixture, hyperboles resulted. Extrapola-



TEXT-FIG. 2. Extinction coefficients of dialyzing bath plotted against methyl green/P_{DNA} of original mixture after dialysis for fixed periods of time (see text for description of experiment). Note that amount of dye which dialyzes out is directly proportional to excess of dye (*i.e.*, when dye/P, molar exceeds 0.1).

tion of the "straight" portion of the curves to zero dye dialysis gave a dye/P of 0.1 (Text-fig. 2). It was found that under these conditions of dialysis, in which the volume of the dialyzing bath was small and the bath was not changed during the course of the experiment, a state of equilibrium was reached in 6 to 15 days. At the end of a month, when it was apparent that no further dialysis of stain was occurring (contrast the situation in which maintaining the concentration of dye in the bath at a minimum by continuous flow dialysis, favored continuous slow dissociation of the DNA-stain complex), the contents of the dialyzing bags were analyzed for methyl green and nucleic acid phosphorus (the first was measured spectrophotometrically; the latter was obtained by two independent methods: (1) Allen's method (5) and (2) spectrophotometrically: since the absorption coefficient of a 1 mg./cc. DNA solution in 4.1 acetate buffer at 260 mμ is 20 and of methyl green it is 10 per cent of that at its maxi-

mum, the nucleic acid content of a DNA-methyl green solution in mg./cc. $= \frac{E_{260} - .1E_{645}}{20}$). The methyl green in the sac was corrected for free methyl green by the concentration found in the bath. The results are given in Table XIV.

From Table XIV it is apparent that when the original ratio of dye/P_{DNA} molar was less than 0.099, the final ratio at equilibrium was within 10 per cent of the original value (indicating no significant loss of methyl green). With original mixtures of 0.099 dye/P_{DNA} or higher, the change is greater than 10 per cent. It is, therefore, our opinion that the mixture in which the dye/P_{DNA} was 0.099, represented approximate stoichiometric equivalence. If we consider the final dye/P_{DNA} values of all mixtures whose predialysis values fell by more than 10 per cent, we find values in the range 0.084 to 0.11 with an average of 0.095. This result is in agreement with those found on analysis of washed

TABLE XIV

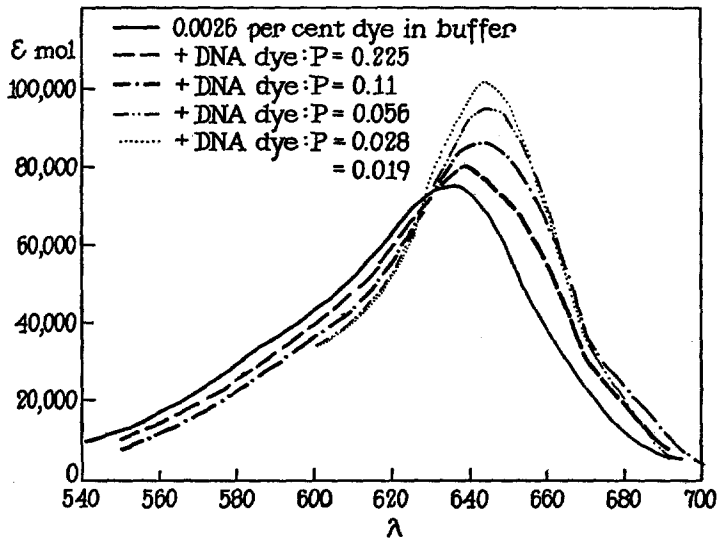
Original mixture before dialysis Dye/P_{DNA}, molar	After dialysis Dye/P_{DNA}, molar	Original dye/P_{DNA}, molar	Final dye/P_{DNA}, molar
0.055	0.052	0.136	0.1
0.078	0.076	0.156	0.11
0.084	0.082	0.182	0.11
0.091	0.087	0.218	0.091
0.099	0.084		
0.109	0.09		
0.121	0.096		

stained precipitates, by measurement of the amount of dye removed from solution on precipitation of known amounts of DNA, and by extrapolation to zero of a curve of the amount of dye dialyzed from known mixtures of dye and nucleic acid.

As was noted in the preceding paper (1), solutions of DNA and methyl green were emerald green, whereas the dye alone gave blue-green solutions. When the DNA was in excess, so as to insure complete modification of the dye, the absorption maximum was found to have been shifted from 635 mμ to 645 mμ and the molecular extinction coefficient to have increased from 74,400 to 100,000. Whereas mixtures containing 0.1 dye molecule per P demonstrated the completely modified absorption spectrum in shape, the addition of DNA up to a ratio of 0.05 mol dye per P caused a slight increase in the absorption at 645 mμ (Text-fig. 3). Further increments of DNA had no measurable influence on either the shape or the height of the curve. At first sight, this observation would appear to contradict the stoichiometric relationship of 1 dye molecule per 10 P_{DNA}. However, the observations from the dialysis experiments that when the free methyl green concentration is kept at zero, continuous dissocia-

tion of the DNA-methyl green complex occurs, whereas when the free methyl green is not continuously removed, a stable equilibrium is reached, indicate that the reaction should be written:

$$10\ P_{DNA} + 1\ \text{methyl green} \rightleftarrows (P_{DNA})_{10}\ \text{methyl green}$$



TEXT-FIG. 3. Effect of added DNA on absorption spectrum of methyl green.

TABLE XV

DNA Source	Dye/P 0.0128 per cent dye	Dye/P 0.05 per cent dye
Calf thymus chromosomes (saline)	0.075	0.081
Calf thymus chromosomes (sucrose)	0.079	0.089
Calf liver chromosomes	0.074	0.093
Shad sperm	0.069	0.086

Therefore, an excess of DNA would be expected to shift the reaction to the right, the concentration of free methyl green approaching zero as an asymptote. As a consequence, not all the methyl green is bound when the mixture contains the stoichiometric amounts of methyl green and DNA. When the DNA concentration exceeds twice the stoichiometric amount, the methyl green approximates 100 per cent bonding.

To determine whether or not preparations of DNA from several sources demonstrated the same stoichiometric relationship on staining with methyl

green, samples of DNA prepared from calf thymus chromosomes which had been isolated in saline, from thymus chromosomes isolated in 30 per cent sucrose, from shad sperm, and from beef liver chromosomes isolated in saline were compared (Table XV). The shad DNA appears to be more highly polymerized than the others since solutions of equal concentration are much more viscous.

In each case, solutions of the nucleic acid containing 0.3 mg. P_{DNA} were stained in 0.0128 per cent buffered methyl green and in 0.05 per cent buffered methyl green (final volume 4.8 cc.). After 2 hours, 0.2 cc. 1.5 per cent lanthanum acetate was added to each, the fibrous precipitates removed with a glass rod, and the suitably diluted supernates compared at 635 mμ (upon precipitation of the DNA, as has been mentioned (1), the solution reverts to the spectrum of methyl green alone) with controls containing no DNA. In each case, the dye/P, molar, found averaged 0.075 for the lower dye concentration and 0.089 for the higher concentration.

As has already been reported in the preceding paper (1), it is possible to determine the DNA content of isolated nuclei by measuring the amount of methyl green which they removed from a solution, since the DNA bound 1 molecule of dye per 10 P after removal of the histone with dilute HCl.

In a subsequent paper (4) a quantitative histochemical method for DNA based upon these stoichiometric observations will be reported.

CONCLUSIONS

Study of the stoichiometry of the DNA-methyl green reaction by dialysis, precipitation of stain-nucleic acid mixtures, and the staining of nuclei of known DNA content, indicate that the compound consists of one dye molecule per 10 P. The significance of this result was discussed in the preceding paper (1). Histone and lanthanum (and probably other multivalent cations (3)) compete with the dye for the nucleic acid molecule, indicating a common site of attachment, presumably the phosphoric acid groups.

With care in the avoidance of procedures which might depolymerize DNA, and the use of a buffer at about pH 4.1, a quantitative histochemical method for DNA by the use of methyl green is possible. Pyronin staining appears to be of qualitative significance only. Slight differences in degree of polymerization, as between the shad and mammalian DNA appear to have no effect on methyl green staining. It may be that a critical level of polymerization for DNA staining exists. This level must exceed 20 nucleotides to account for the 10 P to 1 dye molecule and the effect on the methyl green absorption spectrum; but it may be considerably greater. Beyond this critical level, whatever it may be, further polymerization probably has no influence on staining.

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