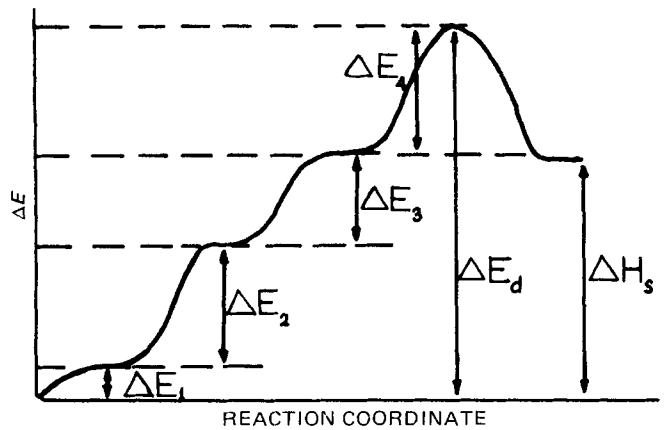

Figure 5—*Energy diagram for the dissolution of I.*

of ΔE_d based on the proposed energy diagram is quite good, considering the errors inherent in the graphical determination of energy values. This finding indicates that the proposed scheme of dissolution should reflect accurately the overall mechanism of the dissolution process. The sum of the energy terms of ΔE_1, ΔE_2, and ΔE_3, all of which are related to the process at the interface between the solid and solvent, is larger than ΔE_4, which is related to the dispersion process. This finding may also indicate that the energy barrier for dissolution of I is the interaction between the solvent and solid at the interface and not the mass transfer of solute into the bulk solution.

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Effect of Laboratory Light on Tetrazolium Reaction and on Stability of Formazans in Various Solvents

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Abstract □ Studies on purified formazans of blue tetrazolium and triphenyltetrazolium showed that ordinary laboratory fluorescent light of about 52-foot-candle intensity provided sufficient energy for phototransformation in certain nonpolar solvents. Slow reoxidation of formazan to tetrazolium was observed in all solvents tested in the presence or absence of light. Water greatly inhibited the oxidation of the red formazan of blue tetrazolium. The blue formazan of blue tetrazolium isomerized to the red formazan in protic solvents in the presence and absence of light. The photochromic phenomena for the blue formazan of blue tetrazolium and the formazan of triphenyltetrazolium in cyclohexane were completely reversible when exposed to alternate periods of intense light and darkness. Under irradiation, the formazans of blue tetrazolium and triphenyltetrazolium in chloroform solution formed their respective photoformazans; darkness reversed the process, and continued exposure of the same solutions for extended times or at a higher light intensity created a solution whose spectrum was no longer reversibly affected by the presence or absence of light. When alcohol USP is used as the solvent for the quantitative determination of corticosteroids, no light precautions need be observed, although reagent blanks have higher absorbances than light-protected solutions. Exact compensation is made by running samples and standards *versus* the reagent blank as recommended by the USP. When other solvents and/or tetrazolium reagents are used, special precautions may be required to keep the reagents, developing solutions, and final formazans protected from light to ensure a quantitative reaction.

Keyphrases □ Tetrazolium reagents—effect of laboratory light, various solvents □ Formazans—of blue tetrazolium and triphenyltetrazolium, stability, effect of laboratory light, various solvents □ Phototransformation—formazans of blue tetrazolium and triphenyltetrazolium, effect of various solvents □ Stability—formazans of blue tetrazolium and triphenyltetrazolium, effect of laboratory light, various solvents

The blue tetrazolium reaction is widely used for the analysis of corticosteroids. USP XIX (1) and NF XIV (2) indicate a slightly modified Mader and Bück procedure (3) for corticosteroid analysis. Blue tetrazolium (I) [3,3′-(3,3′-dimethoxy -4,4′- biphenylylene)bis(2,5-diphenyl-2*H*-tetrazolium chloride)] and triphenyltetrazolium (II) (2,3,5-triphenyl-2*H*-tetrazolium chloride) oxidize the α-keto moiety of the C_{17} side chain in strongly alkaline solution and are reduced quantitatively to highly colored formazans, which are measured spectrophotometrically.

The analytical procedure is subject to several variables such as temperature (4–6), solvent (1–3, 7–13), and concentrations of base (14), water (6, 14), and tetrazolium (6), as well as the steric configuration of the corticosteroid molecule (15). The effect of these variables is minimized by analyzing reagent blanks, standards, and samples concurrently.

Light may or may not influence the tetrazolium reaction. For example, both II (16, 17) and II formazan (10, 12, 16–19) were reported to be light sensitive. Photochemical reoxidation of II formazan to the tetrazolium salt in the presence of intermittent UV irradiation was observed (20). The I formazan was reported (21) to be photosensitive in chloroform solution, and the sensitivity of I to light in 60% chloroform was observed (11) to present problems in the assay. The use of actinic glassware was recommended in

Table I—Effect of Light on Reaction of I with Hydrocortisone

Sample	Net Absorbance at 90 min Light[a]	Dark[b]
1	0.577	0.578
2	0.579	0.579
3	0.580	0.579
4	0.578	0.579
5	0.578	0.577
6	0.578	0.579
7	0.579	0.578
8	0.579	0.576
9	0.579	0.579
10	0.581	0.579
Blank[c] (90 min)	0.066	0.044
Blank[c] (130 min)	0.076	0.051
Mean[d]	0.579	0.578
$SD^{d,e}$	0.001	0.001

[a] Exposed to laboratory fluorescent light of 52 ft-c. [b] Aluminum foil wrapped. [c] Measured against alcohol as reference. [d] Values for blanks are not included in mean and standard deviation figures reported. [e] Calculated by the method of Dean and Dixon (23).

a study of the effect of light and other parameters on the reaction of II with corticosteroids (12). In contrast, several studies (6, 22) found that the reaction of I with corticosteroids was not sensitive to light.

This paper reports the effect of laboratory fluorescent lighting on the compendial (1, 2) reaction of I with corticosteroids. The effect of fluorescent and incandescent light on I and II formazans in various solvents also is discussed.

EXPERIMENTAL

Apparatus—The following were used: UV–visible recording spectrophotometers[1] with 1-cm stoppered quartz matched cells, an electrobalance[2], a TLC apparatus[3], a light meter[4], an IR instrument[5], and a microscope light[6] equipped with a transformer.

Materials—The following solvents were used: distilled-in-glass acetone, acetonitrile, absolute methanol, chloroform, and cyclohexane; analytical reagent grade absolute ethanol, cyclohexanol, cyclohexene, carbon tetrachloride, dimethylformamide, dimethyl sulfoxide, *n*-hexane, and methylene chloride; alcohol USP; sodium borohydride[7], 0.8-cm (0.3-in.) pellets; blue tetrazolium[8]; triphenyltetrazolium[9]; 10% aqueous tetramethylammonium hydroxide[9]; and USP reference standard hydrocortisone and prednisone.

Reagents—Tetramethylammonium hydroxide (1%) was prepared by diluting 5.00 ml of 10% aqueous reagent to 50.0 ml with alcohol USP. Blue tetrazolium (5 mg/ml) was prepared by dissolving 250.0 mg of I in 50.0 ml of alcohol USP. A standard solution containing 0.010 mg of hydrocortisone/ml of alcohol USP was prepared.

General Procedure—All studies were performed in 25-ml glass-stoppered flasks, which were placed on white paper during irradiation. Unless otherwise indicated, the solutions were irradiated with laboratory fluorescent light of 52-foot-candle (ft-c) intensity. Spectrophotometric scans were made from 720 to 250 nm *versus* the same solvent as the blank unless otherwise noted.

Formazan Isolation—Actinic glassware and aluminum foil-wrapped columns were used to protect the formazans from light during the isolation procedures.

I Blue Formazan—One pellet of sodium borohydride was added to a solution containing 53 mg of I in 100 ml of absolute ethanol, and this mixture was placed in the dark for several days. The precipitate was filtered, washed with absolute ethanol, dissolved in chloroform, and evaporated to dryness to obtain the blue formazan.

I Red Formazan—Three milliliters of 10% tetramethylammonium hydroxide was added to a solution containing 75 mg of prednisone and 1.53 g of I in 300 ml of alcohol USP. The mixture was placed in the dark for 30 min and then transferred to a separator containing 600 ml of water and 100 ml of chloroform. This solution was shaken vigorously for 1 min, the layers were allowed to separate, and the chloroform layer was filtered through a 12-cm length of sodium sulfate. An additional 50-ml portion of chloroform was used to complete the extraction and to wash the formazan from the sodium sulfate column. The chloroform extracts were combined and evaporated to obtain the red formazan.

II Formazan—One pellet of sodium borohydride was added to a solution of 100 mg of II in 20 ml of absolute ethanol. The solution was kept in the dark for 24 hr; it was then transferred to a separator containing 80 ml of water and extracted repeatedly with chloroform until the aqueous phase became colorless. The combined chloroform extracts were filtered through chloroform-washed cotton and evaporated to dryness to obtain a pure crystalline formazan.

Effect of Light on I–Hydrocortisone Reaction—Ten 20.0-ml aliquots, each containing 0.200 mg of USP reference standard hydrocortisone in alcohol USP plus a reagent blank, were treated in accordance with the official procedure (1). Each of the 10 hydrocortisone standard solutions was scanned *versus* the reagent blank from 720 to 490 nm exactly 90 min after addition of the tetramethylammonium hydroxide reagent. The reagent blank was scanned *versus* alcohol USP exactly 90 and 130 min after addition of the tetramethylammonium hydroxide. All reagents and solutions were protected from light at all times. The experiment was repeated with the exception that all reagents and solutions were exposed to laboratory fluorescent lighting during the entire study.

Photochemical Effect of Laboratory Lighting on Tetrazolium Formazans—For each experiment involving formazans, aliquots of chloroform stock solutions were taken to dryness carefully, and these residues were dissolved in an appropriate volume of solvent. Care was taken to protect all solutions from light before the experiment was initiated.

Solutions of I blue formazan (0.0086 mg/ml), I red formazan (0.0647 mg/ml), and II formazan (0.0103 mg/ml) in each of several different solvents were exposed to laboratory lighting for 1 hr and to office lighting (78 candle power) for 19 hr, with spectra being obtained after 0, 20, and 60 min and 20 hr. These solutions were then exposed to intense incandescent light of approximately 1×10^4-ft-c intensity for 90 min and rescanned. Certain red formazan solutions were exposed to laboratory lighting for an additional 11 days. These solutions were then scanned and evaporated to dryness. Each residue was dissolved in alcohol USP, a pellet of sodium borohydride was added, and the absorption spectrum of the resulting solution was obtained.

Reversibility of Photoeffect on I and II Formazans—*I Blue Formazan in Chloroform*—Duplicate solutions containing 0.0687 mg of I blue formazan in 10.0 ml of chloroform were prepared; one solution was placed in the light and the other was kept in the dark for 24 hr. During this period, absorption spectra were determined periodically on each solution. Both solutions were subsequently exposed to alternating periods of laboratory lighting and darkness, and their visible absorption spectra were obtained periodically for an additional 976 hr. The solutions were then evaporated to dryness; each residue was dissolved in alcohol USP, a pellet of sodium borohydride was added, and the absorption spectrum of the resulting solution was obtained.

I Blue Formazan in Cyclohexane—A solution containing 0.0687 mg of I blue formazan in 10.0 ml of cyclohexane was exposed alternately to light and darkness for 960 hr, and absorption spectra were obtained during this time.

I Red Formazan in Chloroform—A solution containing 0.496 mg of

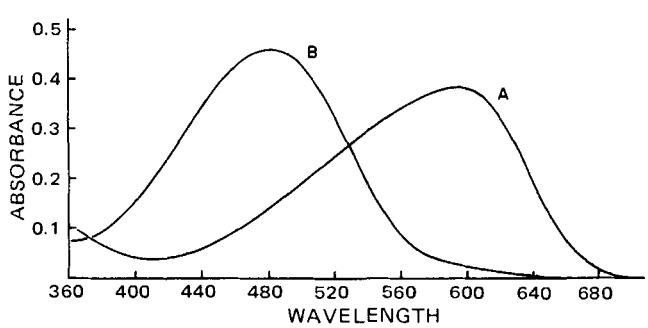


Figure 1—*Spectra of I blue formazan (A) and its photoformazan (B) in cyclohexane.*

[1] Cary models 15 and 17.
[2] Cahn model G-2.
[3] Eastman No. 6060 silica gel.
[4] Weston model 703-60.
[5] Perkin-Elmer model 337.
[6] Bausch & Lomb model 282.
[7] Alpha Products.
[8] Dajac Laboratories.
[9] Eastman Organic Chemicals.

Table II—Effect of Extent of Exposure on the λ_{max} and Absorbance Values of I Blue Formazan in Selected Solvents

Solvent	Minutes at 52 ft-c			19 hr at 78 ft-c	90 min at 1×10^4 ft-c
	0	20	60		
Absolute ethanol[a]	595 (0.140)[b]	595 (0.139)	595 (0.136)	575 (0.120)	570 (0.302)
Absolute methanol[a]	590 (0.055)	585 (0.051)	585 (0.045)	530 (0.108)	530 (0.150)
Acetone	594 (0.619)	594 (0.617)	593 (0.615)	590 (0.592)	573 (0.436)
Acetonitrile[a]	592 (0.340)	590 (0.350)	590 (0.350)	585 (0.340)	560 (0.365)
Alcohol USP[a]	580 (0.060)	580 (0.068)	575 (0.069)	550 (0.087)	550 (0.236)
Chloroform	595 (0.590)	592 (0.584)	588 (0.573)	592 (0.582)	575 (0.401)
Cyclohexane[a]	590 (0.176)	580 (0.149)	578 (0.123)	463 (0.360)	460 (0.470)
Dimethyl-formamide	602 (0.621)	602 (0.625)	602 (0.624)	602 (0.598)	595 (0.511)
Dimethyl sulfoxide	605 (0.518)	605 (0.577)	605 (0.584)	605 (0.573)	602 (0.525)
n-Hexane[a]	575 (0.114)	570 (0.112)	570 (0.105)	460 (0.195)	453 (0.463)

[a] The amount of I blue formazan taken was not completely dissolved in the volume of solvent used. [b] Values are the λ_{max}, nanometers, with the absorbance in parentheses.

I red formazan in 10.0 ml of chloroform was exposed to laboratory light and scanned periodically for 485 min. It was then treated as follows, with the absorption spectrum taken at the end of each step: dark, 88 hr; light, 24 hr; dark, 168 hr; light days and dark nights, 5 days; 78-ft-c light, 24 hr; dark, 48 hr; and the same light intensity, 18 hr.

Two additional solutions containing 0.496 mg of I red formazan in 8.0 and 10.0 ml of chloroform were prepared. The former solution was scanned, exposed to incandescent light of approximately 1×10^4 ft-c for 65 min, and scanned immediately after being exposed to light. The volume was adjusted to 8.0 ml after light exposure before scanning. The latter solution was kept in the dark for 23 days, and absorption spectra were obtained periodically during this time.

II Formazan in Chloroform—Ten milliliters of chloroform containing 0.161 mg of II formazan was exposed for 34 days to several cycles of laboratory light and darkness, and the absorption spectrum was obtained after each cycle. The experiment was repeated with a duplicate aliquot irradiated with approximately 1×10^4 ft-c of light intensity.

II Formazan in Cyclohexane—Ten milliliters of cyclohexane was pipetted into each of two flasks containing 0.161 mg of II formazan. One solution was exposed to laboratory lighting continuously for 53 hr; the other was wrapped in aluminum foil for the first 5 hr before exposure to light for 48 hr. Nine absorption spectra were made of these samples during this time. These solutions were then exposed to a light of approximately 1×10^4-ft-c intensity for short periods, scanned, placed in the dark overnight, and rescanned. This latter procedure was repeated through several cycles of light and darkness.

Rate Studies—*Light Effect*—A 10.0-ml chloroform solution containing 0.496 mg of I red formazan was exposed to laboratory light, and absorption spectra were obtained periodically during 485 min to follow the conversion of I red formazan to its photoformazan.

Dark Effect—A 4.0-ml chloroform solution containing 0.496 mg of I red formazan was exposed to laboratory fluorescent light of 78 ft-c intensity until the maximum amount of its yellow photoform was generated. The solution was placed in a stoppered spectrophotometric cell in the dark, and absorption spectra were made every 15 min for 8 hr.

Correlation of Phototransformation of Formazans to Photoformazans—Chloroform solutions of I red, I blue, and II formazans were scanned, irradiated with light to complete the conversion to their respective photoformazans, and then rescanned. The wavelength of the absorption maximum for each species was determined.

Photochemical Effect of Laboratory Lighting on Tetrazoliums—*Blue Tetrazolium*—A saturated solution of I in absolute ethanol and a concentration of 5 mg of I/ml of alcohol USP were exposed to laboratory fluorescent lighting for 4 days, after which time the absorption spectrum of each solution was taken.

Triphenyltetrazolium—A colorless solution of II in absolute ethanol and another in alcohol USP (both 5 mg/ml) were exposed for 24 hr to laboratory lighting and then scanned. The absolute ethanol solution was then divided into two parts; one part was used as a control, and enough water was added to the other to change it to the equivalent of alcohol USP. These solutions were exposed to light for 1 additional week and rescanned.

RESULTS AND DISCUSSION

The effect of laboratory fluorescent light upon the reaction of I with hydrocortisone is shown in Table I. The average net absorbance (0.578 ± 0.001 *SD*) of 10 determinations of hydrocortisone not exposed to light by the USP XIX (1) method was essentially the same as that (0.579 ± 0.001 *SD*) obtained for the same number of hydrocortisone determinations exposed to laboratory lighting. These results indicate that the net absorbance of this reaction is not affected by ordinary laboratory light. The reagent blank of I was found previously (10, 21) to be affected by light. This study found that the absorbance of the light-exposed blanks was approximately 1.5 times as great as the blank protected from light at 90 and 130 min from the start of the reaction. However, the procedure of running sample *versus* blank compensated for any deviation in the reagent blank caused by light.

The influence of light on the stability of I and II formazans in various solvents is listed in Tables II–IV. In general, the formazans were converted to photoderivatives in nonpolar solvents such as cyclohexane, *n*-hexane, and chloroform. Exposure of these solutions to laboratory lighting resulted in hypsochromic shifts of the absorption maxima. Figures 1 and 2 show typical absorption spectra of I blue and red formazans

Table III—Effect of Extent of Exposure on the λ_{max} and Absorbance Values of I Red Formazan to Light

Solvent	Minutes at 52 ft-c			19 hr at 78 ft-c	90 min at 1×10^4 ft-c
	0	20	60		
Absolute ethanol	522 (0.532)[a]	523 (0.529)	523 (0.546)	523 (0.480)	525 (0.426)
Absolute methanol	517 (0.521)	517 (0.526)	517 (0.522)	518 (0.521)	518 (0.490)
Acetone	525 (0.511)	520 (0.513)	520 (0.510)	520 (0.480)	ND[b]
Acetonitrile	515 (0.517)	515 (0.504)	512 (0.499)	512 (0.476)	500 (0.380)
Alcohol USP	522 (0.520)	522 (0.513)	522 (0.513)	525 (0.512)	520 (0.482)
Chloroform	525 (0.497)	500 (0.438)	465 (0.451)	440 (0.854)	525 (0.660)
Cyclohexane			Insoluble		
Dimethyl-formamide	535 (0.466)	535 (0.459)	535 (0.456)	535 (0.433)	535 (0.322)
Dimethyl sulfoxide	542 (0.486)	542 (0.483)	542 (0.483)	542 (0.482)	542 (0.452)
n-Hexane			Insoluble		

[a] Values are the λ_{max}, nanometers, with the absorbance in parentheses. [b] ND = not determined.

Table IV—Effect of Extent of Exposure on the λ_{max} and Absorbance Values of II Formazan in Selected Solvents

Solvent	Minutes at 52 ft-c			19 hr at 78 ft-c	90 min at 1×10^4 ft-c
	0	20	60		
Absolute ethanol	483 (0.566)[a]	483 (0.569)	483 (0.570)	483 (0.564)	483 (0.535)
Absolute methanol	480 (0.513)	480 (0.551)	480 (0.553)	480 (0.525)	480 (0.472)
Acetone	482 (0.572)	482 (0.533)	482 (0.573)	480 (0.492)[b]	480 (0.276)
Acetonitrile	480 (0.560)	480 (0.560)	478 (0.530)	405 (0.570)[b]	480 (0.302)
Alcohol USP	483 (0.558)	483 (0.569)	482 (0.561)	482 (0.560)	483 (0.510)
Carbon tetrachloride	490 (0.555)	488 (0.411)[b]	415 (0.470)[b]	489 (0.370)[b]	483 (0.070)
Chloroform	485 (0.540)	433 (0.414)[b]	406 (0.751)[b]	404 (0.749)[b]	486 (0.400)
Cyclohexane	487 (0.573)	486 (0.486)[b]	486 (0.496)[b]	487 (0.556)	400 (0.665)[b]
Cyclohexanol	488 (0.572)	487 (0.564)	487 (0.567)	487 (0.550)	486 (0.380)
Cyclohexene	490 (0.569)	490 (0.553)	489 (0.542)	489 (0.532)	489 (0.552)[c]
Dimethylformamide	485 (0.487)	485 (0.492)	483 (0.490)	483 (0.450)	484 (0.350)
Dimethyl sulfoxide	485 (0.496)	485 (0.495)	485 (0.496)	485 (0.469)	485 (0.367)
n-Hexane	478 (0.567)	480 (0.558)	480 (0.551)	413 (0.421)[b]	480 (0.235)
Methylene chloride	490 (0.561)	483 (0.465)[b]	403 (0.659)[b]	403 (1.007)[b]	482 (0.393)[b]

[a] Values are the λ_{max}, nanometers, with the absorbance in parentheses. [b] A second λ_{max} was observed in these solvents. [c] The yellow photosolution reverted completely to orange before the spectrum could be obtained.

and their corresponding yellow derivatives. The conversion of II formazan to its photoderivative was accompanied by a change in the color of the solution from orange to yellow. Small amounts of the three formazans were converted to the corresponding photoformazans in acetonitrile. The insolubility of I red formazan in cyclohexane and *n*-hexane prevented the investigation of the effect of light in these systems.

In the protic solvents, absolute methanol, absolute ethanol, and alcohol USP, I blue formazan was partially converted to I red formazan in the presence or absence of light. During this process, a gradual diminution in the absorption maxima at about 585 nm and a corresponding increase at approximately 525 nm were observed. The amount of conversion of the blue form to the red form decreased as the solvent was varied from absolute methanol to alcohol USP to absolute ethanol. These results are consistent with the conclusion of Graham *et al.* (13) that water and methanol stabilize the *trans-anti*-configuration of I red formazan to a larger extent than ethanol due to the smaller size and greater hydrogen-bonding ability of methanol and water.

Of all solvents studied, I red formazan was the most stable in absolute methanol in the presence of light, even though methanol inhibits the formation of I formazan in the reaction of I with corticosteroids. By using the procedure described previously (13), the formazans were reoxidized to tetrazolium in varying degrees in all solvents listed in Tables II–IV.

To study the influence of water on the stability of I red formazan toward light, selected solutions of this formazan were exposed to laboratory light for 12 days. After this time, the absorbance of the absolute ethanol solution was reduced to 47% of its original value; the absorbance of the alcohol USP solution was reduced to only 93%. Construction of Dreiding models revealed that the *trans-syn*-configuration, III, of I blue formazan would be more readily reoxidized to I than the *trans-anti*-configuration, IV, of I red formazan. Water stabilizes I red formazan (13) and thus acts to prevent the rearrangement of IV to III and the subsequent oxidation of III to I.

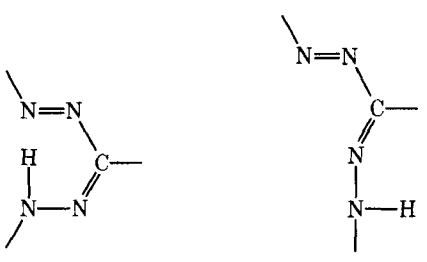
The reversibility of the photoeffect observed in solutions of I and II formazans was studied by exposing these solutions to alternating periods of light and darkness and obtaining spectra at appropriate times during each cycle. In cyclohexane, I blue formazan (λ_{max} 595 nm) and its photoderivative (λ_{max} 487 nm) were completely interconverted by the action of light and darkness during 960 hr. During this time, a small portion (21%) of I blue formazan was reoxidized to tetrazolium, as shown by a decrease in absorbance.

An immediate scan of II formazan in cyclohexane yielded an absorption spectrum of typical shape with a maximum at 486 nm. Further scans of the solution during light exposure produced time-related curves in which an absorption maximum of the photoderivative appeared at about 400 nm. The absorbance of this wavelength increased with time and reached a maximum after 30 min of light exposure. However, further scans during 53 hr of continued light exposure produced curves in which the photoderivative absorption maximum gradually disappeared to yield a spectrum similar to that of the original II formazan. The absorption spectrum of II formazan in a duplicate cyclohexane solution that was not exposed to light exhibited no change during 5 hr; however, when this solution was exposed to light for an additional 48 hr, periodic scans yielded spectra similar to those observed for the first solution. Figure 3 shows spectra typical of those obtained for II formazan in cyclohexane when irradiated with laboratory light.

Irradiation of a solution of II formazan in cyclohexane with approximately 1×10^4 ft-c of light intensity produced a light-stable yellow photoderivative (λ_{max} 400 nm), which could be converted to the original II formazan by darkness. These two species were readily interconvertible over several cycles of alternating intense light and darkness.

Five alternate periods of exposure of I blue formazan in chloroform to laboratory light and darkness during 1000 hr showed partial reversibility of the photoformazan. The wavelength and absorbance at the maximum returned to the starting value after each period of darkness, but there was less conversion to the photoform with each period of light exposure, as shown by decreased absorbance at 487 nm. This result indicates that two forms of I blue formazan exist and have similar absorbance maxima in chloroform. One form apparently is photolabile and converts gradually to the other, which is not further affected by light.

Spectra of the aliquot exposed to light in the initial 24 hr revealed that I blue formazan was converted mainly to its yellow photoform. A dupli-



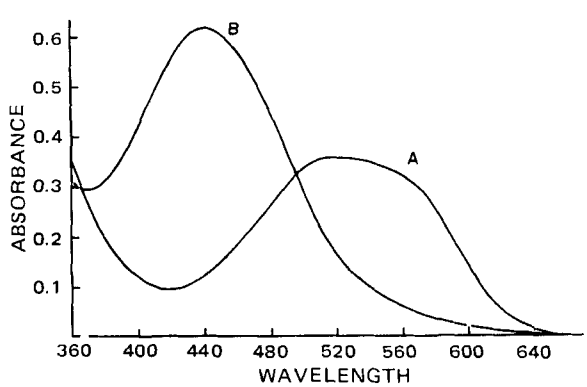


Figure 2—*Spectra of I red formazan (A) and its photoformazan (B) in chloroform.*

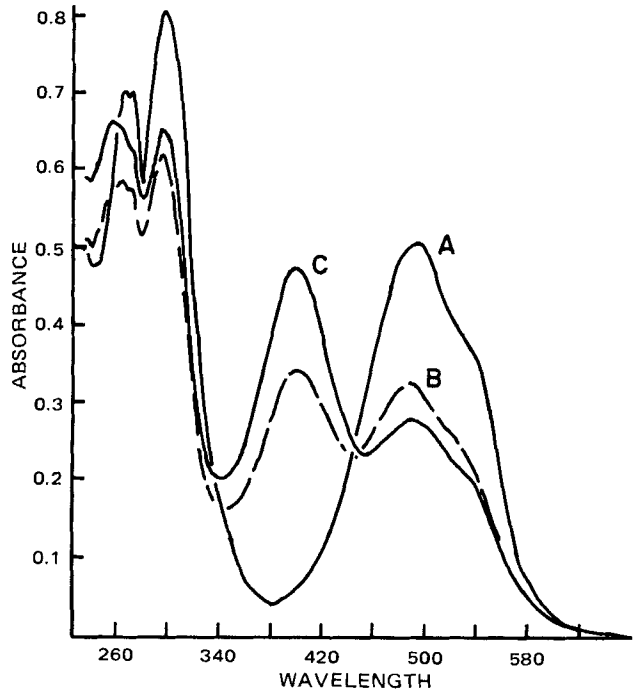


Figure 3—*Spectral changes of II formazan exposed to laboratory lighting. Key: A, identical spectra of II formazan and of II orange photoformazan; and B and C, intermediate spectra of the yellow photoderivative of II after different exposure periods.*

cate aliquot kept in the dark during the same period showed no spectral changes. However, when the latter solution was exposed to light, a similar photoeffect was observed. During the observation period, the absorbances of both solutions gradually decreased until they were free of color and contained only I. The addition of a sodium borohydride pellet to an alcoholic solution of the final tetrazolium residue yielded a mixture of I red and blue formazans (λ_{max} 555 nm). TLC confirmed the presence of both colored formazans in these solutions.

Exposure of a chloroform solution of I red formazan (λ_{max} 525 nm) to ordinary laboratory fluorescent lighting of approximately 52-ft-c intensity produced the corresponding yellow photoform (λ_{max} 440 nm). This photoform readily interconverted to I red formazan by exposure to alternating periods of light and darkness. Evaporation of the yellow photoform solution afforded the crystalline I red formazan, as determined by TLC, IR, and its reconversion to the yellow photoform derivative upon being redissolved in chloroform and exposed to laboratory light. During the evaporation process, the color of the solution changed from yellow to red immediately before dryness.

Exposure of a chloroform solution of the yellow photoform of I red

Table V—Change of Absorbance at 525 nm of I Red Formazan when Exposed to Laboratory Lighting

Minutes	Observed Absorbance[a]	Absorbance of Yellow Photoformazan[b]	Net Absorbance of Red Photoformazan
0	0.357	0.000	0.357
10	0.342	0.005	0.337
28	0.304	0.014	0.290
50	0.271	0.024	0.247
70	0.251	0.034	0.217
105	0.210	0.051	0.159
130	0.190	0.063	0.127
160	0.180	0.078	0.102
190	0.164	0.092	0.072
220	0.155	0.107	0.048
250	0.149	0.121	0.028
280[c]	0.136	0.136	0.000
310	0.136	0.136	0.000
485	0.136	0.136	0.000

[a] Includes the absorbance of I red formazan plus its photoformazan. [b] Absorbance due to the yellow photoformazan of I red formazan calculated from maximum absorbance (0.136), assuming that the rate of formation of the photoformazan is constant. [c] Complete conversion to photoformazan occurs by this time.

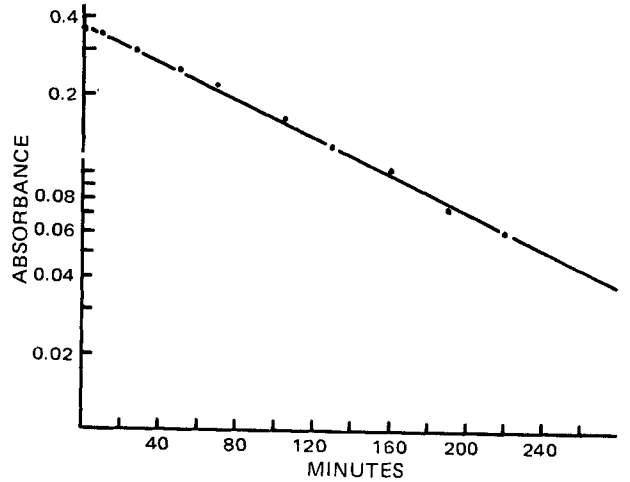


Figure 4—*Rate of change of I red formazan to its yellow photoform in chloroform when exposed to laboratory lighting.*

formazan to ordinary office light of approximately 72 ft-c for about 16 hr converted it to a red color with a spectrum like the original. This spectrum does not change upon further exposure to alternate periods of light and darkness. Exposure of a chloroform solution of I red formazan to intense incandescent light of approximately 1×10^4 ft-c produced a yellow color within 2 min, which remained unchanged for 1 hr under continuous irradiation and then quickly turned (5 min) to red. The spectrum of the final red solution was similar to the original, but the absorbance was 44% higher in the final solution as compared to the original. The higher absorbances and the lack of response of this photoform to successive periods of light and darkness indicate that it may be a different high energy conformer than the original I red formazan. A chloroform solution of the I red formazan that was kept in the dark and scanned periodically showed no change in the spectrum during 23 days.

The II formazan in chloroform demonstrated a photoeffect during the first 2 hr of exposure to laboratory fluorescent lighting, as shown by a change in color from orange to yellow and by a hypsochromic shift of the maximum from 487 to 406 nm. Within the next 5 hr of exposure, the solution returned to an orange color with a spectrum similar to the original and a bathochromic shift of the maximum back to 487 nm. No change was observed in the spectra following successive exposures of the solution to light and dark during the next 33 days, except for a gradual reduction in the intensity of the color due to the oxidation of the orange photoformazan back to II. A chloroform solution of II formazan, when irradiated with approximately 1×10^4 ft-c, changed to a yellow color within 1 min and back to orange within 5 min. The spectrum of the latter color was similar in absorbance and shape to the original formazan but was not reversible when exposed to additional periods of light and dark.

Table V lists the spectral data obtained from the rate study of the conversion of I red formazan to its photoderivative in chloroform in the presence of laboratory light. The observed absorbance values at 525 nm had to be corrected for a small amount of overlap of the absorption maximum of the yellow photoform of I red formazan (Fig. 2). From the graph of the corrected absorbance *versus* time (Fig. 4), the rate of conversion of I red formazan to its photoderivative in chloroform was found to be 1.4×10^{-3} absorbance unit/min.

A sequence of spectrophotometric scans of the regeneration of I red formazan from its yellow photoformazan in chloroform in the dark was taken at 15-min intervals over 8 hr. These scans revealed isosbestic points at 370 and 490 nm, indicating that these two species were in equilibrium with each other. The rate for the regeneration process calculated either from the decrease in absorbance of the yellow form or from the increase in absorbance of the red form corrected for overlap by the yellow form yielded the same value, 2.5×10^{-3} absorbance unit/min.

The difference in energy of the transformation of a formazan to its photoderivative may be calculated from:

$$\Delta E = \frac{hc(\lambda_2 - \lambda_1)}{\lambda_2 \lambda_1} \qquad \text{(Eq. 1)}$$

where ΔE is the difference in energy in ergs at the longer wavelength (λ_2) of absorption of the formazan and the shorter wavelength (λ_1) of the photoform, h is Planck's constant, and c is the speed of light. By using

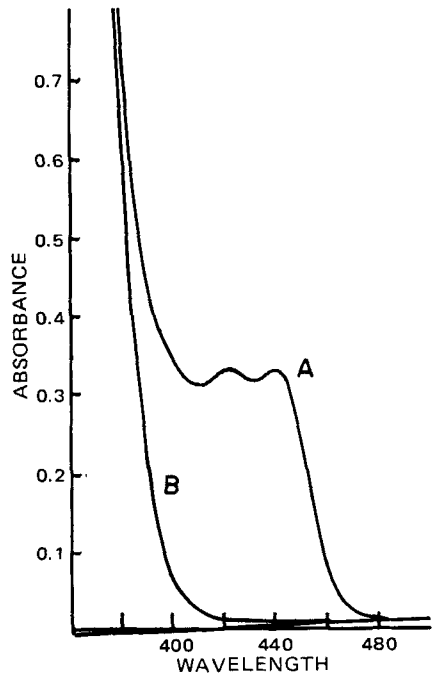
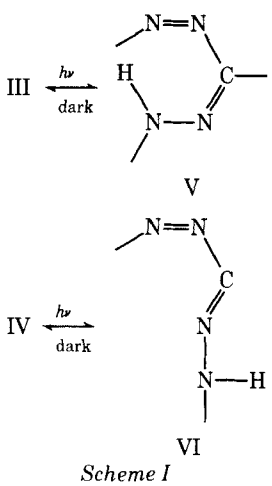


Figure 5—*Spectra of II (B) and its photoderivative (A) in absolute ethanol.*

the maximum absorption values for the hypsochromic shifts observed in chloroform solutions of 595–487 nm for I blue formazan, 525–440 nm for I red formazan, and 487–407 nm for II formazan, the following ΔE values were calculated: 7.31×10^{-13} erg for the I blue formazan transformation, 7.40×10^{-13} erg for the I red formazan transformation, and 8.02×10^{-13} erg for the II formazan transformation. These calculated values indicate that the transformation process is similar for all three formazans.

A possible mechanism for the phototransformation of I blue and red formazans is suggested in Scheme I. The *trans–syn*-configuration, III, of I blue formazan is isomerized to the *cis–syn*-configuration, V, and the *trans–anti*-configuration, IV, of I red formazan is isomerized to the *cis–anti*-configuration, VI, by absorption of light by the N=N moiety. Construction of Dreiding models revealed that steric effects prevent coplanarity of the conjugated π electronic system in the photoderivatives, V and VI, which would account for the hypsochromic shifts observed in these phototransformations.



Scheme I

Finally, the stability of I and II in the presence of light was studied. Solutions of I in absolute ethanol and alcohol USP showed no apparent change in their absorption spectra after extended exposure to laboratory light. However, when II reagent in absolute alcohol was exposed to laboratory lighting, the solution changed from colorless to yellow within a few hours. The absorption spectrum of this solution (Fig. 5) shows the presence of two additional peaks with maxima at 420 and 440 nm. Addition of water to a portion of the yellow photosolution of II sufficient to make a 95% ethanol–water mixture (USP) caused the solution to become colorless within 1 week of continuous light exposure. The other portion of the absolute ethanol solution remained yellow. A control solution of II in absolute ethanol kept in the dark as well as a similar concentration of II in alcohol USP exposed to laboratory light for the same period remained colorless.

These experiments showed that ordinary laboratory fluorescent lighting of approximately 52–74 ft-c provided sufficient energy for phototransformations of the formazans studied to take place in certain nonpolar solvents. Alcohol USP, recommended by the compendia (1, 2), is the solvent of choice for both the tetrazolium reagent and the I reaction with corticosteroids since no special light precautions need be observed, although reagent blanks have higher absorbances than light-protected ones. Exact compensation is made by running samples and standards *versus* the blanks in accordance with the official procedure. If other solvents and/or tetrazolium reagents are used, special precautions may be required to keep the reagents, developing solutions, and final formazans protected from light to ensure a quantitative reaction.

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