This finding would artifactually lead one into concluding that absorption was occurring by two consecutive first-order processes. It should not be concluded that averaging of individual data is inappropriate, but its primary function should be to reduce the variance so that an accurate theoretical interpretation becomes more feasible.

CONCLUSION

Minor random deviations from averages of ideal data have been shown to be capable of producing results similar to those expected from enterohepatic recycling when the ideal data exclude that phenomenon. Thus, the definition of enterohepatic recycling from real pharmacological data probably requires an experimental study before confirmation can be made.

REFERENCES

(1) R. K. Nayak, R. D. Smyth, J. H. Chamberlain, A. Polk, A. F. DeLong, T. Herczog, P. B. Chemburkar, R. S. Joslin, and N. H. Reavey-Cantwell, *J. Pharmacokinet. Biopharm.*, **2**, 107(1974).

(2) R. D. Schoenwald, *J. Pharm. Sci.*, **63**, 411(1974).

(3) A. G. Hibble, P. Isaac, and D. G. Grahame-Smith, *Lancet*, **2**, 90(1972).

(4) J. Lindenbaum, M. H. Mellow, M. O. Blackstone, and V. P. Butler, Jr., *N. Engl. J. Med.*, **285**, 1344(1971).

(5) T. R. D. Shaw, M. R. Howard, and J. Hamer, *Lancet*, **2**, 303(1972).

(6) J. G. Wagner, M. Christensen, E. Sakmar, D. Blair, J. D. Yates, P. W. Willis, III, A. J. Sedman, and R. G. Stoll, *J. Amer. Med. Ass.*, **224**, 199(1973).

(7) H. Gold, M. Cattell, T. Greiner, L. W. Hanlon, N. T. Kwit, W. Modell, E. Cotlove, J. Benton, and H. L. Otto, *J. Pharmacol. Exp. Ther.*, **109**, 45(1953).

(8) E. R. Garrett, *J. Pharmacokinet. Biopharm.*, **1**, 341(1973).

(9) W. J. Dixon and F. J. Massey, Jr., "Introduction to Statistical Analysis," 2nd ed., McGraw-Hill, New York, N.Y., 1957, pp. 366–380, 457–461.

(10) J. C. K. Loo and S. Riegelman, *J. Pharm. Sci.*, **57**, 918(1968).

(11) D. Perrier and M. Gibaldi, *ibid.*, **62**, 225(1973).

(12) G. Levy and L. E. Hollister, *ibid.*, **53**, 1446(1964).

ACKNOWLEDGMENTS AND ADDRESSES

Received December 16, 1974, from the *Department of Pharmacy, School of Pharmacy, University of California, San Francisco, CA 94143*

Accepted for publication May 7, 1975.

Presented in part at the Basic Pharmaceutics Section, APhA Academy of Pharmaceutical Sciences, New Orleans meeting, November 1974.

Supported in part by Project Grant GM 16496 from the Institute of General Medical Sciences, National Institutes of Health. Ms. Gustafson received support from Training Grant in Pharmaceutical Chemistry GM 00728 from the National Institutes of Health. Ms. Gustafson also received support as a Fellow of the American Foundation for Pharmaceutical Education during 1974–1975.

* Present address: Alcon Laboratories, Fort Worth, TX 76101

ˣ To whom inquiries should be directed.

Anaerobic Photodecomposition of an Acridan Drug through Energy Transfer

GEORGE A. DIGENIS ˣ, SHUKRI SHAKHSHIR *, M. A. MIYAMOTO ‡, and H. B. KOSTENBAUDER

Abstract □ Anaerobic irradiation of 2-chloro-9-(3-dimethylaminopropyl)acridan phosphate (1:1) (III*a*) with filtered visible light (300–400 nm) resulted in its quantitative conversion to its acridine derivative IV. Photoproduct IV exerted significant product catalysis on the reaction rate at concentrations of 5×10^{-6} *M*. The anaerobic photodecomposition of III*a* to IV was catalyzed by the monosodium salt of riboflavin 5-phosphate (I*a*). Loss of I*a* was insignificant relative to that of III*a*, and reagents known to serve as quenchers of the triplet state of I*a* retarded the reaction. Fluorescence spectra of I*a* in the presence of III*a* and 1×10^{-4} *M* KI indicated no quenching of the I*a* singlet excited state. No deuterium isotope effect was noticed when III*a* and its deuterated derivative III*b* were subjected to anaerobic photodecomposition in the presence of I*a*. It is suggested that the anaerobic photodecomposition of III*a* by visible light in the presence of I*a* proceeds *via* a triplet–triplet energy transfer from I*a* to III*a*.

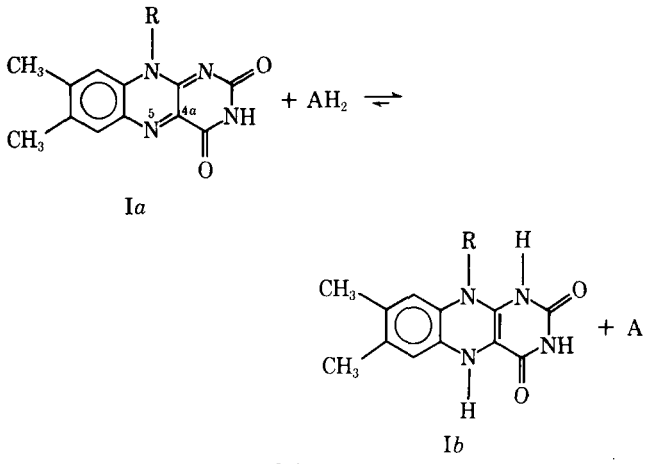
Keyphrases □ Photodecomposition, anaerobic—acridan derivative converted to acridine derivative in visible light through energy transfer, riboflavin 5-phosphate as catalyst □ Acridans—2-chloro-9-(3-dimethylaminopropyl)acridan phosphate converted to acridine derivative in visible light through energy transfer □ 2-Chloro-9-(3-dimethylaminopropyl)acridan phosphate—anaerobic photodecomposition to acridine derivative

Flavin coenzymes participate in various enzymatic dehydrogenation reactions. The overall dehydrogenation can be represented by Scheme I, where I*a* and A are the oxidized forms of riboflavin and substrate, respectively, and I*b* and AH_2 represent the reduced forms of the coenzyme and substrate, respectively (R = ribityl group).

Several classes of such reactions are known including: (*a*) alcohol dehydrogenation (glucose oxidase and lactate dehydrogenase); (*b*) amine dehydrogenation, commonly referred to as oxidative deamination (amino acid oxidases); (*c*) dehydrogenation alpha and beta to a carbonyl (succinate dehydrogenase); and (*d*) dihydronicotinamide dehydrogenation (NADH dehydrogenase).

BACKGROUND

Based on model studies, a mechanism was proposed for amine and alcohol dehydrogenations. This mechanism involves the formation of a carbanion-like intermediate (II, Scheme II) formed after the addition of an alcohol or amine at the C_{4a}-position of the flavin (1).



Scheme I

The partial reaction sequence depicted in Scheme II is an example of covalent catalysis (2). This type of catalysis involves an intermediate, such as II, in which substrate and coenzyme become covalently bonded. The isoalloxazine moiety of flavins has been implicated in the formation of covalent intermediates. The existence of such intermediates is substantiated by several examples including the photoinduced decarboxylation of phenylacetate (3), the photocatalyzed formation of 3-benzyl-5-(2-thiolyl)-1,5-dihydroflavin from 3-benzyllumiflavin and tetrahydrothiophene (4), the nucleophilic addition of sulfite ion at the N-5 position of the flavin coenzyme in D- and L-amino acid oxidases (5), and the oxidation of β-chloroalanine to chloropyruvate by D-amino acid oxidase (6).

It was previously reported that, in UV light, the antipsychotic drug 2-chloro-9-(3-dimethylaminopropyl)acridan (III*a*) is photooxidized to an acridine (IV) as the initial photoproduct (Scheme III). Compound III*a* is relatively stable to visible light (>400 nm); but in the presence of riboflavin phosphate, exposure to visible light results in rapid photooxidation of III*a* without comparable destruction of riboflavin (7). The present article presents evidence to show that the riboflavin-mediated photooxidation of III*a* in visible light occurs *via* energy transfer involving a flavin triplet excited state.

The participation of drugs such as III*a* in energy transfer reactions in aqueous solution has obvious implications with respect to the stability of the drug when it is combined with an agent that absorbs visible light and that has a triplet state favorable to energy transfer. Also, the ability of III*a* to quench the flavin triplet excited state suggests that the drug might also intercept biological electron transport *in vivo* at the NADH dehydrogenase (flavoprotein) level by quenching a flavin excited state. Published data from these laboratories indicate that III*a* interferes with electron transfer in the NADH dehydrogenase segment of the respiratory chain in *Pseudomonas saccharophila*, a facultative autotrophic bacterium (8).

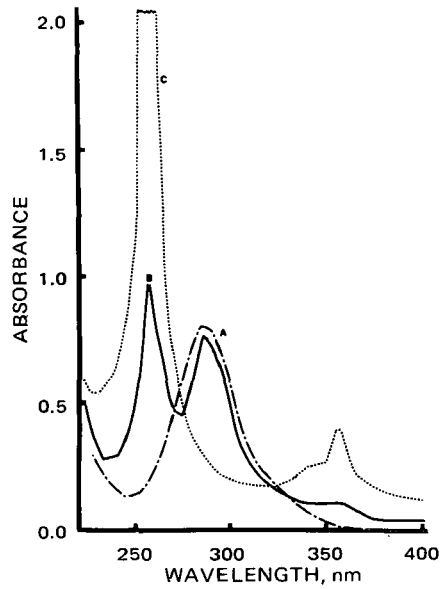
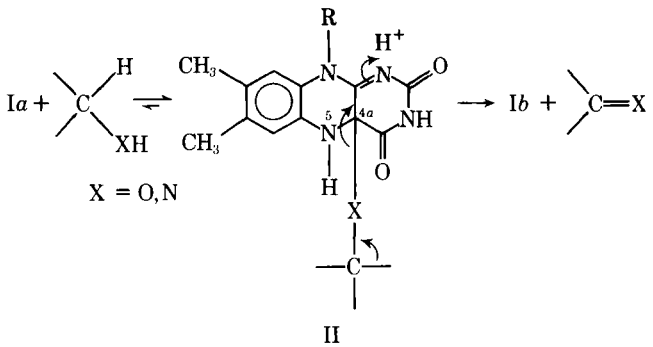


Figure 1—*Progressive loss of III*a *(282 nm) upon irradiation with filtered sunlamp light source (300–400 nm) and appearance of the acridine photoproduct (IV). Key: A, 5 × 10⁻⁵ M of III*a *prior to irradiation; and B and C, appearance of IV.*



Scheme II

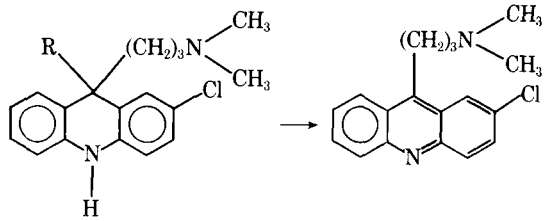
EXPERIMENTAL

Reagents—Compound III*a*[1] [2-chloro-9-(3-dimethylaminopropyl)acridan phosphate (1:1)] and its acridine derivative[1] [2-chloro-9-(3-dimethylaminopropyl)acridine dihydrochloride] (oxidized form, IV) were used as supplied. Riboflavin 5-phosphate monosodium salt[2] (I*a*) was recrystallized from 95% ethanol. Potassium iodide, USP grade, was recrystallized from methanol.

Spectroscopy[3]—The disappearance of III in the presence of riboflavin phosphate (I*a*) was determined by following the decrease in absorbance at 292 nm near the 282-nm maximum for III. Measurement at 292 nm, an isosbestic point for riboflavin (I*a*) and its photoproducts (9), facilitated correction for contribution of flavin mononucleotide to the total absorbance.

Riboflavin Photooxidation of III*a* and III*b*—Unless otherwise specified, solutions were prepared daily in degassed double-distilled water and reactions were run in a 1-cm quartz Thunberg cell, equipped with a bulb (2 ml) and a side arm fitted to a stopcock. The Thunberg cell was put under nitrogen by repeated (at least seven times) evacuation and introduction of nitrogen. Immediately prior to irradiation, the riboflavin phosphate (I*a*) was introduced anaerobically into the cell, from the bulb, by tilting the Thunberg cell.

The light source for the photocatalyzed oxidation of III*a* by I*a* was the unfiltered light from a 500-w projection lamp. The cell was placed in a water bath (27°) 155 cm from the center of the light source. The thickness of the glass wall of the water bath was 0.31 cm. For the photooxidation of III*b* by I*a*, filtered visible light[4] (300–400 nm) was utilized. The cell was placed 6.5 and 155 cm



III*a*: R = H
III*b*: R = D

IV

Scheme III

[1] Smith Kline and French Laboratories, Philadelphia, PA 19101
[2] Mann Research, Orangeburg, NY 10962
[3] Absorbance was measured with a Cary 15 recording spectrophotometer in the 220–500-nm region. Kinetic studies were performed with a Gilford spectrophotometer (model 240).
[4] General Electric 275-w sunlamp.

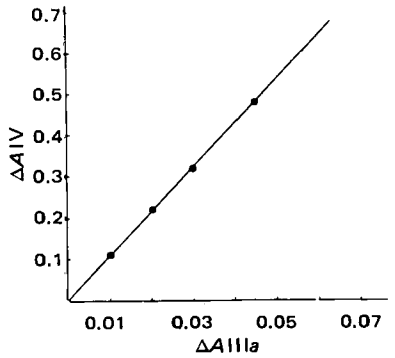


Figure 2—*Plot of increase in absorbance of photoproduct (IV) (ΔA IV) at 255 nm* versus *loss of IIIa (ΔA IIIa) at 282 nm, resulting from the anaerobic irradiation of IIIa with filtered sunlamp light.*

from the filter and light source, respectively.

Photolysis of IIIa Alone or in Presence of IV by Filtered Visible Light—Photolytic studies in the presence of filtered light[4] (300–400 nm, filter placed 6.25 cm from cell) were performed in double-distilled water in a Thunberg cell under anaerobic conditions and at 25°. The Thunberg cell was placed 155 cm from the light source.

Isolation and Identification of Acridine (IV) from Anaerobic Photodecomposition of IIIa and Ia—One gram (2.5 mmoles) of IIIa and 2.7 g (5.25 mmoles) of riboflavin 5-phosphate monosodium salt (Ia), dissolved in 600 ml of a methanol–water (1:1) mixture, was introduced into a flask fitted through a glass joint to a nitrogen inlet. The reaction mixture was put under nitrogen by repeated (at least seven times) evacuation and introduction of nitrogen. After 23 hr of irradiation under a sunlamp, the reaction mixture was flash evaporated to remove its methanol content.

The remaining aqueous mixture was first adjusted to pH 8 by adding 10% NH_4OH and then extracted twice with 2 liters of chloroform. The chloroform extracts were then dried and evaporated to dryness with reduced pressure, and the residue was chromatographed on fine silica gel G TLC plates (0.5 mm) with chloroform–methanol (9:1) as the eluent. A large band was visualized (UV lamp) at the same level (R_f 0.45) as that from an authentic solution of acridine (IV), obtained by neutralizing the solution with

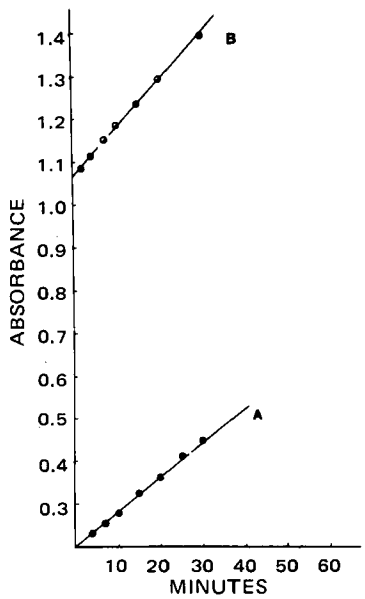


Figure 3—*Rate of appearance of photoproduct IV (at 252 nm) upon anaerobic irradiation of IIIa with filtered sunlamp light. Key: A, 5 × 10⁻⁵ M IIIa (slope 0.008); and B, 5 × 10⁻⁵ M IIIa and 5 × 10⁻⁶ M IV (slope 0.0115).*

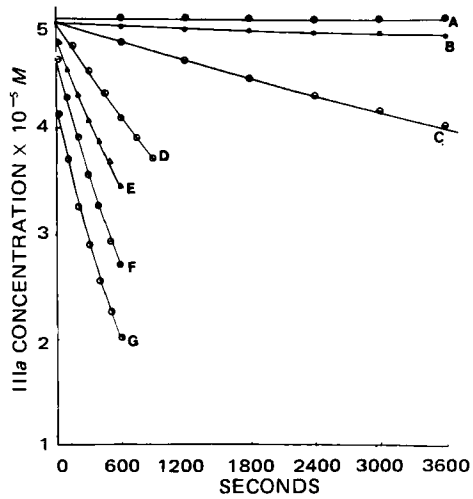


Figure 4—*Riboflavin- (Ia) photocatalyzed decomposition of IIIa with visible light (anaerobic conditions). Key: A, 5 × 10⁻⁵ M IIIa; B, 5 × 10⁻⁵ M IIIa and 5 × 10⁻⁷ M Ia; C, 5 × 10⁻⁵ M IIIa and 5 × 10⁻⁶ M Ia; D, 5 × 10⁻⁵ M IIIa and 5 × 10⁻⁵ M Ia; E, 5 × 10⁻⁵ M IIIa and 1 × 10⁻⁴ M Ia; F, 5 × 10⁻⁵ M IIIa and 1.5 × 10⁻⁴ M Ia; and G, 5 × 10⁻⁵ M IIIa and 5 × 10⁻⁴ M Ia.*

10% NH_4OH. Elution of the band (R_f 0.45) with chloroform and subsequent evaporation of the solvent yielded 0.76 g (2.5 mmoles, quantitative yield) of a thick orange-brown liquid. The latter exhibited identical IR and UV spectra with an authentic sample of IV, obtained in its free amine form by neutralization of its aqueous solution with 10% NH_4OH and subsequent extraction with chloroform.

Preparation of 9-D-IIIb—Two grams of IV was dissolved in water and extracted with ether after it was made alkaline with 10% NH_4OH. The ether extracts were reduced to 250 ml under reduced pressure. The ethereal solution was added slowly into a previously stirred (for 1.5 hr) mixture of 3–4 g of lithium aluminum deuteride[5] ($LiAlD_4$) and 450 ml of ether. The mixture was heated under reflux with stirring under nitrogen for 24 hr. At the end of this time, the excess hydride was decomposed by adding a 50% aqueous solution of sodium potassium tartrate with cooling (5°). The mix-

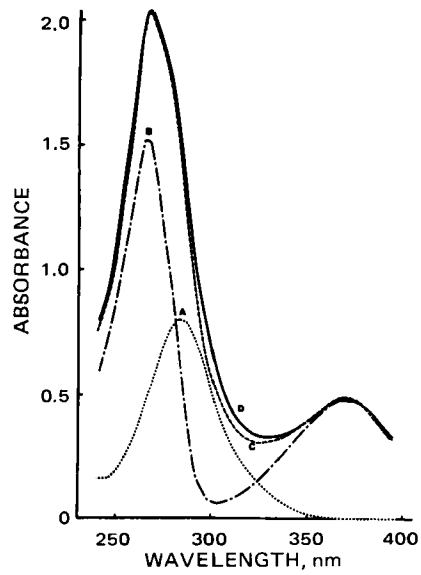


Figure 5—*UV spectra of IIIa and Ia. Key: A, 5 × 10⁻⁵ M IIIa; B, 5 × 10⁻⁵ M Ia; C, theoretical summation spectra of A and B; and D, spectrum of a mixture of 5 × 10⁻⁵ M IIIa and 5 × 10⁻⁵ M Ia.*

[5] International Chemical and Nuclear Corp., Irvine, CA 92664

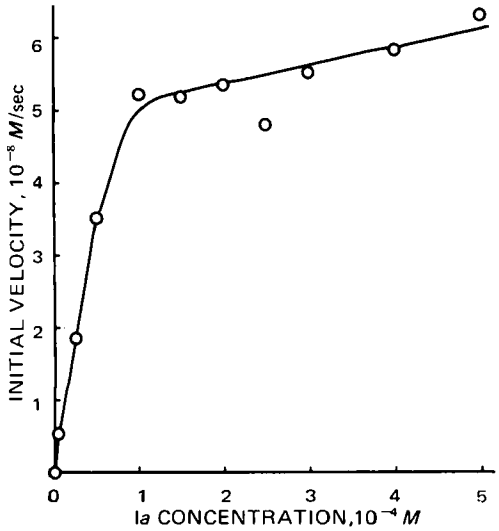


Figure 6—*Influence of riboflavin phosphate Ia concentration on the rate of anaerobic photodecomposition (visible light) of IIIa (5×10^{-5} M).*

ture was filtered, and the aqueous layer was extracted with ether.

Distillation of the solvent under reduced pressure at 25° yielded a yellowish-brown oil. The mass spectrum of this product exhibited major fragments at *m/e* 301 (m), 257, 256, and 254; the free amine base of IIIa exhibited fragments at *m/e* 300 (m), 256, 255, and 254. The NMR spectrum of the deuterated product showed the absence of the 9-H proton and the presence of the 10-H(—NH) proton, which was found to undergo a facile exchange with deuterium oxide in accordance with other acridans (10). Treatment of the yellowish-brown oil with aqueous phosphoric acid led to the production of 9-D-IIIb, mp[6] 205–208°, in 70% yield.

RESULTS AND DISCUSSION

The progressive loss of absorbance at 282 nm and the increase in absorbance at 255 and 360 nm when IIIa is irradiated with light of predominantly 300–400 nm are shown in Fig. 1. It is clear that the loss of IIIa can be measured in the presence of its acridine photoproduct IV. Figure 2 shows that a plot of the increase in absorbance at 255 nm *versus* the loss of absorbance at 282 nm is linear

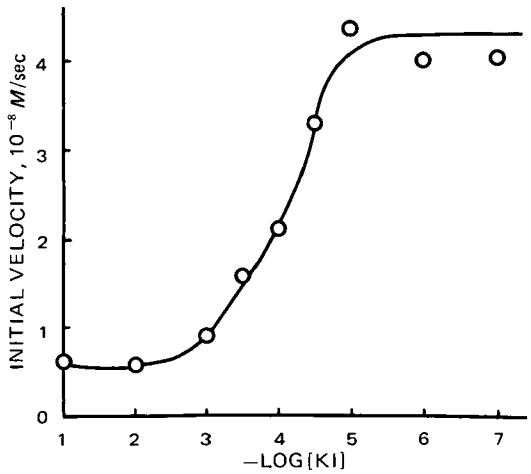


Figure 7—*Effect of potassium iodide on the riboflavin-catalyzed (5×10^{-5} M Ia) anaerobic photodecomposition (visible light) of IIIa (5×10^{-5} M).*

[6] Melting points were taken in a Fisher-Johns apparatus and are uncorrected. Compound IIIa exhibited a melting point at 203–206°.

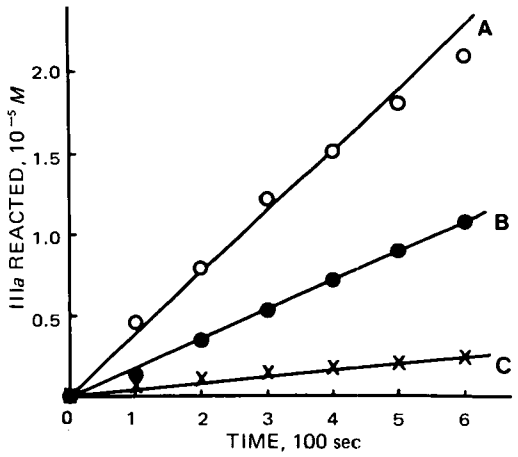


Figure 8—*Effect of quenchers of riboflavin phosphate (Ia, 5×10^{-5} M) photodecomposition (visible light) of IIIa (5×10^{-5} M). Key: A, IIIa and Ia, anaerobic; B, IIIa and Ia, aerobic; and C, IIIa, Ia, and 10^{-2} M KI, anaerobic.*

and that the slope (10:1) corresponds to the ratio of the molar absorptivity of IV (1.7×10^5) at 255 to the molar absorptivity of IIIa (1.6×10^4) at 282. The isolated photoproduct (IV) was shown through comparison of UV and IR spectra and TLC to be identical with an authentic sample of IV.

As seen from Fig. 3, the presence of the acridine photoproduct catalyzes the photodecomposition of IIIa in the absence of oxygen. To avoid the complication of product catalysis of the reaction, all rate data were obtained during the first 6.4% of decomposition of IIIa, where concentration of the photoproduct was such that it did not influence the reaction rate.

Compound IIIa was stable to visible light in the absence of riboflavin. However, in the presence of riboflavin, there was photodecomposition of IIIa as indicated by a loss of absorbance at 292 nm, an isosbestic point for riboflavin and reduced riboflavin (9) (Fig. 4). Although riboflavin is notoriously light sensitive (11–13), the photodecomposition of riboflavin was markedly inhibited by IIIa. Riboflavin loss due to photoreduction was insignificant relative to loss of IIIa, and the photolysis of IIIa proceeded to completion with IIIa concentrations 100 times those of riboflavin. Therefore, the riboflavin-catalyzed photooxidation of IIIa is not a coupled oxidation–reduction involving production of reduced flavin. There also was no spectral evidence for complex formation between riboflavin and IIIa, since spectra for mixtures of riboflavin and IIIa exhibit the expected additivity (Fig. 5).

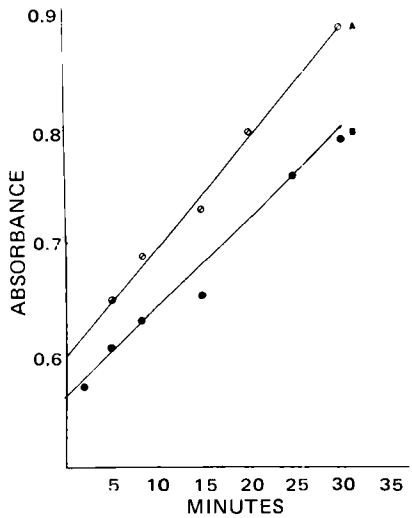


Figure 9—*Rate of appearance of photoproduct IV (at 252 nm) upon anaerobic irradiation of III with filtered sunlamp light. Key: A, 1×10^{-4} M IIIa; and B, 1×10^{-4} M IIIb.*

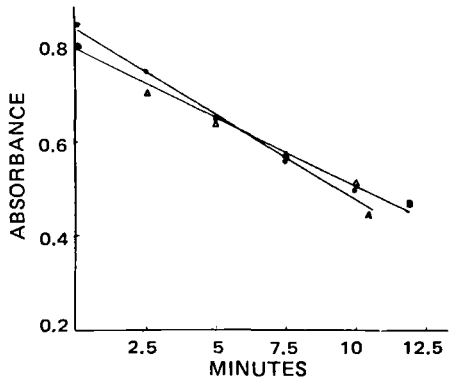


Figure 10—*Rate of disappearance of III (at 292 nm) in presence of Ia upon anaerobic irradiation with filtered sunlamp light. Key: A, 5 × 10⁻⁵ M IIIa and 5 × 10⁻⁵ M Ia; and B, 5 × 10⁻⁵ M IIIb and 5 × 10⁻⁵ M Ia.*

The rate of anaerobic photodecomposition of IIIa, at an initial concentration of 1×10^{-5} *M*, was directly proportional to riboflavin concentration up to about 1.0×10^{-4} *M* flavin (Fig. 6). At flavin concentrations above 1×10^{-4} *M*, concentration quenching of a flavin excited state (14) led to less efficient catalysis.

Fluorescence spectra of 5×10^{-5} *M* riboflavin in the presence of up to 1×10^{-4} *M* IIIa show no evidence of quenching of the riboflavin singlet state by IIIa. Figure 7 illustrates the inhibition of the riboflavin-catalyzed anaerobic photooxidation by 10^{-4} *M* iodide ion, a concentration of iodide recognized as effective in quenching riboflavin triplet species, but not shorter-lived riboflavin singlet species, in a 10^{-4}–10^{-5} *M* riboflavin solution (15). Inhibition of the photooxidation by oxygen, as shown in Fig. 8, also is explicable as oxygen quenching of a triplet excited state of riboflavin.

On the basis of these observations, the following mechanism is proposed to account for the influence of riboflavin on IIIa photodecomposition. Ground-state riboflavin (Ia^0) absorbs visible light and is promoted to a singlet excited state (Ia) (Scheme IV). Riboflavin triplet (Ia^3) species are generated *via* radiationless transition from the singlet (Scheme IV); energy transfer occurs between the riboflavin triplet and ground state of $IIIa^0$ with generation of the $IIIa^3$ triplet and ground-state riboflavin (Scheme V). The $IIIa^3$ triplet may then undergo decomposition to its oxidized form IV (Scheme VI).

Compound IIIa and its deuterated derivative IIIb (deuterium atom at 9-position of the acridan nucleus) were exposed to filtered visible light under anaerobic conditions. The rate of production of IV from both isomers was nearly identical; *i.e.*, it failed to exhibit a kinetic isotope effect (Fig. 9). Similarly, no kinetic isotope effect was noticed when IIIa and IIIb were exposed to anaerobic irradiation with filtered visible light in the presence of 5×10^{-5} *M* Ia (Fig. 10). These results show that the elimination of hydrogen from III, which results in its conversion to photoproduct IV (Scheme VI) in the presence and absence of Ia is a kinetically fast step.

Walsh *et al.* (6) found that in the D-amino acid oxidase-catalyzed conversion of β-chloroalanine to β-chloropyruvate, substitution of the α-hydrogen of the former by deuterium or tritium results in kinetic isotope effects which slow down its conversion to pyruvate. The work of these investigators suggests that the conversion of chloroalanine to pyruvate by D-amino acid oxidase, a riboflavin-dependent enzyme, involves an intermediate similar to II in which the α-hydrogen of the substrate is removed as a proton. Furthermore, this step is rate determining in the formation of the intermediate. The absence of the deuterium isotope effect in the photocatalyzed conversion of IIIa and IIIb to IV indicates that an intermediate such as V is not involved in the rate-determining step of the reaction.

Furthermore, the results indicate that the photooxidation does not involve a covalent bond catalysis with Ia in the manner represented by Structure II.

$$Ia^0 \underset{\text{fluorescence}}{\overset{h\nu}{\rightleftharpoons}} Ia^1 \rightarrow Ia^3$$

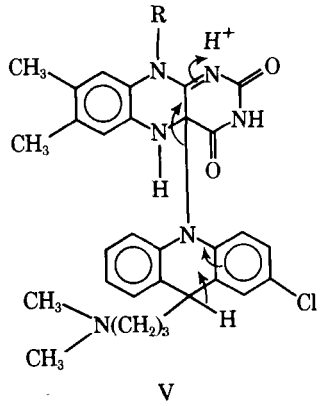
Scheme IV

$$Ia^3 + IIIa^0 \rightarrow Ia^0 + IIIa^3$$

Scheme V

$$IIIa^3 \rightarrow IV + H_2$$

Scheme VI



V

REFERENCES

(1) L. E. Brown and G. A. Hamilton, *J. Amer. Chem. Soc.*, **92**, 7225(1970).

(2) W. P. Jencks, "Catalysis in Chemistry and Enzymology," McGraw-Hill, New York, N.Y., 1969, p. 42.

(3) P. Hemmerich, V. Massey, and G. Weber, *Nature*, **213**, 728(1967).

(4) W. R. Knappe and P. Hemmerich, *FEBS Lett.*, **13**, 293(1971).

(5) V. Massey, F. Muller, R. Feldberg, M. Schuman, P. A. Sullivan, L. G. Howell, S. G. Mayhen, R. G. Matthews, and G. P. Foust, *J. Biol. Chem.*, **244**, 3999(1969).

(6) C. T. Walsh, A. Schonbrunn, and R. H. Abeles, *J. Biol. Chem.*, **246**, 6855(1971).

(7) M. A. Miyamoto, H. B. Kostenbauder, D. Walstad, and G. A. Digenis, *FEBS Lett.*, **7**, 101(1970).

(8) G. A. Digenis, D. L. Walstad, M. Ishaque, and M. I. H. Aleem, *Biochem. Pharmacol.*, **20**, 919(1971).

(9) H. Beinert, in "The Enzymes," vol. II, part A, 2nd ed., P. D. Boyer, H. Lardy, and K. Myrback, Eds., Academic, New York, N.Y., 1960, p. 379.

(10) G. A. Digenis, *J. Pharm. Sci.*, **58**, 335(1969).

(11) O. Warburg and W. Christian, *Naturwissenschaften*, **20**, 980(1932).

(12) G. Oster, J. S. Bellin, and B. Holmstrom, *Experientia*, **18**, 249(1962).

(13) H. Beinert, in "The Enzymes," vol. II, part A, 2nd ed., P. D. Boyer, H. Lardy, and K. Myrback, Eds., Academic, New York, N.Y., 1960, p. 356.

(14) Q. H. Gibson, V. Massey, and N. M. Atherton, *Biochem. J.*, **85**, 369(1962).

(15) J. Posthuma and W. Berends, *Biochim. Biophys. Acta*, **122**, 422(1966).

ACKNOWLEDGMENTS AND ADDRESSES

Received March 12, 1975, from the *College of Pharmacy, University of Kentucky, Lexington, KY 40506*

Accepted for publication May 13, 1975.

* Present address: Arab Pharmaceutical Manufacturing Co., Salt, Jordan.

‡ Present address: Sumitomo Chemical Co., Ltd., Osaka, Japan.

ˣ To whom inquiries should be directed.