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Studies of the Interaction of Sodium Polyphosphates with Acridine Orange*1

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The interaction of sodium polyphosphates with acridine orange has been studied by means of absorption spectra, fluorescence, and viscosity measurements. Polyphosphates with a variety of molecular weights were used in order to investigate the effect of the degree of polymerization ($\bar{n}$) on the interaction. No spectral change was observed in the case of polyphosphates with $\bar{n}<5$. In the case of $\bar{n}=9$, an absorption band corresponding to the dye dimer was observed, whereas in the case of larger $\bar{n}$ values those corresponding to higher aggregates were also found. The maximum metachromasy occurred at $P/D=3$—6. Fluorescence quenching was observed, accompanied by an absorption change. Such behavior may be considered to be characteristic of the dye molecules bound to the long-chain polyphosphates; the bound-dye molecules seem to exhibit almost no fluorescence. The quenching constants of the fluorescence increased with the increase in $\bar{n}$ values. The binding energies were estimated to be 5—6 kcal/mol independent of the $\bar{n}$ values. In the presence of acridine orange, the reduced viscosity of Kurrol's salt decreased remarkably, probably as a result of the neutralization of the charged phosphate groups. Further, its reduced viscosity decreased remarkably with time; this may be attributed to the catalytic action of the dye on the hydrolytic degradation of polyphosphates.

There are many cationic dyes which do not obey Beer's law; that is, the apparent absorption peak in the visible region shifts to a shorter wavelength as the dye concentration increases. This deviation from Beer's law has been ascribed to the formation of dimers and higher polymers in solution.[1,2] Even in dilute aqueous solutions of such dyes, the addition of an appropriate polyanion causes a change in the absorption spectra of dyes similar to that in their concentrated solutions. This phenomenon has been interpreted as follows[3]: when dye cations are bound to a polyanion, the

*1 A part of this study was presented at the Meeting of the Chugoku & Shikoku Branch of the Chemical Society of Japan, Hiroshima, November, 1965.

1) E. Rabinowitch and L. F. Epstein, *J. Am. Chem. Soc.*, **63**, 69 (1941).
2) V. Zanker, *Z. physik. Chem.*, **199**, 225 (1952).
3) L. Michaelis, *J. Phys. & Colloid Chem.*, **54**, 1 (1950).

dye aggregates are formed. Such a spectral change was termed "metachromasy" by Ehrlich.

Acridine orange (hereafter abbreviated as AO), which tends to associate easily, even in a dilute solution, has been widely used to stain biological tissues; it has been found that AO has a great affinity for such polyanions as nucleic acids and synthetic polynucleotides. In the presence of suitable polyanions, it shows a metachromatic color which has a spectrum similar to that at its highest concentration, while its fluorescence is simultaneously quenched due to the formation of non-fluorescent complexes. These interactions have been extensively studied by spectrophotometry and fluorescence techniques.[4-10] Such studies give important information about the nature of the binding sites, the electronic state of bound-dye molecules, polymer configuration in solution, and so on. Therefore, it is of great interest to study the dye-macromolecule systems systematically in relation to the photochemical behavior of bound-dye molecules and the nature of macromolecules.

For this paper, the interaction between AO and sodium polyphosphates has been studied over a wide range of polymer-dye ratios by means of absorption spectra, fluorescence quenching, and viscosity measurements. The metachromatic reaction of hexametaphosphate with toluidine blue had been studied by Wiame,[11] but the molecular weight of the phosphate had not been characterized. We used sodium polyphosphates with a variety of molecular weights and paid special attention to the effect of the degree of polymerization on the interaction with the dye. Furthermore, the measurements of the absorption spectra were extended to the ultraviolet (UV) region in order to see the overall electronic transition of the bound dye. Viscosity measurements of polyphosphate-AO complexes clarify how the configurations of polyphosphates in solution change when AO molecules are bound to them.

Experimental

Purification of AO. An excess of 0.1 N sodium hydroxide was added to an aqueous solution of AO hydrochloride (Tokyo Kasei Co.). The resulting free base was recrystallized two times from a 1 : 1 ethanol-water mixture. The orange needles thus obtained were washed with cold water and then dried in a vacuum at 80°C. To check the purity of the dye, its methanol solution was chromatographed on a silica gel strip. The chromatogram showed only a single component. The purified free base of AO was converted to the monocationic form by adding 0.1 N hydrochloric acid sufficient to neutralize it, after which the dye solution was stored below 5°C in a dark place.

Reagents. Sodium polyphosphate glasses (NaPP-G) and sodium Kurrol's salt, $(NaPO_3)_x$, were prepared according to the usual methods.[12,13] Three kinds of samples of NaPP-G were used. Their molecular weights, which were determined by endgroup titration, were 590 ($\bar{n}$=5.5), 960 ($\bar{n}$=9), and 8700 ($\bar{n}$=85), where $\bar{n}$ denotes the number-average degree of polymerization.

Kurrol's salt seemed to contain low molecular weight species. Therefore, the fibrous crystals were picked up and briefly washed with water, methanol, and ether successively, and then dried in a vacuum. The molecular weight of Kurrol's salt was determined by light-scattering and viscosity measurements in a 0.35 N sodium bromide solution. Its molecular weight and radius of gyration were 4.3×10^6 and 1.3×10^3 Å respectively.

An aqueous solution of Kurrol's salt was prepared by stirring the dry salt in water for 24 hr and by then removing insoluble species by ultracentrifugation or filtration. After the solution had stood at room temperature, the reduced viscosity and the Rayleigh ratio considerably decreased as a result of hydrolytic degradation. Therefore, the solutions of NaPP-G and Kurrol's salt were stored at 0—4°C in order to minimize this degradation, and the measurements were carried out within a week after the solutions had been prepared. The phosphate concentrations of the stored solutions of NaPP-G and Kurrol's salt were determined by titrating the orthophosphate produced by boiling the solutions about 10 hr in *ca.* 1 N hydrochloric acid with sodium hydroxide.

Ortho-, pyro-, tri-, trimeta-, and tetra-phosphates were purified by crystallization from water or by precipitation by adding methanol to aqueous solutions of the phosphates.

Sodium-poly-α-L-glutamate (NaPG; molecular weight, 5.1×10^4) was used without further purification.

The inorganic salts used were of a reagent grade.

Deionized distilled water was used for the preparation of all solutions.

The organic solvents were distilled according to the usual methods.

Apparatus and Procedures. *Absorption Spectra.* The absorption spectra were measured with a Hitachi Perkin-Elmer 139 spectrophotometer over the range from 220 to 550 mμ. The measurements were made at low ionic strength (10^{-3} M buffer), since this factor affects the interaction between AO and polyanions. Solutions of higher ionic strengths were prepared by adding sodium chloride to 10^{-3} M buffer solutions. The spectrophotometric titrations were performed by adding micro-volumes of a concentrated polymer solution to

4) R. F. Steiner and R. F. Beers, Jr., *Science*, **127**, 335 (1958).
5) R. F. Steiner and R. F. Beers, Jr., *Arch. Biochem. Biophys.*, **81**, 75 (1959).
6) D. F. Bradley and M. K. Wolf, *Proc. Natl. Acad. Sci. U. S.*, **45**, 944 (1959).
7) A. L. Stone and D. F. Bradley, *J. Am. Chem. Soc.*, **83**, 3627 (1961).
8) A. F. Harris, A. Saifer and S. K. Weintraub, *Arch. Biochem. Biophys.*, **95**, 106 (1961).
9) R. Boyle, S. S. Nelson, F. R. Dollish and M. J. Olsen, *ibid.*, **96**, 47 (1962).
10) G. Weill and M. Calvin, *Biopolymers*, **1**, 401 (1963).
11) J. M. Wiame, *J. Am. Chem. Soc.*, **69**, 3146 (1947).
12) M. Miura, S. Otani, Y. Abe and C. Fukumura, This Bulletin, **36**, 1091 (1963).
13) M. Miura, A. Hasegawa and T. Fukui, *ibid.*, **39**, 1432 (1966).

a dye solution with magnetic stirring so as to obtain the desired P/D values, where P/D represents the ratio of the number of binding sites of the polymer to the number of the dye molecules. The dye concentration used was 2×10^{-5} M.

Fluorescence Quenching Measurements. The relative fluorescence intensities were measured with a Shimadzu light-scattering photometer, using a 436 mμ mercury line as an exciting light. Since the fluorescence was observed at right angles to the incident beam, it was necessary to correct for the absorption of the incident light in the cell. For the fluorescence light, a Fuji gelatine filter or a Toshiba color filter was used. Two 1-cm cells, one containing a dye solution (reference cell) and the other a dye-and-polymer solution (sample cell), were placed in turn in the light path. From the readings of a galvanometer, the F_0/F_Q values were calculated, where F_0 and F_Q are the intensities of fluorescence in the absence and in the presence of a quencher respectively. The solutions were prepared by the same procedures as were used in the case of spectrophotometric titrations. Fluorescence measurements were made immediately after the preparation of the solution in order to avoid any photochemical reaction. In order to control the temperature of the solution, water from a thermostat was circulated through a water jacket surrounding the cell. The temperature was measured with a calibrated thermistor. Since the self-quenching became remarkable above 5×10^{-6} M,[14] all the measurements were made at dye concentrations lower than 3×10^{-6} M, where AO is considered to exist predominantly in the monomer form.[2,14]

The fluorescence spectra were measured by the same spectrophotometer with a fluorescence attachment; hence, they are not genuine fluorescence spectra. The pH measurements were carried out with a Hitachi-Horiba pH meter. The light-scattering measurements were made with a Shimadzu light-scattering photometer using a cylindrical cell.[15]

All the measurements were carried out in a constant-temperature room maintained at 25±1°C.

Viscosities were measured at 25±0.01°C using Ubbelohde-type viscometers, the flow times of which ranged from 59.8 to 321.2 sec for water.

Results and Discussion

Absorption Spectra. *Absorption Spectra of AO.* Figure 1 shows the absorption and fluorescence spectra of AO in an acetate buffer (pH 5.5). The deviation of AO spectra from Beer's law has been well characterized by Zanker,[2] mostly in the visible region. As the dye concentration increases, the monomer band (492 mμ) falls and is displaced by new bands, namely, the dimer band (464 mμ) and the bands of the shorter wavelengths corresponding to the higher aggregates. Parallel with the spectral change in the visible region, it was found that the molar absorbance in the UV region decreases and the peak shifts slightly to a shorter wavelength.

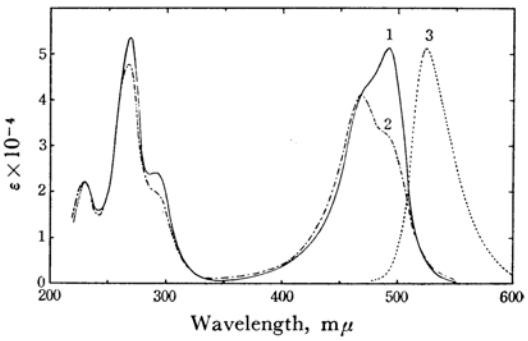


Fig. 1. Absorption and fluorescence spectra of acridine orange in acetate buffer (pH 5.5).
(1) Absorption spectrum, $[AO]=2\times10^{-5}$ M
(2) Absorption spectrum, $[AO]=2\times10^{-4}$ M
(3) Fluorescence spectrum, $[AO]=2\times10^{-6}$ M

As may be seen in Fig. 1, the fluorescence spectrum is a mirror image of the absorption spectrum at low concentrations of AO. With an increase in the concentration of AO, the maximum shifts to a longer wavelength, as has been described by Zanker.[2,16]

Absorption Spectra of AO in the Presence of Sodium Polyphosphates. In the pH 3—8 region, no pH dependency of the metachromatic reaction was found. It may, therefore, be considered that the dissociation of the end groups of polyphosphates has little influence on metachromasy. However, metachromasy was considerably affected by the degree of polymerization of polyphosphates, especially in the cases of low $\bar{n}$ values.

Polyphosphates with $\bar{n}<5$ gave no metachromasy. In the case of $\bar{n}=5.5$, a small decrease in absorbance at 492 mμ was observed; this is probably due to some of the polyphosphates with a higher degree of polymerization involved.

As may be seen in Figs. 2 and 3, when $\bar{n}$ is greater than 9, the metachromatic reaction becomes remarkable and gives absorption spectra characteristic of a strong coupling between the bound-dye molecules.[6] In the case of $\bar{n}=9$, only the dimer band (464 mμ) was observed, while in the case of higher $\bar{n}$ values, the bands corresponding to the higher aggregates were observed, accompanied by intermediate bands at appropriate P/D values. The spectral changes in the case of NaPP-G ($\bar{n}=85$) were similar to those in the case of Kurrol's salt; no pronounced difference due to the degree of polymerization was found. As may be shown in Fig. 3, the absorption peak corresponding to maximum metachromasy appears at 450 mμ; this is in accordance with the value of 451 mμ at the highest concentration of AO (9.1×10^{-2} M).[2]

14) Y. Kubota and M. Miura, *J. Sci. Hiroshima Univ., Ser. A-II,* **30**, 49 (1966).
15) M. Miura, Y. Kubota and T. Masuzukawa, This Bulletin, **38**, 316 (1965).
16) V. Zanker, M. Held and H. Rammensee, *Z. Naturforsch.,* **14b**, 789 (1959).

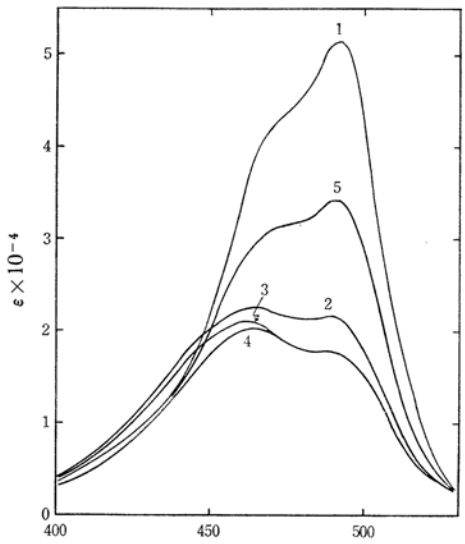


Fig. 2. Absorption spectra of acridine orange in the presence of NaPP-G ($\bar{n}=9$) in 10^{-3} M acetate buffer (pH 5.5).

[AO]$=2\times10^{-5}$ M; (1) pure dye, (2) $P/D=5$, (3) $P/D=10$, (4) $P/D=100$, (5) $P/D=1000$

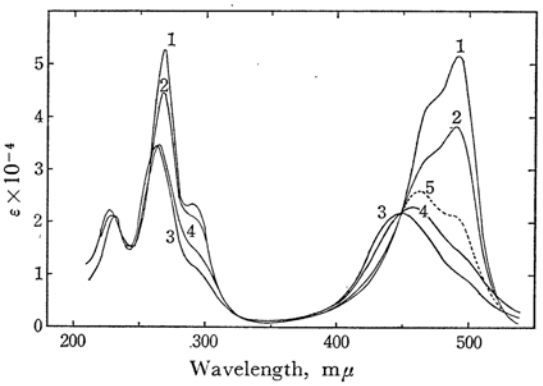


Fig. 3. Absorption spectra of acridine orange in the presence of $(NaPO_3)_x$ in 10^{-3} M acetate buffer (pH 5.5).

[AO]$=2\times10^{-5}$ M; (1) pure dye, (2) $P/D=1$, (3) $P/D=5$, (4) $P/D=100$, (5) NaPP-G ($\bar{n}=85$), $P/D=1000$

Therefore, we may define the strength of metachromasy as $\varepsilon_{450}/\varepsilon_{492}$, where ε_{450} and ε_{492} express the molar extinction coefficients at 450 mμ and 492 mμ respectively. In Fig. 4, both $\varepsilon_{450}/\varepsilon_{492}$ and the progressive change in absorbance at 492 mμ are plotted against the logarithm of the P/D values. As may be seen in this figure, metachromasy reaches maxima at $P/D=3$ and 6 for $(NaPO_3)_x$ and NaPP-G ($\bar{n}=85$) respectively, and remains constant up to $P/D=20$—30. Wiame,[11] in a study of the interaction between toluidine blue and sodium hexametaphosphates, has found that

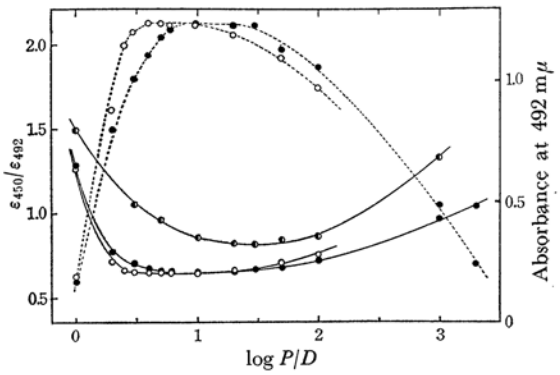


Fig. 4. $\varepsilon_{450}/\varepsilon_{492}$ (broken line) and absorbance at 492 mμ (solid line) *vs.* log P/D.

◐: NaPP-G ($\bar{n}=9$), ●: NaPP-G ($n=85$), ○: $(NaPO_3)_x$

maximum metachromasy occurs not at $P/D=1$ but at 8. Such behavior agrees with our results for the cases of AO and sodium polyphosphates. These conditions, under which the electrostatic force balances the van der Waals' force, may be optimum for establishing the distance of 4—5 Å necessary for the coupling between two parallel dye molecules.[17,18] In the case of $\bar{n}=9$, higher aggregates than the dimer can not be formed because of its short chain length. In the case of $\bar{n}$ values lower than 5, not even the dimer can be formed. On the basis of the above facts, it can be concluded that the spectral change is characteristic of the aggregates of AO bound to long-chain polymers.

As is shown in Fig. 4, in all cases, metachromasy decreases with a further increase in the concentration of polyphosphates. In the presence of a large amount of polyphosphates, there exist a large number of available binding sites, so the dye molecules may disperse over the polymer chains and so may no longer be close to one another enough to form the dye aggregates.[6,11] Furthermore, the increase in counter ions may result in the dissociation of bound-dye molecules since the metachromatic reaction occurs in the competition between the dye molecule and the counter ion. The decrease in metachromasy may be expected for the above two reasons.

Some interesting results were obtained by extending the measurements to the UV region. As is shown in Fig. 3, the main band in the UV region shows a large decrease in molar absorbance and a slight blue shift of the maximum in the presence of polyphosphates, as has been observed in the concentrated free-dye solutions. This change in absorption also indicates that the bound-dye

17) S. E. Sheppard and A. L. Geddes, *J. Am. Chem. Soc.*, **66**, 1995 (1944).
18) C. S. Levinson, W. T. Simpson and W. Curtis, *ibid.*, **79**, 4314 (1957).

molecules form the higher agregates on the polyphosphate chain in a manner similar to that found in the concentrated solutions of dye.

Fluorescence Quenching of AO in the Presence of Sodium Polyphosphates. When AO molecules are bound to polyphosphate anions, the fluorescence intensity of the dye remarkably decreases, accompanied by a change in its absorption spectra. Even in the low concentration of AO, the absorption spectra in the presence of polyphosphates showed bands corresponding to dimer and higher aggregates, though no significant changes were observed in the shape of the fluorescence spectra.

Figure 5 shows the fluorescence quenching of AO over a wide range of polymer-dye ratios. In Figs. 6 and 7, the values of F_0/F_Q are plotted against the phosphate concentration expressed by the monomer unit. As may be seen in these figures,

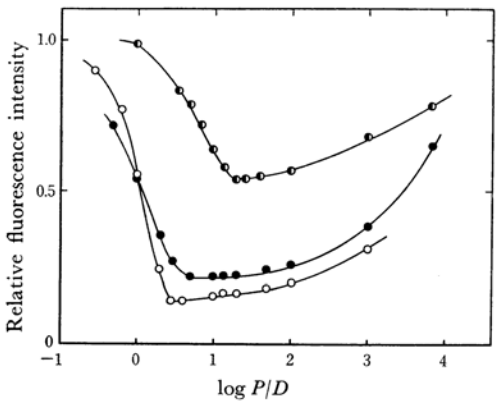
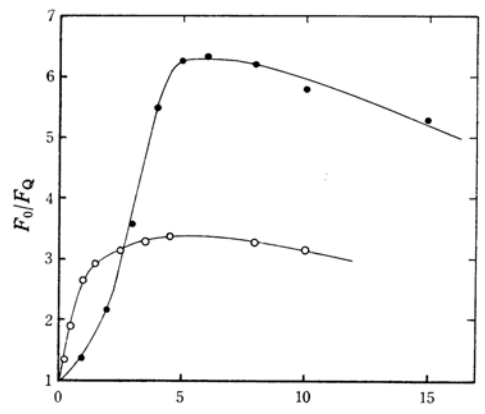


Fig. 5. Fluorescence quenching of acridine orange by sodium polyphosphates in 10^{-3} M acetate buffer (pH 5.5) at 25°C.

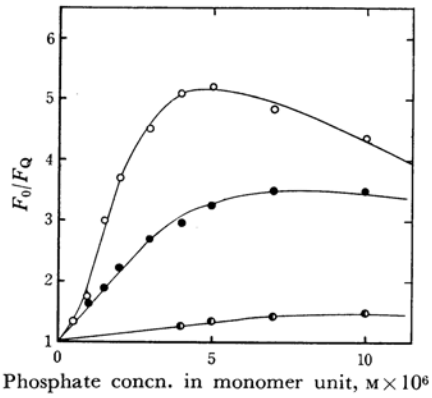
[AO]=3×10^{-6} M; ◐: NaPP-G (n=9),
●: NaPP-G ($\bar{n}$=85), ○: $(NaPO_3)_x$



Phosphate concn. in monomer unit, M$\times10^6$

Fig. 6. Fluorescence quenching curves at 25°C.

○: $(NaPO_3)_x$, [AO]=10^{-6} M
●: $(NaPO_3)_x$, [AO]=2×10^{-6} M



Phosphate concn. in monomer unit, M$\times10^6$

Fig. 7. Fluorescence quenching curves at 7.5°C.

[AO]=10^{-6} M; ◐: NaPP-G ($\bar{n}$=9),
●: NaPP-G ($\bar{n}$=85), ○: $(NaPO_3)_x$

maximum quenchings occur at P/D=2—3 and 5—6 for $(NaPO_3)_x$ and NaPP-G ($\bar{n}$=85) respectively, and the fluorescence intensity gradually increases with a further increase in the concentration of polyphosphates. Such behavior is similar to that in the metachromatic reaction. It can be concluded from these facts that the dye molecules bound on the polyphosphate chain exhibit almost no fluorescence and that the increase in the fluorescence intensity at the higher concentrations of polyphosphates is due to the dissociation of the bound-dye molecules.

The quenching data were analyzed according to the methods of Oster[19] and Heiweil and Van Winkle.[20] The quenching constant in l/mol in a 10^{-3} M acetate buffer (pH 5.5) increased with the increase in the degree of polymerization of polyphosphates; its values were 1.2×10^6, 5.9×10^5 and 7.0×10^4 at 25°C for $(NaPO_3)_x$, NaPP-G ($\bar{n}$=85) and NaPP-G ($\bar{n}$=9) respectively. The quenching constant for NaPG, which was dependent on pH, was 2.6×10^5 under the same conditions. Such large values of quenching constants, the order of which agrees with those obtained for DNA-acriflavine systems,[19,20] may be attributed to the strong interaction between dye and polymer. The temperature dependence of quenching constants was negative; thus, the reaction is exothermic. Plots of the logarithm of the quenching constants obtained at 7.5, 25, and 40°C against the reciprocal of the absolute temperature gave a straight line; from its slope the binding energies were calculated to be 6.4 kcal/mol and 5.5 kcal/mol for $(NaPO_3)_x$ and NaPP-G ($\bar{n}$=85) respectively. These values seem to be almost independent of the degree of polymerization of polyphosphates and are relatively smaller than those obtained for DNA-acriflavine interaction. Since quenching constants

19) G. Oster, *Trans. Faraday Soc.*, **47**, 660 (1951).
20) H. G. Heilweil and Q. Van Winkle, *J. Phys. Chem.*, **59**, 939 (1955).

markedly decrease with an increase in the ionic strength, the interaction may be basically electrostatic.

Viscosity of Kurrol's Salt in the Presence of AO. The degree of the extension of the polyelectrolyte chain in an aqueous solution can be estimated from viscosity measurements. $(NaPO_3)_x$ in a dilute solution behaves as a typical polyelectrolyte, and its dilute solution is very viscous. The plot of the reciprocal of the reduced viscosity (η_{sp}/C) against the square root of the polymer concentration (C, in g/100 ml) gave a straight line, and the intrinsic viscosity ($[\eta]$) was estimated to be 3300 in 100 ml/g by extrapolating it to infinite dilution. The large value of $[\eta]$ and its viscous behavior may be attributed to the high molecular weight of $(NaPO_3)_x$.

Figure 8 shows the variation in the reduced viscosity with the concentration of AO at a constant phosphate concentration. The reduced viscosity pronouncedly lowers with an increase in the concentration of AO; its further increase leads to the precipitation of polyphosphate-AO complexes. In all cases, spectral changes characteristic of the dye aggregates were observed. The decrease in reduced viscosity was much more remarkable than in the presence of sodium bromide. In a dilute solution the polyphosphate chain is considered to take a fully-extended configuration as a result of the repulsion between similarly-charged groups on the same chain. When the dye molecules are bound to the charged phosphate groups, they may neutralize the charges on the fully-extended chain, resulting in a shrinking of the polymer chain; furthermore, as the bound-dye molecules have a tendency to establish a distance suitable for dye-dye coupling, they may more and more allow a polyphosphate chain to coil up.

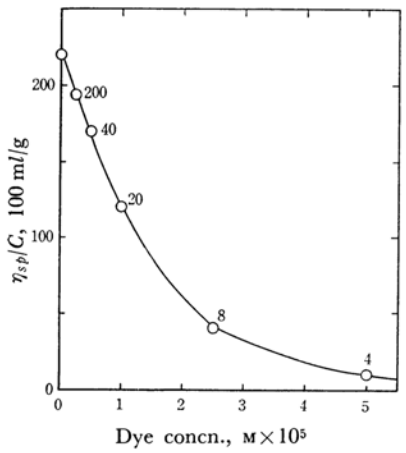


Fig. 8. Reduced viscosity, η_{sp}/C, of $(NaPO_3)_x$ in the presence of acridine orange.
Phosphate concentration, 0.02 g/100 ml; each figure represents P/D value.

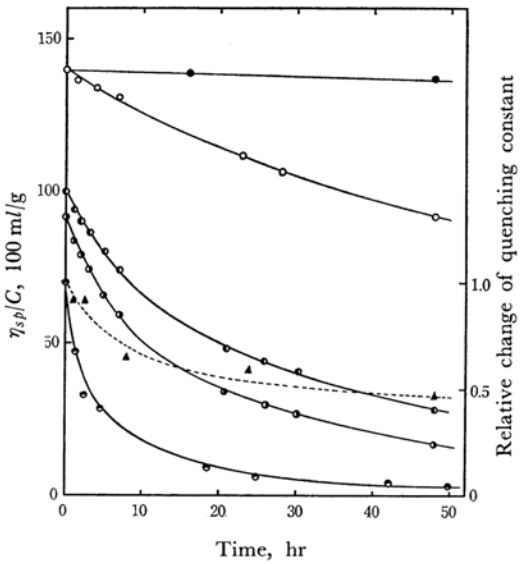


Fig. 9. Variation of reduced viscosity of $(NaPO_3)_x$ in the presence of acridine orange with time.

Phosphate concentration, 0.05 g/100 ml
●: no dye, pH 11
○: no dye, pH 6
◐: [AO]=10^{-5} M, P/D=500
◑: [AO]=2×10^{-5} M, P/D=250
◓: [AO]=5×10^{-5} M, P/D=100
▲: relative change of quenching constant for the case of [AO]=5×10^{-5} M

In addition to the viscosity change mentioned above, it was found that, when the solution is left standing, the reduced viscosity in the presence of AO remarkably decreases with time, as may be seen in Fig. 9. This phenomenon may be attributed to the hydrolytic degradation of polyphosphate, since the fluorescence quenching constants decrease simultaneously with the viscosity change, as is shown in Fig. 9 for the case of [AO]=5×10^{-5} M; as has been mentioned above, the quenching constant becomes smaller with a decrease in the degree of polymerization. Figure 9 shows that the polyphosphate is stable in an alkaline solution, whereas it is considerably degradated in a neutral solution. It also shows that the rate of degradation increases with an increase in the concentration of dye, that is, the polymer-dye ratios. From the analysis of the degradated phosphates by means of paper chromatography, however, the low molecular weight species could not be detected in any significant amount.

In view of the above facts, it seems reasonable to assume that the degradation occurs at random in the phosphate groups where AO molecules are bound, probably due to the neutralization of the change or the strain of the chain, and that the degradation near the middle of the chain mainly contributes to the large change in the viscosity. It seems of

interest that the dye, as polyvalent cations[21] such as Al^{3+}, has a catalytic action on the degradation of polyphosphates.

21) W. Wieker and E. Thilo, *Z. anorg. Chem.*, **306**, 48 (1960).

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