# Physicochemical Characteristics of Patent Blue Violet Dye

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**Abstract** □ Physicochemical data for patent blue violet dye (I) are reported. The pKa for protonation of the first diethylanilino group was 2.78 ± 0.03. The absorptivity values calculated for a 1% (w/v) solution of previously dried I at pH 7.4 were 1650, 170, and 250 at 638, 412, and 309 nm, respectively. A table of wavelength maxima and observed solution color as a function of pH and $H_o$ and five spectra of I at certain pH and $H_o$ values are included. The solution chemistry of I is explained, and a scheme showing its two protonated carbonium ions and its triphenylcarbinol derivative is presented. The distribution coefficients of I in *n*-octanol or chloroform and pH 7.4 phosphate buffer systems were 0.013 and 0.12, respectively. The approximate solubilities at 25° of I in six organic solvents and the solubility analysis of I in distilled water are reported. Results of the latter analysis suggest that I forms a lyotropic mesophase in high aqueous concentrations. Compound I is poorly lipid soluble. Samples of 1.000% I in 0.9% NaCl, formulated with and without 1% (v/v) benzyl alcohol and autoclaving, varied not more than 5% from the initial I content during storage in the dark and under constant fluorescent light at 25 ± 5° for 20 months. Data from the TLC of I in several eluents indicated a high degree of purity of the dye. The half-lives for the loss of color in $5 \times 10^{-4}$% I solutions in potassium hydroxide solutions of pH 13.7, 12.7, 11.3, and 10.0 were 1.2 hr, 17.0 hr, 9.5 days, and 180 days, respectively. The fraction of I bound to 4% (w/v) human serum albumin at 37° and pH 7.4 ranged from 0.05 to 0.83, corresponding to unbound I in the postdialysis concentration range of $1.7 \times 10^{-4}$ to 2.0% (w/v). A Scatchard plot of the albumin binding data of I revealed one high-affinity binding site, $K = 6235\ M^{-1}$, and five low-affinity sites, with average affinity constants of $33\ M^{-1}$. The data support the fact that the spectrophotometric determination of I at 639 ± 2 nm appears to comprise a stability-indicating assay.

**Keyphrases** □ Patent blue violet—physicochemical characteristics □ Dyes—patent blue violet, physicochemical characteristics

Patent blue violet (I) is the monosodium salt of a disulfonated triphenylmethane vital stain, which is used to delineate the patency of lymph vessels in the dorsum of the hands or feet prior to injection of an iodinized contrast medium and subsequent lymphangiography (1–3). Certain injectable formulation and nomenclature[1] problems (1), as well as some structural formula and purity discrepancies of I, have been reviewed (4).

The purpose of this study was to determine values for several physicochemical characteristics of I that pertain to its pharmaceutical analysis and unique clinical use. The concentration of I used for most research studies and all injectable solutions prepared under the investigational new drug application for administration to humans was limited to 1% (w/v) or less because higher concentrations were reported (5–8) to produce sarcomas in rodents at the sites of repeated subcutaneous injections. The usual human dose of I injected subcutaneously to assist in visualizing lymph vessels is 2–3 ml of a 1% solution normally prepared in 0.9% NaCl.

[1] Synonyms for I (as the 2,4-disulfonate) include patent blue V, blue VRS, alphazurine 2G, sulfan blue, and acid blue 1.

## EXPERIMENTAL

**Materials**—Purified patent blue violet[2] was used as received unless otherwise specified. Human serum albumin[3] and sterile water for irrigation[4] were used as received, and other reagents and solvents were analytical grade.

**Procedures**—Spectral data and the pKa titration were obtained[5] in $5 \times 10^{-4}$% solutions of I[6]. Chloroacetic acid (0.01 *M*), adjusted to the appropriate pH with 0.50 *N* NaOH or HCl, was used for samples in the pKa studies, and dilutions of 1.00 *N* KOH or $H_2SO_4$ (ACS reagent) in distilled water were used for other spectral studies. The pKa was calculated (9) using the absorbance values of I solutions determined at 638 nm:

$$\text{pKa} = \text{pH} + \log [(A_0 - A)/(A - A_+)] \qquad \text{(Eq. 1)}$$

where $A_0$ is the absorbance of the nonprotonated species[7], $A_+$ is the absorbance of the protonated species[8], and $A$ represents the absorbance values of the I solutions adjusted to pH values in the range of approximate pKa ± 1 unit.

Loss on drying was determined by placing three accurately weighed samples of ~0.5 g of I into tared glass containers with ground-glass caps unaffixed, heating at 135° for 3 hr, tightly placing the caps on the containers, allowing the samples to cool to ambient temperature in a desiccator, and determining the weight until it was constant at ±0.5 mg.

Distribution coefficients were determined with triplicate samples of $5 \times 10^{-4}$ and $5.0 \times 10^{-3}$% solutions of I in 0.1 *M* phosphate buffer (pH 7.40) previously saturated with *n*-octanol or chloroform. A 7.0-ml portion of aqueous I was transferred to a 15-ml glass tube, 7.0 ml of either *n*-octanol or chloroform saturated previously with the phosphate buffer was added, a polyethylene-lined screw cap was tightly affixed, and the tube was rotated at 45 rpm for 15 min at 25 ± 0.5° in a water bath in a bottle apparatus similar to that described previously (10). The samples were allowed to stand at 25° until phase separation occurred. The absorbance of the aqueous layer or an appropriate dilution thereof was determined at 638 nm, the concentrations of I in both phases were computed, and the lipid–aqueous distribution ratio was calculated.

The approximate solubilities of I in certain organic solvents were determined in triplicate by combining accurately weighed samples of I, ~1.5 g with 95% ethanol and 0.1 g with other solvents, with a specific volume of solvent, 5.0 ml of 95% ethanol and 50.0 ml of chloroform, ether, ethyl acetate, hexane, and toluene, in a glass tube of appropriate size. The mouths of the tubes were covered with aluminum foil, polyethylene-lined screw caps were tightly affixed, and the samples were rotated for 24 hr at 45 rpm and 25° in the bottle apparatus. The samples were filtered through a 0.45-μm membrane[9], and 1.0 ml of the 95% ethanol solution and 25.0 ml of the other solutions were evaporated and dried to constant weight in a tared dish. The weights of the solute residues then were determined to the nearest 0.1 mg.

The equilibrium phase solubility analysis of I in water for irrigation[4] was conducted as follows. Accurately weighed samples of I ranging from

[2] Product P-1888 FD, lot 4-1-1, monosodium salt, dark-green luminous powder, dye content of ~85%, mol. wt. 566.67, Sigma Chemical Co., St. Louis, Mo.

[3] Product A 9511, lot 34C-8120, mol. wt. 66,300, Sigma Chemical Co., St. Louis, Mo.

[4] Lot 89-388-DE-5, Abbott Laboratories, North Chicago, Ill.

[5] A Beckman DB-GT, Perkin-Elmer Hitachi 200, or Bausch & Lomb Spectronic 2000 double-beam spectrophotometer was used to record spectra and absorbance values.

[6] The specimen of I was previously dried to constant weight at 135°.

[7] $A_0 = 0.82$ over the pH range of 5.0–11.0 in freshly prepared solutions.

[8] $A_+ = 0$ in 2.36 *N* $H_2SO_4$ ($H_o - 0.4$).

[9] MF-Millipore, cellulose esters, Millipore Corp., Bedford, Mass.



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**Table I—Spectral Data for Patent Blue Violet (I) at $5 \times 10^{-4}$% in 0.05 *M* Phosphate Buffer (pH 7.40) at 25°**

| Wavelength, nm | Absorptivity[a] | |
|---|---|---|
| | I as Received | I Dried Previously[b] |
| 638 ± 1 | 1540 | 1650 |
| 412 ± 1 | 170 | 170 |
| 309 ± 1 | 250 | 250 |

[a] Calculated for a 1% solution of I in a 1-cm quartz cell. [b] Dried to constant weight at 135°.

~0.1 g to 5.5 g in 0.5-g increments were combined with 10.0 g of water in a 15-ml glass tube. A polytef-lined screw cap was tightly affixed, and the cap and tube neck were sealed with silicone rubber cement, which was allowed to cure under ambient conditions for 12–16 hr. The samples then were rotated at 45 rpm and 25 ± 0.5° for 24 hr. They were centrifuged at 2000 rpm for 10 min, and 7–8 ml of the supernate was removed and filtered through a 0.45-μm membrane[9]. A 5.0-ml tared flask was filled to volume (±0.1 ml) with the filtrate and weighed, and the contents were transferred quantitatively to a tared evaporating dish and dried to constant weight at 70–75° in an oven. The weights of the solute residues were recorded to the nearest 0.1 mg.

A 2.5000-g sample of I was diluted to 250.0 ml with 0.9% NaCl or 1.0% (v/v) benzyl alcohol in 0.9% NaCl. This solution was filtered through a 0.22-μm membrane[9] into clean type I glass vials[10] and sealed with rubber closures[11] and one-piece aluminum seals. Half of each lot was autoclaved at 121° and 15 psi for 30 min. Several vials from each formulation group then were stored at 25 ± 5° in the dark or 35 cm from a 15-w fluorescent bulb illuminated continuously. The absorbance at 638 nm of a sample representing each set of formulation variables was determined on six occasions during a 20-month storage period.

Solutions of I[6] at $5 \times 10^{-4}$% were prepared in 0.900, 0.090, 0.009, and $9 \times 10^{-4}$ *N* KOH and had pH values of 13.71, 12.67, 11.31, and 10.04, respectively. The solutions were stored in amber glass bottles sealed with plastic screw caps, and their absorbance at 638 nm was recorded at appropriate times to quantitate the rate of color fading of I in alkaline solution at 23 ± 2°.

Seventeen solutions of I, $2 \times 10^{-4}$–5.00% (w/v), were prepared in 0.067 *M* phosphate buffer ($\mu = 0.2$) to suppress the Donnan effect (pH 7.40 ± 0.05). A 4.00% (w/v) human serum albumin solution also was prepared in the phosphate buffer. Exactly 1.0 ml of sample solution was delivered into one side of a dialysis cell[12] partitioned by a cellulose membrane[13],

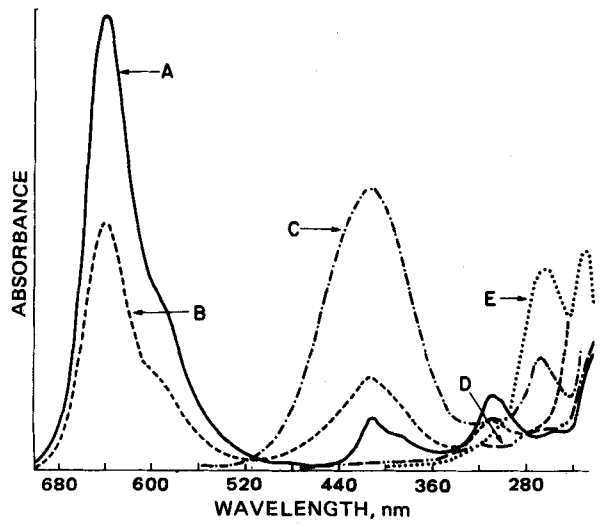



**Figure 1**—*Spectra of patent blue violet (I) at $5 \times 10^{-4}$% (A–C) or 1.0 $\times 10^{-3}$% (D and E) at specified pH or $H_o$. Key: A, pH 7.00; B, pH 2.75; C, 1.0 ≥ $H_o$ ≥ −5.8; D, −7.0 ≥ $H_o$ ≥ −10.0; and E, after 24 hr in 0.90 N KOH (pH 13.7).*

[10] Wheaton 400, 5-ml serum bottle, Wheaton Scientific, Millville, N.J.
[11] Type S-63, formulation 1816 gray butyl rubber, West Co., Millville, N.J.
[12] Constructed of plexiglass, Technilab Instrument Inc., Pequannock, N.J.
[13] Nominal pore size = 4.8 nm, impermeable to molecules with mol. wt. >6000, Technilab Instrument Inc., Pequannock, N.J.

**Table II—Observed Colors of Patent Blue Violet (I) Solutions over Certain pH and $H_o$ Ranges**

| pH or $H_o$ Range | Observed Color | Approximate Wavelength Maximum, nm[a] |
|---|---|---|
| 4.0–11.0 | Blue | 638 |
| 2.7–3.5 | Green-blue[b] | 638, 412 |
| 1.5–2.5 | Yellow-green[b] | 412 |
| −5.8–1.0[c] | Yellow | 418 |
| −10.0–−7.0[c] | Colorless | 273 |

[a] The 273-nm value was determined in 0.01% I; other values were determined in $5 \times 10^{-4}$% I. [b] Colors change from left to right corresponding to pH shifts. [c] $H_o$ values *via* dilutions of sulfuric acid.

and 1.0 ml of albumin solution was placed into the other cell compartment. The dialysis samples were incubated for 48 hr at 37 ± 1° in an oven. An accurately measured portion of I solution was removed from the cell, an appropriate dilution was made when necessary, and the absorbance at 638 nm was recorded. The percent of I bound to albumin was determined by reading the concentration of the dialyzed I solution from a linear Beer's plot for I and subtracting this value from the predialysis concentration of I.

The TLC of I was conducted with 250-μm silica gel plates[14] on which 2 μl of 0.01% methanolic I was spotted and allowed to dry before elution. The TLC plates were eluted with several single- and multicomponent solvent systems for a distance of 10.0 cm in a developing tank lined with paper to ensure saturation of the atmosphere with eluent vapor. After developing and drying under ambient conditions, the plates were observed in a chamber[15] under incandescent and UV light of ~254 and 375 nm. In some eluent systems, the TLC plate was developed a second time at a 90° angle to the first trial to assess any interaction between I and the eluent. The $R_f$ values of all samples were measured to the nearest 1.0 mm.

## RESULTS AND DISCUSSION

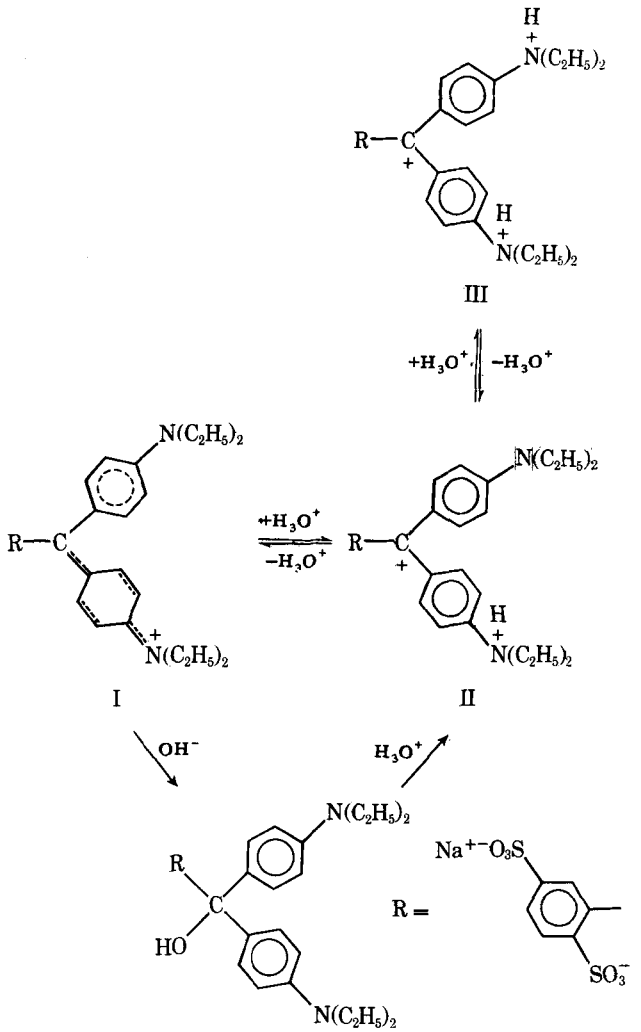
The structure of I shown in Scheme I is that of its recently proposed 2,5-disulfonate derivative[16] (11). However, most publications have assigned sulfonation to positions 2 and 4 (12–14). As depicted by the dotted lines, the cationic nitrogen atom exists in resonance between the two diethylanilino groups until either the diethylanilino groups are protonated (II and III) or the central carbon atom is hydroxylated (IV). The mesomerism of diamino- and triaminotriphenylmethane dyes producing quinoidal and benzenoidal (carbonium) ions *via* resonance equilibrium in aqueous solutions has been demonstrated and rationalized (12, 15–20).

Spectral data and color descriptions of I solutions are reported in Tables I and II. Figure 1 shows the spectra of several I solutions. The pKa for the equilibrium between I and II (Scheme I) was determined spectrophotometrically at 25° to be 2.78 ± 0.03 based on seven samples of pH 2.78 ± 0.56. The titration spectra exhibited an isosbestic point at 505 ± 2 nm. The protonation of one of the diethylanilino groups decreases by 50% the length of the quinoid–benzenoid resonance system in I. This result would be expected to reduce the overlapping of electron orbitals, thereby producing the hypsochromic absorbance shift from 638 to 418 ± 2 nm evidenced in Fig. 1 and Table II as the acidity of I solutions increases from pH 4 to $H_o$ −5.8. As indicated in Scheme I, the protonation of I also generates a carbonium ion (II) from the central carbon atom of the triphenylmethane molecule.

It has been shown that the second *p*-diethylamino substituent further stabilizes the already stable triphenylcarbonium (trityl) ion (20). In sulfuric acid solutions of $H_o$ −7.0 and stronger, and the second diethylanilino group of I is protonated, thereby precluding quinoidal resonance in the trityl ion (III) (15), which causes a hypsochromic shift from 418 (II) to 273 nm. The pKa for the equilibrium between II and III was not quantitated accurately, possibly because of the interfering association of the sulfonate groups, in the solutions of $H_o < -5.8$.

Finally, the effect of ethylating the anilino nitrogens and *ortho*-sulfonating the nonanilino ring of I is to cause a bathochromic shift, as

[14] Redi-Plates, silica gel GF, 5 × 20 cm or 20 × 20 cm, Fisher Scientific Co., Fair Lawn, N.J.
[15] Model CC-20 Chromato-Vue Cabinet, Ultra-Violet Products Inc., San Gabriel, Calif.
[16] The proposed USAN is isosulfan blue.



*Scheme I*

compared, for instance, to the quinoidal form of 4,4′-diaminotriphenylmethane (12, 13, 15, 20). One sulfonate group of I confers water solubility on the dye, whereas the second group forms a zwitterion with a diethylamino group during the final synthetic oxidation step (13).

Three samples of I dried at 135° to constant weight showed a loss on drying of 8.65 ± 0.15%. This weight loss apparently represents water since thin-layer chromatograms and UV spectra of ether extracts of I showed no evidence of a major starting compound, diethylaniline.

The distribution coefficients ($P$) for I in $n$-octanol–pH 7.4 buffer and chloroform–pH 7.4 buffer were 0.013 and 0.12, respectively. The experimental value of $P$ in the chloroform system was 20 times larger than that calculated using the value of $P$ obtained from the $n$-octanol system (21):

$$\log P_{\text{chloroform}} = 1.276 \log P_{n\text{-octanol}} + 0.171 \qquad \text{(Eq. 2)}$$

The poor lipophilicity of I may be attributed to its highly polar nature in solutions at physiological pH, *i.e.*, two dissociated sulfonate groups and one diethylanilinium ion. These results corroborate the microscopic observations that I enters frog capillaries *via* pores between endothelial cells and not *via* diffusion through the tissue (22) and that it does not stain the spinal cord or brain tissues of small mammals (23).

The approximate solubilities of I in several solvents are reported in Table III. As expected from its $P$ values in $n$-octanol and chloroform, I is virtually insoluble in organic solvents with low dielectric constants. The data from the phase solubility analysis of I in distilled water are illustrated in Fig. 2. This graph of solute *versus* I added appears to be analogous to that expected from a plot of a colligative property *versus* electrolyte concentration in which more extensive ion association elicits a progressive diminishing of the colligative effect (24). It is not discernible why at 25° the solubility of sodium chloride is 26.5% (w/w) (25) whereas

**Table III—Approximate Solubilities of Patent Blue Violet (I) in Various Solvents at 25°**

| Solvent | Solubility, % w/v[a] |
|---|---|
| Alcohol USP | 9.8 ± 0.4 |
| Distilled water | ≥42 |
| Chloroform | $4 \times 10^{-5}$ |
| Ether | $<1 \times 10^{-5}$ |
| Ethyl acetate | $<1 \times 10^{-5}$ |
| Hexanes | $<1 \times 10^{-5}$ |
| Toluene | $<1 \times 10^{-5}$ |

[a] A ±2% volume error in reading the meniscus of alcohol and water solutions of I.

that of I appears to exceed 37% (w/w) (Fig. 2). Furthermore, the molecular weight ratio of I to sodium chloride is 9.7. One possibility is that the commercially obtained sample of I consisted of various triphenylmethane isomers with diethylamino groups in other than just the 4,4′-positions on two of the benzene rings and/or sulfonate groups in other than positions 2 and 5 on the third ring. A mixture of such isomers could result in a phase solubility plot comprised of several linear segments (26–28). However, the following evidence appears to discredit such conjecture.

1. The data points on the curve of Fig. 2 cannot be plotted readily as a series of, for instance, three- or four-point segments, the intersections of which can be delineated clearly.

2. The precision of the pKa value, 2.78 ± 0.03, for the equilibrium between I and II (Scheme I), as well as the isosbestic point of 505 ± 2 nm for the pKa titration, indicates that the diethylamino group in only a single substituent position, *i.e.*, *para* to the central triphenylmethyl carbon atom, was protonated. The transition from I to II causes a hypsochromic shift of ~220 nm, from blue to yellow. This shift could result only from shortening of the I resonance system. Furthermore, the intense blue color of aqueous I can be attributed only to the quinoidal resonance system, which occurs when the two diethylamino groups are *para* to the central triphenylmethyl carbon atom (12, 13, 15–20). The occurrence of *meta*-quinones is highly unlikely, and the *para*-forms are more favored than the *ortho*-forms (12, 29).

3. The dialkylamino group is a highly activating, predominantly *para*-directing, stable quinoidal intermediate, which, when augmented by steric hindrance effects, virtually precludes competitive electrophilic substitution at the *ortho*- and, less likely, *meta*-positions (29). The synthesis of I requires condensing benzaldehyde-2,5-disulfonic acid with diethylaniline (13). It is ostensible that steric hindrance of the former to *ortho*- and *meta*-attack on diethylaniline should be expected.

The negative curvature of the Fig. 2 plot most likely is the result of a phenomenon involving the association of solvated solute molecules or the presence of impurity (26–28). The terms solid solutions (26, 27), liquid crystals, mesophases, and lyotropic mesomorphism have been used to define or describe these complex systems (30–34). It is relevant that the centrifuged supernates of those aqueous phase solubility analysis samples of I that exceeded ~15% (w/w) required an unusually high force on the syringe plunger to effect filtration through the 0.45-μm membrane. Furthermore, the filtered supernates of the aqueous I solubility samples,

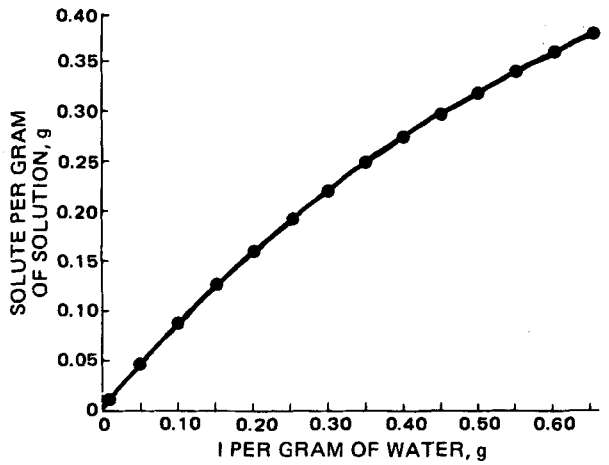



**Figure 2**—*Plot of phase solubility analysis data for patent blue violet (I) in distilled water at 25 ± 0.5°.*

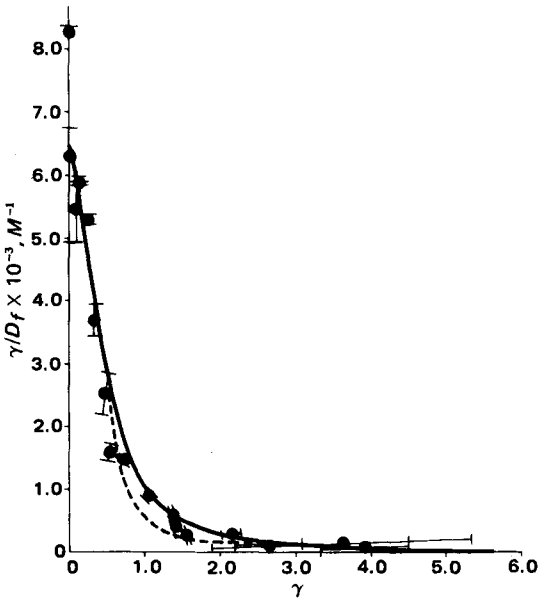



**Figure 3**—*Scatchard plot of* $\gamma/D_f$ versus $\gamma$ *for patent blue violet (I) over the concentration range of* $2 \times 10^{-4}$–*5.00% (w/v) (*$3.0 \times 10^{-6}$–*0.075 M) I dialyzed against 4% (w/v) (*$6.03 \times 10^{-4}$ *M) human serum albumin at pH 7.4 and 37°.*

when wiped onto a tissue paper, possessed a luster and did not exhibit capillary migration, which is inherent to isotropic liquids.

Derivatives of naphthylaminedisulfonic acid have been shown to produce lyotropic mesophases in water but not in alcohol (30, 35). Furthermore, none of the aqueous and all of the alcoholic I solubility analysis samples were observed to contain undissolved solid material following both rotation at 45 rpm for 24 hr and centrifugation at 2000 rpm for 10 min. This finding suggests that the alcoholic samples consisted of two distinct phases whereas the aqueous samples were comprised of a single mesophase. Lyotropic mesophases are characterized by unusually high viscosity (30, 36) and occurrence in high concentrations of extensively hydrated solutes with large dipole moments (24, 30, 37). Because I appears to be highly dipolar (Scheme I) and its aqueous solutions were atypically viscous in concentrations exceeding the solubility of sodium chloride, it was concluded that the plot in Fig. 2 represents a lyotropic mesophase of I. Several sulfonated and other dyes have shown lyotropic mesomorphism in aqueous systems (32).

The I concentration of samples formulated with 1.000% of I with and without 1.0% (v/v) benzyl alcohol, autoclaved and not autoclaved, and stored in the dark or under continuous illumination from a fluorescent bulb varied from −0.01 to 0.04% (w/v) of I during 20 months of storage at 25 ± 5°. However, autoclaving at 121° and 15 psi for 30 min appeared to cause a 3% initial decrease in the I concentration of samples formulated with and without 1.0% (v/v) benzyl alcohol. Similarly, a decrease of ~10% in the benzyl alcohol concentration[17] was detected upon autoclaving. Interaction of I and benzyl alcohol with the rubber closures of the samples could be one reason for the decrease in the solution concentrations that resulted from autoclaving.

The half-lives for the loss of absorbance at 638 nm of $5 \times 10^{-4}$% I dissolved in 0.900, 0.090, 0.009, and $9 \times 10^{-4}$ *N* KOH at 23 ± 2° are reported in Table IV. The loss of I absorbance at 638 nm in strongly alkaline solutions was accompanied by increasing absorbance at ~265 nm and followed apparent first-order kinetics. The conversion rate of the quinoidal form of triphenylmethane dyes to their colorless carbinol or leuco bases in alkaline media is a recognized means of assessing color fastness (12, 38). Although reversible, the conversion of I to IV (Scheme I) can be rapid in strong alkali, whereas the conversion from IV to II is comparatively slow, even in more concentrated acid. For instance, $1.0 \times 10^{-3}$% I in 0.80 *N* KOH was 99.9% converted to IV in 20 hr at 23 ± 2°. However, when this solution was prepared in 3.60 *N* $H_2SO_4$ ($H_o$ −0.7, equivalent I concentration of $8.9 \times 10^{-4}$%), the dehydroxylation of IV to regenerate II required 30 days. This slowness of the triphenylcarbinol (IV) to trityl ion (II) conversion was reported elsewhere (12). Fastness to color fading of I in alkaline media is enhanced in decreasing order by the addition of a sulfonate, chloro, or methyl group *ortho* to the central triphenylmethyl carbon atom (12).

[17] Determined *via* UV absorbance at 258 nm.

**Table IV—Half-Lives for the Loss of Absorbance at 638 nm of 5 × $10^{-4}$% Patent Blue Violet (I) in Potassium Hydroxide Solutions at 23°**

| Potassium Hydroxide Concentration, *N* | pH | Half-Life |
|---|---|---|
| 0.900 | 13.7 | 1.2 hr |
| 0.090 | 12.7 | 17.0 hr |
| 0.009 | 11.3 | 9.5 days[a] |
| $9 \times 10^{-4}$ | 10.0 | 180 days[a] |

[a] One day = 24.0 hr.

It has been demonstrated that sulfonated triphenylmethane vital stains are bound to serum albumin prior to their endocytosis by macrophages (39). Furthermore, the weaker association between I and albumin compared to other dyes resulted in its more rapid clearance from, and lesser toxicity to, local tissues following repeated subcutaneous injections of 1% solutions (6).

A Scatchard plot (40–42) of the data for the binding of I to human serum albumin appears in Fig. 3. Because transmembrane equilibrium time increased proportionately with I concentration, the data were normalized to 100% equilibrium *via* the results of dialyzing I solutions against albumin-free phosphate buffer. These blank specimens also accounted for contributions of I binding to the dialysis membrane, which did not exceed 2% in any case.

The numbers of binding sites in two classes, $n_1$ and $n_2$, and their corresponding affinity constants, $K_1$ and $K_2$, were estimated by an iterative procedure of drawing ordinate ($\gamma/D_f$) and abscissa ($\gamma$) asymptotes to the original data plot (41, 43). These asymptotic lines were omitted from Fig. 3 to avoid obscuring the raw data (solid line) and fitted (dashed line) curves. One graphical iteration sufficed to produce a curve that was superimposable with a subsequent iteration of the method used (41, 43). This method normalized the raw binding data to yield the fitted curve for the special case of $n_{H1} = n_{H2} = 1$ (41, 42):

$$\gamma = \frac{n_1(K_1D_f)^{nH1}}{1 + (K_1D_f)^{nH1}} + \frac{n_2(K_2D_f)^{nH2}}{1 + (K_2D_f)^{nH2}} \qquad \text{(Eq. 3)}$$

where $\gamma$ is the molar concentration ratio of albumin-bound I to that of albumin, $D_f$ is the molar concentration of unbound I at dialysis equilibrium, $n_{H1}$ and $n_{H2}$ are the binding cooperativity or Hill coefficients, and $n_1$, $n_2$, $K_1$, and $K_2$ are the parameters described earlier. The molarity of I was based on its nominal 85% purity[2], and that of human serum albumin was based on a molecular weight of 66,300 (44, 45).

The error bars on the solid curve in Fig. 3 represent $\chi \pm 1\sigma$ for triplicate dialysis samples. These bars become more horizontal with increasing $\gamma$ because they extend radially in the direction of constant $D_f$ (41). Variability of the data tended to increase with increasing values of $\gamma/D_f$ because of the greater sensitivity of $\gamma/D_f$ to small changes in $D_f$ attributed to experimental imprecision. Several error bars at high values on the

**Table V—Fraction of Patent Blue Violet (I) Bound to 4% (w/v) Human Serum Albumin at 37° and pH 7.4 Corresponding to Concentrations of I before and after Equilibrium Dialysis**

| Fraction of I Bound | Concentration of I, % w/v | |
|---|---|---|
| | Predialysis | Postdialysis[a] |
| 0.83 | $2.0 \times 10^{-3}$ | $1.7 \times 10^{-4}$ |
| 0.79 | $3.1 \times 10^{-3}$ | $3.4 \times 10^{-4}$ |
| 0.77 | $9.8 \times 10^{-3}$ | $1.1 \times 10^{-3}$ |
| 0.76 | $4.8 \times 10^{-3}$ | $5.7 \times 10^{-4}$ |
| 0.75 | $1.95 \times 10^{-2}$ | $2.4 \times 10^{-3}$ |
| 0.68 | $3.24 \times 10^{-2}$ | $5.2 \times 10^{-3}$ |
| 0.59 | $5.23 \times 10^{-2}$ | $1.07 \times 10^{-2}$ |
| 0.48 | $7.57 \times 10^{-2}$ | $1.98 \times 10^{-2}$ |
| 0.46 | 0.1020 | $2.72 \times 10^{-2}$ |
| 0.34 | 0.2030 | 0.0068 |
| 0.26 | 0.3490 | 0.1300 |
| 0.19 | 0.4930 | 0.1980 |
| 0.13 | 0.7700 | 0.3340 |
| 0.14 | 1.0100 | 0.4350 |
| 0.09 | 1.9500 | 0.8880 |
| 0.07 | 3.2000 | 1.4800 |
| 0.05 | 4.9600 | 2.3500 |

[a] Refers to unbound I.

$\gamma$-axis could have resulted from experimental uncertainty in reading the meniscuses of I solutions exceeding 0.5% (w/v); they are colored so intensely as to preclude light transmission.

The abscissa intercept was extrapolated asymptotically to be 0.8, indicating a single class of binding site, *i.e.*, $n_1 = 1$. The total $\gamma$-axis intercept was 5.6, indicating between five and six total binding sites. Thus, by difference, the $\gamma$-axis intercept for a second class of lower affinity binding sites was 4.8, thereby indicating that five binding sites occurred in this class, *i.e.*, $n_2 = 5$. The total ordinate intercept from the fitted curve was 6400 $M^{-1}$, and $K_1$ was extrapolated asymptotically to be 6235 $M^{-1}$. The intercept for the second class of binding sites was, by difference, 165 $M^{-1}$. Thus, each of the five binding sites in the second class was assigned an average $K_2 = 33\ M^{-1}$.

It was established elsewhere that protein binding has a clinically significant effect on *in vivo* drug distribution only when $K \geq 1 \times 10^4\ M^{-1}$ (46). Therefore, it is understandable why, with $K_1 \simeq 6000$, I can be observed in the urine of lymphangiography patients within minutes after subcutaneous injection[18] (47) and has been used to measure renal clearance (48). In fact, when 5–10% (w/v) solutions of I were used in the past for lymphangiography, the rapid secretion of I into saliva and tears was regularly seen[18].

Table V lists the albumin-bound fractions of I at varying concentrations of I before and after equilibrium dialysis. A plot of the fraction of albumin-bound I *versus* the concentration of unbound I at dialysis equilibrium yielded a curve similar to the curves in Fig. 3. If the total I in a 2–3-ml injection of 1% solution were distributed in plasma and extracellular fluid, ~12 liters in an adult (49), then the I concentration *in vivo* would be in the range of $1.7 \times 10^{-4}$–$2.5 \times 10^{-4}$% (w/v), for which Table V indicates >80% of I to be albumin bound. Other brief studies of I binding to albumin appear to be clinically irrelevant because the I concentration used ranged from 0.1 to 11.0% (8, 50).

Based on derived thermodynamic parameters, it has been proposed that the binding of sulfonated dyes to albumin involves electrostatic attraction between the sulfonate anions and the cationic arginine and lysine residues of albumin as well as hydrogen bonds and van der Waals forces (51–53). The evidence for both hydrophobic and electrostatic bonding was provided in a study (51) that found no binding of a sulfonated dye to the unpolymerized amino acids arginine and lysine, whereas significant binding to bovine serum albumin and salmine occurred. Salmine is a protamine with an arginine content of 87% (51), while human serum albumin contains 23 and 58 residues of arginine and lysine per molecule, respectively (45).

Hill plots (42, 54, 55), *i.e.*, $\log (\bar{y}/1 - \bar{y})$ *versus* $\log D_f$, where $\bar{y} = \gamma/(n_1 + n_2)$, were constructed for both the 17 experimental data points on the solid curve and the 17 points on the fitted (dashed) curve corresponding to the same $D_f$ values (Fig. 3). Regression lines computed for these plots had slopes of 0.615 and 0.613 and correlation coefficients of 0.992 and 0.989, respectively. When solved for several sets of coordinates describing the locus of the fitted curve (Fig. 3), the values of the Hill coefficients in Eq. 3 were $n_{H1} = n_{H2} = 1$, which mathematically confirmed this curve to be a hyperbola. Furthermore, the condition of $n_{H1} = n_{H2} = 1$ occurs for two different classes of binding sites only when there is no cooperativity within the ligand–protein binding system (42, 54, 55). Therefore, it was concluded that neither positive nor negative cooperative binding occurred between I and human serum albumin, as evidenced by the insignificant difference between the slopes of the Hill plots of the two curves in Fig. 3; *i.e.*, the experimental (solid) data curve represents a nearly hyperbolic function where $n_{H1} = n_{H2} \simeq 1$.

The compositions of Eluents A, B, and C, used for the TLC of I, are reported in Table VI. Systems A (4) and B[19] were used in other TLC studies, and System C is similar to that reported elsewhere (56). The $R_f$ values for the TLC of I, spotted as 2 $\mu$l of a 0.01% (w/v) solution in methanol and developed over 10.0 cm, allowed to dry for 24 hr, and redeveloped at a 90° angle in Eluents A–C, are reported in Table VII. Only a single major spot, indicating a high degree of organic content purity in I, was observed following initial development in each eluent. The second development in Eluent B resulted in a 0.10-unit decrease in the $R_f$ value, which could be attributed to the formation of some IV (Scheme I) during the first trial, although only a single visible spot was observed under incandescent and UV lamps. The second TLC elution of I with Eluent C produced a second, faintly visible spot with $R_f$ 0.31, the explanation for which is not evident. However, it is apparent from Scheme I and evidence supporting it that the exposure of I to TLC eluents containing acid or alkali could produce I derivatives of different polarity.

**Table VI—Compositions of Eluents Used for TLC of Patent Blue Violet (I)**

| | Composition, parts by volume | | | | | |
|---|---|---|---|---|---|---|
| Eluent | Acetone | Acetic Acid | Ammonium Hydroxide | 1-Butanol | Ethanol | Water |
| A[a] | — | 1 | — | 4 | — | 5 |
| B | 37 | — | 19 | 37 | — | 7 |
| C | — | 1 | — | 60 | 15 | 15 |

[a] Upper phase of system used.

**Table VII—$R_f$ Values of Patent Blue Violet (I) Spotted as 2 $\mu$l of 0.01% (w/v) Methanolic Solution and Developed for 10.0 cm in Eluents A–C**

| | $R_f$ Values | |
|---|---|---|
| Eluent[a] | First Trial | Second Trial[b] |
| A | 0.26 | 0.26 |
| B | 0.58 | 0.48[c] |
| C | 0.26 | 0.26[d] and 0.31 |

[a] Compositions are listed in Table VI. [b] Developed at a 90° angle to the first trial. [c] Because of eluent volatility, the second trial was conducted with fresh eluent. [d] Major spot.

As expected from its lipophobic nature, no measurable $R_f$ values were observed when the TLC of I was conducted in the following solvents of increasing dielectric constant: toluene, ether, chloroform, and ethyl acetate. However, the TLC of I in the more polar solvents *n*-butanol, acetone, and methanol produced visible spots with $R_f$ 0.05, 0.42, and 0.72, respectively. Preliminary TLC studies showed that 2-$\mu$l spots of methanolic I solutions exceeding 0.01%, although suggested (56), elicited trailing and tailing effects which impaired the resolution of $R_f$ values. Perhaps this effect is why 2-$\mu$l samples of $5 \times 10^{-4}$% aqueous I specimens were evaluated by TLC in an earlier study (4).

## CONCLUSIONS

Based on the results of spectrophotometric studies of I solutions prepared in solvents ranging from 0.90 *N* KOH (pH 13.7) to 96% (w/w) $H_2SO_4$ ($H_o$ −10.0), the quantitative determination of I at 639 ± 2 nm appears to represent a stability-indicating assay. The results of distribution coefficient, solubility analysis, and human serum albumin binding studies corroborate other evidence suggesting that the *in vivo* distribution of I is limited to plasma and extracellular fluid. Based on the pKa for the equilibrium between I and II (Scheme I) and on the results of TLC determinations, I appears to be essentially pure with respect to its organic compound content.

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# Utility of Chloranil in Assay of Naphazoline, Clemizole, Penicillin G Sodium, and Piperazine

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**Abstract** □ A simple and sensitive spectrophotometric method is described for the assay of naphazoline, clemizole, penicillin G sodium, and piperazine. The method was based on the formation of a charge transfer complex between these drugs as $n$-donors and chloranil, the $\pi$-acceptor. Conformity to Beer's law enabled the assay of dosage forms of these drugs. Compared with official methods, the results obtained were of equal accuracy. A more detailed investigation of the naphazoline–chloranil complex was made with respect to its composition, association constant, and free energy change.

**Keyphrases** □ Naphazoline—spectrophotometric assay in dosage forms using complex formation with chloranil □ Clemizole—spectrophotometric assay in dosage forms using complex formation with chloranil □ Penicillin G sodium—spectrophotometric assay in dosage forms using complex formation with chloranil □ Piperazine—spectrophotometric assay in dosage forms using complex formation with chloranil

Naphazoline and clemizole (antihistamines), penicillin G sodium (antibiotic), and piperazine (anthelmintic) are widely used in pharmaceutical practice. The official compendia describe a nonaqueous titration for naphazoline (1), an iodometric titration (2) and microbiological assay (3) for penicillin G sodium, and a gravimetric method (1) and nonaqueous titration (3) for piperazine. Among the methods described for the assay of naphazoline are UV

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