Fluorescence quenching is used extensively in studies of molecular interactions. In fluorescence quenching studies two aspects demand attention: (i) quenching by diffusion-controlled collisional interaction between the fluorescer and quencher; and (ii) quenching by non-fluorescent complex formation (static quenching) between the reactants in the ground state. Boaz and Rollefson were able to explain deviations from Stern-Volmer (S-V) equation by postulating either association of the quencher with fluorescer or the self-association of the quencher. However, positive deviation from linearity is also expected at high quencher concentration even when static quenching is absent.

The interaction of oxytetracycline and phenol with organic acids has attracted a great deal of interest owing to its biological importance. Weber and Rosenheck studied the quenching effect of carboxylic acids on the fluorescence of phenol and concluded that the process involved the formation of a ground state nonfluorescent complex. On the other hand, Ricci and Nesta noted that fluorescence quenching by carbonyl compounds was primarily dynamic in nature. Steinar and Kirby found that amino acids quench dynamically. However, in order to understand the mechanism of fluorescence quenching, more detailed results on direct observation of the quenching process is required. In the present work we have studied steady state fluorescence quenching of oxytetracycline (OTC) in alkaline medium and that of phenol in neutral aqueous medium, by a number of amino acids. The rate constants of fluorescer and quencher interactions have been determined and the observed deviations from Stern-Volmer (S-V) plots are discussed on the basis of stronger interaction via a proton transfer from the fluorescer molecule to the carboxylate ion of the quencher.

**Materials and Methods**

Phenol (Eastman Kodak) was repeatedly distilled before use. Oxytetracycline samples was obtained as gifts from Pfizer Ltd, and Hindustan Antibiotics Ltd. The samples were purified by recrystallization from hot water and dried in vacuo before use. The amino acid quenchers used were: glycine (I), β-alanine (II), L-asparagine (III), L-glutamine (N), L-histidine (V), DL-methionine (VI), N-acetylglucine (VII), N-glycylglucine (VIII); 4-aminobenzoyl glycine (IX), glycine ester hydrochloride (X), L-glutamic acid (XI) and L-aspartic acid (XII). Amino acids obtained from E Merck, Sigma Chemical and Fluka AG were used as such. Triply distilled water was used for the preparation of solutions. Analytical grade sodium hydroxide was used. The electronic absorption spectra were recorded on a Cary 17D spectrophotometer. A Systronics digital pH meter (model 335) was used for pH measurements. Fluorescence spectra were recorded on a Perkin-Elmer MPF 44A fluorimeter. The excitation wavelengths for OTC and phenol were 380 and 270 nm re-
Table 1 - Quenching rate constant ($k_q$) for phenol amino acid system ($\tau_0 = 6.5/\text{ns}, \text{pH} = 7, \lambda_{\text{exc}} = 270 \text{ nm}$)

<table>
<thead>
<tr>
<th>Quencher</th>
<th>$[\text{Quencher}]$ (7 mol dm$^{-3} \times 10^2$)</th>
<th>$k_q$ (dm$^3$mol$^{-1}$g$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>6.5-32.3</td>
<td>3.1 $\times 10^6$</td>
</tr>
<tr>
<td>II</td>
<td>1.1-8.80</td>
<td>2.1 $\times 10^6$</td>
</tr>
<tr>
<td>III</td>
<td>5.0-11.4</td>
<td>4.5 $\times 10^6$</td>
</tr>
<tr>
<td>IV</td>
<td>1.9-5.70</td>
<td>4.6 $\times 10^6$</td>
</tr>
<tr>
<td>V</td>
<td>3.0-13.5</td>
<td>4.8 $\times 10^6$</td>
</tr>
<tr>
<td>Lysine</td>
<td>2.5-12.2</td>
<td>5.6 $\times 10^6$</td>
</tr>
<tr>
<td>Methyl amine</td>
<td>0.05-1.9</td>
<td>1.9 $\times 10^{11}$</td>
</tr>
<tr>
<td>Acetic acid</td>
<td>49.0-391.0</td>
<td>7.8 $\times 10^7$</td>
</tr>
</tbody>
</table>

Rate constant for diffusion-controlled quenching reaction, $k_d(=8RT/30V)=7.6 \times 10^6$ dm$^{-3}$ mol$^{-1}$ s$^{-1}$ in water.

Results and Discussion

Phenol fluorescence quenching

The intensity of phenol fluorescence gradually decreases with increase in [amino acid] without any appreciable change in spectral profiles. Moreover, no evidence of a ground state complex formation could be detected in the absorption spectra in the amino acid concentration up to $\sim 10^{-2}$ mol dm$^{-3}$. The quenching data have been treated by the usual Stern-Volmer (S-V) relationship (Eq. 1), where AA stands for amino acid and other terms have their usual meanings$^9$. The calculated values of $k_q$ are listed in Table 1. A point to note here is that, fluorescence quenching is not observed when any of the amino acid under study is added to a solution of an analogue of phenol, e.g. 2,5-dimethylanisole. Accordingly, when $-\text{COO}^-$ group is a good proton acceptor, the phenol fluorescence quenching may be ascribed due to an interaction of H-bond type between the phenolic proton and carboxylate ion in the excited electronic states.

It has been recently shown that the phenol fluorescence quenching process is partly due to association between amino acid and phenolic compound in its ground state$^{10}$ and is not entirely due to deactivation of excited state. However, White$^{11}$ observed that the carboxylate ion was a very strong quencher and reported that the decrease in the intensity of phenol fluorescence with increase in [carboxylate ion] could be adequately explained by collisional quenching. Moreover, when the S-V plots using intensity data are quite linear (not shown in figure) phenol fluorescence quenching may be postulated as dynamic quenching. Furthermore, the $k_q$ values presented in Table 1 tend to
suggest that this quenching reaction proceeds more or less via diffusion-controlled process.  

An interesting observation about phenol fluorescence quenching is that when a stronger electron donor like methylamine is used as the quencher the effect is large and distinct positive deviation from S-V plot is observed. Moreover, the kg value (obtained from the linear part of the S-V plot) is about two order of magnitude higher (Table 1) than the corresponding kg values obtained with amino acids and an absorption spectral shift of about 1949 cm$^{-1}$ is detected in this case. All these facts clearly indicate that this efficient quenching by amino acids is the result of ground state complexation. Kunimitsu et al.\textsuperscript{15} suggested that association in the ground state was mainly responsible for the departures from S-V kinetics and any alternative mechanism involving diffusion-controlled rates was inapplicable. However, in the present case we are unable to detect any significant evidence of ground state complexation. Lakowiez and Weber\textsuperscript{16} observed large positive deviations even though there was no detectable change in absorption spectrum. According to Moriya\textsuperscript{17} the S-V plots with high quenching ability generally deviated from linearity. In our study quenching is postulated to involve an OH$^–$ – OOC H-bond.

The H-bond of such a complex accounts for the instantaneous de-excitation of the excited state\textsuperscript{15} and a very rapid quenching process may not concern with the stationary diffusion process\textsuperscript{18}. However, this instantaneous quenching process may be described by the modified form of the S-V equation (Eq. 2)\textsuperscript{19}.

$$1 - \frac{(1/L_0)}{[AA]} = k_t \tau_0 I_0 + \frac{1 - W}{[AA]}$$  \hspace{1cm} \text{(2)}

The fraction, W, decreases from unity in contrast to the simple S-V equation (Eq. 1) where $W = 1$. In order to get more information about the validity of Eq. (2), it is quite reasonable to calculate the values of W and $k_t$. The plots of $1 - (1/L_0)/[AA]$ versus $I_0$ (Fig. 4) have quite large intercepts and these become strictly zero if the linear condition, i.e. $W = 1$ is satisfied. An interesting point to note here is that the value of $k_t$ presented in Table 3 does not differ much from the $k_q$ va-
Fig. 3-Stern-Volmer plots for quenching of OTC by XI(□); XII(▼); IX(○); X(●); and VIII(△).

Fig. 4 - Plots of 1-(I/I₀)/[AA] versus I/I₀ (Quencher 0(IX); □(V); △(XI); ▼(XII); □(X) and ●(VIII))

Table 3 - Values of quenching rate constants (kₚ/dm³ mol⁻¹ s⁻¹)

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>kₚ (dm³ mol⁻¹ s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VIII</td>
<td>8.5 x 10¹⁰</td>
</tr>
<tr>
<td>IX</td>
<td>1.2 x 10¹¹</td>
</tr>
<tr>
<td>X</td>
<td>9.6 x 10¹⁰</td>
</tr>
<tr>
<td>XI</td>
<td>1.1 x 10¹¹</td>
</tr>
<tr>
<td>XII</td>
<td>1.0 x 10¹¹</td>
</tr>
</tbody>
</table>

values. Moreover, range of W values (~0.82-0.96) obtained from the intercepts of the above plots and Eq. 2 are very close to unity. Thus, it may be presumed that static quenching by ground state complexation is not the main quenching mechanism for the deviations in S-V plots and higher values of kₚ, though there is considerably large intercepts in Fig. 4.

The high values of kₚ in the case of amino acids VI-XII may be explained as follows: The electron distribution in amino acid is strongly influenced by the regions of positive and negative charge in the zwitterions. It has also been shown earlier that protonated amino group is able to reduce the electron density of −COO⁻ group, with concomitant decrease in electron donating and proton accepting abilities of amino acids. However, the extent of fluorescence quenching or proton accepting ability of −COO⁻ group may increase by the number of substituted groups attached to it and this may bring about increased basicity through an inductive effect via −COO⁻ group. Moreover, −COO⁻ group in alkaline medium, is a poor electrophile and a good proton acceptor. Thus, when NH₂ moity is farther from carboxylate ion and hence less efficient to enter in a charge transfer interaction, −COO⁻ group may become a quite efficient quencher in OTC fluorescence quenching. Nevertheless the fact that remarkably efficient electronic quenching is observed suggests that relatively strong interaction takes place between excited OTC and the amino acids VII-XII even in the presence of NH₂ group. Therefore, this may be the main reason for anomalous behaviour of OTC fluorescence quenching and deviation from linearity in S-V plots. How-
ever, two features in this work are apparent: (i) the plots in the case of phenol fluorescence quenching are linear in contrast to upward curvatures normally seen for OTC quenching. (ii) the concentrations of added quenchers are approximately two order of magnitude higher than those used for OTC quenching. At high pH the ionized carboxylic groups will be large in number due to the increased flexibility of the chain of ionized group and each carboxylate ion becomes a strong quencher at high pH. This effect may be manifested in an increase in the quenching rate constant and hence \( k_q \) values in the case of OTC/amino acid interactions are always higher than the corresponding values in the case of phenol (Tables 2 and 3).

Some more significant facts in the present study are: (i) From the \( k_q \) values of glycine and N-acetylglucine (Table 2) it may be said that the degree of electrophilicity has decreased due to acylation of the amino group. (ii) The quenching of fluorescence varies with [amino acid] indicating that this quenching is essentially an intermolecular type and it is not due to an intramolecular effect. (iii) OTC fluorescence remains almost unaffected by the presence of a strong electron donor like methyl amine; however, moderately strong quenching is observed when acetic acid is spiked with OTC in aqueous alkaline medium. This may mean that the dissociated carboxylic group is essential and reveals the importance of COO\(^-\) group as the quenching centre in amino acids in this study. White\(^{11}\) has pointed out that fluorescence of phenolic compounds could be quenched by carboxylic ion via an proton transfer mechanism and proton transfer is facilitated by the increase in the anion field.\(^{21}\) Moreover, occurrence of a strong H-bond leads to a loosening of the OH bond and a shift of the proton towards the carboxylate oxygen.\(^{22}\) Accordingly, a proton transfer mechanism can not be ruled out in the present case of OTC fluorescence quenching, particularly when the interaction is stronger.\(^{23}\) Finally, the amino acid proton is not essential for quenching as the ester derivative is found to retain its quenching ability to a great degree (Table 2).

In conclusion, the present results show unambiguously that carboxylate ion has an important role on spectral properties of OTC and phenol, and the quenching by amino acids involves interaction between COO\(^-\) group of amino acid and phenolic proton of fluorescer compound. Therefore, H-bonding interaction has been shown to be predominant quenching mechanism for the singlet states of fluorescer compounds with amino acids in excited electronic states. Deviations from S-V plots in the case of OTC fluorescence quenching seem to be the result of increased quenching efficiency or strong interaction between the reactant through the inductive effects of the substituents.\(^{24,25}\) Finally, our findings combined with other evidences strongly support the hypothesis of dynamic quenching mechanism in the case of all the systems under study.

References