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Studies of the Quenching of the Fluorescence of Acridine Orange from the Standpoint of Charge-Transfer

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The quenching action of *N*, *N*-dimethylaniline, nitrobenzene, *s*-trinitrobenzene and tetracyanoethylene on the fluorescence of acridine orange (AO) has been studied using ethanol and dioxane as solvents. In these solvents AO exists as cations or neutral molecules. In the former solvent, the quenching actions of the above substances on these two species have been investigated separately. The quenching constants, K's (in reference to the Stern-Volmer equation) of the above substances (in the above sequence) in ethanol are (34—35), (19.6), (53.6) and (~90) for cations and (8.4), (72.8), (—), and (—) for neutral molecules (in l./mol.). Thus, when quenchers are electron acceptors, K increases with the accepting power: nitrobenzene < *s*-trinitrobenzene < tetracyanoethylene. Further, acceptors are more efficient quenchers on neutral molecules than on cations (nitrobenzene), whereas the reverse is true for donors (*N*, *N*-dimethylaniline). In dioxane the quenching constants are generally somewhat smaller than in ethanol, but a basic similarity has been confirmed. All the results support the view that the present quenching phenomena are closely related to a charge-transfer process.

An electron transfer mechanism for the quenching of fluorescence has been postulated for a long time, but it was only in 1961 that the first evidence for it was presented by Weller.[1]

According to this mechanism, it is expected that a fluorescer with an electron-donating (accepting) power is quenched by an electron-accepting (donating) substance, and that the quenching constant runs parallel with the electron-accepting (donating) power of a quencher. This paper will deal, from such a viewpoint, with the quenching action of a few substances on the fluorescence of acridine orange, which may act as an electron donor or acceptor as the case may be. Besides, acridine orange exists as cations (acid) or neutral

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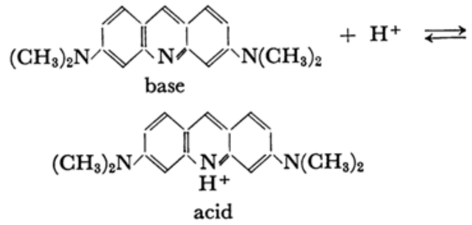
[1] H. Leonhardt and A. Weller, *Z. physik. Chem., N. F.*, **29**, 277 (1961); *Ber. Bunsges. Phys. Chem.*, **61**, 791 (1963).

molecules (base) according to the experimental conditions (see below); it is, therefore, interesting to compare the quenching efficiency of each substance on these two species.



Experimental

Materials.—Acridine Orange: a double salt of Tokyo Kasei, G. R. grade, was purified by precipitating the free base from a dilute sodium hydroxide solution, dissolving the precipitate in chloroform (G. R.), and chromatographing it on activated silica-alumina treated with hydrochloric acid. The main band was collected, dissolved in chloroform-methanol, concentrated, and then cooled to −20°C. The precipitate thus obtained was filtered and dried in air and finally in vacuo for several hours at 70°C.[2] M. p. 181°C.

N, N-Dimethylaniline of a G. R. grade of Tokyo Kasei was dried over potassium carbonate, distilled under reduced pressure in a nitrogen atmosphere, and finally submitted to vacuum distillation. The sample was stored in a sealed ampoule.

Triethylamine of a G. R. grade of Tokyo Kasei was dreid over sodium, distilled under reduced pressure in a nitrogen atmosphere, and then stored in a sealed ampoule.

Nitrobenzene, G. R. grade of Tokyo Kasei, was dried with phophorus pentoxide and distilled under reduced pressure. [5.7°C]. *s*-Trinitrobenzene, G. R. grade of Tokyo Kasei, was recrystallized from water two times. After being dried, it was further recrystallized from carbon tetrachloride two times.

Tetracyanoethylene, G. R. grade of Tokyo Kasei, was purified by sublimation three times. The solution soon got a tint of reddish violet, perhaps from an impurity.

Trichloroacetic acid of Kanto Kagaku, G. R. grade, was purified by recrystallization from benzene three times.

Ethanol of Wako Junyaku, G. R. grade, was fractionally distilled. B. p. 78°C.

Dioxane, G. R. grade of Wako Junyaku, was boiled under reflux for a few days over sodium and then distilled twice. B. p. 101°C.

Benzene of Wako Junyaku, G. R. grade, was dried over sodium and then distilled. B. p. 80.1°C.

Methanol of Wako Junyaku, G. R. grade, was fractionally distilled. B. p. 64.5°C.

Procedures.—The fluorescence intensities, fluorescence spectra and action spectra were measured by an Aminco-Bowman spectrofluorimeter. Correction for sensitivity dependence on the wavelength was not made on the fluorescence spectra. The absorption spectra were measured with a Hitachi EPS III autorecording spectrometer.

Results and Discussion

The Absorption and Fluorescence Spectra of Acridine Orange.—According to Zanker,[2] association becomes apparent above 10^{-5} M of acridine orange in an aqueous solution; Mataga reported similar association phenomena in pyridine, acrylonitrile and ethanol.[3] The recent results of Olszowski and Ruziewicz,[4] however, are quite different from Mataga's results. They assigned the two bands with peaks at ~20000 cm^{-1} and ~23400 cm^{-1} which appear separately or simultaneously in various solvents to cations (acid) and neutral molecules (base) respectively. This assignment was confirmed by the present investigation. As is shown in Fig. 1, acridine orange 7.35×10^{-5} M in ethanol gives a single peak at 495 mμ, with a shoulder[5] near 460 mμ while the fluorescence spectrum obtained by 490 mμ excitation has a single peak at 522 mμ. When ethanol which has been made alkaline by adding a few pieces of sodium hydroxide is used as a solvent, the absorption peak is at 433 mμ with $\varepsilon_{max}=24900$ and the fluorescence spectrum obtained by 430 mμ excitation has a peak at 510 mμ. Figure 2 shows the change in the absorption spectra when trichloroacetic acid or triethylamine is added to a 1.96×10^{-5} M solution of acridine orange in

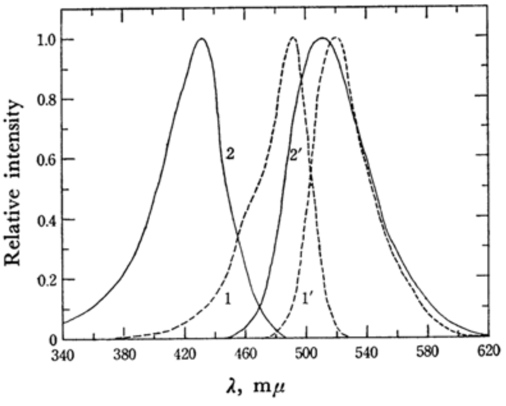


Fig. 1. Fluorescence and absorption spectra of acridine orange (7.35×10^{-6} M) in ethanol.

1 Absorption spectra
1′ Fluorescence spectra } (in ethanol)
2 Absorption spectra
2′ Fluorescence spectra } (in alkaline ethanol)

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[2] V. Zanker, *Z. physik. Chem.*, **199**, 225 (1952); **200**, 250 (1952).

[3] N. Mataga, This Bulletin, **30**, 375 (1957).

[4] A. Olszowski and Z. Ruziewicz, ibid., **37**, 1883 (1964).

[5] Although the above shoulder seems to suggest the existence of a dimer, Zanker made it clear that there is no sign of association in a dilute ethanol solution. Cf. V. Zanker, Dissertation, T. H. Munchen (1948).

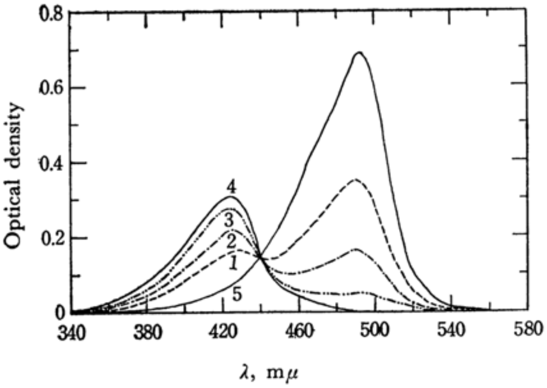

Fig. 2. Absorption spectra of acridine orange (1.96×10^{-5} M) in dioxane.

1) Without addition of a third component
2) With 2.8×10^{-5} M triethylamine
3) With 2.8×10^{-4} M triethylamine
4) With 7.11×10^{-4} M triethylamine
5) With 4.14×10^{-5} M trichloroacetic acid

dioxane. When more than 7.1×10^{-3} M of triethylamine is added, only the peak at 426 mμ due to neutral species becomes notable, whereas upon the addition of trichloroacetic acid of 4.14×10^{-5} M, only the peak of the cation at 492 mμ survives. There is an isosbestic point at 442 mμ. Thus, it is in complete accord with the general behavior of the acid-base system. The molar absorbances for cationic and neutral species were estimated to be 4.29×10^4 and 1.55×10^4, respectively. Figure 3 shows a similar change in the fluorescence spectra obtained by the excitation at the isosbestic point. The neutral and cationic species have fluorescence peaks at, respectively, 500 and 530 mμ. From the intensity ratio when excited by the light of the isosbestic point, the ratio of the apparent fluorescence quantum yields of the two species was decided to be neutral: cation = 1 : 2.5. Using the data taken from these two figures, the fraction of the cation, x_C can be calculated from the following equations:

$$x_C=\frac{[\text{cation}]}{[\text{total AO}]}=\frac{D_i-D_{iB}}{D_{iC}-D_{iB}} \tag{1}$$

$$x_C=\frac{F_i-0.4F_{iB}}{F_{iC}-0.4F_{iB}} \tag{2}$$

where D_i, is the optical density of the solution in question, where D_{iC} and D_{iB} are, respectively, the optical densities of the cation and the base when they exist in a pure form and at the same concentration (all at a certain wavelength, i), where F_i is the fluorescence intensity of the solution in question, and where F_{iB} and F_{iC} are the relative intensities when the intensity for each species at the peak of the fluorescence spectra is taken as unity. It should be added that Eq. 2 is derived on the assumption that there is no shift of equilibrium during the lifetime. Figure 4, which shows the relation between the two sets of x_C-values obtained from Eqs. 1 and 2, verifies that no essential shift occurs during the lifetime of the excited state.

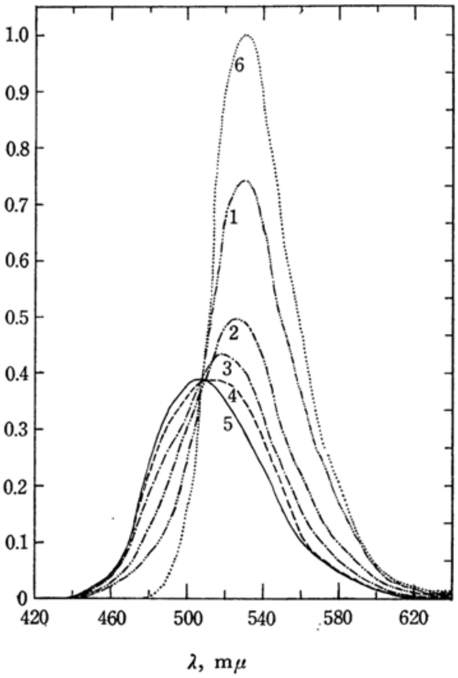

Fig. 3. Fluorescence spectra of acridine orange (1.96×10^{-5} M) in dioxane.

1) Without a third component
With triethylamine of
2) 2.8×10^{-5} M
3) 5.6×10^{-5} M
4) 2.8×10^{-4} M
5) 8.4×10^{-4} M
6) With trichloroacetic acid of 4.14×10^{-5} M

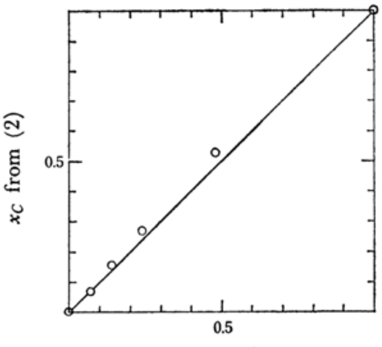

Fig. 4.

The Quenching Action of the Added Substances in Ethanol. — This was examined separately on cations and on neutral molecules. The quenching constants on cations were obtained by measuring the relative height of the fluorescence spectra at 522 mμ upon excitation at 490 mμ. The quenching constants for neutral molecules were similarly determined by measuring the fluorescence intensity in an alkaline ethanol solution at 510 mμ, with the exciting wavelength at 430 mμ. In all cases it was confirmed that the addition of a quencher does not appreciably affect the absorption spectra of acridine orange. *N, N*-Dimethylaniline gives a weak absorption, extending from 380 to 450 mμ, when the concentration is large. This is due to its interaction with the dissolved oxygen,[6] but the excitation in this wavelength region does not give any sign of emission. The absorption spectra of nitrobenzene extend to around 450 mμ; this was taken into account in the measurement in the alkaline solution. *s*-Trinitrobenzene and tetracyanoethylene, both of which are strong electron acceptors, also do not affect the absorption nor the fluorescence spectra of acridine orange. This rules out any specific interaction between fluorescer and quencher. A small band of trinitrobenzene in the 450—600 mμ region is also not affected by the addition of acridine orange. The same holds for the other bands. The absorption spectra of tetracyanoethylene in ethanol is also not essentially affected by the addition of acridine orange. However, trinitrobenzene in the alkaline solution reacts chemically, and the solution becomes red in color. The addition of tetracyanoethylene to the alkaline solution of acridine orange, on the other hand, causes the fluorescence spectra to change into cationic-type spectra. Since tetracyanoethylene is a strong π-acid, some kind of ion-pair may be formed. At any rate, the above situations prevent the determination of the quenching constants of trinitrobenzene and tetracyanoethylene in an alkaline solution.

The results are given in Table I, while some examples of the Stern-Volmer plot are given in Fig. 5.

The results given in Table I can be well comprehended on the basis of a charge-transfer mechanism of quenching. Thus, the order of quenching efficiency on cations, — nitrobenzene < trinitrobenzene < tetracyanoethylene—agrees with that of the electron-accepting power of these substances. Further, *N, N*-dimethylaniline, which is considered to act as an electron donor, is a more efficient quencher on cations than on neutral molecules, whereas the reverse is true for nitrobenzene.

The Quenching Action of the Added Substances in Dioxane.—The quenching phenomena were studied with a solution containing both cation and neutral species of acridine orange; suitable

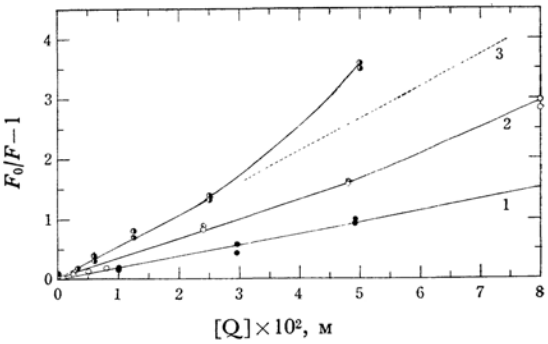

Fig. 5-a. $F_0/F-1$ vs. concentration of quenchers
quencher: 1) Nitrobenzene
2) *N, N*-Dimethylaniline
3) *s*-Trinitrobenzene

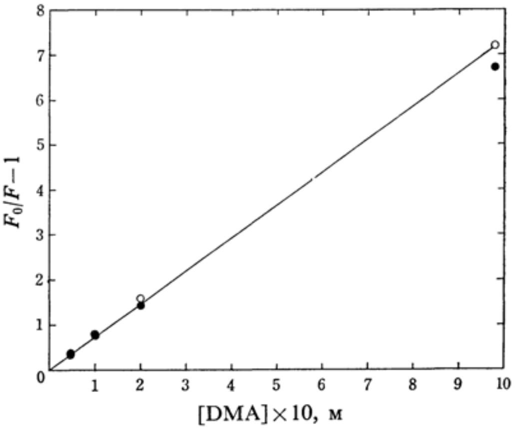

Fig. 5-b. $F_0/F-1$ vs. concentration of nitrobenzene in alkaline ethanol (EtOH+NaOH).
Concentration of AO, 7.35×10^{-6} M
Excitation wavelength—open circle: 440 mμ;
filled circle: 420 mμ

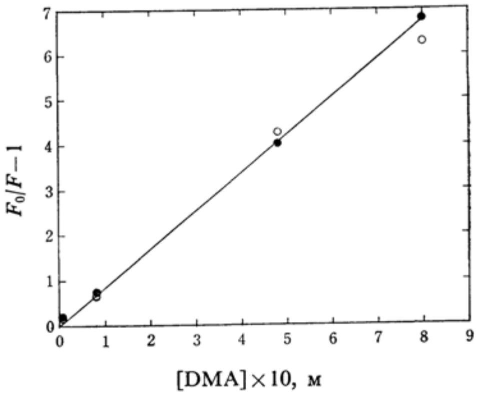

Fig. 5-c. $F_0/F-1$ vs. concentration of *N, N*-dimethylaniline in alkaline ethanol (EtOH+NaOH).
Concentration of AO, 4.90×10^{-6} M
Excitation wavelength—open circle, 450 mμ;
filled circle, 430 mμ

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[6] D. F. Evans, *J. Chem. Soc.*, **1953**, 345; **1957**, 1351, 3885; **1959**, 2753; *Proc. Roy. Soc.*, **A255**, 55 (1960).

TABLE I. QUENCHING CONSTANTS OF SOME SUBSTANCES ON THE FLUORESCENCE OF AO IN ETHANOL

Quencher	Concn. of AO M	Concn. range of quencher, M	Quenching for cation	Const. K*(l. mol^{-1}) for neutral species
N,N-Dimethylaniline	4.90×10^{-5}	2.4×10^{-3}—4.8×10^{-2}	34.5	
	4.90×10^{-6}	8.0×10^{-3}—8.0×10^{-1}		8.4
Nitrobenzene	7.35×10^{-6}	4.9×10^{-3}—9.8×10^{-2}	19.6	
	7.35×10^{-6}	4.9×10^{-3}—9.8×10^{-2}		73
s-Trinitrobenzene	7.35×10^{-6}	3.1×10^{-3}—5.0×10^{-2}	53.6	
Tetracyanoethylene	7.35×10^{-6}	5.6×10^{-3}	(90)	

* $K=(F_0/F-1)/[Q]$

F_0 and F are respectively the fluorescence intensities in the absence and in the presence of a quencher.

exciting wavelengths were chosen and the fluorescence intensities at different wavelengths were compared. The data were analyzed on the basic assumption that no essential shift of equilibrium occurs during the lifetime of the excited state. When the wavelength of the exciting light, i, is not the one at the isosbestic point, F_j, the fluorescence intensity at the j wavelength is approximately given by:

$$F_j = x_C F_{Cj}\frac{\varepsilon_C}{\varepsilon_O} + \frac{1}{2.5}(1-x_C)F_{Bj}\frac{\varepsilon_B}{\varepsilon_O} \qquad (3)$$

where ε_C, ε_B and ε_O are, respectively, the molar absorbance of cation and base at the i wavelength and that at the isosbestic point. From this equation it was confirmed that, except in very extreme cases, the fluorescence behavior of cation and base can be studied separately by choosing the exciting lights at 490 and 430 mμ respectively and by measuring the intensity at 530 and 490 mμ respectively. In some cases the change in the fluorescence spectra and in the action spectra caused by the addition of a quencher were made use of in getting semi-quantitative information on quenching.

***N, N*-Dimethylaniline.**—Figure 6 gives the Stern-Volmer plot for cation and neutral species. The quenching constant for neutral species was determined by the use of a solution containing 2.80×10^{-4} M of triethylamine. The results are, for cation, K=20 l./mol. and for base, K=0 l./mol.

Nitrobenzene.—With the increase in the concentration of nitrobenzene, the superposition of the absorption of nitrobenzene becomes notable in the shorter wavelength region. The fluorecscence spectra observed when the excitation wavelength is 490 mμ, as well as the action spectra obtained at 530 mμ of the fluorescence spectra, are not essentially affected by the addition of nitrobenzene.

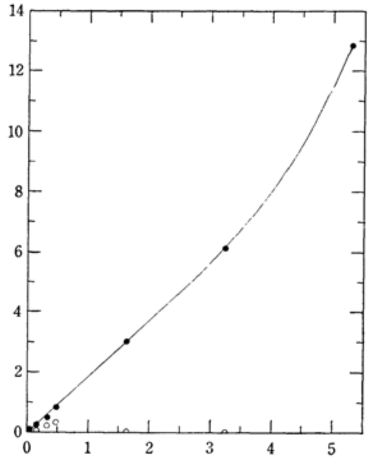

Fig. 6. $F_0/F-1$ vs. concentration of N,N-dimethylaniline in dioxane.
Concentration of acridine orange 1.96×10^{-5} M
Excitation wavelength—filled circle: 490 mμ; open circle, 430 mμ
Fluorescence wavelength—filled circle, 530 mμ; open circle 500 mμ

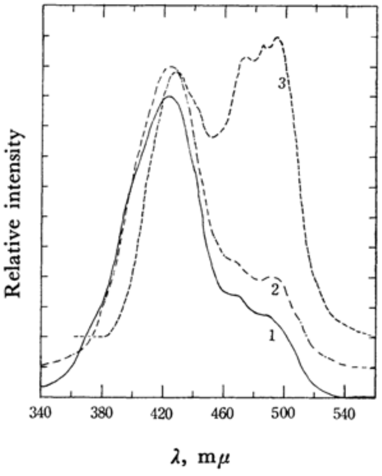

Fig. 7. The effect of the addition of nitrobenzene on the fluorescence excitation spectra of AO (1.95×10^{-5} M) in dioxane.
Concentration of nitrobenzene: (1) 0 M, (2) 9.8×10^{-2} M, (3) 9.8×10^{-1} M
Fluorescence wavelength, 500 mμ
Some structures near 500 mμ of curve 3 are due to the characteristics of the Xe - arc lamp emission.

This implies that the fluorescence of the cation is not quenched very much by nitrobenzene. On the other hand, the action spectra taken at 500 mμ of the fluorescence spectra, as Fig. 7 shows, depend on the concentration of nitrobenzene to a great extent. Thus, there is scarcely any doubt that nitrobenzene quenches neutral molecules more efficiently than cations. The quenching constants evaluated from the intensities at 530 and 490 mμ with the exciting lights at 495 and 430 mμ respectively, are ~10 and ~30 l./mol.

Trinitrobenzene.—As in ethanol, a weak absorption from 550 to 600 mμ is not due to the interaction of trinitrobenzene and acridine orange, but to trinitrobenzene itself. The measurement of the fluorescence intensities at 530 and 490 mμ, with the exciting lights at 490 and 430 mμ respectively, gives, as the quenching constants for cations and neutral species respectively, ~55 and ~70 l./mol. The fluorescence spectra taken with exciting lights of shorter wavelengths and the action spectra taken at 490 and 510 mμ of fluorescence spectra also indicated that the neutral species is more quenched than the cation.

Tetracyanoethylene.—With the addition of tetracyanoethylene, the absorption spectra change into cationic-type spectra, just as in ethanol. The fluorescence spectra also become cationic-type spectra; however, their peaks shift slightly toward blue. The quenching constant of tetracyanoethylene on the ion-pair is ~70 l./mol. from the Stern-Volmer plot. The appoximate quenching constants in the dioxane solution are summarized in Table II.

TABLE II. APPROXIMATE QUENCHING CONSTANTS OF SOME SUBSTANCES ON THE FLUORESCENCE OF AO IN DIOXANE

Quencher	*K* for cation l. mol^{-1}	*K* for base l. mol^{-1}
N, *N*-Dimethylaniline	~20	~ 0
Nitrobenzene	~10	~30
s-Trinitrobenzene	~55	~70
Tetracyanoethylene	70	

Relations similar to those in ethanol are apparent in this case. All the quenching constants in dioxane are somewhat smaller than in ethanol. This is consistent with the results reported by Miwa and Koizumi.[7)]

Conclusion

All the results obtained in the present experiment strongly support a charge-transfer mechanism of quenching. Besides, it has been confirmed that the acid-base equilibrium does not shift essentially during the lifetime of the excited state.

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7) T. Miwa and M. Koizumi, This Bulletin, **39**, 2588 (1966).