The Quenching of Fluorescence in Solution. III. The Nature of the Quenching Process

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In two previous papers we have shown that[1] the quenching of fluorescence in solution may be considered as a bimolecular reaction which requires little or no energy of activation. The effects of temperature and the viscosity and dielectric constant of the solvent on the rate have also been studied. In this paper we wish to present experimental evidence concerning the mechanism of the quenching action and discuss these results in relation to the theories which have been presented in the literature.

One possible action of a quencher molecule is that it combines with the absorbing molecule to form an addition compound which does not fluoresce when photoactivated. In our first paper we cited as evidence against this mechanism the fact that in the systems we were studying the absorption is not changed appreciably by the addition of the quencher. Since a number of people have expressed the opinion that that criterion is not sufficiently sensitive we have collected more decisive evidence. It is readily shown that if such an addition compound is formed, the quenching constant, k, in the expression $I_0/I = 1 + k$ (concn. of quencher), is proportional to the equilibrium constant for the reaction which forms this compound. Let us compare the observed properties of this quenching constant with those to be expected for an equilibrium constant. In the first place if a compound of sufficient stability to account for the observed quenching is formed, it is to be expected that some heat would be evolved in the formation. Therefore the equilibrium constant, and hence the quenching constant, should decrease as the temperature is raised whereas experimentally it has been found that the quenching constant increases as the temperature is raised. A second point is that the quenching constant is decreased by increasing the viscosity of the solvent. An equilibrium constant must be independent of viscosity since the principle of microscopic reversibility requires that the rates of the direct and reverse reactions be affected to the same extent by the viscosity. These two facts alone are strong evidence against formation

of an addition compound before photoactivation but to obtain even more conclusive evidence we have determined the relative activities of the fluorescent molecule in the presence and absence of the quencher. The method consisted of establishing the distribution equilibrium of the fluorescent substance between the aqueous solutions and benzene. This procedure can be applied only if the fluorescent substance is a neutral molecule, hence our experiments of this type were limited to fluorescein and acridone. A typical experiment with fluorescein was carried out as follows: two 250-cc. solutions of the same dye concentration in 0.001 M perchloric acid were prepared, one contained 0.5 M potassium nitrate, the other 0.5 M potassium iodide. The fluorescence intensity of the latter was found to be approximately 5% of that of the former. Each of these solutions was shaken with 25 cc. of benzene, allowed to stand and the two layers separated. Each benzene layer was shaken with 250 cc. of 0.01 M potassium hydroxide, a procedure which removed practically all of the dye in the form of the divalent negative ion. The concentration of dye in each of these solutions was determined by measuring the intensity of the fluorescence which could be excited in them. They were found to contain exactly the same quantity of dye, approximately 30% of that present in the original solutions. If the iodide had formed a complex with the fluorescein to the extent of 95% (as would be required to account for the quenching) the quantity extracted from the solution which contained the iodide should have been less than 10% of that extracted from the nitrate solution. Hence we can conclude that complex formation before the photoactivation cannot account for the quenching of the fluorescence of fluorescein by iodide ion. Similar results were obtained for acridone with potassium iodide and with silver nitrate. On the basis of all these observations we feel justified in concluding that for systems of the type we are studying the action of the quenchers cannot be attributed to complex formation before photoactivation.

The nature of the action of the quencher on

(1) THIS JOURNAL, **61**, 2634 (1939); **62**, 2264 (1940).

the photoactivated molecule has been the subject of some speculation. Before discussing the theories which have been proposed let us consider some of the experimental facts. In the first place it might be supposed that there would be some correlation between the quenching efficiency and the magnitude of the separations of the energy levels in the quencher as compared to the energy to be given up by the activated molecule. Little is known about these energy levels for most of the ions which have been used as quenchers. In the case of iodide ion and several others, which have been used as quenchers for visible fluorescence excited by near ultraviolet light, the levels associated with the light absorption are separated so far that the absorption band lies on the short wave length side of 2500 Å. There is certainly no close correlation in these cases between the energy to be taken up from the excited molecule and the energy difference indicated by these absorptions. It is true that if we consider the series iodide, bromide, and chloride as quenchers the best quencher is the one with the absorption nearest the visible. However, for most of the fluorescent substances which have been studied, nitrate ion is a much poorer quencher than these, yet it has an absorption maximum at 3000 Å. and a second absorption region begins about 2600 Å. If the rule which applies to the halides was valid generally nitrate should be a better quencher than the halides. Furthermore, on the basis of any correlation with the position of the ultraviolet absorption, it would be predicted that oxalate and acetate should be better quenchers than bromide and chloride with the former approximately the same as iodide. Actually these ions rate below chloride in efficiency. It is possible, of course, that there are other energy levels to which transitions by the absorption of radiation are forbidden which are responsible for the quenching action. In the absence of evidence concerning such levels their introduction must be considered an *ad hoc* assumption. Even if such levels do exist the case of nitrate ion already cited suggests that an approximate coincidence of energy differences is not sufficient to bring about quenching. In order to obtain data in cases in which there is a much closer agreement in the known energy differences of the quencher and fluorescent molecule we have tested some of the rare earth ions.

These ions are known to have absorption in

the same region of the spectrum as the fluorescence of the substances we have studied. The rare earth salts used in these experiments and the results obtained are listed in Table I together with the results of tests with manganous sulfate, and gallium nitrate. The positive effects observed were very small compared with the quenching by an ion such as iodide and may have been only the absorption of the fluorescent light rather than any real quenching. On the basis of these experiments we conclude that the existence of electronic states which differ in energy by the same amount as those in the fluorescing molecule is not sufficient to make a molecule serve as a quencher for that fluorescence.

Since the existence of a suitable difference between energy levels is not sufficient to make a substance act as a quencher it becomes necessary to consider many different examples of quenching in order to see if the necessary and sufficient characteristics of a quencher can be identified. The most extensive studies on quenching have been carried out with quinine sulfate, sodium fluoresceinate, uranyl salts and other substances which are quenched by negative ions. The efficiencies of these are roughly in the order

$$ I^- > CNS^- > Br^- > Cl^- > C_2O_4^= > $$
$$ C_2H_3O_2^- > SO_4^- > NO_3^- > F^- $$
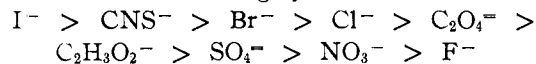
However, Weber[2] found that the halides have little effect on the fluorescence of sodium naphthionate but nitrate ion is a good quencher. We have confirmed this observation and have tried a number of other substances. The result is that we find that substances which fluoresce in solution may be divided into two classes each of which has its characteristic group of quenchers. This division is shown in Table II. Only negative ions are listed as quenchers in this classification since we found that both classes are quenched by silver, cupric, and ferrous ions. The fluorescent substances are those which we tested and must be looked upon only as examples of each class and not a complete list for either. Most of the observations on class II substances have been directed toward finding examples of fluorescent substances in this class and quenchers. Both the intensity of fluorescence and the quenching are affected by the hydrogen ion concentration, but this may be due to changes in the molecular species in solution, as has been shown to be the case for class I substances. As a tentative order for

(2) Weber, *Z. physik. Chem.*, **B15**, 18 (1931).

the efficiencies of the quenchers we may write $IO_3^- > BrO_3^- > NO_3^- > S_4O_6^- > AsO_4^= > ClO_3^-$. This list may be divided into sub-groups; the first three have nearly the same quenching constants, the next two are quite appreciably smaller and chlorate ion is a very poor quencher. Iodide does quench some of these substances also but the efficiency is of the same order as that of nitrate for class I substances. The magnitude of the quenching in the case of sodium naphthionate is indicated by the following I/I_0 values with the quenchers listed: 0.05 M strontium nitrate 0.41, 0.1 M potassium bromate 0.35, 0.5 M potassium iodide 0.83. The quenching action of 0.01 M potassium bromate is the same as that of the 0.5 M potassium iodide.

TABLE I

EFFECT OF CERTAIN POSITIVE IONS ON THE FLUORESCENCE OF SEVERAL DYES IN AQUEOUS SOLUTION

		Dye (Concn. $= 10^{-4}\,M$)			
Quencher	M	Quinine sulfate	Anthracene sulfonic acid	Acridone sulfonic acid	Fluorescein in H^+
$La(NO_3)_3$	0.1	0	0	0	?
$Er_2(SO_4)_3$	$\sim .2$	+	0	0	0
$Sm(NO_3)_3$	$\sim .2$	0	?	+	?
Didymium Sulfate $(Pr,Nd)_2$- $(SO_4)_3$	$\sim .1$	?	?	+	?
$Ga(NO_3)_3$	$\sim .1$	0	0	0	0
$MnSO_4$	~ 1.0	?	0	?	?

0 = no noticeable effect.　? = apparently slight effect. + = definite but small effect.

TABLE II

CLASSIFICATION OF FLUORESCENT SUBSTANCES ACCORDING TO THE NATURE OF THE QUENCHING AGENT

Class I
　Quenching agents: I^-, Br^-, Cl^-, CNS^-, $S_2O_3^=$, $SO_3^=$, etc.
　Fluorescing substances: acridone, α- and β-anthracene sulfonic acid, quinine sulfate, fluorescein, eosine, rhodamine β, rhodamine 6 g.

Class II
　Quenching agents: IO_3^-, BrO_3^-, NO_3^-, $S_4O_6^=$, etc.
　Fluorescing substances: anthranilic acid, sodium sulfanilate, sodium naphthionate, α-naphthol, 1,4-naphthol sulfonic acid.

Both classes are quenched by Ag^+ and Cu^{++}.

The existence of these two groups of fluorescent substances each with its characteristic group of quenchers is positive evidence that any adequate theory of the quenching action must be based on something far more specific than the mere existence of similar differences in the energy levels of the activated molecule and the quencher. In

our experiments both groups have been excited by the same frequency and the fluorescence lies at least approximately in the same region of the spectrum. Much of the theoretical discussion concerning fluorescence quenching in solution makes use of the term "resonance transfer" and refers to the paper of Franck and Levi.[3] Neither in this paper nor any of the others in which that term is used is any clear picture of such a process or the conditions controlling it presented. From the reference of similarity to gaseous systems it might be implied that the energy transfers are considered as occurring by the same mechanism as those in the mercury sensitized fluorescence of sodium[4] or the quenching of the mercury fluorescence by various gases.[5] In both of these great stress is laid on the correlation of the similarity in magnitude of the energy differences in the quencher and the fluorescing molecule and the quenching efficiency. We have seen that such a correlation is not sufficient in solution, hence any "resonance transfer" theory, if it is to be of value, must be amplified so as to show what other factors are involved. A more specific action has been proposed by Weiss[6] who suggested that the quenching involved the transfer of an electron to the activated molecule from the quencher. In some published discussion he has suggested that the transfer may be limited to a resonance between two forms which may be represented as (DA) and (D^-A^+) in which D represents the dye and A the quencher molecule. Such a modification of the theory serves no useful purpose since it is impossible to distinguish between such a resonance and any other process which might occur when the dye and quencher molecules come together. The more specific process originally given probably is correct in some cases. Certainly it must be considered that if an actual electron transfer occurs, $i.\ e.$, there is oxidation or reduction of the dye, the fluorescence probably is prevented. The only question we wish to raise on this hypothesis is whether or not it is necessary in all cases.

According to the electron-transfer theory the distinction between the two classes of fluorescent substances which we have described is that those in Class I require the addition of an electron for

(3) Franck and Levi, Z. physik. Chem., **B27**, 409 (1935).
(4) Beutler and Josephy, Z. Physik, **53**, 747 (1929).
(5) Zemansky, Phys. Rev., **36**, 919 (1930).
(6) Weiss, Trans. Faraday Soc., **35**, 48 (1939); Weiss and Fischgold, Z. physik. Chem., **B32**, 135 (1936).

quenching whereas those in Class II require removal. In favor of this view is the fact that the quenchers for Class I are the reduced forms and those for Class II the oxidized forms of the oxidation–reduction couples to which they belong. On the other hand, there seems to be no correlation between the efficiency of a substance as a quencher and either its strength or rate of reaction as an oxidizing or reducing agent. Some regularity seems to exist in that the sequence $I^- > CNS^- > Br^- > Cl^-$ holds for both quenching action and reducing power. On the other hand, oxalate is readily oxidized by the halogens and hence is a better reducing agent than the halides, but it is a weaker quencher. Furthermore, with Class II substances the order $IO_3^- > BrO_3^- > NO_3^- > S_4O_6^-$ is not even qualitatively in the order of oxidizing strengths regardless of what half reaction is written for the oxidizing agent. (Provided we limit ourselves to known half reactions.) Lack of correlation between rates of other reactions of the quenching ions and the quenching ions is not surprising when one considers that only very rough correlations are ever obtained in the comparison of rates.

Another point to consider is that there is nothing in the formulas of the dyes which have been studied which would enable us to predict to which of the two classes any other dye would belong. Nor have we been able to find any change with time in the absorption of the dye or in the intensity of fluorescence which would correspond to the transformation of some of the dye into either an oxidized or reduced form. However, if only 1 or 2% of the dye was in such a form in the photostationary state we would not have detected it with certainty.

The intensity of the light which we used to excite the fluorescence was such that 6.5×10^{-6} einstein passed through the 40-cc. cell per minute. Since the concentration of the dye was 10^{-4} molal or less, this means that enough light was put in to activate all of the molecules in one minute. For 50% quenching this would mean that half of the dye would be either reduced (or oxidized) every minute according to the electron transfer theory.

It has been known for some time that the dyes, the fluorescence of which is quenched by iodide, cause the liberation of iodine on illumination of solutions which contain the dye and potassium iodide. We have found that if the solution is freed from oxygen by bubbling nitrogen through

it for some time before illumination, then this liberation of iodine does not occur nor is the absorption of the solution or intensity of fluorescence altered appreciably by this treatment.

The quenching of both classes of fluorescent substances by silver and copper ions may involve electron transfers. The energy present in the photoactivated molecules of Class I is sufficient to oxidize either silver or copper to the known higher states of oxidation. On the other hand, silver and cupric ions are good oxidizing agents and could remove electrons from the substances in Class II. In the case of the quenching of anthracene sulfonic acid by cupric ion a rapid decrease in the absorption of the exciting radiation during illumination indicated that the dye was being destroyed by a photochemical reaction.

In this discussion we have stressed the difficulties involved in the application of current views on fluorescence quenching to our data. The results which we have presented in this and the two preceding papers show that the quenching constant has all of the properties of a specific reaction rate. It follows that the theoretical problems involved are the same as for the calculation of absolute reaction rates. Up to the present time we have not found any such theory which would predict the existence of the two classes of fluorescent substances we have pointed out.

Summary

It has been shown by means of measurements of the activity of a fluorescent substance in the presence of a quencher that the quenching action cannot be attributed to complex formation before photoactivation. The effect of temperature and viscosity changes on the quenching process furnishes additional support for this idea. The simple resonance transfer theory has been shown to be inadequate because (1) ions known to have levels separated by the amount of energy which must be removed from the fluorescent molecule do not serve as quenchers; (2) substances which are excited by the same radiation and fluoresce in the same region of the spectrum can be divided into two classes each of which has its characteristic group of quenchers. The electron transfer theory has been discussed and it has been pointed out that it does not aid in the prediction of the type of quencher or quenching efficiency to be expected in any given case.