added to precipitate the crude product. Recrystallization from petroleum ether gave 1.1 g. (87%) of 3-methoxy-2-pyrone as a hygroscopic solid, m.p. 61°; reported[4] m.p. 60°.

The infrared absorption spectrum for 3-methoxy-2-pyrone shows absorption at the following wave numbers: 3200 (medium-narrow), 1725 (strong), 1645 (strong), 1575 (strong), 1465 (medium), 1413 (weak).

4-Arylhydrazono-2,3-pyronones.—The procedure which follows for coupling of 3-hydroxy-2-pyrone with diazotized *p*-chloroaniline was typical of that used for the preparation of the 4-arylhydrazone derivatives listed in Table I with a few exceptions. *p*-Nitroaniline, 2-methyl-4-nitroaniline, *p*-aminobiphenyl and *p,p'*-methylenebisdianiline were diazotized as their hydrochlorides in acetic acid with solid sodium nitrite. 2,6-Dichloro-4-nitroaniline was diazotized by the addition of powdered sodium nitrite to a concd. sulfuric acid solution of the amine.

4-(*p*-Chlorophenylhydrazono)-2,3-pyronone.—A solution of 1.0 g. (0.007 mole) of *p*-chloroaniline in 8 ml. of hydrochloric acid and 15 ml. of water was cooled to 0°. To this solution was added enough 5% aq. sodium nitrite solution to give a positive starch–iodide test. This reaction mixture containing the diazotized amine was then added to a cold solution of 0.5 g. (0.005 mole) of 3-hydroxy-2-pyrone and 5 g. of sodium acetate in 500 ml. of water. The temperature was held at 0–5° at all times. The red precipitate which forms on mixing the two solutions was collected on a filter and recrystallized from toluene to give 1.0 g. (77%) of pure product, m.p. 220°.

In addition to the analytically pure products listed in Table I, products were obtained from the following amines which could not be purified or gave analytical data not in accord with the arylhydrazone structure: *p,p'*-bismethylenebisdianiline, 4-aminobiphenyl, α-naphthylamine and *p*-iodoaniline.

Absorption Data.—Infrared spectra were determined using potassium bromide pellets in a Baird double beam recording infrared spectrometer.

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[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, UNIVERSITY OF CALIFORNIA, LOS ANGELES]

The Binding of Safranine O by Tobacco Mosaic Virus[1]

BY WILLIAM GINOZA AND DANIEL E. ATKINSON

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Tobacco mosaic virus ("common" strain) contains about 3.4×10^3 apparently identical sites per particle which bind safranine O. Removal of C-terminal threonine residues does not affect the number of these sites, but exposes an approximately equal number of new sites which appear to be identical among themselves but have a higher affinity for the dye. In the presence of *p*H 7 phosphate buffer the native virus can bind about 1.0×10^4 dye ions. The results are discussed in terms of the repeating sub-unit hypothesis of tobacco mosaic virus structure.

The demonstration that some strains of tobacco mosaic virus (TMV) bind a host nucleoprotein while others do not[2] suggested the desirability of quantitative studies on the binding of ions of known structure by the virus. Its large size makes TMV particularly well suited for such studies, since the virus and any bound component can be removed by centrifugation after equilibration. No dialysis is required and no membrane effects are involved. Despite these advantages and the wide application of ion-binding methods in the study of small protein,[3] there appears to be only one reported study of ion binding by TMV. Oster and Grimsson[4] reported that the virus bound 1.3×10^{-4} mole of acriflavin per g. of virus and presented a plot of extent of binding against free dye concentration.

Safranine O has been used in purification of TMV.[5] An insoluble complex was formed which was dissociated readily by addition of competing anions. The virus activity was fully restored on dissociation, implying that the dye–virus interaction was reversible and produced no permanent chemical or structural change in the particles. This dye has also been used at high *p*H for the determination of total acidic groups in TMV and other proteins.[6] It appeared to combine stoichiometrically with such groups, since the results with small proteins agreed well with the number of acidic groups indicated by amino acid analysis.

This paper reports quantitative studies on the binding of safranine O by native TMV and by the same strain of TMV after enzymic removal of its C-terminal threonine residues.

Experimental

Virus.—TMV strain U-1 ("common TMV") was purified by differential centrifugation.[7] Samples were stored either in distilled water or in phosphate buffer at 4° or at −30°. No observable change in binding behavior resulted from storage under any of these conditions. Virus concentration was estimated by nitrogen analysis (Nessler), using a factor of 6.25 g. of protein per g. of nitrogen[8] and a particle weight of 50,000,000.[9] Samples were dialyzed against dilute phosphate buffer, *p*H 7.0, or distilled water before use. When phosphate-stored samples were to be used for equilibrations in water, they were sedimented by centrifugation, resuspended in distilled water, and dialyzed against several changes of distilled water for six days on a shaker at 4°. The specific conductivity of the resulting solutions was about 15 μmhos per cm.

Terminal threonine residues were removed by treatment with carboxypeptidase.[10] After alternate slow and fast centrifugation to remove the enzyme, the virus was resuspended in distilled water and dialyzed against several changes of distilled water. Samples so treated will be referred to as "dethreonized" virus.

Dye.—Safranine O (National Aniline) was recrystallized once from water and dried under vacuum with sulfuric acid

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as desiccant. Phenosafranine dyes have little tendency to polymerize,[11] and the molar absorptivity of safranine O has been reported[6] to be constant at least to 3×10^{-5} M. Using solutions of 2.4×10^{-6}, 2.4×10^{-5} and 2.4×10^{-4} M safranine O in cells with optical paths of 10, 1 and 0.1 cm., we obtained molar absorptivities at the wave length of maximum absorbance, 520 mμ, of 35,400, 33,800, and 28,700, respectively. A shift of the absorptive peak to 505 mμ was observed for the most concentrated solution. These results suggest that some polymerization may occur in this concentration range.

Procedure.—The dye–virus equilibrations were performed by mixing TMV and dye solutions in 25 × 100 mm. tubes. The final volume was 10 ml., containing from 7.5 to 11.6 mg. of virus (0.075 to 0.12% final concentration), and the initial dye concentrations were usually between 0.15×10^{-4} and 4.2×10^{-4} M. The tubes were sealed with Parafilm (Marathon Corporation, Menasha, Wis.) and shaken at 108 oscillations per minute at 4° for 24 hours. This period was adequate for equilibration, since no further uptake resulted from longer treatment. The solutions were transferred in the cold to Lusteroid centrifuge tubes and centrifuged at 4° for 1 hour at 40,000 r.p.m. in the no. 40 rotor of a Spinco Model L ultracentrifuge. The supernatant solutions were analyzed photometrically at 520 mμ after dilution, when necessary, to a suitable concentration range. From the equilibrium free dye concentration, c, the average number of dye ions bound per virus particle, r, was calculated. Since about 4% of the dye was adsorbed by the tube and tube cap assembly, standard curves were prepared from dye solutions treated in the same manner as in the test runs but without virus.

Results

Treatment of Data.—The case of multiple binding of ions by a protein with all binding sites having the same intrinsic association constant and with bound ions not influencing subsequent bindings is described by the Langmuir isotherm, which may be put in the form[12,13]

$$\frac{r}{c} = nk - rk$$

where r denotes the average number of dye ions bound per protein molecule, n the total number of binding sites per protein molecule, c the equilibrium free dye concentration, and k the intrinsic association constant. It follows that a plot of r/c against r will be linear when a single type of binding site is involved and there is no interaction between sites.

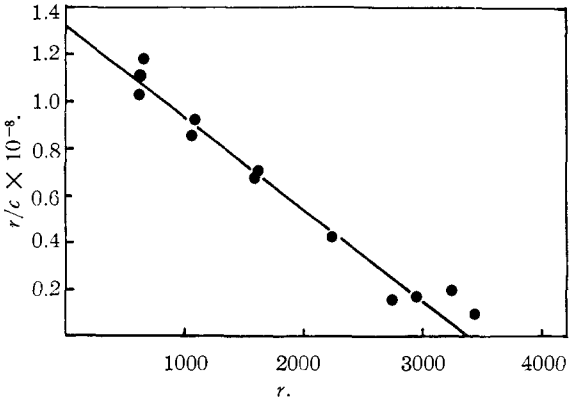

Fig. 1.—Plot of r/c *vs.* r for the binding of safranine O by native TMV in distilled water; virus concentration, 0.85 or 1.16 mg./ml.

From such a linear plot, n and k can be evaluated graphically.

Equilibration in Distilled Water.—The results of equilibrations in distilled water gave a linear plot of r/c against r (Fig. 1). This demonstration of similar association constants suggests that all binding sites may be chemically identical, and that they are sufficiently separated from each other to obviate electrostatic interaction between them. Extrapolation to the r axis yields a value for n of about 3380. The curve is a composite of points obtained from two separate experiments a month apart using different virus preparations. The association constant, k, is estimated to be 3.9×10^4 liters/mole.

The pH values at equilibrium varied between 6 and 7. This variation, which was not surprising in the absence of added buffer, had no detectable effect on the extent of binding. The ionic strengths of the solutions also varied with concentrations of dye and virus. In other experiments this variation in ionic strength was minimized by the addition of potassium chloride. The results are presented in Fig. 2. Although in the presence of 10^{-3} M potassium chloride the amount of dye bound at nearly saturating values of c (beyond the range plotted) was essentially identical to that in the absence of salt, the shape of the curve suggests competitive inhibition of dye binding by added electrolyte at lower values of c. This effect was much more pronounced at higher salt concentrations. Oster and Grimsson observed a similar inhibition of acriflavine binding by TMV on addition of sodium chloride.[4]

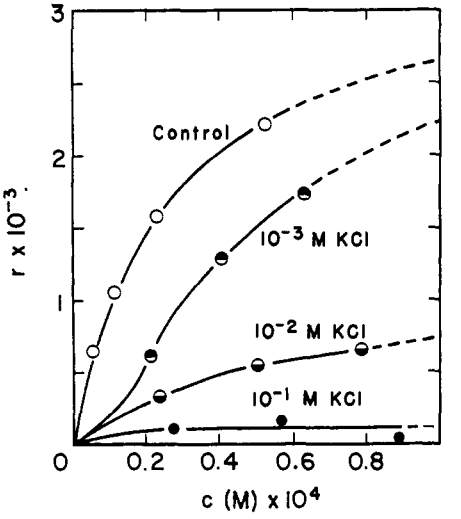

Fig. 2.—Effect of added KCl on the binding of safranine O by native TMV; virus concentration, 0.75 or 0.89 mg./ml.

Equilibration of "Dethreonized" Virus with Dye in Distilled Water.—Since the only terminal α-carboxyl group in TMV appears to be that of threonine,[10] it seemed desirable to observe the effect of removal of threonine on safranine binding. The results of equilibrating the dye with "dethreonized" virus in distilled water are presented in Fig. 3. The plot is clearly a composite of two linear curves and thus suggests two sets of binding sites, with each set of sites binding the dye molecules in a sta-

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tistical manner. Extrapolation of the curve to the r-axis gives an n value of 6600, which is approximately twice the number of binding sites found for the native virus. Moreover, extrapolation of the steeper curve gives an n value of 3570, in fair agreement with the value for native TMV. The association constants are estimated as 1.6×10^5 and 3.2×10^4 liters/mole.

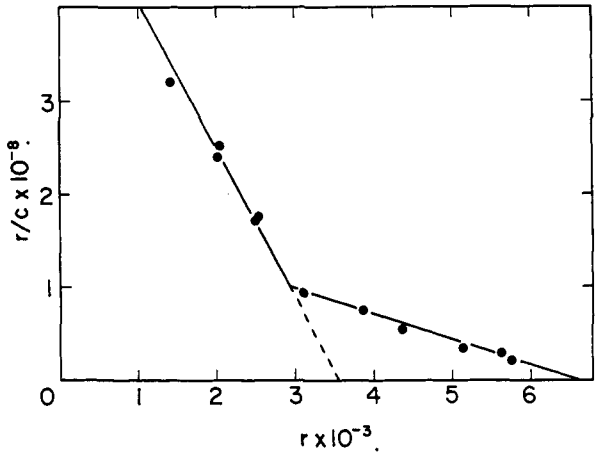

Fig. 3.—Plot of r/c *vs.* r for the binding of safranine O by "dethreonized" TMV in distilled water; virus concentration, 0.91 mg./ml.

Equilibration in Phosphate Buffer.—The interaction between safranine and TMV in phosphate buffer is more complex than in distilled water. The plot of r/c *versus* r for an experiment in 0.01 M phosphate at pH 7.0 (Fig. 4, curve A) suggests competition either between the cationic dye and potassium ion for binding sites on the virus or between phosphate ions and the anionic sites of the virus for the dye ions. The initial positive slope leads to a peak at an r value of about 3500 and then is followed by the expected negative slope which extrapolates to an n value of about 10,000. The results of equilibrations in 6×10^{-4} M phosphate are shown as curve B, Fig. 4. The anomaly apparent in curve A is absent. Although the points are scattered they indicate a curvature which is typical of that shown by proteins having heterogeneous binding sites. Again the curve extrapolates to an n value close to 10,000.

Discussion

The safranine O binding experiments in distilled water indicate that there are approximately 3400 binding sites per TMV particle. The sites appear to be sufficiently separated so that there is no interaction between them. Since the intrinsic association constant appears to have the same value for all sites, it is likely that the binding groups are chemically identical. Removal of threonine does not affect the number of these sites or the strength of binding, but unmasks an approximately equal number of new sites which bind safranine more strongly. The plot of the resulting data (Fig. 3) is unique in being made up of two linear portions and thus in permitting the estimation of n and k for two chemically different groups of binding sites from equilibrations with a single dye.

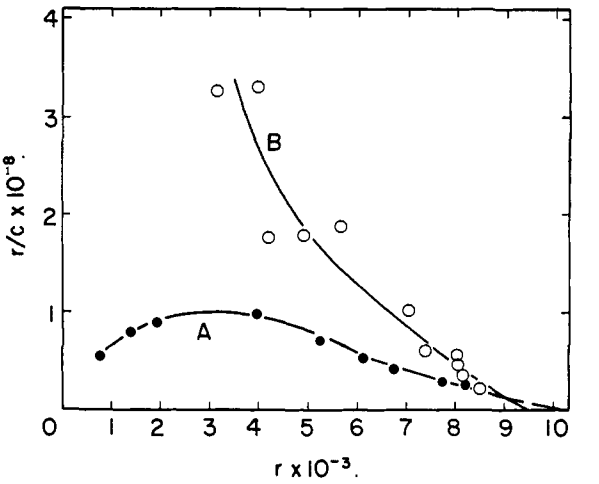

Fig. 4.—Plot or r/c *vs.* r for the binding of safranine O by native TMV in phosphate buffer, pH 7.0. Virus concentration: A, 1.0 mg./ml.; B, 0.79 mg./ml. Phosphate concentration: A, 0.01 M; B, 6×10^{-4} M.

These observations may be rationalized in terms of the repeating sub-unit structure of the virus particle proposed on the basis of X-ray diffraction studies by Bernal[14] and recently elaborated by Watson[15] and Franklin.[16] Results obtained by the latter two workers suggest that the TMV particle is built of about 3300 identical or nearly identical protein sub-units[16] helically arranged around a nucleic acid core.[15] Electron micrographs of partially disrupted TMV particles support this general picture.[17,18] Harris and Knight[19] suggest that the approximately 2900 molecules of threonine released per TMV particle on treatment with carboxypeptidase[19,20] arise from C-terminal threonine residues of a corresponding number of possibly identical units. Several other results support the sub-unit hypothesis:[21] 3400[22a] and 2800[22b] C-terminal threonine residues determined chemically; 3100 C-terminal alanine residues after threonine removal[23]; 2900 cysteine residues[24]; and 3000 amino end groups[25] (the significance of this last determination has been questioned[26]).

Our results thus suggest that each sub-unit of the native virus binds one safranine ion, and that after removal of threonine each sub-unit binds two dye

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(16) R. Franklin, *Nature*, **175**, 379 (1955).

(17) G. Schramm, G. Schumacher and W. Zillig, *ibid.*, **175**, 549 (1955).

(18) R. G. Hart, *Proc. Nat. Acad. Sci., U. S.*, **41**, 261 (1955).

(19) J. I. Harris and C. A. Knight, *J. Biol. Chem.*, **214**, 215 (1955).

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(21) These values have been recalculated where necessary on the basis of a particle weight of 5.0×10^7.

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ions, one at the original site and one at a site made available by threonine removal. The fair agreement between the k value for native TMV (3.9×10^4) and the lower of the two constants for "dethreonized" virus (3.2×10^4) supports the suggestion that the same set of sites is present in both of these virus preparations. The finding of equal numbers of sites of the two types and the lack of interference between sites (spatial separation) agree with the sub-unit hypothesis and strengthen the case for chemical similarity of the units. The number of sub-units estimated from dye binding studies agrees satisfactorily with the number indicated by X-ray diffraction.

In the presence of phosphate ions the situation is less simple, but a value of 10,000 sites per particle or 3 per sub-unit is definitely indicated. It seems likely that phosphate ions are bound to positively charged groups on the protein, and that they in turn bind safranine ions, or in some way permit dye binding at otherwise unreactive neighboring sites. Whatever the mechanism, the attempt to control pH and ionic strength by addition of buffer leads to complications which obscure the true interaction between dye and protein.

There is some evidence that safranine O begins to dimerize or polymerize at concentrations above about 2×10^{-5} M. However, the effect of such aggregation should be negligible except at higher values of c in the case of "dethreonized" virus. Further, the effect is probably small even in this case since (1) the plot shows no obvious deviation from linearity and (2) safranine O at twice the highest concentration used in these experiments has been successfully employed as a titrant in the determination of acidic groups in proteins.[6] This application appears to rule out the binding of dimeric or polymeric forms of the dye.

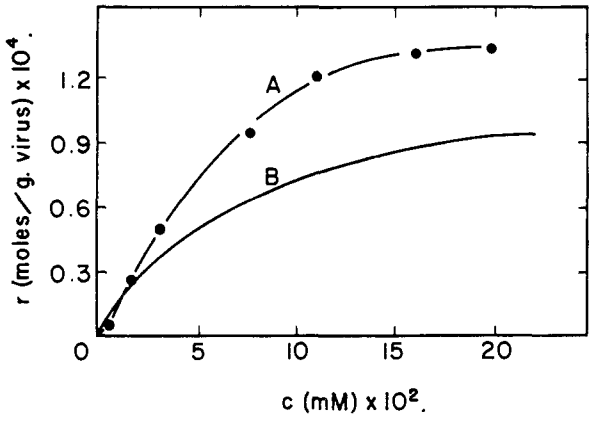
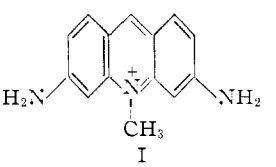


Fig. 5.—A, the binding of acriflavine by TMV (from ref. 3); B, the plot of the Langmuir equation $r = nck/(ck + 1)$, using the values of n and k given in ref. 3.

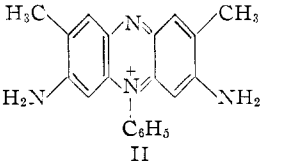
In the only previously reported study of TMV–dye interaction, Oster and Grimsson[3] reported the binding of 1.3×10^{-4} mole of acriflavine per g. of virus (6500 dye molecules per particle of mass 50,000,000) in distilled water. The relation between amount of dye bound and equilibrium free dye concentration was said to follow a Langmuir isotherm and thus to indicate that bound ions did not affect subsequent binding. The curve presented differs considerably from that obtained on plotting the Langmuir equation, however, and indicates that bound ions did markedly affect subsequent binding, as seen by the increasing discrepancy between the experimental and calculated curves with increasing values of r and c (Fig. 5). Curve B, calculated from the value of k given, would reach 90% of saturation when $c = 7 \times 10^{-4}$ M (70 in the units used in Fig. 5). The divergence from statistical binding could alternatively be expressed in terms of the apparent association constant, k, calculated from the experimental points using the equation

$$k = \frac{r}{c(n - r)}$$

The values of k obtained in this way (using the reported value of n, 1.3×10^{-4} mole per g. of virus) increase with increasing dye concentration from $10^{4.1}$ (the value given by the authors) at low concentrations to about $10^{5.1}$ when $c = 1.1 \times 10^{-4}$ M and to meaninglessly high values at the two highest concentrations of dye plotted. The simplest explanation of this interaction, in view of the results with safranine, is that only about 3300 primary binding sites were involved in acriflavine binding, but that the presence of the first bound ion permitted the binding of a second at or near the same site. The second ion might, for example, become attached to the dye ion already bound. Such dimerization appears more probable for acriflavine (I) than for safranine O (II) because of the number and arrangement of groups in the latter ion capable of possessing a partial positive charge.



I



II

The increase in apparent association constant with increasing acriflavine concentration would then reflect an increase in the number of sites binding dye dimer rather than monomer, and the maximum number of ions bound would be twice the number of binding sites.

LOS ANGELES, CAL.