Effect of Daylight on the Reaction of Thiols with Ellman's Reagent, 5,5'-Dithiobis(2-Nitrobenzoic acid)

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The reaction of 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB, Ellman's reagent) with thiols is sensitive to daylight, in particular to ultraviolet radiation at wavelengths around 325 nm. Exposure to light at the absorbance maximum of the yellow product (the thionitrobenzoate ion) at 410 nm had no effect on the reaction. The light-sensitive species is apparently the DTNB, because a spectral-irradiation experiment showed that the wavelength of light that produced the maximum rate of absorbance change coincided with the peak absorbance of DTNB, and it was well separated from the thionitrobenzoate absorbance peak. Ascorbate is ineffective as a stabilizer and can produce an apparent increase in the rate of DTNB destruction. In a practical example we found the light interference to be severe when hydrolysis of propionylthiocholine by plasma cholinesterase (EC 3.1.1.8) was measured after a 20-min incubation. The apparent cholinesterase activity in clear glass or plastic tubes exposed to diffuse daylight could be decreased to 25% of the value obtained for samples in light-excluded tubes. We recommend the reaction be carried out in artificial room light, with total elimination of daylight, because window glass does not sufficiently attenuate 325-nm wavelength irradiation.

Additional Keyphrases: photolysis · cholinesterase · ascorbate · variation, source of
We therefore investigated light interference in DTNB solutions at concentrations typical of those used in assays of cholinesterase, and found that daylight laboratory illumination sufficiently degrades DTNB in solution as to cause a serious decrease in the apparent cholinesterase activity. We recommend that strict exclusion of daylight is essential for reliable results.

Materials and Methods

Phosphate buffer salts were from Fluka AG, Buchs, Switzerland; all other chemicals were from Sigma Chemical Co., St. Louis, MO. Water was distilled and de-ionized. All reactions were carried out in phosphate buffer (67 mmol/L, pH 7.4), with DTNB in concentrations of 70, 140, 210, and 280 μmol/L. In instances where we used ascorbate to investigate the free-radical nature of the decomposition, we added it immediately before initiating each experiment, to give final concentrations of 0.5 to 5.0 mmol/L. For experiments involving a DTNB/TNB mixture, we added cysteine to the DTNB solution to give an absorbance at 410 nm in the range of 0.9 to 1.1. We measured absorbances with either an SP8-100 or an SP6-550 spectrophotometer (Pye-Unicam Ltd., Cambridge CB1 2PX, U.K.). In temperature-controlled experiments, glass reaction tubes were placed on reflective aluminum foil and immersed in 20 to 30 mm of water in a water bath that was fully illuminated through a closed window but with no direct sunlight. Contents of additional tubes were kept dark by wrapping the tubes in at least two layers of thick aluminum foil, and these tubes were placed adjacent to the irradiated tubes. We allowed a 5-min pre-incubation for the solutions to reach thermal stability.

For selective wavelength experiments, we irradiated 1.0 mL of DTNB/TNB solution in absorbance cells (cross section 2 mm × 10 mm) in the sample compartment of an Amino-Bowman spectrophotofluorometer (American Instrument Co., Silver Spring, MD), using a 1-mm entrance slit and a xenon arc lamp as a light source. According to the manufacturer's specifications the intensity of light emerging from the excitation monochromator should vary less than 20% over the range of wavelengths used.

In a practical demonstration of photolytic interference, we used a modification of a plasma cholinesterase method (8), prolonging the original 3-min incubation at 37 °C to 20 min to facilitate working with large batches of samples. The plasma was diluted 61-fold in water, and a 50-μL aliquot was added to 2.5 mL of DTNB in phosphate buffer. We monitored substrate autolysis and noncholinesterase hydrolysis of substrate in blank tubes containing physostigmine. For the tests we assayed five plasma samples, each in duplicate, firstly in a 37 °C bath of clear water in daylight, and secondly under subdued fluorescent lighting with daylight totally excluded. Test reactions were stopped at exactly 20 min by the addition of physostigmine, and the tubes were kept in the dark until their absorbances at 410 nm were measured.

Results

Figure 1 summarizes a typical experiment comparing rates of change in absorbance at 410 nm in tubes with DTNB/TNB in the absence and presence of ascorbate. Little change is noted in tubes kept dark. A steady decrease in absorbance is seen in light-exposed tubes without ascorbate; but ascorbate, far from stabilizing the absorbance of TNB, caused it to increase at least as fast as the decrease seen in light-exposed tubes without added ascorbate. We observed this phenomenon throughout the ascorbate concentration range from 0.5 to 5.0 mmol/L, at DTNB concentrations of 0.14 mmol/L and above, and at 17 and 37 °C. We did note some evidence of thermal decomposition of DTNB, the rate of decrease of 410 nm absorbance of tubes kept dark being slightly greater at 37 °C than at 17 °C.

The lower lines in Figure 2 show that there was no change in TNB concentration when a solution of DTNB was irradiated unless ascorbate was present. To monitor DTNB during this experiment, we added aliquots from all three sets of tubes to excess cysteine solution, to convert the remaining DTNB to TNB, and corrected the results for the preformed TNB. The upper lines in Figure 2 show that the DTNB was destroyed under irradiation, but apparently more rapidly when ascorbate was present. We therefore suggest that ascorbate prevents the re-formation of DTNB from its photolytic fission products.

The relative rates of change in absorbance at 410 nm of a DTNB/TNB mixture with added ascorbate were 0.0062, 0.0071, 0.0086, and 0.0096 A/min for initial DTNB concentrations of 40, 60, 110, and 250 μmol/L, respectively. These results, the means of triplicate estimates each over 5 min, show that the rate of change of TNB concentration increased with DTNB concentration, albeit nonlinearly.

The spectral absorbance curves for DTNB and TNB are displayed in Figure 3. Also shown is an action spectrum, or plot of the rates of decrease in absorbance of solutions of DTNB/TNB as a function of irradiation wavelength, relative to the rate at 350 nm. The similarity between the action spectrum and the absorbance curve for DTNB establishes that TNB is not susceptible to irradiation damage; rather, it is the parent compound, DTNB, that is affected.

Light-exposed and light-excluded hydrolysis of propionylthiocholine by plasma cholinesterase (Figure 4) showed that not only was there a relative decrease in the test absorbances, there was also an increase in the blanks when the tubes were exposed. There was also poorer precision, and the apparent activities in the exposed tubes ranged from 78% down to 25% of those in the light-excluded tubes.

Fig. 1. The absorbance at 410 nm when DTNB/TNB mixtures were irradiated with diffuse daylight, at 17 °C (left) and 37 °C (right) The upper lines show the effect of ascorbate addition (ASC), the middle lines are for solutions kept dark (DARK), and the lower lines are for light-exposed (EXP) ascorbate-free solutions.
Discussion

Disulfides are photolyzed readily in aqueous solution (6), and we have demonstrated that DTNB is a typical example of this, whereas TNB is relatively stable. The effect is much less under fluorescent light, which suggests that such light sources have little intensity around 325 nm in comparison with daylight. Exposure to direct sunlight leads to virtual bleaching of color from the cholinesterase hydrolysis tubes by 20 min—qualitative evidence that DTNB photolysis increases with light intensity. The extent of photolysis is also related to DTNB concentration, although our attempt to characterize the relationship was hindered by the strong molar absorptivity of DTNB: ε = 18,000 at 325 nm (2).

Details of the reaction mechanisms of disulfide photolysis are not fully understood (6). However, light fission of dithiols produces thyl radicals (5). In the absence of ascorbate, interaction between thyl radicals would largely result in the re-formation of DTNB. However, radical scavenging by ascorbate would protect the TNB already formed, but not prevent photolysis of DTNB. Possibly propionylthiocholine reacts with DTNB photolysis products, which might explain the high blanks in the plasma cholinesterase hydrolysis experiment; it is similarly possible that intra-chain cholinesterase dithiols may be susceptible to increased disruption.

Sensitivity of DTNB to light has clear practical consequences. We have demonstrated that it is a potentially serious interference in lengthy incubation as, for example, in batch methods for plasma cholinesterase, including the original Ellman method, in which 15–20 min or longer incubations were suggested (4). Similarly, estimations of protein thiols will be inaccurate if the samples are not protected from light. In addition, other dithiol chromogens (e.g., dithiopyridyl) are likely to be similarly susceptible at their respective wavelengths of peak absorbance.

The obvious way to eliminate the photolysis of DTNB is to exclude daylight. Our experience confirms another report (8) that DTNB solutions stored refrigerated in brown-glass bottles are stable for several weeks or months. We recommend that DTNB solutions be used only under subdued artificial lighting, or alternatively, that total light shields be placed around automatic analyzers if reaction cuvettes are exposed during any part of the hydrolysis incubation.

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Sensitivity of the Direct Oxalate Oxidase Assay of Urinary Oxalate Improved

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The direct colorimetric method for urinary oxalate has been modified to improve its sensitivity. Oxalate is precipitated overnight with calcium chloride and ethanol, the precipitate is redissolved, and the oxalate is measured by use of oxalate oxidase (EC 1.2.3.4), methylbenzothiazolone hydrazone, and dimethylaniline. The color developed is more intense, analytical recovery averages 102%, and the overall imprecision is <5%. To assess the accuracy of the method, we used a gas-chromatography comparison method and control sera. Interference from ascorbate is negligible. The modified method retains its simplicity and is less expensive.

Additional Keyphrases: enzymatic method · colorimetry · gas chromatography compared

Our previously reported direct colorimetric method for measurement of urinary oxalate with use of commercial oxalate oxidase (1), although simple, lacks sensitivity at the oxalate concentration usually encountered in normal urines and is inaccurate if the ascorbate concentration in the urine is high. We have improved the sensitivity of the assay by including an overnight precipitation step, which also decreases interference due to ascorbate.

Materials and Methods

Reagents

3-Methyl-2-benzothiazolone hydrazone (MBTH) and \(N,N\)-dimethylaniline (DMA) (both from Sigma Chemical Co., St. Louis, MO 63178) were stored at 4 °C at respective 3.0 and 2.5 g/L concentrations in 0.1 mol/L HCl. Peroxidase (from horseradish), Grade 1 suspension in ammonium sulfate, 2500 kU/L (cat. no. 108073) and oxalate oxidase (from barley seedling), 10 U/vial (cat. no. 567698) were both purchased from Boehhringer Mannheim Australia, Pty. Ltd., Adelaide, SA 5063.

Dissolve the contents of a 10-U vial of the oxalate oxidase in 1.0 mL of glass-distilled water and store in 0.1-mL portions at −20 °C; prepare working enzyme solution by dissolving the frozen aliquot in 1.0 mL of glass-distilled water. Surplus working enzyme solution can be refrozen.

Prepare bromcresol-purple indicator by dissolving 50 mg of the dye in 50 mL of ethanol.

Prepare citrate buffer (100 mmol/L, pH 4.4) by dissolving 5.2 g of citric acid monobydrate and 7.43 g of sodium citrate dihydrate in 500 mL of water; store at 4 °C.

Prepare 0.3, 0.6, and 0.9 mmol/L oxalic acid standards in 0.1 mol/L HCl; these standards are stable for several months at 4 °C.

Prepare color reagent just before assay. The following proportions are suitable for assays involving 10 tubes: Add 10 mL of citrate buffer to 0.4 mL of DMA solution and 0.1 mL of MBTH solution, followed by 40 μL of peroxidase and 0.5 mL of working oxalate oxidase solution.

Clean the glass tubes (12 × 100 mm) used for color development by boiling in (e.g.) Deconex 15 PFE detergent (Borer Chemical Ltd., CH 4528 Zuchwil, Switzerland), 150 mL/L, for 5 min; then let cool for 30 min and rinse with tap water and distilled water.

Procedure

The 24-h urine specimen must be collected in acid (2), e.g., 15 mL of concentrated HCl. Then proceed as follows:

Add 1.0 mL of urine or standards to 17 × 110 mm, 10-mL graduated centrifuge tubes, followed by 1.0 mL of aqueous 5 g/L CaCl\(_2\) solution and two drops of indicator. Adjust the pH of each tube to about 6.8 (stable purple color) with NaOH, 0.5 mol/L, added dropwise with constant mixing. Without delay, add ethanol to the 10-mL mark (approximate), seal the tube with Parafilm, and mix by inversion. Keep the tubes at room temperature overnight.

The next day, centrifuge the tubes (1300 × g, 10 min) decant the supernate, and leave the tubes inverted over paper tissue to drain for 2–3 min. Carefully dry the inside of each tube with paper tissue, without touching the precipi-