New Fluorescence Probes for Drug–Protein Binding Studies

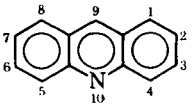
JOSEPH K. H. MA, PAR-LIN HSU, and LOUIS A. LUZZI[x]

Abstract □ 9-(4′-Carboxyanilino)-6-chloro-2-methoxyacridine and 9-anilino-6-chloro-2-methoxyacridine were synthesized from 6,9-dichloro-2-methoxyacridine. The fluorescence characteristics of these compounds along with *N*-phenylsulfanilate in various solvents and in their bovine serum albumin mixtures were studied using a fluorescent technique. The results show that the fluorescence of these acridine derivatives is essentially a combination of the fluorescence of acridine and the substitution effects. In polar solvent, the fluorescence is mainly characterized by the acridine residue; in nonpolar solvents and in bovine serum albumin mixtures, the substitution effects are the dominant factors. It was found that 9-(4′-carboxyanilino)-6-chloro-2-methoxyacridine and *N*-phenylsulfanilate possess the main features of fluorescence probes. In binding to bovine serum albumin, the quantum yields of 9-(4′-carboxyanilino)-6-chloro-2-methoxyacridine and *N*-phenylsulfanilate increased 20-fold and 26-fold, respectively. However, neither 9-anilino-6-chloro-2-methoxyacridine nor diphenylamine showed significant interaction with bovine serum albumin. These findings indicate that, as an anion, the probes may interact with positively charged residues on the protein molecules. The potential for using 9-(4′-carboxyanilino)-6-chloro-2-methoxyacridine as a fluorescence probe was tested with several drug compounds.

Keyphrases □ Acridine derivatives—synthesized, evaluated as potential fluorescence probes for drug-protein binding studies □ 9-(4′-Carboxyanilino)-6-chloro-2-methoxyacridine—synthesized, evaluated as potential fluorescence probe for drug-protein binding studies □ Fluorescence probes, potential—synthesis of acridine derivatives, evaluation in drug-protein binding studies □ Drug-protein binding—synthesis, evaluation of acridine derivatives as fluorescence probes

Fluorescence probes have been defined as: "small molecules which undergo changes in one or more of their fluorescence properties as a result of noncovalent interaction with protein or other macromolecules" (1). In general, the fluorescence of a probe varies in intensity and in spectral characteristics with the polarity of the solvent. The emission maximum is shifted toward the blue, and the fluorescence increases as the polarity of solvent decreases (2, 3). In aqueous solution, the same effects occur with the addition of protein. These effects have been shown to indicate that the binding of the probe to the protein is hydrophobic in nature. Because of the large molecule of the protein molecule and the rather complicated binding process, it is the authors' belief that more than one probe is required for the complete study of certain binding systems. The number of probes available at this time, however, is limited, and the finding of new probes became necessary. Therefore, several acridine compounds and *N*-phenylsulfanilate were studied in this laboratory.

The fluorescence of acridine (I) and its derivatives is varied by different substitutions on the acridine residue. It was reported (4, 5) that acridine shows pronounced shifts in fluorescence maximum with increasing dielectric constant of the solvent. However, the introduction of an anilino group or of certain substituted anilino groups at the 9-position (I) results in a compound that is nonfluorescent in aqueous solution but strongly fluorescent when absorbed in organic solution or on the solid phase (6). Similar compounds in which the anilino group is replaced by a benzylamino or a phenyl group are brightly fluorescent in aqueous solution, in contrast with the anilino-substituted compounds (6).



I

Acridine fluoresces very weakly in cyclohexane and benzene but shows intense fluorescence in ethanol, butanol, and water (4, 5). These solvent effects were interpreted (7) to indicate that an electron of the unshared electron pair on the nitrogen atom in acridine undergoes a transition to an antibonding π molecular orbital. This excited state, in turn, undergoes a radiationless transition to the "triplet state"; consequently, such a compound does not exhibit fluorescence. When the unshared electrons on the nitrogen atom become involved in hydrogen bonding with proton-donating solvents, transitions to the triplet state become less likely or nonexistent and, therefore, enhanced fluorescence is observed in a proton-donating solvent. In other studies (8, 9) the effect of acetic acid and trichloroacetic acid on the absorption and fluorescence of acridine in benzene was also examined and the changes in fluorescence quality was ascribed to hydrogen bond formation.

The substituted acridine compounds mentioned exhibit different features in their fluorescence characteristics. This is probably because the substitutions increase the hydrophobicity of these compounds, and the changes of structures may influence the dipole moment and/or the charge-transfer character of these compounds and, therefore, their fluorescence. The study of fluorescence changes in these compounds brought about by solvent and protein binding effects may provide useful information in understanding the interaction between proteins and small molecules.

EXPERIMENTAL

Materials—The following were used: acridine[1], 3,6-diaminoacridine[1], 6,9-dichloro-2-methoxyacridine[1], *N*-phenylsulfanilic acid sodium salt[2], dansic acid[3], and bovine serum albumin[4]. 9-Anilino-6-chloro-2-methoxyacridine and 9-(4′-carboxyanilino)-6-chloro-2-methoxyacridine were prepared in this laboratory from 6,9-dichloro-2-methoxyacridine by a method previously described (10). Concentrations of the protein were determined by measuring the absorbance at 280 nm and by using $a = 0.66$ ($E_{1\,cm}^{1\%} = 6.6$) (11); the molecular weight of 69,000 was used to determine molar concentrations. Methanol and other solvents were spectroscopic

[1] Aldrich Chemical Co.
[2] Pfaltz & Bauer, Inc.
[3] Pierce Chemical Co.
[4] Nutritional Biochemicals Corp.

Table I—Solvent Effects on Fluorescence of 6,9-Dichloro-2-methoxyacridine (II), 9-Anilino-6-chloro-2-methoxyacridine (III), and 9-(4′-Carboxyanilino)-6-chloro-2-methoxyacridine (IV)[a]

Solvent	Solvent Dielectric Constant[b], debyes	II			III			IV		
		λ_{ex}, nm	λ_{em}, nm	Relative[c] Intensity	λ_{ex}, nm	λ_{em}, nm	Relative Intensity	λ_{ex}, nm	λ_{em}, nm	Relative Intensity
pH 7.4 phosphate buffer	—	400	465	12.7	395	465	1.2	390	465	1.0
Methanol	32.6	400	452	197.3	395	452	1.4	390	450	1.7
Dimethyl-acetamide		400	450	150.3	395	450	1.7	415	515	2.3
Acetone	20.7	400	445	125.0	395	445	1.1	415	492	7.7
Ethyl acetate	6.0	400	440	96.0	415	520	0.9	415	482	46.7
Benzene	2.3	400	440	117.3	415	490	8.7	415	472	75.6

[a] A concentration of 2×10^{-6} *M* was used in all samples. [b] "Handbook of Chemistry and Physics," 51st ed., R. C. Weast, Ed., The Chemical Rubber Co., Cleveland, Ohio, 1970–1971, pp. E61–64. [c] The relative intensities of all solutions are compared to the fluorescence of IV in pH 7.4 buffer, of which the value is taken to be 1.

grade, and all other chemicals were reagent grade or of special purity.

Synthesis—*9-Anilino-6-chloro-2-methoxyacridine Hydrochloride*—A mixture of 6.0 g of 6,9-dichloro-2-methoxyacridine and 30 g of phenol was heated in a flask to 70° over an oil bath. After all of the starting material was dissolved in phenol, 2 g of aniline was added to the mixture. The reaction was completed by refluxing the mixture at 140° for 12 hr with constant stirring using a magnetic stirrer. The product was cooled and washed thoroughly with anhydrous ether to remove phenol. A yellow product was recrystallized from 95% ethanol and dried *in vacuo*, mp 300–301° dec., *m/e* 335.

Anal.—Calc. for $C_{20}H_{16}Cl_2N_2O$: C, 64.70; H, 4.34; Cl, 19.15; N, 7.55. Found: C, 64.55; H, 4.30; Cl, 19.15; N, 7.45.

9-(4′-Carboxyanilino)-6-chloro-2-methoxyacridine Hydrochloride—This compound was prepared from 6,9-dichloro-2-methoxyacridine and *p*-aminobenzoic acid using the same procedures as already described, mp 335–337° dec., *m/e* 378.

Anal.—Calc. for $C_{21}H_{16}Cl_2N_2O_3$: C, 60.74; H, 3.88; Cl, 17.10; N, 6.75. Found: C, 60.64; H, 3.98; Cl, 17.19; N, 6.61.

9-(4′-Carboxyanilino)-6-chloro-2-methoxyacridine Sodium Salt—This compound was prepared by adding an excess amount of sodium hydroxide into the above compound (pH 9). The bright-yellow crystal was recrystallized from aqueous solution and dried *in vacuo*.

Anal.—Calc. for $C_{21}H_{14}ClN_2NaO_3$: C, 62.92; H, 3.50; N, 6.99; Na, 5.74. Found: C, 62.75; H, 3.65; N, 6.82; Na, 5.69.

The purity of these compounds was tested by TLC, and no impurity was found.

Apparatus and Methods—Fluorescence measurements were made with a spectrophotofluorometer[5] equipped with a 150-w xenon lamp and a lp21 photomultiplier tube. The relative fluorescence intensities were recorded directly from fluorometer readings. Spectra were recorded with an *X–Y* recorder.[6] The entrance slit for the excitation light and the exit slit for fluorescence emission were 3 and 4 mm, respectively.

Stock solutions of all probes were prepared using methanol as the solvent. Fluorescence titrations were performed manually with microsyringes[7]. The temperature of all fluorescence measurements was controlled to 25 ± 0.1°. Quantum yield determinations were made using Eq. 1 (12):

$$\Phi_u = \Phi_s\left(\frac{a_u A_s d_s \lambda_s}{a_s A_u d_u \lambda_u}\right) \qquad \text{(Eq. 1)}$$

where:

a = emission peak area corrected for solvent blank
A = absorbance at excitation wavelength
d = dilution factor in dilution of sample used for absorbance measurements to concentration used in fluorescence measurements
λ = excitation wavelength
Φ = quantum yield
s, u = subscripts denoting standard (s) and unknown (u)

Although the quantum yield can be obtained by absolute methods (13), it is more convenient to use comparative methods in which compounds of known quantum yield are used as reference standards. Dansic acid (5-dimethylamino-1-naphthalenesulfonic acid) was used as the reference standard, taking its quantum yield to be 0.36 at an excitation wavelength of 320 nm (14).

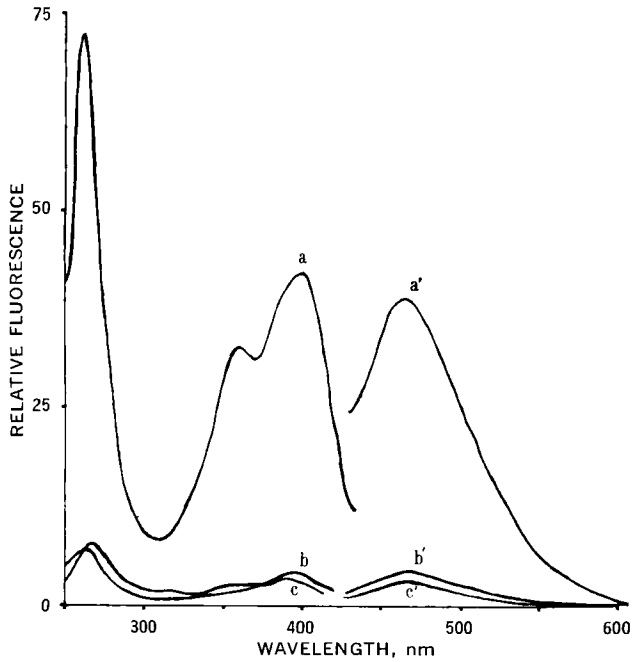


Figure 1—*Excitation and emission spectra of 2 × 10⁻⁶ M probes in pH 7.4 phosphate buffer. Key: a and a′, excitation (λ_{em} = 465 nm) and emission (λ_{ex} = 400 nm) curves of 6,9-dichloro-2-methoxyacridine; b and b′, excitation (λ_{em} = 465 nm) and emission (λ_{ex} = 395 nm) curves of 9-anilino-6-chloro-2-methoxyacridine; and c and c′, excitation (λ_{em} = 465 nm) and emission (λ_{ex} = 390 nm) curves of 9-(4′-carboxyanilino)-6-chloro-2-methoxyacridine.*

RESULTS AND DISCUSSION

The excitation and emission spectra of 6,9-dichloro-2-methoxyacridine (II), 9-anilino-6-chloro-2-methoxyacridine (III), and 9-(4′-carboxyanilino)-6-chloro-2-methoxyacridine (IV) measured in pH 7.4 phosphate buffer are shown in Fig. 1. 6,9-Dichloro-2-methoxyacridine was highly fluorescent in the buffer solution, but the intensity was quenched considerably when the 9-chloro group was replaced by an anilino or a carboxyanilino group. The emis-

[5] Aminco-Bowman, American Instrument Co., Silver Spring, Md.
[6] Omnigraphic, Houston Instrument, Bellaire, Tex.
[7] Hamilton.

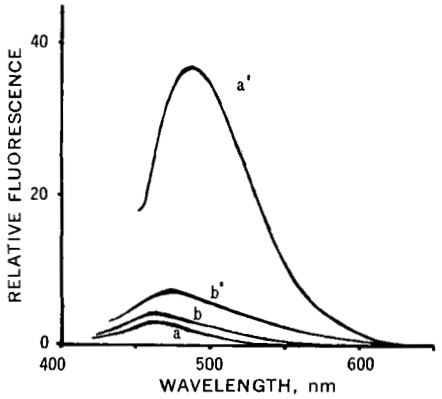


Figure 2—*Emission spectra of 2 × 10⁻⁶ M probes in the presence and absence of 6.9 × 10⁻⁶ M bovine serum albumin. Key: a and a', emission curves of 9-(4'-carboxyanilino)-6-chloro-2-methoxyacridine in the absence and presence of bovine serum albumin; and b and b', emission curves of 9-anilino-6-chloro-2-methoxyacridine in the absence and presence of bovine serum albumin.*

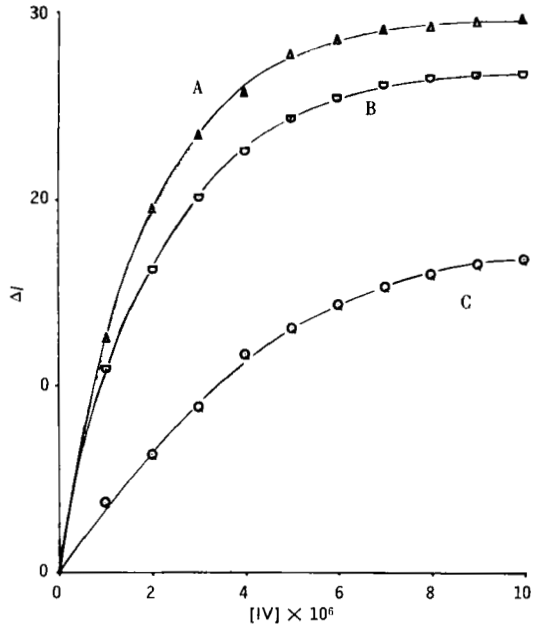


Figure 3—*Plots of* ΔI versus *9-(4'-carboxyanilino)-6-chloro-2-methoxyacridine (IV) concentration for solutions containing 1.38 × 10⁻⁶ M bovine serum albumin in the absence and presence of drug compound, where* ΔI *is the corrected fluorescence intensity of the solution from the residue fluorescence of bovine serum albumin and the fluorescence of IV in the absence of bovine serum albumin at the same wavelengths* (λ_{ex} = *420 nm,* λ_{em} = *485 nm). Key: A, in the absence of drug; B, in the presence of 5 × 10⁻⁵ M salicylic acid; and C, in the presence of 5 × 10⁻⁵ M sodium warfarin.*

sion wavelength, however, remained the same at 465 nm. The excitation maxima for II, III, and IV were 400, 395, and 390 nm, respectively. Acridine fluoresced much stronger than II and had excitation and emission maxima at 355 and 442 nm in pH 7.4 buffer, respectively.

The fluorescence characteristics of II in various solvents are shown in Table I. The emission maximum shifted toward the blue as the polarity of solvent decreased. The intensity changes, however, did not follow the probe pattern. As shown in Table I, the relative intensities in methanol, dimethylacetamide, and acetone are higher than those in either more polar or more nonpolar solvents. Since the fluorescence of acridine has been known to decrease as the polarity of solvent decreases (4, 5), it is concluded that the fluorescence of II is essentially a combination of the fluorescence of acridine and the substitution effects. In contrast to acridine, the presence of the chloro and methoxy groups of II apparently causes an increase of intensity with a decrease in the polarity of the solvent as a result of an increase in its hydrophobicity. This is further substantiated by the results obtained for III and IV (Table I). The fluorescence of III is quite similar to II with the exception of having a much lower intensity in the same solvent. Compound IV is practically nonfluorescent in buffer solution but brightly fluoresces in nonpolar solvents. Furthermore, in buffer and methanol the spectral characteristics of II, III, and IV are very similar, but in less polar solvents the emission maxima of III and IV are shifted to longer wavelengths. Further increases in the polarity of the solvent result in a blue shift accompanied by an increase in emission intensity. This effect is particularly clear in the results for IV.

The interactions of acridine and some derivatives with bovine serum albumin also were studied. The emission spectra of III and IV in the presence of 6.9 × 10⁻⁶ *M* bovine serum albumin in pH 7.4 buffer are shown in Fig. 2. The emission maxima for III and IV are 475 and 485 nm, respectively. The quantum yield data of II, III, and IV measured in pH 7.4 phosphate buffer and the data of their bovine serum albumin mixtures are listed in Table II. Compound IV produced the highest quantum yield increases in binding to bovine serum albumin (20-fold). The protein mixture of II had a twofold increase in quantum yield, whereas III increased about three times in the presence of protein. The manifold increase in fluorescence accompanied by the blue shifts of the emission maximum in various solvents and in the protein mixture indicates that IV, 9-(4'-carboxyanilino)-6-chloro-2-methoxyacridine, should be useful as a probe for drug–protein binding studies.

The fluorescence of the bovine serum albumin mixtures of acridine and 3,6-diaminoacridine was also examined. These compounds do not seem to interact with bovine serum albumin. In polar solvents, the fluorescence of II, III, and IV is mainly charac-

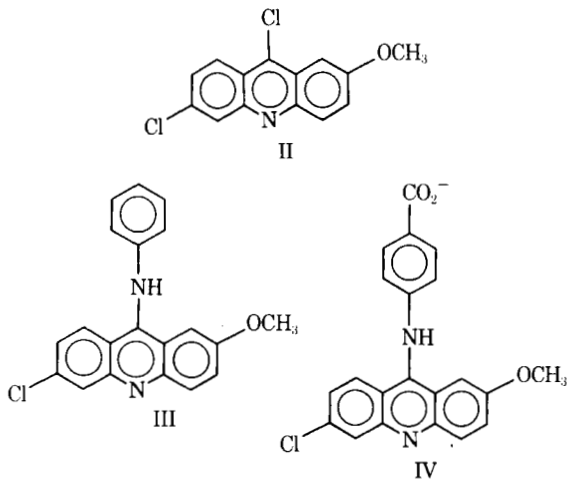


Table II—Quantum Yield of 6,9-Dichloro-2-methoxyacridine (II), 9-Anilino-6-chloro-2-methoxyacridine (III), 9-(4'-Carboxyanilino)-6-chloro-2-methoxyacridine (IV), and *N*-Phenylsulfanilate (V) and of Their Bovine Serum Albumin Mixtures in pH 7.4 Phosphate Buffer

Compound	Concentration, *M*	Bovine Serum Albumin Concentration, *M*	Excitation, nm	Emission, nm	Quantum Yield
II	2 × 10⁻⁶	—	400	465	0.044
II	2 × 10⁻⁶	6.9 × 10⁻⁶	400	460	0.092
III	2 × 10⁻⁶	—	395	465	0.021
III	2 × 10⁻⁶	6.9 × 10⁻⁶	400	475	0.060
III	2 × 10⁻⁶	69.0 × 10⁻⁶	400	475	0.053
IV	2 × 10⁻⁶	—	390	465	0.006
IV	2 × 10⁻⁶	6.9 × 10⁻⁶	430	485	0.120
IV	2 × 10⁻⁶	69.0 × 10⁻⁶	430	485	0.119
V	4 × 10⁻⁴	—	332	457	0.006
V	4 × 10⁻⁴	6.9 × 10⁻⁶	338	405	0.155

Table III—Fluorescence of *N*-Phenylsulfanilate in pH 7.4 Phosphate Buffer

Concentration, *M*	Excitation, nm	Emission, nm	Relative Intensity
1×10^{-5}	300	465	11.0
2×10^{-5}	304	467	17.0
5×10^{-5}	311	460	25.0
15×10^{-5}	324	465	29.1
25×10^{-5}	328	460	30.0
35×10^{-5}	332	458	30.0
45×10^{-5}	332	457	30.2
55×10^{-5}	332	456	30.6

terized by the acridine residue; in nonpolar solvents and in protein mixtures, the substitution effects are the dominant factors.

The fluorescence of a probe is affected by the solvent or protein environment through dipole–dipole interaction, hydrogen bonding, and charge transfer, *etc.* Probes with greater dipole moment in the excited state than the ground state show solvent-dependent emission spectra reflecting the mobility and polar character of the solvent (15–18). This is true since solvent molecules may relax about the excited state prior to emission and this, in turn, tends to lower the energy of the excited state, resulting in a red shift at the fluorescence emission (15–18). Other probes, such as acridine, may be sensitive to the proton-donating or proton-accepting character of the solvent.

The fluorescence of acridine is characterized by the hydrogen-bonding effect of the solvent (4, 5). This effect, however, is reduced by the chloro, methoxy, anilino, and carboxyanilino substitutions as in the cases of II, III, and IV. These substituents, which are more sensitive to the polarity of the solvent, tend to change the dipole moment of II, III, and IV in the excited state and, therefore, alter their fluorescence. As shown in Table I, III still shows the hydrogen-bonding effect by the solvent, whereas IV clearly shows a solvent-relaxation effect. This difference may be due to the difference of the intramolecular charge-transfer character of these compounds. The fluorescence changes of IV by the solvent and protein environment are very similar to those of *N*-arylaminonaphthalinesulfonates, the probes that were reported (19–21) to possess the intramolecular charge-transfer character.

By carefully examining the interactions of III and IV with bovine serum albumin, it is believed that the binding occurs on the anilino group rather than on the acridine residue. The carboxylic group in IV must be directly involved in the binding. Hydrophobic forces do exist in the binding, but the difference in binding affinity of III and IV is somewhat greater than can be accounted for solely on the basis of hydrophobic interaction. This fact is further strengthened by the binding studies of diphenylamine and *N*-phenylsulfanilate to bovine serum albumin. The results will be discussed later.

It was reported (1, 22) that the strong interactions between 5-dimethylaminonaphthalene-1-sulfonamide and carbonic anhydrase were due to the loss of amino proton by 5-dimethylaminonapthalene-1-sulfonamide; *i.e.*, the binding species is the anion rather than the neutral molecule. In fact, most probes used in drug–protein binding studies have an anionic group while, on the other hand, proteins such as bovine serum albumin are known to bind strongly with small organic molecules with anionic groups (23). These acid groups may interact with positively charged residues in the protein to form complexes, and the binding is reinforced by the hydrophobic interactions between the protein and the small molecule. The most strongly hydrophobic sites on the protein are probably primarily involved in the binding. The fluorescence change of the probe when bound to protein could be due to changes in the charge of protein molecules (24).

Use of 9-(4′-carboxyanilino)-6-chloro-2-methoxyacridine (IV) as a probe was tested with several anionic drugs, two of which are reported here. As an example, the competitive displacements of the probe by salicylic acid and sodium warfarin in binding to bovine serum albumin are shown in Fig. 3. Other drugs such as phenylbutazone, dicumarol, and the sulfonylureas can also displace the probe from its binding sites on bovine serum albumin.

Fluorescence studies of diphenylamine and *N*-phenylsulfanilate were also carried out in this laboratory. Table III shows the concentration effects of *N*-phenylsulfanilate on the fluorescence measured in pH 7.4 phosphate buffer. At low concentrations, the excitation maximum of *N*-phenylsulfanilate is close to that of bovine serum albumin (excitation maximum 290 nm). At 3.5×10^{-4} *M*, the excitation maximum ceases to increase with increasing excitation. Therefore, the concentration chosen for quantum yield and spectral measurements was 4×10^{-4} *M*, where the excitation maxima of *N*-phenylsulfanilate in the absence and presence of bovine serum albumin are 332 and 338 nm, respectively.

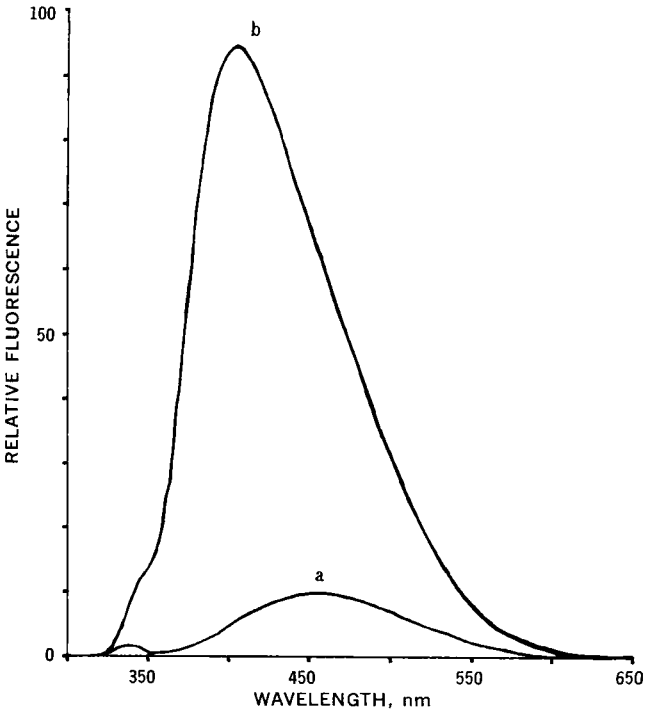


Figure 4—*Emission spectra of* 4×10^{-4} M *N-phenylsulfanilate. Key: a, in the absence of bovine serum albumin* (λ_{ex} = 332 nm, λ_{em} = 457 nm); *and b, in the presence of* 6.9×10^{-6} M *bovine serum albumin* (λ_{ex} = 338 nm, λ_{em} = 405 nm).

Diphenylamine is highly fluorescent in nonpolar as well as polar solvents. The addition of bovine serum albumin did not affect the fluorescence of diphenylamine. In the case of *N*-phenylsulfanilate, however, the fluorescence was changed dramatically upon addition of bovine serum albumin. The emission spectra of *N*-phenylsulfanilate in the absence and presence of 6.9×10^{-6} *M* bovine serum albumin are shown in Fig. 4. In binding to the protein, the quantum yield of *N*-phenylsulfanilate increased from 0.006 to 0.155 while its fluorescence emission maximum shifted from 457 to 405 nm. The fact that *N*-phenylsulfanilate and 9-(4′-carboxyanilino)-6-chloro-2-methoxyacridine strongly interact with bovine serum albumin, whereas diphenylamine and 9-anilino-6-chloro-2-methoxyacridine do not, suggests that the acid groups of these compounds are indeed involved in the binding.

REFERENCES

(1) G. M. Edelman and W. O. McClure, *Accounts Chem. Res.*, **1**, 65(1968).

(2) L. Stryer, *J. Mol. Biol.*, **13**, 482(1965).

(3) W. O. McClure and G. M. Edelman, *Biochemistry*, **5**, 1908(1966).

(4) N. Mataga, Y. Kaifu, and M. Koizumi, *Bull. Chem. Soc. Jap.*, **29**, 373(1956).

(5) B. B. Van Duuren, *Anal. Chem.*, **32**, 1436(1960).

(6) G. Weber and D. J. R. Laurence, *Biochem. J.*, **56**, xxxi(1954).

(7) M. Kasha, *Discuss. Faraday Soc.*, **9**, 14(1950).

(8) H. Kokubun, *Z. Phys. Chem.*, **13**, 386(1957).

(9) N. Mataga and S. Tsuno, *Bull. Chem Soc. Jap.*, **30**, 368(1957).

(10) A. Albert, "The Acridines," 2nd ed., St. Martin's Press, New York, N.Y., 1966, p. 306.

(11) E. Daniel and G. Weber, *Biochemistry*, **5**, 1893(1966).
(12) "Bulletin 8885," G. K. Turner and Associates, Palo Alto, Calif., 1966.
(13) W. H. Melhuish, *J. Phys. Chem.*, **65**, 229(1961).
(14) C. M. Himel and R. T. Mayer, *Anal. Chem.*, **42**, 130(1970).
(15) E. Lippert, *Z. Elektrochem.*, **61**, 962(1957).
(16) E. Lippert, *Angew. Chem.*, **73**, 695(1961).
(17) N. Mataga, Y. Kaifu, and M. Koizumi, *Bull. Chem. Soc. Jap.*, **29**, 465(1956).
(18) *Ibid.*, **36**, 654(1963).
(19) L. Brand, C. J. Seliskar, and D. C. Turner, *Probes Struct. Funct.*, **1**, 17(1971).
(20) C. J. Seliskar and L. Brand, *J. Amer. Chem. Soc.*, **93**, 5405(1971).
(21) M. Kasha and R. H. Rawls, *Photochem. Photobiol.*, **7**, 561(1968).
(22) R. F. Chen and J. C. Kernohan, *J. Biol. Chem.*, **242**, 5813(1967).
(23) I. M. Klotz, H. Triwush, and F. M. Walker, *J. Amer. Chem. Soc.*, **70**, 2935(1948).
(24) L. Brand and J. R. Gohlke, *Ann. Rev. Biochem.*, **41**, 843(1972).

ACKNOWLEDGMENTS AND ADDRESSES

Received May 29, 1973, from the *School of Pharmacy, University of Georgia, Athens, GA 30601*

Accepted for publication September 12, 1973.

[x] To whom inquiries should be directed.

Vaginal Odors: GLC Assay Method for Evaluating Odor Changes

A. DRAVNIEKS[x], L. KEITH*, B. K. KROTOSZYNSKI, and J. SHAH

Abstract □ A GLC assay technique, previously applied to the evaluation of the influence of nitrofurazone on pathological vaginal odors, was refined and tested in a study of vaginal secretion odors in normal women before and after administration of a suppository. The principal methodological refinements included considering changes in the concentrations of seven frequently encountered malodorants and testing results for their statistical validity. The technique was sufficiently sensitive to indicate statistically significant ($p < 0.05$) changes in a group of 10 subjects.

Keyphrases □ Vaginal odors—method and GLC assay technique for evaluating odor changes, suppository induced □ Odors, vaginal—method and GLC assay technique for evaluating odor changes, suppository induced □ GLC—detection of changes in vaginal odors

Established techniques exist to determine the type and the number of microorganisms that may produce odors in the vaginal secretions. However, formation of malodorous compounds *in vivo* may depend also on the secretions of changing composition. Reduction in bacterial or fungal counts *in vitro* or *in vivo* cannot be *a priori* equated with the activity in odor suppression; lack of such correlations in axillary odors was reported (1, 2).

Earlier work showed that a combination of GLC and sensory techniques permits an evaluation of the reduction of infection-induced vaginal odors by nitrofurazone (3, 4), as well as a comparison of the number of malodorous compounds in normal secretions (5). Even such a simple indicator as the number of malodorous compounds was sufficient to assess gross changes.

It was felt that further and more quantitative refinements in the odor evaluation technique were desirable. The degree of the malodorosity of each component should be considered. Changes should be objectively quantified by observing the concentration changes (GLC peak areas) of the same odorant. Finally, spurious and arbitrary interpretations should be avoided by application of some simple statistical tests so that an estimate of the significance of the changes can be obtained.

The usefulness of these refinements was investigated in a study of odor changes following an intravaginal administration of a suppository.

EXPERIMENTAL

Sampling—Samples of vaginal secretions were obtained using heat-sterilized and deodorized Teflon inserts (Fig. 1). The insert carried 480 circular smooth-edged pits, each 0.15 cm (0.06 in.) wide and 0.07 cm (0.03 in.) deep. A Teflon slip-ring collar allowed adjustment of the depth of insertion. The pits and the surface of the insert served as mechanical traps for mucus. After 20 min, the insert was removed, placed in a clean glass container (Fig. 1), and stored in a freezer until analyzed.

Analysis—Inserts, still in their glass containers, were brought to room temperature. A total volume of 6000 ml of high purity ("zero" grade) helium was passed through the vessel at a rate of 100 ml/min. The collection arrangement is shown in Fig. 1. The vapor-saturated helium then entered a vapor collector, containing 5 g of highly purified, high surface area (>300 m^2/g), nonpolar styrene–divinyl copolymer[1], where organic vapors were absorbed but water vapor was retained to a negligible extent. Vapors then were removed from this collector and transferred in one single injection into a gas chromatograph, using procedures and devices described previously (6).

The chromatographic partition column was a 6.09-m (20-ft), 0.31-cm (0.125-in.), stainless steel tubing packed with 2.5% Carbowax 20M on 60–80-mesh Chromosorb G support, acid washed and dimethylsiloxane treated. The carrier gas was high purity helium introduced at 60 ml/min; the temperature was elevated linearly from 60 to 180° at 2°/min. The effluent was split, with one half flowing to a hydrogen flame-ionization detector and the other half flowing to a sniffing port supplied with a controlled flow of air humidified to reduce nose irritation (7).

An analyst, specialized in the sensory evaluation of GLC effluents, characterized the odors of the separated components as

[1] Chromosorb 102, Johns-Manville Celite Division.