(22) F. Franks, in "Water A Comprehensive Treatise," vol. 2, F. Franks, Ed., Plenum, New York, N.Y., 1973, pp. 1–41.

(23) D. Winne and J. Remischovsky, *J. Pharm. Pharmacol.*, **22**, 640(1970).

(24) R. J. Pietras and E. M. Wright, *Nature (London)*, **247**, 222(1974).

(25) B. Sandstrom, *Cytobiology*, **3**, 293(1971).

(26) C. H. van Os, M. D. de Jong, and J. F. G. Slegers, *J. Membr. Biol.*, **15**, 363(1974).

(27) W. R. Galey and J. T. Van Bruggen, *J. Gen. Physiol.*, **55**, 220(1970).

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Chromatographic, Mass Spectral, and Visible Light Absorption Characteristics of Toluidine Blue O and Related Dyes

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Abstract □ Contrary to earlier literature reports, the impurities in toluidine blue O were shown by column chromatography, TLC, and mass spectrometry to be *N*-methyl homologs of 2-methylthionine rather than *N*-methyl homologs of thionine. Small amounts of 2-methyl-3-amino-7-methylaminophenothiazine and 2-methyl-3,7-diaminophenothiazine were identified in commercial samples of toluidine blue O. However, sample handling and a warm alkaline environment can cause rapid demethylation of the dye.

Keyphrases □ Toluidine blue O and related dyes—chromatography, mass spectra, and visible light absorption □ Dyes—chromatographic, mass spectral, and visible light absorption characteristics of toluidine blue O and related dyes □ Mass spectrometry—impurities, toluidine blue O and related dyes

In recent years there has been increased interest in the medical use of the dye toluidine blue O (3-amino-7-dimethylamino-2-methylphenazathionium chloride, C.I. Basic Blue 17). It recently has been used for the identification of the parathyroids during surgery (1, 2) and as an aid in the diagnosis of small oral cancers (3) and has been suggested as a potential parathyroid and pancreatic scanning agent if labeled with a suitable radionuclide (4).

BACKGROUND

During an investigation into the synthesis of radioactive analogs of toluidine blue O, some problems were encountered in obtaining pure dye samples. Elemental analysis, column chromatography, TLC, and mass spectrometry all indicated the presence of impurities which could not be separated from the dye.

Similar difficulties in analyzing samples of toluidine blue O by TLC were reported (5, 6). The investigators observed three spots for the commercial dye, two blue and one violet. They identified the violet spot as thionine (I) on the basis of its R_f value and visible light absorption spectrum. Extraction of one of the two blue spots and rechromatography again produced two blue spots, which were attributed to different ionic forms of the dye or "solvent effects." In addition, Apgar and Patel (6) reported that visible light absorption experiments indicated that toluidine blue O lost a methyl group (in this case an aryl methyl group) and was converted to azure A (III) upon standing in aqueous solution.

Normann and Normann (7) succeeded in isolating five contaminants from a sample of tritiated toluidine blue O by TLC. The resulting spots were extracted from the thin-layer chromatograms and identified by their absorption maxima (Table I). Their identification of various compounds on the basis of the absorption maxima was not consistent with earlier work (8), which is summarized in Table II.

In 1953, Ball and Jackson reported on chromatographic and spectrophotometric studies of various samples of toluidine blue O and azure A (9). Descending paper chromatograms[1] developed in butanol–acetic acid–water (40:10:50 v/v) indicated that the various samples, supplied by different manufacturers, were indeed not the same dyestuff. Column chromatography on aluminum oxide with varying solvents yielded a number of fractions.

The confusion in the assignment of consistent visible light absorption maxima to the various related dyes was disturbing. In the authors' laboratory, commercial toluidine blue O shows a significant change in absorption characteristics as the pH of the solution is changed. In saline at pH 2, toluidine blue O exhibits a λ_{max} value of 630 nm, while at pH 10 a broad absorption maximum from 550 to 630 nm is observed. Differences in reported λ_{max} values (6, 7) may have been due to sample preparation, since the pH of the solutions studied was not mentioned. In addition, the assignment of the structure of azure A (III) to the degradation product of toluidine blue O implies an aryl-demethylation rather than the more probable *N*-demethylation.

The purposes of the present study were to explain the chromatographic behavior of toluidine blue O and to clarify the interpretation of the visible light absorption data.

EXPERIMENTAL[2]

Column Chromatography—Columns [5.1-cm (2-in.) diameter] were packed with dry silica gel (70–325 mesh) to a depth of 45.7 cm (18 in.). Samples were dissolved in ethanol and applied to the tops of the dry columns. Columns were eluted with ethanol followed by

[1] Whatman No. 1.

[2] The dyes used were obtained from National Aniline and Eastman Kodak and were labeled 80–90% pure. All other chemicals were reagent grade and were used as received. Elemental analyses were performed by Schwarzkopf Microanalytical Laboratory, Woodside, N.Y., and by Strauss Microanalytical Laboratory, Oxford, England.

Table I—Fractions of ^3H-Labeled Commercial Toluidine Blue O Separated by TLC[a]

Dye	Absorption Maximum, nm	Approximate R_f Values[b]
Thionine (violet)	600	0.81
Toluidine blue O (blue)	616	0.64
Azure A (blue green)	632	0.36
Methylene blue (green)	652	0.17
Methylene blue (gray)	—	0.00

[a] From Ref. 7. [b] R_f values were determined by measurement of a photograph of the chromatogram provided in the text. Values were not reported in Ref. 7.

ethanol–concentrated hydrochloric acid (99:1 v/v). Fractions obtained from the columns were evaporated under reduced pressure.

TLC—Commercially prepared aluminum-backed silica gel plates were used. Developing solvents were: I, methanol–2 *N* HCl (93:7 v/v); and II, ethanol–concentrated hydrochloric acid (99:1 v/v).

Mass Spectrometry—Mass spectra (70 ev) were obtained on a high-resolution mass spectrometer[3] coupled to a computer[4]. All samples were introduced on a direct-insertion probe. Source temperatures varied from 150 to 250°. The computer allowed the spectrometer magnet to decay and recorded multiplier current *versus* time at 85-μsec intervals.

The center of gravity of each peak was determined and compared to perfluorokerosene peaks of known *m/e* values. High-resolution *m/e* values and empirical formulas were calculated with software under control of the operator. Only formulas within ±3 mmass of calculated values are presented here.

Rapid Scan Mass Spectrometry—Rapid, repetitive mass spectra were taken from *m/e* 350 to 180 every 35 sec while under computer control. Scanning commenced when the sample (on the direct-insertion probe) was placed into the source and continued until the monitor current returned to preinsertion levels. Data were stored on magnetic tape and processed like other spectra.

Visible Light Absorption Spectrometry—Visible light absorption spectra were obtained on a recording spectrophotometer[5]. All samples were dissolved in water at pH 3.5. A path length of 1 cm was used.

RESULTS AND DISCUSSION

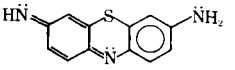
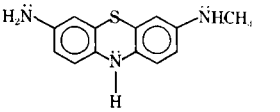
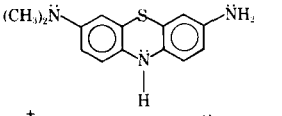
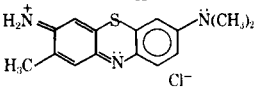
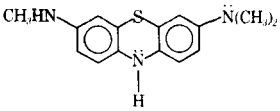
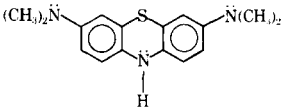
Elemental Analysis of Toluidine Blue O—Elemental analysis of the commercial dye revealed 51.6% C, 5.45% H, 10.46% N, and 9.22% S. This composition yields an empirical formula of $C_{15}H_{19}N_{2.6}S$ (toluidine blue O should be $C_{15}H_{15}N_3S$). Since it was suspected that the depression of nitrogen from the calculated value was due to incomplete burning, a repeat analysis was requested. This analysis included drying the sample to constant weight, burning over vanadium pentoxide, and estimating the chloride ion. This analysis revealed 48.6% C, 4.92% H, 11.86% N, 11.19% S, and 18.24% Cl, which calculates for $C_{11.6}H_{13.9}N_{2.4}SCl_{1.5}$.

TLC of Commercial Toluidine Blue O—TLC (Solvent System I) revealed that the commercial preparation was separable into three colored fractions with R_f values equal to 0.55 (intense blue), 0.69 (blue), and 0.72 (faint violet observable only when rather large amounts of the commercial dye were spotted). Extraction of the intense blue spot (R_f 0.55) with methanol–2 *N* HCl, evaporation of the solvent under reduced pressure at 70–100°, and rechromatography of the blue residue yielded an identical chromatogram to that obtained originally, with the exception that the violet band was not visible.

Treatment of the violet spot at R_f 0.72 in a like manner yielded a chromatogram with only a single spot at R_f 0.72. Other solvent systems were tried with analogous results. Elemental analysis of the fractions was impossible to interpret due to the large amounts of silica gel that dissolved in the solvent and could not be removed from the dye.

[3] AEI MS-9.
[4] PDP-12.
[5] Cary model 14.

Table II—Absorption Maxima for Related Phenothiazine Dyes[a]

Dye	Absorption Maximum, nm	Structure
Thionine (I)	602	
Azure C (II)	611	
Azure A (III)	638	
Toluidine blue O (IV)	635	
Azure B (V)	652	
Methylene blue (VI)	667	

[a] From Ref. 8.

Mass Spectrometry of Toluidine Blue O and Related Dyes—To identify and confirm the presence of contaminants in commercially prepared toluidine blue O, a series of mass spectra (Table III) was obtained for a group of related phenothiazine dyes: toluidine blue O (IV), thionine (I), azure A (III), azure B (V), azure C (II), and methylene blue (VI). For the sake of simplicity, only unit masses are presented in the discussion.

Toluidine Blue O—Inspection of the mass spectrum of commercial toluidine blue O indicates peaks at *m/e* 299 ($C_{17}H_{21}N_3S$), 285 ($C_{16}H_{19}N_3S$), 271 ($C_{15}H_{17}N_3S$), 257 ($C_{14}H_{15}N_3S$), and 243 ($C_{13}H_{13}N_3S$). Since the molecular weight of toluidine blue O is 271, it was apparent that the mass spectrum indicated the presence of contaminants. Since the major fragments were separated by 14 mass units (CH_2), they could not arise by fragmentation, say from the *m/e* 299 peak, because loss of methylene is highly improbable.

It was possible that thermal transfer of methyl groups had occurred in the high temperature source (250°) used to vaporize the samples. Thermal transfer of methyl groups has been reported (10), but data to be presented later indicate that this is probably not the case with toluidine blue O.

Azures A, B, and C—Mass spectra of the azure dyes[6] A (III), B (V), and C (II) are shown in Table III. These spectra are consistent with the presence of contaminating homologs of the parent structure. This finding is easily understood since they are prepared commercially by the oxidative demethylation of methylene blue with chromic acid (11).

Methylene Blue—The methylene blue spectrum (Table III), like the others, indicated that the dye contained a contaminant at *m/e* 299 ($C_{17}H_{21}N_3S$).

Phenothiazine Dyes—In general, phenothiazine systems are characterized by the high abundance of the molecular ion, which is due to the resonance stabilization of the radical ion. Phenothiazine ($C_{12}H_9NS$) fragments primarily to $C_{12}H_9N$ (53%), $C_{12}H_8NS$ (22%), $C_{12}H_8N$ (19%), and $C_{11}H_7S$ (3%) (12). Addition of an exocyclic amine, as in the case of thionine, increases the probability of losing 27 mass units (presumably by the loss of HCN) from the molecular ion.

Addition of *N*-methyl groups favors fragmentation by loss of CH_3; however, the spectra are confusing, due, evidently, to contamination by homologs of the parent with at least one or two ad-

[6] Eastman Kodak.

Table III—Mass Spectra (70 ev) of Some Related Phenothiazine Dyes.

m/e	Empirical Formula	Base Intensity, % Commercial Toluidine Blue O	Thionine	Azure A	Azure B	Azure C	Methylene Blue
299	$C_{17}H_{21}N_3S$	4.	—	—	12.	—	26.
287		1.	—	2.	4.	2.	5.
286		5.	—	6.	17.	6.	22.
285	$C_{16}H_{19}N_3S$	21.	—	29.	92.	37.	100.
284	$C_{16}H_{18}N_3S$	6.	—	7.	25.	8.	42.
283	$C_{16}H_{17}N_3S$	3.	—	—	5.	—	7.
282	$C_{16}H_{16}N_3S$	—	—	—	—	—	3.
273		4.	—	6.	5.	7.	3.
272		17.	—	15.	19.	17.	9.
271	$C_{15}H_{17}N_3S$	100.	—	85.	100.	100.	47.
270	$C_{15}H_{16}N_3S$	22.	—	31.	54.	31.	43.
269	$C_{15}H_{15}N_3S$	8.	—	20.	27.	27.	27.
268	$C_{15}H_{14}N_3S$	—	—	9.	13.	16.	19.
259		—	—	7.	3.	6.	—
258		14.	—	16.	7.	19.	—
257	$C_{14}H_{15}N_3S$	86.	—	100.	41.	100.	9.
256	$C_{14}H_{14}N_3S$	34.	—	50.	44.	54.	17.
255	$C_{14}H_{13}N_3S$	15.	—	36.	18.	50.	11.
254	$C_{14}H_{12}N_3S$	8.	—	19.	15.	33.	11.
253	$C_{14}H_{11}N_3S$	—	—	—	—	6.	4.
252	$C_{14}H_{10}N_3S$	—	—	—	—	8.	5.
245		1.	—	5.	—	2.	—
244		6.	—	10.	2.	9.	—
243	$C_{13}H_{13}N_3S$	39.	—	52.	10.	48.	2.
242	$C_{13}H_{12}N_3S$	34.	—	47.	17.	48.	7.
241	$C_{13}H_{11}N_3S$	6.	—	19.	9.	21.	8.
240	$C_{13}H_{10}N_3S$	—	—	18.	9.	18.	6.
229	$C_{12}H_{11}N_3S$	—	12.	19.	3.	12.	2.
228	$C_{12}H_{10}N_3S$	8.	19.	32.	6.	21.	3.
227	$C_{12}H_9N_3S$	9.	100.	10.	10.	—	5.
226	$C_{12}H_8N_3S$	4.	5.	6.	6.	—	4.
225		3.	—	—	7.	—	—
202		—	4.	—	—	1.	—
201	$C_{11}H_9N_2S$	—	10.	9.	—	5.	—
200	$C_{11}H_8N_2S$	—	78.	6.	—	6.	—
199	$C_{11}H_7N_2S$	—	10.	—	—	—	—
174		—	3.	—	—	—	—
173		—	5.	—	—	—	—
172	$C_{10}H_6NS$	—	7.	—	—	—	—

ditional methyl groups (*i.e.*, differing in empirical formula by one or two CH_2 groups). The loss of HCN (27 mass units) was not observed with the parent structures of azure A, toluidine blue O, and methylene blue.

Rapid Sequence Mass Spectrometry of Commercial Toluidine Blue O—A sample of commercial toluidine blue O was subjected to rapid sequence mass spectral analysis (Fig. 1). The spectra are characterized by the change in the relative abundance of the *m/e* 285 ($C_{16}H_{19}N_3S$), 271 ($C_{15}H_{17}N_3S$), and 257 ($C_{14}H_{15}N_3S$) peaks, which is consistent with a mixture of materials having different volatilities in the source of the mass spectrometer.

Chromatography of Toluidine Blue O—TLC with Solvent System II consistently separated toluidine blue O into four fractions (Fig. 2). A faint violet band at R_f 0.67 (A), a faint blue band at R_f 0.59 (B), an intense blue band at R_f 0.4 (C), and a very faint blue band at R_f 0.17 (D) were routinely observed.

Column chromatography with silica gel and 95% ethanol yielded small amounts of the violet band, A, and somewhat larger quantities of the light-blue fraction, B. The intense blue band, C, was eluted from the column by the addition of hydrochloric acid to the solvent.

The mass spectrum of C is presented in Fig. 3 and seems to be identical to other determinations of the commercial dye. Evidence for all homologs of toluidine blue O was present. Masses at *m/e* 299 ($C_{17}H_{21}N_3S$), 285 ($C_{16}H_{19}N_3S$), 271 ($C_{15}H_{17}N_3S$), and 257 ($C_{14}H_{15}N_3S$) were all present as before.

The mass spectrum of the light-blue fraction, B (Fig. 4), indicated that the mass at *m/e* 257 ($C_{14}H_{15}N_3S$) was the only significant homolog present. This finding was confirmed by rapid sequence

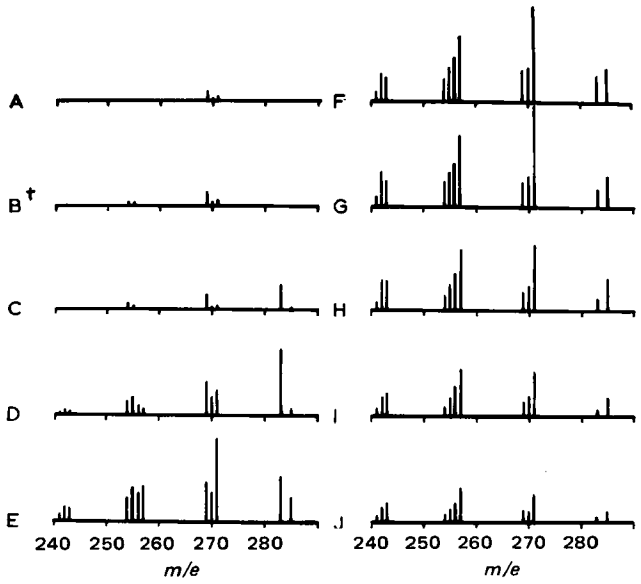


Figure 1—*Rapid sequence mass spectra (70 ev) of commercial toluidine blue O. Peak heights are shown relative to the highest peak seen during an experiment. (†The start of scan B was 35 sec after the beginning of scan A and so on through scan J.)*

mass spectral scans. The scans indicated that the sample did not behave like those in other studies with toluidine blue O; essentially only *m/e* 257 ($C_{14}H_{15}N_3S$) was seen to any extent during the experiment.

Examination of the leading violet band, A, was not successful as the sample could not be volatilized from the direct-insertion probe of the mass spectrometer.

Degradation of Commercial Toluidine Blue O in Aqueous Base—A sample of the light-blue fraction, B, isolated from the commercial dye, previously identified as being $C_{14}H_{15}N_3S$, was ad-

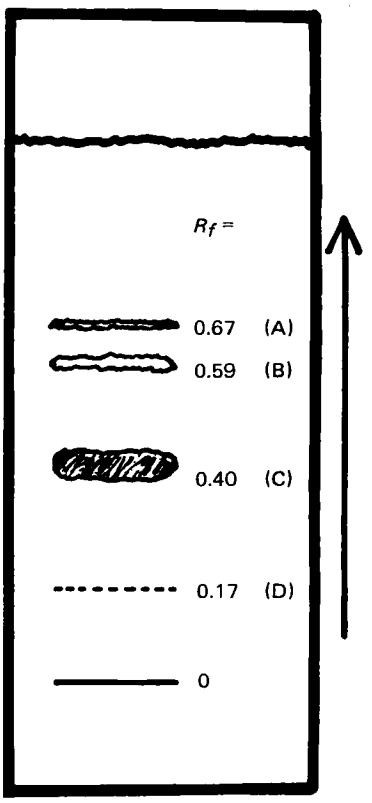


Figure 2—*Thin-layer chromatogram of toluidine blue O [silica gel, ethanol–concentrated hydrochloric acid (99:1 v/v)].*

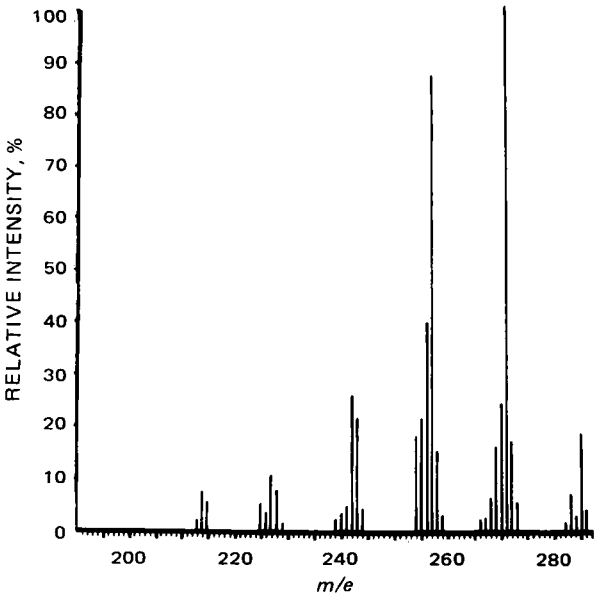


Figure 3—*Mass spectrum (70 ev) of Fraction C isolated by column chromatography from commercial toluidine blue O.*

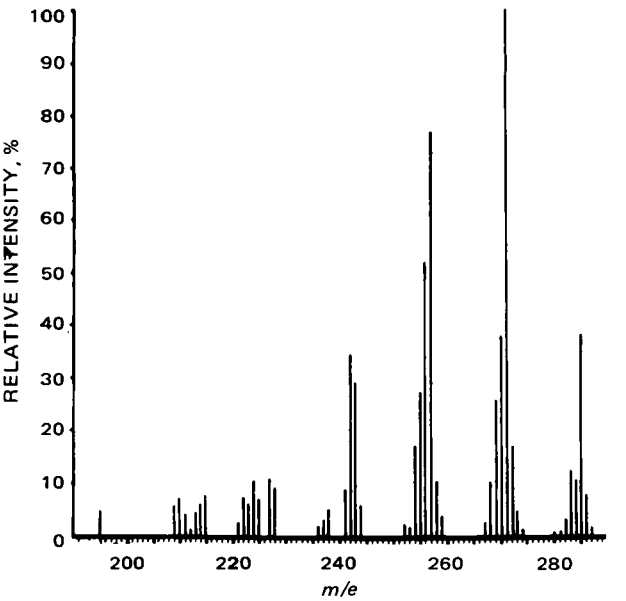


Figure 5—*Mass spectrum (70 ev) of Fraction C isolated from the alkaline degradation of toluidine blue O.*

justed to pH 9 to neutralize the hydrochloric acid used in the chromatography and was evaporated to dryness over a steam bath. Inadvertently, the sample was left on the bath overnight. In the morning the sample was examined and was violet to red in color. Chromatography indicated that B had been converted to the leading violet fraction, A.

Attempts to duplicate this experiment by heating acidic solutions of B and commercial toluidine blue O failed. However, addition of small amounts of base, pH 8–9, showed almost instant degradation of B to A and degradation of toluidine blue O to larger quantities of B and A as well as a red fraction at R_f 0.69. Attempts to isolate large samples of the various impurities from the commercial dye were abandoned in favor of the alkaline degradation of the dye, which did supply large amounts of the principal contaminants for visible light absorption spectroscopy and mass spectrometric studies.

Mass Spectral Analysis of Fractions Isolated from Degraded Commercial Toluidine Blue O—Aqueous solutions of base-treated commercial toluidine blue O were adjusted to pH 1 with hydrochloric acid and were evaporated to dryness under reduced pressure at or below room temperature. The residue was dissolved in a minimum amount of ethanol–concentrated hydrochloric acid, and the fractions were isolated by dry column chromatography. Thin-layer chromatograms indicated that separation was not complete, and the fractions were again chromatographed on silica gel columns.

The fractions were chromatographically (TLC) identical with those isolated directly from the commercial preparation. Mass spectra are presented for the intense blue band, C, the light-blue band, B, and the leading violet band, A (Figs. 5, 6, and 7, respectively). The spectrum of C is consistent with all of the others done on commercial toluidine blue O, with evidence for homologs at *m/e* 257, 271, and 285. However, the spectrum of the light-blue band, B, previously identified as *m/e* 257 ($C_{14}H_{15}N_3S$), contained no higher order homologs.

The spectrum of the leading violet fraction, A (Fig. 7), is similar to the mass spectrum of thionine (Table III). Of note is the +14 mass shift of both the M^+ and M − HCN ions, indicating the addition of a methyl group to the molecule in such a fashion as to provide no additional fragmentation as *N*-methylation would. This observation would be consistent with Structure VII, 2-methylthionine.

By inference, the structure of the light-blue fraction, B, would be VIII, 2-methyl-3-amino-7-methylaminophenothiazine, or IX, 2-methyl-3-methylamino-7-aminophenothiazine.

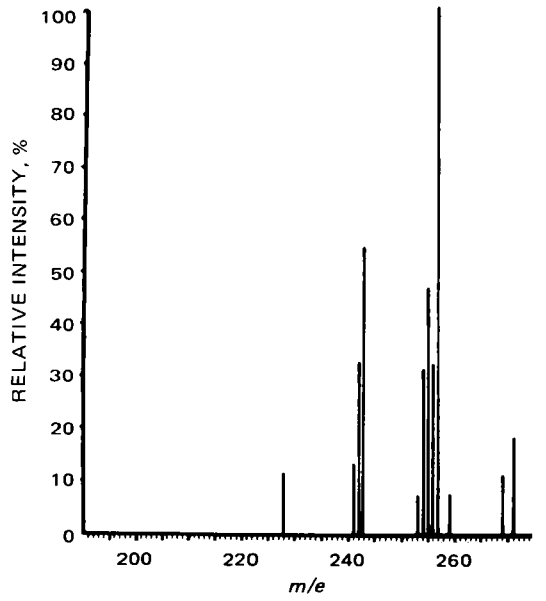


Figure 4—*Mass spectrum (70 ev) of Fraction B isolated by column chromatography from commercial toluidine blue O.*

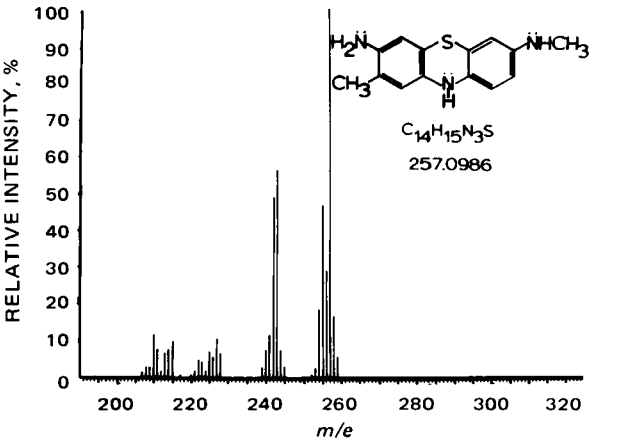


Figure 6—*Mass spectrum (70 ev) of Fraction B isolated from the alkaline degradation of toluidine blue O.*

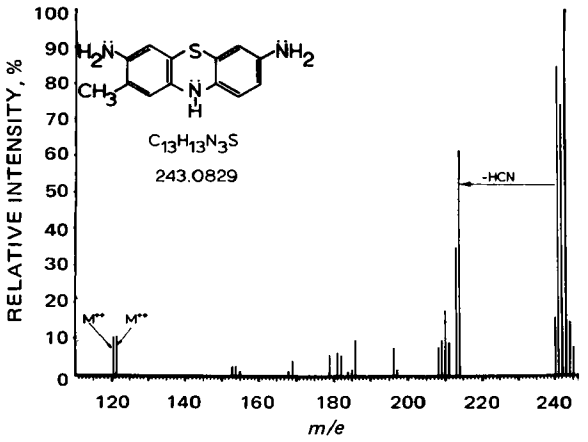


Figure 7—*Mass spectrum (70 ev) of Fraction A isolated from the alkaline degradation of toluidine blue O.*

Mass Spectral Analysis of Red Fraction Isolated from Degradation of Toluidine Blue O in Aqueous Base—In addition to the fractions already mentioned, a fraction with R_f 0.69 was also isolated by TLC with Solvent System II. Mass spectral examination indicated it to be an oxygen-bearing compound of empirical formula $C_{13}H_{10}N_2OS$ (calculated *m/e* 242.0512, found *m/e* 242.0516), potentially of Structure X or XI. Major ions found were $C_{13}H_{10}N_2OS$ (M^+, 100%), $C_{13}H_9N_2OS$ (5%), $C_{12}H_{10}N_2S$ (100%), $C_{12}H_9N_2S$ (38%), and $C_{11}H_8NS$ (48%). Loss of CO to $C_{12}H_{10}N_2S$ would be consistent with the structures drawn.

Visible Light Absorption Spectra of Fractions Isolated from Commercial Toluidine Blue O and Degraded Toluidine Blue O—A summary of the absorption maxima for toluidine blue O, its isolated fractions, and some related dyes is given in Table IV.

Spectra of toluidine blue O at different concentrations are shown in Fig. 8. Of interest is the appearance of an absorption maximum at about 590 nm as the concentration of the dye was increased. Figure 8 is in qualitative agreement with work done by Ohkuma and Furuhata (13). This secondary absorption maximum was reported as being due to dimerization of the dye molecules in solution (14). At the concentrations studied, toluidine blue O apparently does not follow the Lambert–Beer relationship (*i.e.*, the absorbance is not proportional to the concentration of the dye in a linear fashion). Thionine and methylene blue have been reported to not follow the relationship even at concentrations as low as 10^{-7} *M* (15).

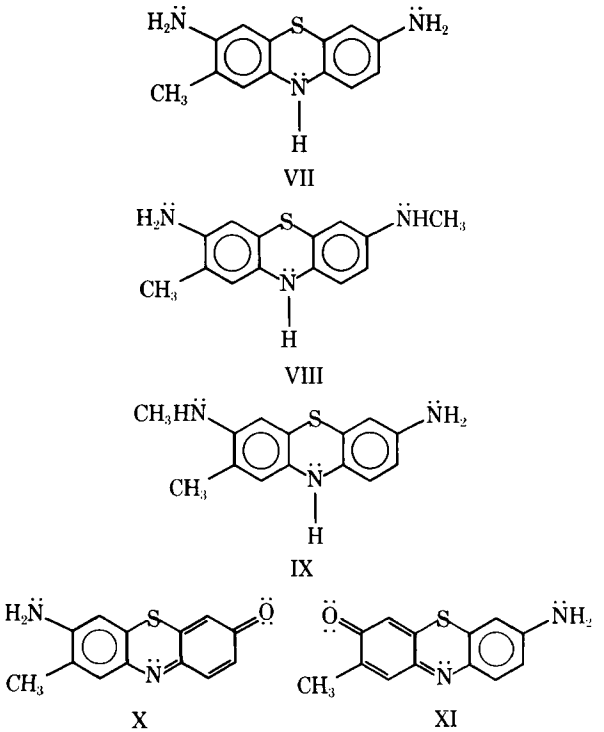
Table IV—Visible Light Absorption Maxima for Toluidine Blue O, Its Isolated Fractions, and Some Related Dyes in Water at pH 3.5

Sample	Absorption Maximum, nm	Concentration, *M*
Commercial methylene blue	652	10^{-4}
Commercial toluidine blue O	634	10^{-5}
Fraction C from commercial dye	638	$\sim 10^{-5}$
Fraction B from commercial dye	618	$\sim 10^{-5}$
Fraction B from degraded dye	613	$\sim 10^{-5}$
Thionine	596	10^{-5}
Fraction A from commercial dye	595	$\sim 10^{-5}$
Fraction A from degraded dye	595	$\sim 10^{-5}$

The mechanism of dimer formation has been discussed previously (15, 16). In dilute aqueous solution, the free energy of dimerization is 4.1 kcal/mole for thionine and 4.9 kcal/mole for methylene blue (15). The association constant has been determined to be about 10^5 for thionine (16).

Interpretation of the Thin-Layer Chromatograms—The data suggest that toluidine blue O and the related dyes are separated on silica gel on the basis of the number of *N*-methyl groups present. Figure 9 gives the chromatographic analysis of the six related dyes. Thionine, which has no *N*-methyl groups and is spectroscopically pure, has the same R_f value as 2-methylthionine (Fraction A) found in toluidine blue O (Fig. 2). 2-Methylthionine also contains no *N*-methyl groups.

Fraction B, isolated from commercial toluidine blue O (R_f 0.59), contains one *N*-methyl group and corresponds (same R_f) to the monomethyl derivative found in azures A and C and, to a lesser extent, azure B. Other bands probably correspond to dimethyl-, trimethyl-, and tetramethylamine derivatives of thionine or 2-methylthionine. The two bands identified as corresponding to dimethylamino derivatives presumably arise from the situation where two methyls are on the same amino group for one compound and one methyl is on each amino group for the other. Since toluidine blue O has both methyls on one amino group, the band at R_f 0.50 probably corresponds to the latter possibility.



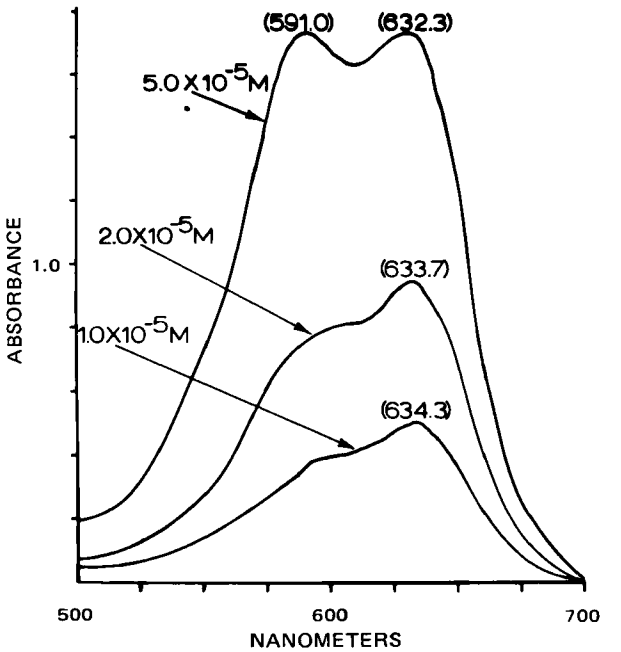


Figure 8—*Visible light absorption spectra of commercial toluidine blue O at various concentrations in water at pH 3.5.*

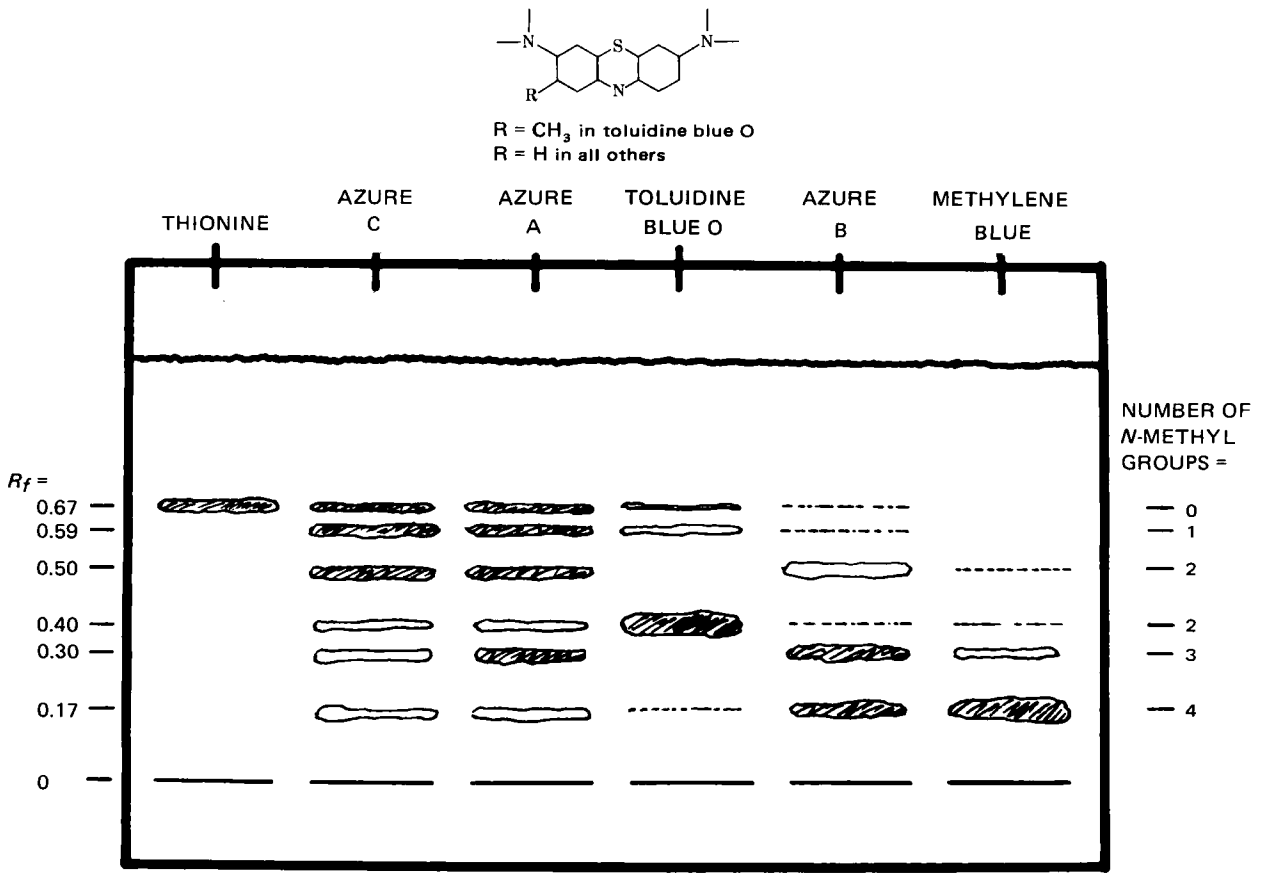


Figure 9—*Thin-layer chromatogram of related phenothiazine dyes [silica gel, ethanol–concentrated hydrochloric acid (99:1 v/v)]. Shaded areas are visibly darker than others. Dotted lines indicate spots that are extremely faint.*

Although the chromatographic correlations mentioned are tentative, they were arrived at by the known purity of thionine, the mass spectral identification of the fractions isolated from commercial toluidine blue O, and the visible light absorption experiments presented here. This system should prove satisfactory for the analysis of the purity of any of the dyes discussed.

Demethylation is known to occur for methylene blue and toluidine blue O by photoreduction (17, 18). It has also been reported that demethylation of methylene blue occurs under basic conditions (19, 20); however, the dye was stable below pH 9.5. Demethylation started between pH 9.5 and 9.8. Azure B was found to undergo demethylation at lower pH values than methylene blue.

As can be seen from Table IV, loss of an *N*-methyl group leads to an approximate 10–20-nm shift in the visible light absorption maximum. The identification of azure A as a contaminant in commercially prepared toluidine blue O-^{3}H at λ_{max} 632 nm by Normann and Normann (7) is undoubtedly an error. The identification of toluidine blue O at λ_{max} 616 nm by Normann and Normann (7) was also consistent with the data presented by Patel (5) and Apgar and Patel (6). Ball and Jackson (9) noted that chromatography of commercial samples of toluidine blue O on aluminum oxide yielded material that gave a rather broad absorption maximum from 590 to 625 nm. These investigators eluted the dye from the column and dried the sample on a water bath at 100°. This experimental technique probably produced a mixture of 2-methylthionine (λ_{max} 595 nm), normethyl-toluidine blue (λ_{max} 613 nm), and toluidine blue O (λ_{max} 638 nm).

Due care must be exercised when one is handling these dyes. The conditions of heat (especially at an alkaline pH) and light must be avoided to assure that demethylation is held to a minimum.

The mass spectral studies of toluidine blue O, isolated from commercial sample by chromatography, still exhibit evidence of a higher homolog at M + 14 (Figs. 4 and 5). Since mass spectral analysis of normethyl-toluidine blue O indicates no higher order homologs (Fig. 6), the possibility of intermolecular methyl transfer taking place in the high temperature source of the mass spectrometer probably is remote. Table III indicates that commercial toluidine blue O contains a small amount of, presumably, a tetramethylated derivative. This fraction could not be separated from the band corresponding to toluidine blue O by column chromatography; consequently, higher order homologs are seen in the mass spectrometer.

SUMMARY

Commercial toluidine blue O has been shown to be a mixture of the normethyl derivative and 2-methylthionine. The synthetic environment used to generate the dye is responsible for a small portion of the contaminants. Heat and alkaline conditions can result in rapid degradation of the parent dye molecule into its demethylated derivatives. Observations made in the literature can be explained on the basis of this demethylation phenomenon. Visible light absorption spectroscopy is a convenient device for estimation of the dyes but is prone to misinterpretation if the purity of the dye is unknown and the deviation from the Lambert–Beer relationship is ignored.

A TLC system has been presented which should prove useful in the estimation of the purity of certain phenothiazine dyes.

REFERENCES

(1) R. M. Yeager and E. T. Krementz, *Ann. Surg.*, **169**, 829(1969).

(2) W. Di Giulio and S. M. Lindenauer, *J. Amer. Med. Ass.*, **214**, 230(1970).

(3) D. P. Shedd and J. F. Gaeta, *Arch. Surg.*, **102**, 442(1971).

(4) R. A. Mortenson and J. McRae, *ibid.*, **100**, 710(1970).

(5) J. Patel, *Amer. J. Hosp. Pharm.*, **26**, 540(1969).

(6) D. A. Apgar and J. Patel, *ibid.*, **26**, 541(1969).

(7) E. Normann and T. Normann, *Scand. J. Clin. Lab. Invest.*, **29**, 403(1972).

(8) H. J. Conn, *Stain Technol.*, **2**, 33(1927); *Ibid.*, **2**, 35(1927).
(9) J. Ball and D. S. Jackson, *Stain Technol.*, **28**, 33(1953).
(10) H. Budzikiewicz, C. Djerassi, and D. H. Williams, "Mass Spectrometry of Organic Compounds," Holden-Day, San Francisco, Calif., 1967, p. 23.
(11) E. Gurr, "Synthetic Dyes in Biology, Medicine and Chemistry," Academic, New York, N.Y., 1971, pp. 49, 58, 72.
(12) J. N. T. Gilbert and B. J. Millard, *Org. Mass Spectrom.*, **2**, 17(1969).
(13) S. Ohkuma and T. Furuhata, *Proc. Jap. Acad.*, **47**, 689(1971).
(14) K. Bergman and C. T. O'Konski, *J. Phys. Chem.*, **67**, 2169(1963).
(15) E. Rabinovitch and L. F. Epstein, *J. Amer. Chem. Soc.*, **63**, 69(1941).
(16) G. R. Haugen and E. R. Hardwick, *J. Phys. Chem.*, **67**, 725(1963).
(17) S. Kato, M. Morita, and M. Koizumi, *Bull. Chem. Soc. Jap.*, **37**, 117(1964).
(18) M. Koizumi, H. Obata, and S. Hayashi, *ibid.*, **37**, 108(1964).
(19) W. C. Holmes and E. F. Snyder, *Stain Technol.*, **4**, 7(1929).
(20) E. Gurr, "Synthetic Dyes in Biology, Medicine and Chemistry," Academic, New York, N.Y., 1971, p. 77.

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Inhibition of Synaptosomal Accumulation of *l*-Norepinephrine I: *N*-Arylalkyl and *N*-Aryloxyalkyl *dl*-Amphetamines and Related Compounds

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Abstract □ The ability of a group of systematically modified amphetamines to inhibit the accumulation of *l*-norepinephrine by nonstriatal synaptosomes was investigated. *N*-Substitution by the proper bulky hydrophobic groups can be well tolerated. Structure–activity relationships generate a qualitative picture of the inhibitor–carrier interaction site.

Keyphrases □ *l*-Norepinephrine accumulation by nonstriatal synaptosomes—inhibition by *N*-arylalkyl- and *N*-aryloxyalkyl-substituted amphetamines □ Amphetamines, *N*-substituted—inhibition of synaptosomal accumulation of *l*-norepinephrine □ Structure–activity relationships—*N*-substituted amphetamine inhibition of synaptosomal accumulation of *l*-norepinephrine

A catecholamine recycling mechanism located at the presynaptic neuronal membrane is thought to play a major role in the termination of noradrenergic nerve transmission and in the conservation of catecholamines (1–4). Antidepressant and stimulant drugs, such as desipramine (5), cocaine (6), and amphetamine (7), have been shown to inhibit this carrier-mediated transport process *in vitro*. Thus, at least part of their pharmacological action is believed to be derived from their ability to prolong effective catecholamine concentrations in the synaptic cleft and, therefore, at the postsynaptic receptors.

On the basis of kinetic (8) and differential inhibition (5, 9) data, it has been proposed that there are at least two different, anatomically separable, neuronal transport mechanisms for the catecholamines in the central nervous system (CNS). One is associated with the hypothalamus, cerebral cortex, midbrain, and medulla oblongata-pons, where *l*-norepinephrine is the major catecholamine present; the other is found in the corpus striatum, where dopamine is thought to be the important catecholamine transmitter. A wide range of tricyclic, sympathomimetic, anticholinergic, and antihistamine drugs (5, 9–11) have been shown to inhibit the *in vitro* uptake of catecholamines by both systems. However, few of these compounds exhibited a substantial preference for one of the transport processes. Even when selective inhibition was observed, as with desipramine, the less active nonstriatal process was usually the most susceptable to blockade.

In light of this evidence, a study was initiated to elucidate the differences and similarities of the two catecholamine uptake processes in the CNS by attempting to map the topography of the different carrier sites through the use of a series of amphetamine-related inhibitors. Structural features, which take advantage of the topographical variances thus uncovered, would then be integrated into the design of new inhibitors to increase specificity.

This report describes the effects of a group of amphetamine-related compounds on the uptake of *l*-norepinephrine by a synaptosome-enriched subcellular fraction from nonstriatal rat brain tissue. First,