# Light—An Essential Factor in the Trihydroxyindole-Spectrophotofluorometric Assay of Norepinephrine

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**When iodine is used as the oxidant in the spectrophotofluorometric assay of norepinephrine, conversion of the oxidation product (noradrenochrome) to the fluorophore (noradrenolutine) requires the presence of adequate light. At a light intensity of 105 foot-candles, a period of 30 minutes is required for maximal development of fluorescence. With the use of intense light (sun light: 8400 foot-candles), maximal development of fluorescence was obtained in 2 minutes. At a light intensity of 40 foot-candles or less, the development of fluorescence was incomplete even when the conversion reaction was allowed the prescribed time of 45 minutes. It is apparent that light is an important factor in the conversion of noradrenochrome to noradrenolutine. Consistent and reproducible results are obtained only when this part of the assay is carried out under an adequate intensity of light for a suitable length of time.**

THE SPECTROPHOTOFLUOROMETRIC method for the assay of norepinephrine described by Shore and Olin (1) involves initial oxidation of the amine to noradrenochrome by iodine at a weakly acidic pH. Noradrenochrome is then rearranged to a highly fluorescent trihydroxyindole (THI) derivative, noradrenolutine, by an alkaline ascorbate solution. Forty-five minutes are allowed for maximal development of fluorescence; the solution is then placed in a spectrophotofluorometer and activated at 400 mμ and the resulting fluorescence read at 520 mμ.

During the course of some early norepinephrine assays in this laboratory, it was observed that fluctuation of daylight in the room, assay at different times of day, and shielding of test tubes from light during the THI reaction yielded erratic results. In the case of samples that were exposed to strong light, development of fluorescence was complete, as suggested by a rapid excursion of the galvanometer indicator-needle to the peak reading. However, in the case of samples that were exposed to weak light or were shielded from bright light, development of fluorescence was incomplete, as suggested by an initial rapid sweep of the indicator to an intermediate point on the galvanometer scale which was then followed by a slower increase in the reading. When a continuous reading was made of these latter solutions, the indicator drifted slowly and reached a peak reading only after approximately 30 minutes, during which time the sample in the spectrophotofluorometer was continuously irradiated by the activating lamp. These early observations suggest that light has a definite effect on this assay. Because there is a paucity of information in the literature regarding the influence of light on this analysis for norepinephrine, studies were undertaken to investigate further the role of light in the procedure.

## EXPERIMENTAL

Norepinephrine,[1] 0.1 mcg./ml. in 0.01 *N* HCl, was used throughout this investigation. The chemical reactions were carried out in the manner described by Shore and Olin (1). In one study, following addition of alkaline ascorbate, test tubes of the sample were exposed to varying intensities of light for a standard period of 45 minutes. In another study, test tubes of the sample were exposed to a standard light intensity for various periods of time. In addition, other test tubes of sample were exposed to direct sunlight (*ca.* 8400 foot-candles) to determine the effect of an intense light on the THI reaction. Measurements of fluorescence were made with the Aminco-Bowman spectrophotofluorometer.[2]

## RESULTS

As illustrated in Fig. 1, completion of noradrenochrome rearrangement requires an adequate light intensity. When the reaction was allowed to take place in the dark for 45 minutes, a negligible amount of fluorescence developed. However, when the reaction was permitted to take place in graded intensities of light, relatively greater degrees of fluorescence developed up to a maximal level. Light intensity of 105 foot-candles was sufficient for producing maximal fluorescence in 45 minutes of exposure; a greater intensity yielded no additional increase in fluorescence. Figure 2 shows that with a light intensity of 105 foot-candles, adequate time is required for completion of the THI reaction. Within the initial 15 minutes, following addition of alkaline ascorbate, there was a rapid development of fluorescence. By 20 minutes the rate of reaction had diminished appreciably and by 30 minutes fluorescence had essentially reached its peak. Readings made after longer intervals of exposure were not significantly different from those obtained at the 30-minute period. In the study which utilized an intense light source, samples that were exposed to direct sunlight showed maximal fluorescence after 2 minutes of exposure. When the samples that had been kept in the dark or exposed to dim light were subsequently exposed to sunlight for 2 minutes, they developed a degree of fluorescence comparable to that seen in the samples exposed for 45 minutes to artificial light of 105 foot-candles. On the other hand, samples that had been allowed

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[1] Norepinephrine bitartrate, supplied by Winthrop Laboratories, was used to prepare the norepinephrine solution. The concentration is calculated and expressed as norepinephrine base.

[2] The galvanometer readings of samples, in which development of fluorescence was incomplete, were made at the end of the initial rapid sweep of the indicator before the onset of its slow drift to a peak value.

to react for 45 minutes in artificial light of 105 foot-candles developed no additional fluorescence upon further exposure to direct sunlight for 2 minutes.

## DISCUSSION

Lund (2), who employed manganese dioxide as the oxidizing agent in the formation of noradrenochrome, indicates that rearrangement of this aminochrome to noradrenolutine occurs almost instantaneously following addition of alkali. Maynert and Klingman (3) report that if potassium ferricyanide is substituted for iodine in the assay of catecholamines, fluorescence may be measured 5–10 minutes after addition of alkali. Udenfriend (4) also indicates that when potassium ferricyanide is used as the oxidizing agent, the conversion of aminochromes to fluorophores is completed very rapidly, 1–2 minutes after addition of alkali. On the other hand, as indicated by the above investigators (3, 4) iodine-oxidized norepinephrine requires a longer period for conversion from noradrenochrome to noradrenolutine. In view of the apparent difference in the rate of rearrangement associated with the use of various oxidizing agents and of the observations made in this laboratory, it is postulated that rapid conversion of noradrenochrome to the fluorophore requires either a metal catalyst or radiant energy. When manganese dioxide or potassium ferricyanide is used as the oxidizing agent, it is conceivable that metallic ions may act as catalysts during the THI reaction. Whereas, when iodine is used as the oxidizing agent, light may supply the energy for accelerating the formation of noradrenolutine; if the intensity of light is exceptionally great (*e.g.*, sunlight), rearrangement may occur at a rate comparable to that of the assay in which one of the metallic salts is used.

It is of interest that reports on the effect of light on the ethylenediamine procedure for the estimation of norepinephrine (5, 6) indicate that daylight causes a *loss* of fluorescence. On the basis of this finding it would appear logical to shield the test tubes from light during the THI reaction in the assay procedure of Shore and Olin (1). However, the present observations demonstrate that when iodine is employed as the oxidant an adequate light intensity is imperative during the THI reaction. Insufficient light during this phase would result in erratic readings; this may account for some of the dissatisfaction voiced by some investigators for the THI-spectrophotofluorometric assay of norepinephrine. On the other hand, one group of investigators (7), who employed iodine as the oxidizing agent, reports 20 minutes (rather than 45 minutes) as adequate for completion of the THI reaction. The shorter time period used by these workers could be explained on the basis of a relatively bright illumination in the laboratory.

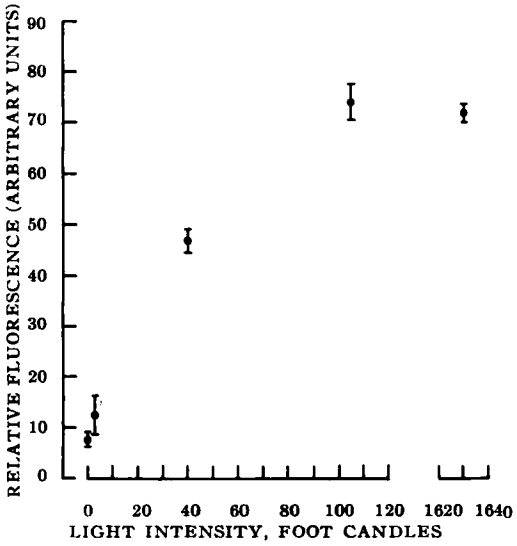


Fig. 1.—Relative fluorescence of norepinephrine samples following oxidation, addition of alkaline ascorbate, and exposure to graded intensities of light for 45 min. Four or more samples were used for each determination. Vertical bracketed lines indicate 95% confidence limits. The reaction at 1630 foot-candles was carried out under sunlight partially shaded with layers of cotton gauze to produce this light intensity; other reactions were carried out in darkness or artificial light (fluorescent light).

## CONCLUSION

The data reported herein show that when iodine is employed as the oxidizing agent in the spectrophotofluorometric assay of norepinephrine, light is essential for the conversion of noradrenochrome to noradrenolutine. This finding supports the warning recently advanced by Udenfriend (4) (with respect to the THI reaction) that when iodine is used as the oxidizing agent shielding the samples from light will produce erratic results. It is obvious that consistent and reproducible results can not be obtained unless the THI reaction is allowed to take place under an adequate intensity of light for a suitable period of time.

## ADDENDUM

Subsequent to completion of the present studies, Sloan and co-workers (8) reported that following the

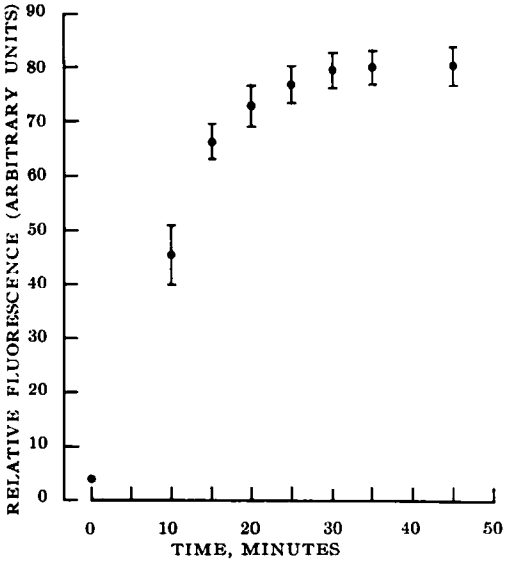


Fig. 2. –Relative fluorescence of norepinephrine samples following oxidation, addition of alkaline ascorbate, and exposure to a standard light intensity of 105 foot-candles (fluorescent light) for various time intervals. Four samples were used for each determination except that one sample was employed for the zero-time determination. Vertical bracketed lines indicate 95% confidence limits.

addition of alkaline ascorbate solution the reaction is extremely photosensitive. These investigators avoided erratic results by placing the tubes of samples inside a box which contains two General Electric 20-watt ultraviolet black light bulbs. They found that rigid control of the lighting conditions enhanced the intensity of fluorescence and improved reproducibility. This finding further supports the contention that light is an essential component in the spectrophotofluorometric assay of norepinephrine during the THI reaction.

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# Cyclized Substituted Thioureas III

## 1-Substituted-tetrazolines-5-thiones

By RONALD E. ORTH

**A convenient synthesis and purification of some 1-substituted-tetrazoline-5-thiones is accomplished. Decreased per cent yields with increased chain length lends some credence to the probability that steric hindrance occurs in the ring closing step. The infrared studies show identifying bands for —N=C=S, C=S, cyclic —N—N=N—, and the tetrazole ring. Low toxicity for these compounds is indicated.**

QUALIFIED success with propylthiouracil (1) and methimazole (2) during hyperthyroid therapy has led to the synthesis of chemically related compounds and to their subsequent *in vitro* and *in vivo* trials. Countless antibacterial and tuberculostatic screening programs have also been carried out using compounds which embody the thiourea moiety.

Some 1-alkyltetrazoline-5-thiones were prepared by synthesizing the methyl ester of *N*-alkyldithiocarbamic acid and refluxing it with sodium azide (3). A more convenient method consists of preparing the sodium *N*-alkyl dithiocarbamate from carbon disulfide, the corresponding amine, and sodium hydroxide. Ethyl chloroformate and the dithiocarbamate give ethyl *N*-alkyldithiocarbamate, which upon decomposition yields carbonyl sulfide, ethanol, and the desired alkyl isothiocyanate (R—N=C=S). The 1-substituted-tetrazoline-5-thiones were isolated following reflux of the isothiocyanate with sodium azide and acidification. The lower yields with increased chain length suggests that there is a steric effect since it has been demonstrated that 1-aryl substituents increases the yields appreciably (4).

Tetrazole and its substitution products usually show only a small amount of end absorption in the ultraviolet region of the spectrum (240–250 mμ) (5). Otting found that tetrazole gave large peaks at 1520, 1270, 1160, 1100, 1020, 915, and 670 cm.$^{-1}$ and a number of characteristic small peaks (6). Lieber, *et al.*, found some 5-substituted tetrazoles to give results interpreted as tetrazole ring absorption bands in the 8.9 to 10 μ region (7). In later work with 1-aryl-tetrazoline-5-thiones more definite tetrazole skeletal assignments were demonstrated. The bands were found at approximately 1100, 1080, 1045, 1020, and 990 cm.$^{-1}$ Frequencies were also assigned for N—C=S, C=S, and cyclic —N—N=N— linkages (8). The spectra for the 1-substituted-tetrazoline-5-thiones summarized in Table I closely approximate the Lieber assigned values and support his hypothesis showing the presence of sulfur in the thione form. Characteristic sulfhydryl bands were not seen for any of the compounds tested from this series. The results of this investigation emphasize the uniformity of the data obtained using 1-aryl and 1-alkyl substituents in the tetrazoline-5-thione system.

$LD_{50}$'s of 207 ± 27 mg./Kg. and 215 ± 23 mg./Kg. body weight were obtained for 1-methyl- and 1-isopropyltetrazoline-5-thiones, respectively, using five groups per compound, each group having 20 young male white mice.

### EXPERIMENTAL

**1-Substituted-Tetrazoline-5-thiones.**—Three-tenths mole (19.5 Gm.) of sodium azide is dissolved in 300 ml. of water and filtered into a 500 ml. round-bottom flask and two-tenths mole of an isothiocyanate is added, followed by refluxing. Cease the operation and remove any unreacted isothiocyanate by ether extraction when the green reaction mixture turns colorless. Acidify the aqueous solution to a pH of 3 with HCl, filter, and extract the filtrate with ether. Wash the ether solution with small portions of ice water, dry over anhydrous sodium sulfate, and decolorize with activated charcoal. The dried acidic ether extract is evaporated to a yellow-brown oil, which in turn forms pale yellow crystals, using the Rinco rotary evaporator and steam bath. These crystals are blotted dry on filter paper, and recrystallized from 100–115° petroleum ether.

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