ing time significantly and can be associated with Category B. With Compound 8 the potentiation was extraordinary; it potentiated the sleeping time four times.

Moreover, during gross screening, Compound 5 appeared to be the most active CNS depressant. However, in the pentobarbital potentiation analysis, Compounds 4, 8, and 10 seemed to be more active than 5; of these, Compound 10 was the most active. It prolonged the sleeping time about three times more than 1-cyclobutanecarbonyl-3,3-dimethylurea. These observations lend further support to the hypothesis that two mechanistically separate modes of action exist for these compounds: one for barbiturate potentiation and the other for depressant ability. However, the data are not sufficient to allow a definite conclusion regarding the mechanism of barbiturate potentiation. The possibility that the compounds potentiate pentobarbital sleeping time *via* the inhibition of the liver microsomal enzymatic system cannot be ruled out.

Myorelaxant activity of the meprobamate type is conveniently studied by ascertaining whether a compound antagonizes strychnine lethality (7–9). In the present series of 10 compounds, all compounds showed myorelaxant activity; in the gross screening and barbiturate potentiation test, only eight were active. This finding suggests that the compounds in question are predominantly myorelaxants and that hypnotic and sedative activity is a secondary effect. Furthermore, these compounds can be classified into two categories. Some are only myorelaxant (Compounds 8 and 9) and belong to a class of compounds exemplified by mephenesin. Others show slight depressant effects along with myorelaxant activity (Compounds 1–7 and 10) and belong to a class of compounds exemplified by meprobamate. At a dose of 1000 mg/kg po, Compounds 5, 8, and 9 protected 100% and Compound 2 protected 60% of the test animals completely from strychnine lethality. Compounds 1, 3, 4, 6, 7, and 10 protected only 20–40% of the population tested. However, as far as partial protection from strychnine lethality is concerned, Compounds 4, 5, and 7–10 protected 100% of the test animals; Compounds 1–3 and 6 protected 60% of the population tested.

All of these compounds were also tested for pentylenetetrazol antagonism (10). At a dose of 1000 mg/kg po, Compounds 3, 4, 7, and 8 protected 100% and Compounds 2, 6, and 9 protected 20–60% of the test animals completely against pentylenetetrazol-induced convulsions. Compounds 1, 5, and 10 did not show antagonism to pentylenetetrazol.

All compounds were tested for an ability to antagonize tremorine-induced, peripheral parasympathetic stimulation and centrally originating parkinsonian-like tremors (11, 12). At a dose of 1000 mg/kg po, the antitremor activity was limited to Compounds 1, 4, 5, 8, and 10. Compounds 1 and 10 protected 20–80% of the test animals moderately, but none was completely protected. However, Compounds 4, 5, and 8 protected 20–80% of the test animals completely from tremors. These compounds were classified as slightly active since a highly active compound should be able to protect 100% of the test animals completely at a dose much lower than used in this test. Standard agents such as atropine, scopolamine, or trihexyphenidyl block tremorine effects in doses of 5–10 mg/kg in mice. The compounds showed no protection against tremorine-induced, peripheral parasympathetic stimulation.

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# Fluorescence Characteristics of Benzodiazepines in Strong Acid


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**Abstract** □ The fluorescence characteristics of 10 substituted 1,4-benzodiazepines in strong acid solution were investigated. The compounds that fluoresce in the Hammett acidity region possess, or can form by enolization, an azomethine linkage in the 1,2- or 4,5-position. All benzodiazepines that fluoresce in strong acid show increases in fluorescence intensity with corresponding blue shifts as acidity increases. Two pKa's in the Hammett acidity region were observed for both the fluorometric and absorptiometric titrations of the benzodiazepines possessing a carbonyl in the 2-position. No evidence of excited state prototropism was observed.



**Keyphrases** □ Benzodiazepines—fluorescence characteristics in strong acid □ Fluorescence characteristics—10 substituted 1,4-benzodiazepines in strong acid, fluorometric and absorptiometric titrations


Of the spectroscopic methods utilized routinely in pharmaceutical analysis, fluorescence techniques are by far the most sensitive. Fluorescence methods are often discarded as analytical methods because many compounds do not appear to possess active fluorescence properties and those that do often provide as-

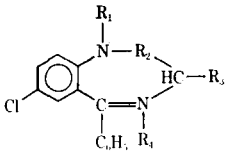


**Table I**—Fluorescence Characteristics and pKa Values of Substituted 1,4-Benzodiazepines Studied

| Compound | $R_1$ | $R_2$ | $R_3$ | $R_4$ | pKa Fluorescence | pKa Absorbance | Fluorescence Spectral Characteristics, $\lambda_{max}$ $H_0 = -10$ | $H_0 = -5$ |
|---|---|---|---|---|---|---|---|---|
| I | — | $=C(NHCH_3)-C(=O)-$ | H | O | −3.5, ∼−9 | −2.5, −8.7 | 497 | 525 |
| II | H | $-C(=O)-$ | H | O | −1.2, ∼−9 | −1.3, −8.6 | 500 | 530 |
| III | $CH_3$ | $-C(=O)-$ | H | O | — | −1.5, −8.0 | No fluorescence | No fluorescence |
| IV | $CH_3$ | $-CH_2-$ | H | O | — | −2.1, −7.2 | No fluorescence | No fluorescence |
| V | H | $-C(=O)-$ | OH | — | −6.6 | −2.4, −8.8 | 482 | 495 |
| VI | H | $-C(=O)-$ | H | — | −2.4, −8.2 | −2.1, −8.0 | 482 | 490 |
| VII | $CH_3$ | $-C(=O)-$ | H | — | −2.5, ∼−9 | −3.0, −7.8 | 465 | 480 |
| VIII | H | $-CH_2-$ | H | — | −5.7 | −1.9, −7.8 | 457 | 457 |
| IX | $CH_3$ | $-CH_2-$ | H | — | −5.6 | −1.5, −7.3 | 460 | 460 |
| X[a] | $CH_3$ | $-CH_2-$ | H | $CH_3$ | — | −1.5 | No fluorescence | No fluorescence |

[a] The azomethine group is saturated in X.

says with poor reproducibility. These problems are often attributed to the well-known dependencies of fluorescence characteristics on the solvent, solvent acidity, and charge of the emitting species. A thorough understanding of the fluorescence characteristics of drug molecules, however, can obviate many apparently undesirable aspects of fluorescence techniques, permitting the analyst to take advantage of the high sensitivity that can be realized from the successful use of fluorometry.

For example, many coumarin derivatives do not fluoresce in the 0–11 pH region but do fluoresce intensely in the Hammett acidity range (1). The analyst who does not realize that the acidity dependence of fluorescence frequently depends on the dissociation constant of the molecule in the excited state rather than (or as well as) in the ground state may well be creating a reproducibility problem where none exists. A knowledge of both pKa and pKa* values is critical to the selection of optimal conditions for fluorometric assays. An excellent review of the theory and problems involved in the choice of such conditions has been presented (2). Systematic studies of the fluorescence characteristics of drug molecules as a function of acidity are needed to provide information useful for prediction of fluorescence behavior of newly synthesized compounds.

The 1,4-benzodiazepines are widely used as tranquilizers. The ground-state acid–base equilibria of a number of 1,4-benzodiazepines have been investigated by absorption spectroscopy in the 1–13 pH region (3). Several reports have appeared concerning the fluorescence properties of 1,4-benzodiazepines (4–8). The fluorescing species in these studies were produced photochemically (4, 5), thermally in acidic solvents (6), and synthetically by derivatization (7). In only one case was the emitting species the parent compound (8), and even here the fluorescence characteristics are difficult to evaluate since the studies were run in alcoholic sulfuric, perchloric, and phosphoric acid solutions.

The physical–chemical characteristics of nitrogen heterocycles is of current interest in this laboratory. This interest resulted in the initiation of the present study—a systematic investigation of the fluorescence behavior of 1,4-benzodiazepines in strong acid solution. A series of benzodiazepines with different structures was chosen to gain insight into the structural requirements needed for fluorescence of these interesting nitrogen heterocycles. Additionally, the study encompassed the prototropic equilibria occurring in the Hammett acidity region.

## EXPERIMENTAL

**Apparatus**—Fluorescence measurements were obtained on a recording spectrofluorometer[1] equipped with a xenon light source. Absorption spectra were recorded using a recording spectrophotometer[2].

**Reagents**—The 1,4-benzodiazepines[3] (Compounds I–X) were used without further purification. Analytical reagent grade sulfuric acid[4] was diluted with distilled deionized water to prepare solu-

[1] Turner model 430, G. K. Turner Associates, Palo Alto, Calif.
[2] Coleman 124, Perkin-Elmer Corp., Maywood, Ill.
[3] Compounds I–IV and VI–X were obtained from Hoffmann-La Roche, Nutley, N.J. Compound V was obtained from Wyeth Laboratories, Philadelphia, Pa.
[4] Mallinckrodt Chemical Works, St. Louis, Mo.

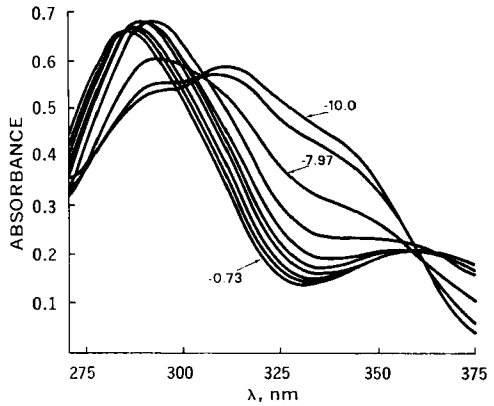



**Figure 1**—*Typical UV spectral changes for VII in sulfuric acid solutions. The curves are labeled as to the Hammett acidity value at which the spectrum was obtained.*

tions of varying acidity for fluorometric and absorptiometric titrations. The corrected Hammett acidity scale of Jorgenson and Hartter (9) was employed for the concentrated sulfuric acid solutions.

**Solutions**—All benzodiazepine solutions were prepared immediately prior to the titration and protected from light. Stock solutions approximately $10^{-2}$ *M* in benzodiazepine were prepared in 95% ethanol. Where the solubility of the compound in ethanol was limited, the stock solution was prepared using 10% sulfuric acid, the lowest acidity solvent used for the titration. Solutions having a final benzodiazepine concentration of approximately $10^{-4}$ *M* were prepared by dilution of the stock solution with the appropriate sulfuric acid solution immediately prior to obtaining the spectrum.

**Stability Studies**—Representative benzodiazepines were checked for stability in the strong acid solutions by the following procedure. A $10^{-3}$ *M* solution of the benzodiazepine was prepared in concentrated sulfuric acid ($H_0 = -10$). After 5 min at room temperature, this solution was diluted 10-fold with water to give a 10% sulfuric acid solution ($10^{-4}$ *M* in benzodiazepine). The UV spectrum was obtained and compared to that for a $10^{-4}$ *M* solution prepared directly in 10% sulfuric acid. The 5-min timespan was several minutes longer than the time usually required to prepare the sample and obtain the spectrum during the titrations. Additionally, the $10^{-3}$ *M* solution in 100% sulfuric acid was heated for 5 min at 80° in an oil bath prior to dilution to a 10% solution and obtainment of the absorption spectrum.

6-Chloro-4-phenylquinazoline-2-carboxaldehyde, a reported acid decomposition product of oxazepam, was prepared according to the method of Bell and Childress (10). The UV absorption spectrum of this compound was also obtained in 10% sulfuric acid solution.

## RESULTS AND DISCUSSION

The basic 1,4-benzodiazepine structure is shown in Table I along with the structural analogs utilized. The compounds studied were chosen to elucidate the structural requirements, if any, necessary to provide fluorescence emission. Of the 10 1,4-benzodiazepines studied, all but three fluoresced to some degree in the strongly acidic sulfuric acid media. The pertinent fluorescence characteristics of the compounds as well as the apparent pKa values obtained from both absorptiometric and fluorometric titration in strong acid are shown in Table I.

Compounds I–IV all contain the *N*-oxide group in the 4-position. Two of the four *N*-oxide benzodiazepines, I and II, exhibit fluorescence in strong acid, although of low intensity relative to other benzodiazepines studied. Compounds III and IV, which do not fluoresce in strong acid, differ significantly in structure from I and II. Compound I possesses a double bond in the 1,2-position. Compound II, through enolization, has the capability of producing the same azomethine fluorophore. Compound III, which has the carbonyl function in the 2-position like II, cannot exist in an uncharged enol form because of the methyl substituent in the 1-position. Compound IV does not possess the carbonyl function.

The remaining benzodiazepines studied reinforce the necessity for the presence of the azomethine group to provide fluorescence

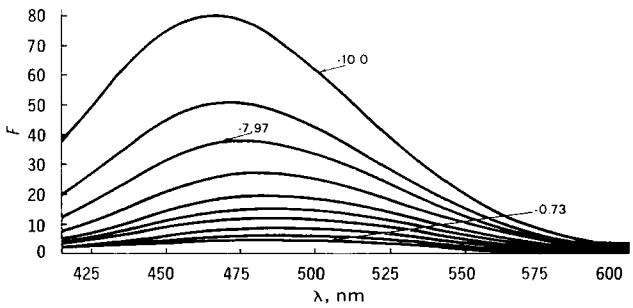



**Figure 2**—*Typical fluorescence spectral changes for VII in sulfuric acid solutions. The curves are labeled as to the Hammett acidity value at which the spectrum was obtained.*

in acid solution. Compounds V–IX fluoresce, whereas X, possessing neither enolization capability nor the double bond in conjunction with the aryl system, shows no fluorescence. These studies indicate that the aryl azomethine function is a requisite for fluorescence of the 1,4-benzodiazepines. The structure of the molecule being excited will also depend on the acid–base equilibria occurring in the ground state. For this reason, the prototropic reactions involved were also studied.

Figures 1 and 2 show representative spectra for the absorptiometric and fluorometric titration of VII in the Hammett acidity region. The spectral changes exhibited are representative of many of the benzodiazepines studied. As demonstrated in Fig. 1, the absorption spectra of the benzodiazepines generally show changes throughout the Hammett acidity region. Changes in the emission spectra as a function of acidity were also noted throughout the same region (Fig. 2). All of the benzodiazepines that fluoresce in strong acid show increases in fluorescence intensity with corresponding blue shifts as the acidity increases.

A typical absorptiometric titration curve for a benzodiazepine exhibiting two apparent pKa's in the Hammett acidity region is shown in Fig. 3. The fluorometric titration curves generally produced the same results as the absorbance data. Several benzodiazepines, however, did not exhibit two clearly distinct pKa's in the fluorometric titration curve when they were observed in the absorptiometric titration (Compounds III–V and VIII–X). An example of this phenomenon is shown in Fig. 4 where the fluorometric titration curve is depicted for the same compound whose absorptiometric titration appears in Fig. 3. Although two pKa's are not distinctly observable, the fluorescence change occurs over a much wider range of Hammett acidity values than would be expected for a single prototropic reaction.

The apparent pKa values determined from the titrimetric graph of the absorption and emission data (11) are given in Table I. No evidence of excited state prototropism was observed since the pKa values determined by fluorescence measurements corresponded to the pKa values obtained in the absorptiometric titrations. Spectral intensity changes were much greater for the prototropic reaction occurring in the lower Hammett acidity region than in the more

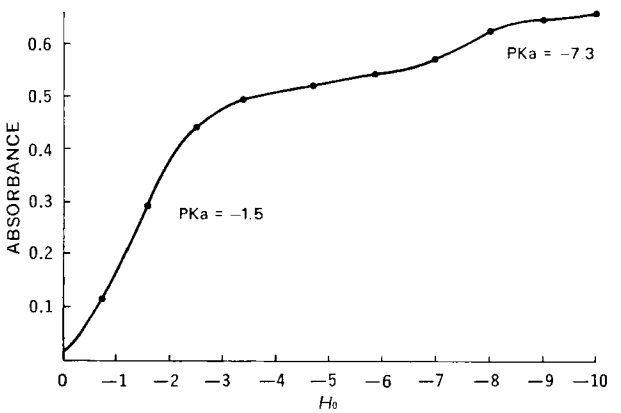
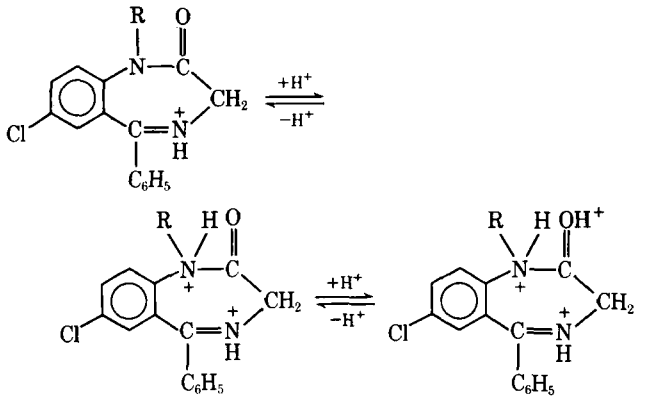



**Figure 3**—*Absorptiometric titration of IX in the Hammett acidity region.*



*Scheme I*

acidic region for I, IV, VIII, and IX (Fig. 3). Conversely, when the benzodiazepine possessed a carbonyl in the 2-position, the pKa in the strongly acidic region was very distinct and intensity changes were more pronounced than for the pKa observed in the lower Hammett acidity region. These observations may be attributed to a two-step protonation reaction for benzodiazepines having the 2-carbonyl. The first involves protonation of the 1-nitrogen—the 4-nitrogen being protonated in the pH region (3)—and the second step is carbonyl protonation. The suggested prototropic reactions are shown in Scheme I.

The benzodiazepines without proton-accepting capability in the 2-position would not be expected to show a pKa in the higher acidity region. Where a pKa is observed, the inflection in the titration curve is not very pronounced. The spectral changes observed in these higher acidities may be due to solvation effects as the medium approaches 100% sulfuric acid. If the observed spectral changes are not solvation artifacts but due to protonation, then the aromatic rings must be involved as the base moiety. This possibility

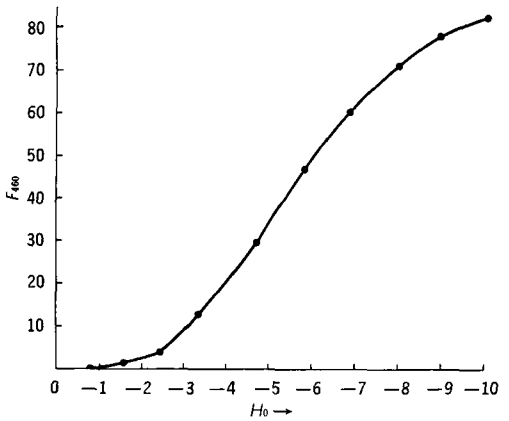



**Figure 4**—*Fluorometric titration of IX in the Hammett acidity region.*

seems unlikely since the positive charges residing in the portion of the molecule in conjugation with these rings should strongly decrease their basic properties. Whether protonation reaction or solvation artifact, the spectral changes are real and would be important for analytical fluorescence or absorption measurements made in the higher Hammett acidity region.

The possibility also existed that the spectral changes in the higher acidity regions were due to chemical instability of the benzodiazepines. Three compounds, V, VI, and IX, representative of the benzodiazepine structures studied were subjected to stability studies described in the *Experimental* section. The spectra obtained in 10% sulfuric acid directly as well as after treatment in 100% sulfuric acid at room temperature and at 80° were identical, indicating no apparent degradation during the timespan studied. In addition, the spectra of V and 6-chloro-4-phenylquinazoline-2-carboxaldehyde, a reported product of the acid-catalyzed isomerization of V (10), in 10% sulfuric acid are distinctly different and the formation of the degradation product would have been observed if present to any significant extent.

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