Pharmacokinetics of Highly Ionized Drugs I: Methylene Blue—Whole Blood, Urine, and Tissue Assays

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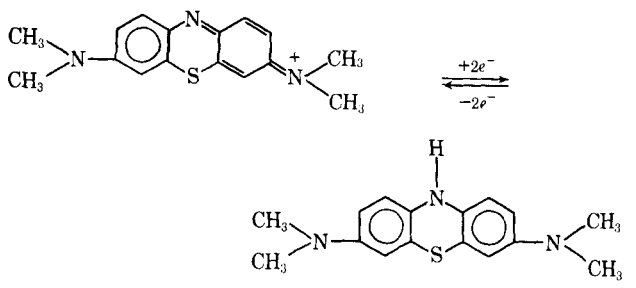
Abstract □ UV spectrophotometric assays which measure both unchanged methylene blue and leucomethylene blue in whole blood and tissues are reported. The major metabolite of methylene blue that appears in urine following oral administration of methylene blue to man was identified as leucomethylene blue stabilized as some salt, complex, or combination form. A UV spectrophotometric assay which allows measurement of both unchanged methylene blue and leucomethylene blue in urine is reported.

Keyphrases □ Pharmacokinetics of highly ionized drugs—UV assay for methylene blue and leucomethylene blue in blood, urine, tissue □ Methylene blue and leucomethylene blue—UV assay in blood, urine, tissue □ Leucomethylene blue, as major methylene blue metabolite—UV assay in blood, urine, tissue □ UV spectrophotometry—assay, methylene blue and leucomethylene blue in blood, urine, tissue

Methylene blue was the first dye to be used as an antiseptic (1) and the first antiseptic dye to be used therapeutically (2). An important therapeutic use is in the treatment of methemoglobinemia, where methylene blue increases the conversion of methemoglobin to hemoglobin (1–3). Current uses of methylene blue include: (*a*) diagnosis of corneal amyloidisis (4), (*b*) detection of *Brucella abortus* in fresh cream (5), (*c*) contrast media in abdominal surgery (6), (*d*) vital stain for specialized tissues of the heart (7), (*e*) facilitation of parotid gland surgery by intraductal installation (8), (*f*) detection of ischemic areas of the heart at operation (9), (*g*) determination of ureteral patency at operation (10), (*h*) vital stain for cornea and conjunctiva (11, 12), (*i*) determination of the viability of muscle after wounding (13), (*j*) determination of sweat pore patterns (14), (*k*) determination of *Pasteurella pestis* (15), (*l*) diagnosis of xanthemalosis (16), (*m*) diagnosis of bacteriuria of pregnancy (17, 18), (*n*) diagnosis of tinea versicolor (19), (*o*) distinction between cancerous and normal tissue (20), and (*p*) detoxification of snake venoms *in vitro* (21–23).

Methylene blue has been used extensively in biological oxidations as an electron acceptor. Its value here lies in the fact that it is colored in its oxidized state (methylene blue) and colorless when reduced (leucomethylene blue). This oxidation–reduction reaction may be represented as in Scheme I. For example, methylene blue acts as an electron acceptor for the oxidation of reduced tri- and diphosphopyridine nucleotides in both aerobic and anaerobic pathways of glucose metabolism (24–26).



Scheme I

In alkaline solutions, methylene blue is stepwise demethylated to other common dyes, namely, trimethylthionine (azure B), dimethylthionine (azure A), and monomethylthionine (azure C) (27–33).

The metabolism and elimination of methylene blue in living organisms were the subjects of many investigations (34–38) in the late 1890's and early 1900's, but since that time very little has been reported. These early investigators did indicate that methylene blue was reduced *in vivo* and eliminated in unchanged form as well as in its leuco-form and as one or more "chromogenic" substances in the urine. These early investigations did not consider the stepwise demethylation of methylene blue in the presence of alkali. Also, at that time, commercial methylene blue was not as pure as in the current product that passes the USP XVIII specifications.

This report describes the assay procedures employed in biological studies (39, 40). It was found that both methylene blue and leucomethylene blue are excreted in human urine following oral administration of methylene blue. Since the leucomethylene blue present in human urine is stabilized as some salt, complex, or combination form, it was possible to assay both methylene blue and leucomethylene blue in human urine. However, in whole blood and tissue the same type of stabilization apparently was not present and only the total of both the methylene blue and leucomethylene blue could be determined. However, in both whole blood and tissue the conversion of methylene blue to leucomethylene blue is extremely rapid; in this case, measurement of the reduction product, leucomethylene blue, is analogous to the usual pharmacokinetic procedure of measuring "unchanged drug."

EXPERIMENTAL[1]

Materials—Methylene blue[2] USP was used as received after purity was verified by the USP tests and TLC. All chemicals and solvents were reagent grade.

Preliminary Investigations—*Common Ion and Ionic Strength Effects*—In the development of the assay methodology for methylene blue in various biological fluids, it was noted that there were both common ion and ionic strength effects on the molar absorptivity of aqueous solutions of methylene blue. The spectrum of aqueous

[1] A Beckman DB spectrophotometer was used to obtain all UV spectra. All Beer's law determinations and assays were performed with a Gilford 2400 spectrophotometer, using a 1-cm. or 10-cm. pathlength cell. Either a Clay Adams Dynac or Sorvall superspeed centrifuge was used for centrifugation steps in the assays. A Sorvall Omnimixer was used for homogenization of tissues.

[2] Fisher Scientific.

methylene blue exhibited maximum absorbance at 660 nm. in the visible region and at 290 nm. in the UV region on the spectrophotometer. The addition of various concentrations of NaCl, up to 2 *M*, did not have any effect on $\lambda_{max.}$ in either the visible or UV region but caused significant fluctuation in the intensity of absorption at the $\lambda_{max.}$ (*i.e.*, the absorptivity). The greatest increase in the absorptivity occurred at 0.125 *M* NaCl in both the visible and UV regions. Figure 1 shows the effect of various concentrations of sodium chloride on the molar absorptivity of methylene blue.

To distinguish common ion effects from ionic strength effects, $NaNO_3$ was employed. Again, no shift in wavelength of maximum absorption occurred. The fluctuation in the absorptivity obtained with $NaNO_3$ exhibited changes somewhat similar to those observed with NaCl. However, the magnitude of these changes in absorptivity were approximately one-half those obtained with NaCl. The greatest increase in the molar absorptivity occurred at 0.5 *M* $NaNO_3$. The changes observed in the absorptivity at 660 nm. indicate how serious errors may result if methylene blue is assayed spectrophotometrically directly in urine.

Partitioning Studies—Methylene blue in dilute aqueous solution will not partition into an equal volume of either chloroform or 1,2-dichloroethane[3]. If urine is spiked with methylene blue, the methylene blue transfers to the organic phase after gentle agitation. That an aqueous solution of methylene blue does not partition or partitions to a minor degree in chloroform was also observed by Mukerjee and Ghosh (41). This effect was first observed with chloroform, and it appeared of interest to see what was causing the methylene blue to enter easily an organic system from urine and not from water. The urinary constituents, urea, creatinine, oxalic acid, and uric acid, were added individually to aqueous solutions of methylene blue, and partitioning into chloroform was attempted. None of these urinary constituents caused any visible partitioning of methylene blue into the chloroform layer. Chloride ion is also a common urinary constituent and its average concentration in the urine, expressed as sodium chloride, ranges from 6.7 to 10 mg./ml. The addition of sodium chloride to an aqueous solution of methylene blue (5 mg./ml.) caused a rapid partitioning of methylene blue into chloroform or 1,2-dichloroethane. Qualitatively, it was shown that this partitioning into 1,2-dichloroethane occurred also when sodium bromide, potassium iodide, and sodium fluoride were added to the aqueous phase. Trichloroacetic acid and sodium taurocholate[4] in very small amounts also caused rapid and efficient partitioning of methylene blue into 1,2-dichloroethane.

Methylene blue solutions, 10 mcg./ml., were made up in various sodium chloride concentrations and extracted with an equal volume of 1,2-dichloroethane. The organic layer was then assayed on a spectrophotometer at the $\lambda_{max.}$ for methylene blue in 1,2-dichloroethane (660 nm.). A plot (Fig. 2) of the absorbance of the methylene blue–1,2-dichloroethane extract *versus* sodium chloride shows that an asymptotic value is reached at approximately 2.75 *M* sodium chloride. This is 161 mg./ml. sodium chloride and far exceeds the amount of sodium chloride normally occurring in urine. Possibly, some other urinary constituent(s) is(are) also contributing to the partitioning effect of methylene blue urine solutions into organic solvents.

Whole Blood Assay—Originally, an assay method was developed for methylene blue in plasma. This should not be used since methylene blue binds avidly to red blood cells. Hence, a whole blood assay is necessary. Several studies of human whole blood spiked with methylene blue indicated that approximately 40–50% of the spiked amount of methylene blue was accounted for when only the plasma was assayed. The remainder was presumably bound to red blood cells.

The assay methodology was developed on outdated human whole blood, but Beer's law plots were obtained from dog whole blood because the dog was the experimental animal used in the *in vivo* studies.

Experiments using heparin as the anticoagulant showed decreased extraction efficiency when compared to whole blood treated with citrated dextrose. The Beer's law slope obtained from heparinized dog blood was significantly lower than the Beer's law slope obtained from citrated whole dog blood. This suggests that heparin apparently binds with the methylene blue and hinders its extraction into 1,2-dichloroethane.

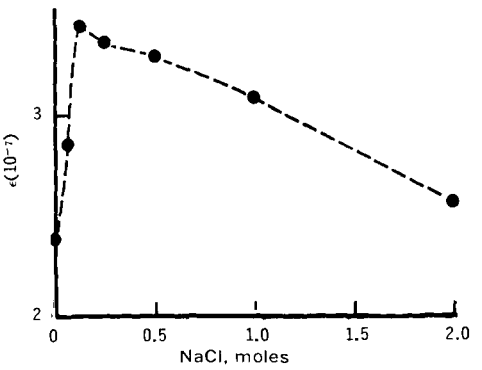


Figure 1—*Effect of sodium chloride on the molar absorptivity of methylene blue in distilled water.*

The addition of sodium chloride to whole blood served a dual function. It caused the hemolysis of the red blood cells, liberating the bound methylene blue, and facilitated the partitioning of methylene blue into 1,2-dichloroethane.

Urine Assay—Methylene blue is excreted in the urine as unchanged drug and as leucomethylene blue stabilized in some salt, complex, or combination form. The assay procedure is based on the fact that this "bound" leucomethylene blue will not extract into 1,2-dichloroethane unless it is first cleaved and that the unchanged methylene blue extracts readily from urine into 1,2-dichloroethane.

Identification of Metabolite—The following experiment was performed to show that the "unchanged drug" in the urine was methylene blue and that the other major component was stabilized leucomethylene blue, which is converted to methylene blue in the assay. A representative urine sample from a subject who had been administered 10 mg. of methylene blue orally was subjected to three treatments and assayed spectrophotometrically. Scans were taken from 200 to 700 nm. and R_f values from TLC were obtained. The treatments were as follows:

I. Five milliliters of the urine sample was extracted with 1,2-dichloroethane, and the absorbance at the $\lambda_{max.}$ was measured.

II. Another 5 ml. of the urine sample was treated with 0.1 ml. of 5 *N* HCl. The tube was placed in a boiling water bath for 2 min.; then the aqueous phase was extracted with 1,2-dichloroethane and the absorbance of the extract at $\lambda_{max.}$ was measured.

III. The urine layer from Treatment I was treated with hydrochloric acid, heated as usual, and extracted with 1,2-dichloroethane. Then the absorbance of the extract at the $\lambda_{max.}$ was measured.

Scans of the 1,2-dichloroethane extracts from the three treatments were all identical in the visible region. The UV region (200–360 nm.) was obliterated in each case, most likely due to extraction of various organic-soluble urine constituents. Freshly voided urine,

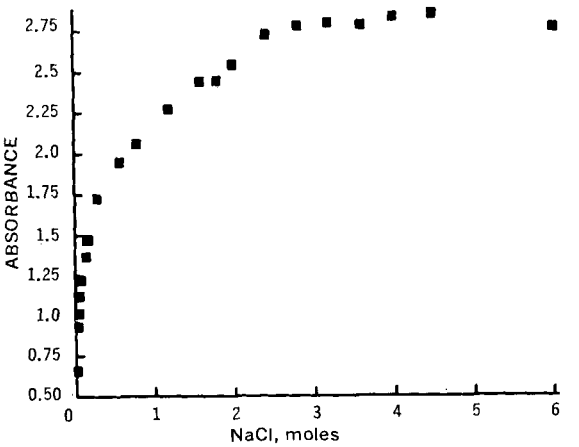


Figure 2—*Absorbances of 1,2-dichloroethane extracts of various aqueous solutions of methylene blue containing different concentrations of sodium chloride.*

[3] Matheson, Coleman and Bell.

[4] Pure sodium taurocholate was generously supplied by T. Parkinson, The Upjohn Co., Kalamazoo, Mich.

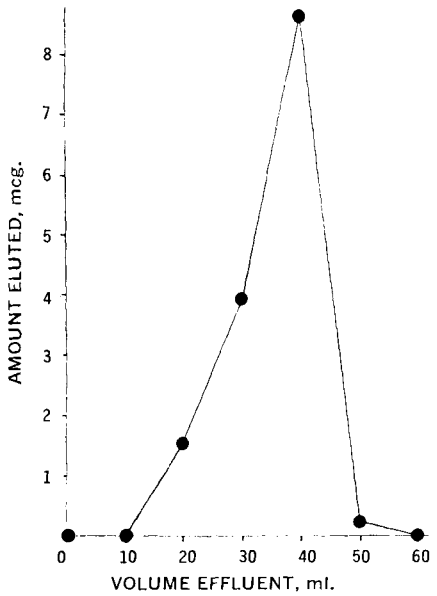


Figure 3—*Separation of leucomethylene blue from urine on a column of cation-exchange resin.*

spiked with methylene blue and treated as described, gave UV spectra identical to the urine of the dosed subject. Treatment II gave the total absorbance of methylene blue in the urine as 0.390. The absorbance values for Treatments I and III were 0.245 and 0.155, respectively. The absorbance of Treatment I plus absorbance of Treatment III should equal the absorbance of Treatment II, *i.e.*, 0.245 + 0.155 = 0.400, and agrees quite well with the observed value of 0.390.

Although purity of methylene blue was determined on Eastman 6060 chromatograms, these were not satisfactory for 1,2-dichloroethane extracts of urine. Thin-layer plates were prepared with silica gel GF_{254}. A 35% w/v slurry was prepared, and plates were activated in a dry oven at 110° 1 hr. before use. The solvent system was butanol–acetic acid–water (4:1:5), and the upper organic layer was used after allowing it to settle for 24 hr. The extracts from the previous spectrophotometric experiment were used for this thin-layer work. These extracts were taken to dryness under nitrogen and made up to a 1-ml. volume with 1,2-dichloroethane. Ten microliters of each sample was spotted on the same plate and developed for several hours. The following R_f values were obtained: spiked urine, 0.17; Treatment I, 0.16; Treatment II, 0.16; and Treatment III, 0.16. Each plate was examined under UV light and showed other spots of varying intensity occurring approximately at the same R_f values as the control spiked sample.

This TLC experiment, coupled with the spectrophotometric experiments, supports that "the leuco-compound" in the urine after oral administration is stabilized leucomethylene blue and that after treatment with acid and heat and extraction into 1,2-dichloroethane, this stabilized compound is converted to leucomethylene blue and then to methylene blue. These experiments also showed that the drug assayed as unchanged methylene blue is indeed methylene blue.

Separation of Stabilized Leucomethylene Blue from Urine—No solvent was found that would preferentially extract only stabilized leucomethylene blue from urine; for this reason, column chromatography was attempted. Separation was accomplished with a cation-exchange polymer[5]. A 3.5 × 15-cm. column was prepared and allowed to settle for 1 day before use. A urine sample containing appreciable amounts of leucomethylene blue and methylene blue was selected for the experiment. A 10-ml. portion of this urine sample was added directly to the column and allowed to reach the top before elution with distilled water. The eluent was collected in 10-ml. volumes. The unchanged methylene blue in the urine bound immediately to the upper portion of the column, and a brownish-yellow zone could be seen separating down the column. The leucomethylene blue zone could be eluted with 2 *M* sodium chloride solution. The aqueous effluents were scanned spectrophotometrically and gave no distinct absorbance in the visible area; the UV region was completely obliterated. When these aqueous effluents were treated with acid and heat and extracted with 1,2-dichloroethane, the leuco-compound was converted to methylene blue.

Figure 3 shows the elution profile of leucomethylene blue from urine. The unchanged methylene blue was not eluted from the column because the samples became quite dilute and difficult to assay. The upper portion (3 cm.) of the column was removed and added to 15 ml. of 2 *M* NaCl, agitated, and centrifuged. The supernate was then made up to 20 ml., and a 5-ml. portion was assayed with and without acid and heat treatment. There was no difference in the absorbance values of either sample, so only unchanged methylene blue was bound to this upper portion of the column. The urine sample, which assayed 1.85 mcg./ml., or a total of 18.5 mcg., was added to the column as methylene blue and stabilized leucomethylene blue. Methylene blue recovered as unchanged methylene blue and leucomethylene blue assayed 4.53 and 14.3 mcg., respectively, giving 18.8 mcg. recovered, or 102%, which is within assay error.

In an attempt to identify what constituent stabilized leucomethylene blue, TLC and paper electrophoresis were carried out on the separated leucomethylene blue samples. This was not successful, because in each case numerous urinary constituents made interpretation futile. The problem as to with what leucomethylene is combined, bound, or complexed is left unsolved at this stage, since more sophisticated means of detection appear necessary. The conducted experiments clearly show that stabilized leucomethylene blue is the major metabolite occurring in urine after oral administration and that it can be assayed as methylene blue as discussed.

Assay Methods—*Whole Blood*—Five milliliters of whole blood sample was placed into a 15-ml. centrifuge tube; 400–600 mg. of sodium chloride was added, and the contents were mixed for 2–3 min. on a rotator. The centrifuge tubes were stoppered with rubber bungs encased with aluminum foil. Seven milliliters of 1,2-dichloroethane was then added, and the contents were mixed on a rotator for 15 min. and centrifuged for 15 min. at 2500 r.p.m. The upper "blood"

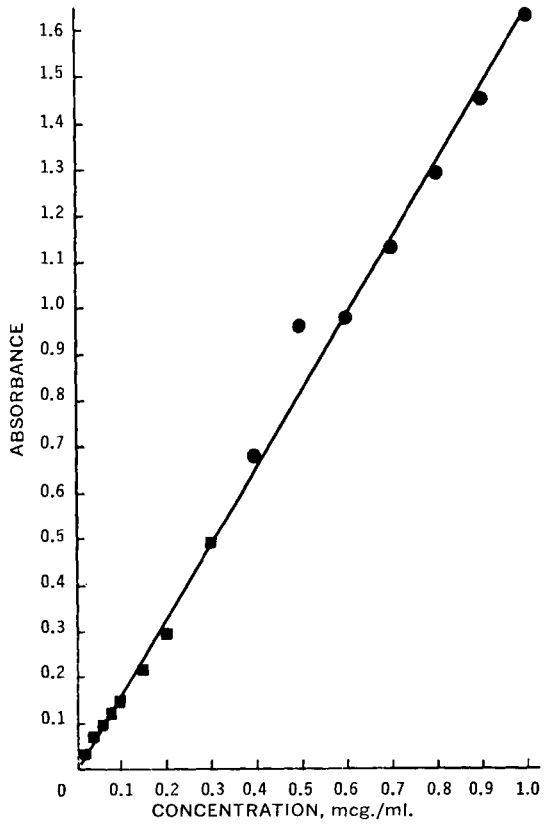


Figure 4—*Beer's law plot of absorbance* versus *concentration of methylene blue for human whole blood. Key:* ■, *10-cm. cell; and* ●, *1-cm. cell.*

[5] CM-Sephadex C-25, Sephadex, Pharmacia Fine Chemicals, Inc., Piscataway, N. J.

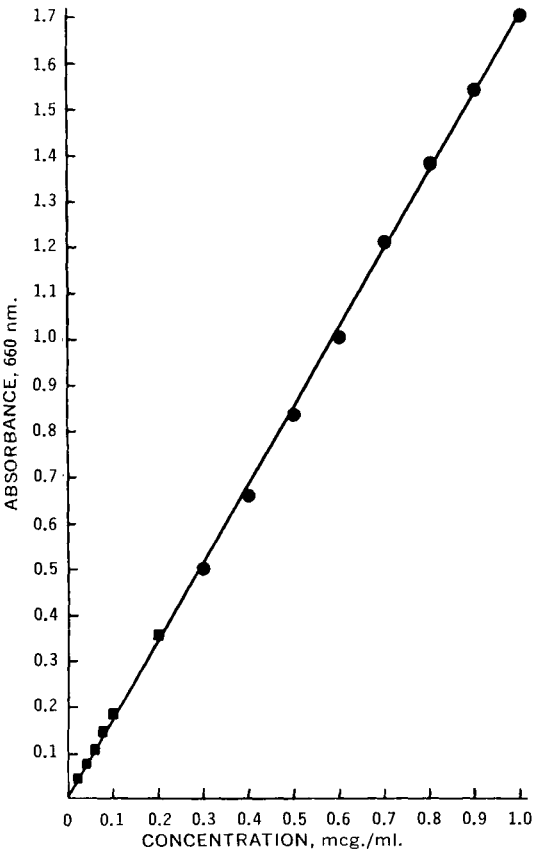


Figure 5—*Beer's law plot of absorbance* versus *concentration of methylene blue for human urine. Key:* ■, *10-cm. cell; and* ●, *1-cm. cell.*

layer was removed and a small hole was made through the plug to obtain the 1,2-dichloroethane extract by disposable pipets. The absorbance of the 1,2-dichloroethane extract was then determined at 660 nm. The Beer's law plot for whole blood is shown in Fig. 4.

Urine—A 5-ml. portion of the urine sample was added to a centrifuge tube containing 300 mg. sodium chloride; 7 ml. of 1,2-dichloroethane was added, and the contents were mixed on a rotator for 15 min. and then centrifuged for 10 min. at 2000 r.p.m. The upper urine layer was removed and the absorbance of the 1,2-dichloroethane extract was determined at 660 nm. This procedure gave the amount of unchanged methylene blue excreted in the urine. The Beer's law plot is shown in Fig. 5.

Next, 300 mg. sodium chloride and 0.1 ml. of 5 *N* hydrochloric acid were added to another 5-ml. portion of the urine sample in a centrifuge tube and the contents were mixed on a cyclomixer. The tube was then placed in a hot water bath for 2–3 min. and allowed to cool. Then 7 ml. 1,2-dichloroethane was added, the contents were mixed on a rotator for 15 min., centrifuged for 10 min. at 2000 r.p.m., and assayed spectrophotometrically at 660 nm. This procedure gave the total amount of methylene blue excreted in the urine as both unchanged and "stabilized" leucomethylene blue. The amount of methylene blue excreted in the urine as stabilized leucomethylene blue was found by difference (*i.e.*, total minus unchanged methylene blue).

Tissue—The desired organs (tissues) were removed from the rats, blotted on filter paper, and weighed[6]. A 5% w/v homogenate was made with 0.1 *N* hydrochloric acid and homogenized at maximum speed for 3 min.[7]. Fifteen milliliters of the homogenate was then added to a Corex centrifuge tube and centrifuged at 20,000 r.p.m. at 0–5° for 1 hr. To 5 ml. of the supernate were added 600 mg. of sodium chloride and 7 ml. of 1,2-dichloroethane; this was mixed on a rotator for 25–30 min. and then centrifuged at 2000 r.p.m. for 10 min. The 1,2-dichloroethane extract was then assayed as already described.

The plug which resulted after centrifugation of the homogenate at 20,000 r.p.m was made up to 10 ml. with 0.1 *N* hydrochloric acid and rehomogenized for 3 min. at maximum speed. The supernate from this rehomogenate was then assayed as already described.

The amount of methylene blue taken up by a given tissue was calculated using Eqs. 1–3:

$$\begin{matrix}\text{total amount of}\\ \text{methylene blue}\\ \text{uptake}\end{matrix} = \begin{matrix}\text{amount of}\\ \text{methylene blue}\\ \text{in supernate}\end{matrix} + \begin{matrix}\text{amount of}\\ \text{methylene blue}\\ \text{in plug}\end{matrix} \qquad \text{(Eq. 1)}$$

where:

$$\begin{matrix}\text{amount in}\\ \text{supernate}\end{matrix} = \frac{A\ (660\ \text{nm.})}{\text{Beer's law slope}} \times \text{volume } q.s. \text{ to give 5\% w/v homogenate} \qquad \text{(Eq. 2)}$$

and:

$$\begin{matrix}\text{amount in}\\ \text{plug}\end{matrix} = \frac{A\ (660\ \text{nm.})}{\text{Beer's law slope}} \times \frac{10\ \text{ml.}}{15\ \text{ml.}} \times \text{volume } q.s. \text{ to give 5\% w/v homogenate} \qquad \text{(Eq. 3)}$$

Assay Control—The zero-hour sample of the whole blood and urine was spiked at 0.5 mcg./ml. and assayed with samples. Table I shows that the individual Beer's law slope values obtained when the tissue assays were run were within the 95% confidence intervals of the Beer's law slope values obtained from controls when blood and urine were assayed at different times.

Assay Sensitivity—The assay procedures for methylene blue in whole blood, urine, and tissue all have a sensitivity of 0.02 mcg./ml.

Table I—Control A/C Values[a] Obtained for Whole Blood and Urine Assays

Whole Blood		Urine	
95% Confidence Intervals, Beer's Law Slope, Dose, mg./kg.	0.157–0.171 Beer's Law Slope	Subject	0.168–0.172 Beer's Law Slope
2	0.168	1	0.168
5	0.156	2	0.166
7.5	0.170	3	0.168
10	0.158	4	0.170
15	0.165	5	0.170
Predose	0.160	6	0.172
Oral	0.156	7	0.170

[a] A = absorbance at $\lambda_{max.}$. C = concentration of methylene blue, mcg./ml.

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[6] Correction for the blood content was neglected (see discussion under tissue assay).

[7] With a Sorval Omnimixer.

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Thermal Dissociation of Sulfonylureas II: Dissociation of Four *N*- and *N'*-Substituted Sulfonylureas in Different Media

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Abstract □ *N-n*-Butyl-*N'*-tosylurea (tolbutamide), *N-n*-butyl-*N'*-methyl-*N'*-tosylurea, *N,N*-pentamethylene-*N'*-tosylurea, and *N,N*-pentamethylene-*N'*-methyl-*N'*-tosylurea were heated at different temperatures in a series of solvents, and the reaction products were submitted to quantitative analysis. The outcome of these experiments, which had the aim of elucidating the mechanism by which sulfonylureas undergo a breakdown when heated in reactive solvents, may contribute to a better understanding of the stability of sulfonylureas in pharmaceutical preparations. The results of the reactions in alcoholic media were in good agreement with a mechanism involving dissociation of the urea into isocyanate and amine, followed by reaction of the isocyanate with the alcohol. The behavior of the four sulfonylureas in water was also in good agreement with the dissociation hypothesis. The breakdown of these compounds in amines seems to follow a more complicated pattern, the reactivity being influenced, among other factors, by the basicity of the amine. Although dissociation seems to be operative at the lower temperature, the occurrence of aminolysis at higher temperatures could not be excluded. The present results point to dissociation, rather than solvolysis, as the most likely mechanism by which sulfonylureas undergo breakdown in alcohols and in water, as well as in amines at relatively low temperatures.

Keyphrases □ Sulfonylureas—thermal dissociation in alcohols, water, and amines, mechanism □ Tolbutamide and related sulfonylureas—thermal dissociation in alcohols, water, and amines, mechanism □ Dissociation, thermal, tolbutamide and related sulfonylureas—in alcohols, water, and amines, mechanism

Sulfonylureas, a class to which belong some important oral hypoglycemic agents, are known to undergo a breakdown under various experimental conditions. Although a hydrolytic process is most frequently observed in such decompositions (1–4), cases of thermal dissociation were reported (5, 6). Symmetrically substituted sulfonylureas such as tolbutamide (I) can theoretically yield on dissociation either an alkyl (path *a*) or an arylsulfonyl isocyanate (path *b*), as indicated in Scheme I. A quantitative dissociation of I to *p*-toluenesulfonamide and *n*-butyl isocyanate (path *a*) was recently reported (5) to take place in inert solvents at 160–180°. Evidence for the dissociation of I in alcohols at 80° according to the alternative path *b* was given in the first paper of this series (6). The purpose of the present work was to provide additional information on the