70.2% range when used at a final concentration of 1×10^{-4} *M* during oxidative deamination of kynuramine. Compound XII produced maximum inhibition. The degree of monoamine oxidase inhibition was influenced by the position of the hydrazide group on the phenyl nucleus attached at the aminomethyl portion of the benzoxazole or benzthiazole moiety. The presence of the hydrazide group at position 4 of the phenyl nucleus produced greater inhibition of monoamine oxidase in benzoxazole or benzthiazole derivatives. The ability of these hydrazides to inhibit monoamine oxidase was greater with 3-benzhydrazide aminomethyl benzthiazole-2-thiones as compared to their corresponding benzoxazole derivatives. All of these hydrazides possessed low anticonvulsant activity, lower than that of their precursor esters (I–VI). The low anticonvulsant activity of these hydrazides corresponded with high pentylenetetrazol mortality, which ranged from 40 to 90% in experimental mice treated with hydrazide derivatives during 24 hr. None of these compounds exhibited any appreciable sedative or central nervous system (CNS) depressant effect nor 24-hr mortality at 100 mg/kg. These observations failed to provide any correlation between *in vitro* enzyme inhibitory activity of these compounds and their low anticonvulsant activity.

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Mechanism for Riboflavin Enhancement of Bilirubin Photodecomposition *In Vitro*

DILIP R. SANVORDEKER* and H. B. KOSTENBAUDERˣ

Abstract □ Riboflavin increases the rate of bilirubin photodecomposition as much as 25-fold under aerobic conditions. In the absence of oxygen, bilirubin photodecomposition is virtually arrested, with or without riboflavin. It is proposed that singlet oxygen generated by the riboflavin triplet species is primarily responsible for the observed rate enhancement. The occurrence of similar photochemical reactions *in vivo* might provide a means for improving the efficiency of bilirubin phototherapy through systemic administration of appropriate agents.

Keyphrases □ Riboflavin—enhancement of bilirubin photodecomposition *in vitro*, mechanism □ Bilirubin, photodecomposition—mechanism for riboflavin enhancement *in vitro* □ Photodecomposition, bilirubin—mechanism of enhanced rates under aerobic conditions with riboflavin *in vitro*

Bilirubin is a tetrapyrrolic dicarboxylic acid produced *in vivo*, mainly by the hepatic and splenic reticuloendothelial systems, from hemoglobin released from senescent erythrocytes (1). Nonhemolytic neonatal hyperbilirubinemia is ascribed to deficient glucuronidation of bilirubin arising from either capacity-limited hepatic uptake of bilirubin (2, 3) or deficiency of the hepatic enzyme bilirubin uridine diphosphate glucuronyltransferase (4), which catalyzes the conjugation of lipophilic bilirubin to its hydrophilic diglucuronide. Facile elimination of this diglucuronide occurs *via* biliary and urinary excretion.

Phototherapy has become a common procedure in neonatal hyperbilirubinemia (5–8). Despite the well-established efficacy of light therapy in reducing plasma bilirubin levels, reservations have been expressed with respect to exposing infants to light intensities of 300–500 foot-candles, often continuously for several days. Conceivably, an enhancement of bilirubin phototherapy may be accomplished by administration of suitable agents which would promote bilirubin photodecomposition *in vivo*. An enhancement of bilirubin phototherapy could mean either a reduction of exposure time or a reduction of light intensity, thereby reducing some potential hazards (9) of this treatment.

In view of its clinical importance, several investigators have studied bilirubin photochemistry in the presence and absence of a complexing polymeric molecule such as a serum albumin (10–12). Recent studies (13) showed that oxygen is essential for bilirubin photodecomposition. In the presence of oxygen, bilirubin undergoes irreversible photooxidation to a variety of products (14–18), including methylvinylmaleimide, biliverdin, and water- or methanol-propentdyopent adducts. Furthermore, under nitrogen and in the presence of a suitable nucleophilic substrate such as *N*-acetyl-1-cysteine, bilirubin undergoes an irreversible reaction with the substrate to give hydrophilic photoaddition products (19, 20). In the presence of oxygen, light-excited bilirubin sensitizes the formation of singlet oxygen which then attacks ground-state bilirubin with resultant bilirubin photooxidation (21). The same author (21) also showed that singlet oxygen-generating compounds

such as methylene blue enhance the rate of bilirubin photodecomposition *in vitro*.

The photochemistry of riboflavin has been extensively studied (22–26). It has been recognized that riboflavin can promote photodecomposition of other compounds in fluid solution *via* several mechanisms, including energy transfer, coupled oxidation–reduction, and sensitized photooxygenation. Energy transfer involves absorption of light by riboflavin and excitation to a higher energy state (triplet) which may, upon collision with an acceptor molecule, decay to the ground state and transfer the electronic excitation energy to the acceptor. Examples of riboflavin promotion of substrate photodecomposition through triplet–triplet energy transfer include photodecomposition of the polyene fungicide pimaricin (27), isomerization of stilbene-4-carboxylic acid and cinnamic acid (28), and photooxidation of the acridan drug clomacran to its acridine derivative (29). Riboflavin might also be expected to promote photodecomposition of a substrate through a coupled oxidation–reduction process involving hydrogen abstraction by riboflavin triplet. The latter mechanism has been used to explain the flavin-sensitized photooxidation of amino acids (30) and of indoleacetic acid (31). Additional mechanisms that have been described for flavin-promoted photooxidation include singlet oxygen-mediated reactions such as riboflavin-sensitized photooxidation of limonene (32) and reactions involving excited flavin–substrate complexes such as the lumiflavin-sensitized photooxidation of nucleotides (33).

It appeared possible that an *in vitro* enhancement of bilirubin photodecomposition might be achieved through use of a suitable promoter and that such a process might be of clinical value. This report constitutes the investigation on *in vitro* enhancement of bilirubin photodecomposition by riboflavin. Evidence that riboflavin can indeed increase the efficiency of bilirubin phototherapy in an animal model was presented previously (34).

EXPERIMENTAL

Reagents—All reagents except nitrogen gas were used as received from the supplier. The following were used: bilirubin[1]; β-carotene[2]; dimethylformamide[3], 99% pure; potassium iodide USP; sodium carbonate, reagent grade; sodium chloride, reagent grade; trichloroacetic acid, reagent grade; bovine serum albumin, fraction V[4]; and vanadyl sulfate, reagent grade.

Nitrogen gas was purified by passing it through a solution of vanadyl sulfate and sulfuric acid containing zinc–mercury amalgam (35) and then through a 10% solution of pyrogallol in dimethylformamide to remove traces of oxygen.

Preparation of Solutions—Stock solutions for photochemical studies were prepared in subdued light. The bilirubin solution (6.6×10^{-5} *M*), the riboflavin monosodium-5′-phosphate solution (1.94×10^{-4} *M*), and the potassium iodide solution (10^{-3} *M*) were prepared in 0.05 *M*, pH 7.4, phosphate buffer. For the nonaqueous study, the bilirubin solution (6.84×10^{-5} *M*), the β-carotene solution (10^{-3} *M*), the riboflavin solution (1.33×10^{-4} *M*), and the potassium iodide solution (10^{-3} *M*) were prepared in dimethylformamide.

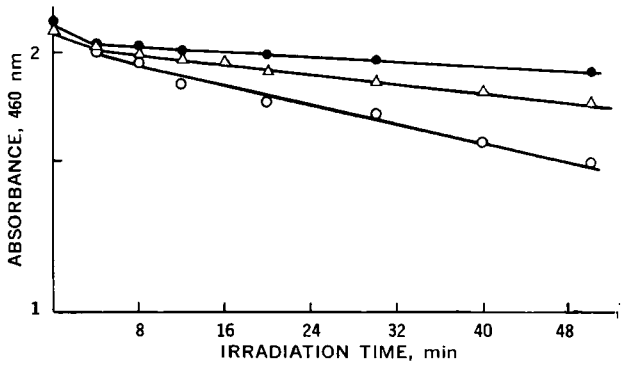

Figure 1—*Effect of oxygen on bilirubin photodecomposition in 1% aqueous serum albumin solution, pH 7.4. Initial bilirubin concentration was approximately 3.4 × 10⁻⁵ M (2 mg %). Key: ●, anaerobic; △, air equilibrated; and ○, oxygen bubbled.*

A stock bilirubin solution in phosphate buffer, pH 7.4, was prepared by dissolving 4 mg of bilirubin in 10 ml of a 1% sodium carbonate solution and then quickly transferring this solution, with constant stirring, to a 150-ml beaker containing a solution of 4 g bovine serum albumin, fraction V, in 60 ml phosphate buffer. The pH of this solution was adjusted to 7.4 with approximately 0.4 ml of 1 *N* hydrochloric acid and the solution was diluted to 100 ml with standard phosphate buffer solution. These solutions were diluted appropriately and used as needed for photochemical studies.

Irradiation Setup and Procedure for Analysis—Photochemical studies were conducted at a room temperature of 22–26°. Solutions were irradiated intermittently in 1-cm quartz cells located 61 cm from a projector equipped with a 500-w tungsten lamp[5]. Studies were conducted both with unfiltered light and with light passed through a filter[6] to exclude wavelengths outside the 360–480-nm range.

Anaerobic studies were conducted in Thunburg and standard spectrophotometric cells fitted with ground-glass stoppers. Aqueous solutions were degassed by a freeze, thaw, and vacuum procedure in three to four cycles. Dimethylformamide solutions were purged for at least 40 min with purified nitrogen prior to irradiation. Absorption spectra were recorded *versus* an appropriate blank solution[7].

To permit determination of riboflavin concentration in aqueous solutions containing unknown quantities of bilirubin, the aqueous solutions were treated with 5% trichloroacetic acid to precipitate the bilirubin–albumin complex. Upon centrifugation, the clear supernatant liquid was diluted and analyzed for riboflavin by a standard fluorometric method (36). Anaerobic solutions of riboflavin containing bilirubin could be analyzed for riboflavin by difference absorption spectrophotometry, since the bilirubin concentration in these solutions remained constant under anaerobic conditions.

To determine the effect of bilirubin on riboflavin photodecomposition under anaerobic conditions, riboflavin solutions in dimethylformamide with and without bilirubin were placed in fluorometric cells and purged with purified nitrogen for at least 40 min. The cells were then quickly stoppered and placed in the spectrophotofluorometer[8]. These solutions were irradiated intermittently with the excitation wavelength set at 440 nm and the slit facing the cell holder removed. The riboflavin concentration in these solutions was monitored by difference absorption spectrophotometry as described previously.

Treatment of Rate Data—The effect of flavin on the rate of bilirubin photobleaching was evaluated by plotting the logarithm of bilirubin absorbance *versus* irradiation time and determining the rate constant for each individual run from the slope of the re-

[1] Lot No. 9324, Nutritional Biochemicals Corp.
[2] Sigma Chemical Co.
[3] Fisher Scientific Co.
[4] Lot No. F32402, Armour Pharmaceutical Co.
[5] Type DAK, Sylvania Electric Products.
[6] CS 560, Corning Glass Works.
[7] Cary 15 spectrophotometer, Cary Instruments Co.
[8] Aminco-Bowman spectrophotofluorometer, American Instrument Co.

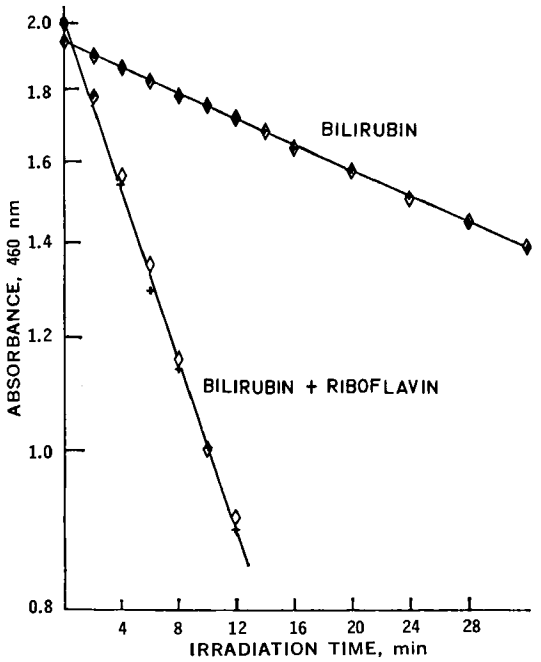

Figure 2—*Effect of 5.3 × 10⁻⁶ M riboflavin on rate of photodecomposition of bilirubin in dimethylformamide solution exposed to unfiltered visible light. Initial bilirubin concentration was approximately 3.4 × 10⁻⁵ M (2 mg %). Key: ◇ and + represent points from duplicate studies.*

sulting linear plot. Control bilirubin solutions containing no flavin were run along with each study of the effect of flavin, and data are reported as the ratio of the rate of bilirubin photodecomposition with flavin to the rate without flavin to compensate for any variation in light intensity from study to study. Each series of studies designed to illustrate the effect of variables such as oxygen concentration was run at the same light intensity.

RESULTS AND DISCUSSION

Figure 1 illustrates the effect of oxygen concentration on the rate of loss of bilirubin absorbance. In both the aqueous serum albumin solution and in dimethylformamide, bilirubin exhibits an increased rate of photofading with increasing concentration of oxygen. The mechanism proposed by McDonagh (21) for bilirubin photooxidation (Scheme I) involves generation of singlet oxygen, *via* light-excited bilirubin, followed by attack of singlet oxygen on ground-state bilirubin. Data obtained in the present study are consistent with such a mechanism.

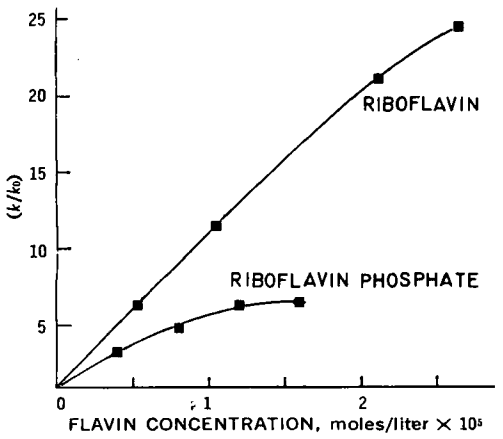

Figure 3—*Influence of flavin concentration on rate of bilirubin photodecomposition in solutions exposed to unfiltered visible light.*

$$B^0 + h\nu \longrightarrow B^1 \text{ (bilirubin in singlet state)}$$
$$B^1 \longrightarrow B^3 \text{ (bilirubin in triplet state)}$$
$$B^3 + O_2^3 \longrightarrow B^0 + O_2^1 \text{ (singlet oxygen)}$$
$$B^0 + O_2^1 \longrightarrow \text{photooxidation products}$$

Scheme I

Figure 2 is a typical plot illustrating the effect of riboflavin on bilirubin photodecomposition in air-equilibrated solutions. At least a 25-fold rate increase may be achieved by riboflavin in dimethylformamide solution, and at least a sixfold rate enhancement may be achieved from riboflavin phosphate in aqueous 1% serum albumin. Potassium iodide, at a concentration of 10^{-4} *M*, reduced the riboflavin enhancement of bilirubin photodecomposition while showing no effect on the rate of bilirubin photodecomposition in the absence of riboflavin. Since potassium iodide at such a concentration is known to be an effective quencher of the flavin triplet state (37, 38), this observation implicates the flavin triplet in the overall enhancement of bilirubin photodecomposition. The lack of any effect of 10^{-4} *M* potassium iodide on the rate of bilirubin photodecomposition suggests that the bilirubin triplet state may be too short lived to collide with potassium iodide at these concentration conditions.

Figure 3 shows a plot of the ratio of first-order rate constants for bilirubin decomposition in the presence of flavin to the rate constant for bilirubin photodecomposition in the absence of flavin under otherwise identical conditions. These data were collected for air-equilibrated solutions exposed to unfiltered visible light. All rate constants were obtained from linear plots of the logarithm of bilirubin absorbance *versus* time. The magnitude of the rate enhancement by flavin varied with light intensity and with wavelength of the light source, but all results were qualitatively similar to those illustrated in Fig. 3. At low flavin concentration, the rate of bilirubin photodecomposition is first order with respect to flavin and becomes independent of change in flavin concentration at high flavin concentration. Such a profile is to be expected for a flavin-sensitized photochemical reaction.

It has become increasingly recognized that many photooxidation reactions proceed *via* singlet oxygen. Kearns *et al.* (39) suggested that the triplet energy of the sensitizers of singlet oxygen should be in the range of 37 kcal. In view of the reported triplet energy level of riboflavin at 46 kcal (40), it is evident that an efficient collisional energy transfer from riboflavin triplet to oxygen triplet (ground state) is possible on energetic grounds. Since riboflavin is capable of generating singlet oxygen (ΣO_2^1 or ΔO_2^1) *via* its excited triplet state (32, 41–43), it is possible that riboflavin might promote bilirubin photodecomposition by this mechanism. β-Carotene, an efficient singlet oxygen quencher at 10^{-4} *M* concentration (43), completely quenched bilirubin photodecomposition in the absence of flavin and partially inhibited the promotion of bilirubin photodecomposition by riboflavin. This observation is consistent with a mechanism (Scheme II) involving generation of singlet oxygen by riboflavin.

As shown in Figs. 1 and 4, absence of oxygen virtually arrests bilirubin photodecomposition, with or without riboflavin. Since there was no evidence for any photodecomposition of bilirubin by riboflavin under anaerobic conditions, the possibility of a coupled oxidation–reduction process involving bilirubin and riboflavin can be rejected.

Figure 5 shows that the presence of bilirubin decreases riboflavin fluorescence. The decrease in riboflavin fluorescence is obtained under both aerobic and anaerobic conditions. Such fluorescence quenching might be attributed either to an energy transfer from light-excited flavin to bilirubin or to an "inner filter" effect (44) arising from the bilirubin absorption spectrum overlapping that of riboflavin from 350 to 550 nm. Without knowledge of the triplet level of bilirubin, the possibility of a triplet–triplet energy

$$Rf^0 + h\nu \longrightarrow Rf^1 \text{ (riboflavin singlet)}$$
$$Rf^1 \longrightarrow Rf^3 \text{ (riboflavin triplet)}$$
$$Rf^3 + O_2^3 \longrightarrow Rf^0 + O_2^1 \text{ (singlet oxygen)}$$
$$B^0 + O_2^1 \longrightarrow \text{photooxidation products}$$

Scheme II

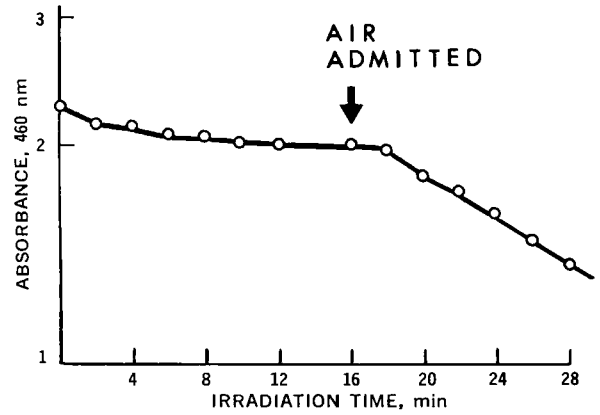

Figure 4—*Effect of oxygen on bilirubin photodecomposition under blue light in the presence of 9.6 × 10^{-6} M riboflavin phosphate in 1% aqueous serum albumin, pH 7.4. Initial bilirubin concentration was approximately 3.4 × 10^{-5} M.*

transfer could not be excluded on energetic grounds. However, if such an energy transfer were to occur, it would be anticipated that it would serve to depopulate the riboflavin triplet and thus retard the photobleaching of riboflavin. As shown in Fig. 6, the presence of bilirubin retards riboflavin photobleaching, but it can be shown that the extent of inhibition does not exceed that to be expected simply from the inner filter effect of overlapping absorption spectra of bilirubin and riboflavin.

To determine whether the effect of bilirubin on flavin fluorescence and photofading is attributable solely to an inner filter effect, a quantitative comparison was made between the effect of bilirubin on the rate of anaerobic photofading of riboflavin and the relative amounts of light absorbed by riboflavin alone and in the presence of bilirubin. If the effect of bilirubin is attributable solely to an inner filter effect, the rate of riboflavin photofading in the presence of bilirubin should decrease in direct proportion to the decrease in the quanta of light absorbed by riboflavin[9]. Based on determination of the absorbances of riboflavin alone and of a solution containing both riboflavin and bilirubin, it can be shown that 1.32 × 10^{-4} *M* riboflavin in the presence of 1.44 × 10^{-5} *M* bilirubin absorbs only 57% as much of the incident light as in the absence of bilirubin. After 30% of the riboflavin is decomposed (range over which the study was conducted), there would be 52% as much light absorbed by riboflavin in presence of 1.44 × 10^{-5} *M* bilirubin. It might, therefore, be anticipated that the rate of photofading of riboflavin, in decreasing the concentration from 1.32 × 10^{-4} to 0.92 × 10^{-4} *M* in the presence of 1.44 × 10^{-5} *M*

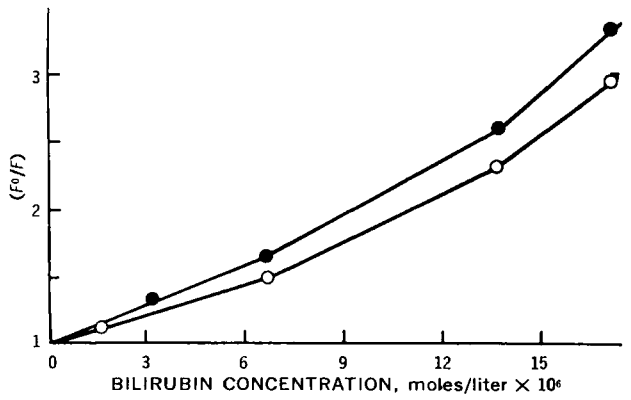

Figure 5—*Effect of bilirubin on flavin fluorescence intensity, where F°/F is the ratio of flavin fluorescence in absence of bilirubin to that in the presence of bilirubin. Key: ●, riboflavin phosphate, 9.6 × 10^{-6} M in 0.2% aqueous serum albumin; and ○, riboflavin, 1.3 × 10^{-5} M in dimethylformamide.*

[9] The comparison is made for the anaerobic data since under these conditions the bilirubin concentration remains constant.

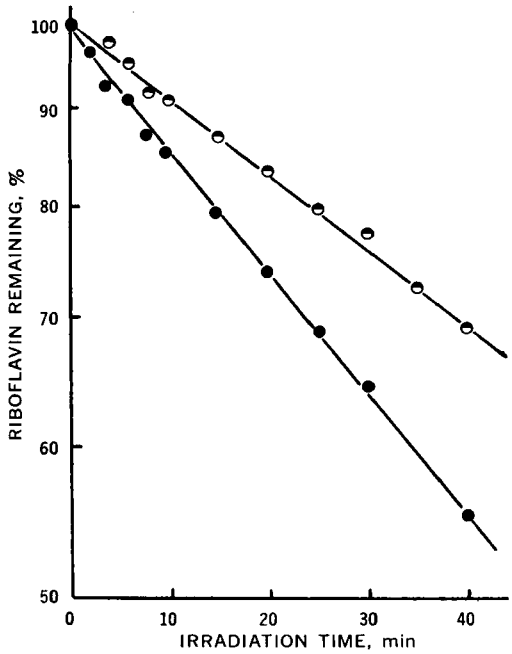

Figure 6—*Effect of bilirubin on rate of riboflavin photodecomposition in deoxygenated dimethylformamide solution. Initial riboflavin concentration was 1.32 × 10^{-4} M, and bilirubin concentration was 1.44 × 10^{-5} M. First-order rate constant is 0.0144 min^{-1} for riboflavin alone and 0.0092 min^{-1} in the presence of bilirubin. Key: ◓, riboflavin plus bilirubin; and ●, riboflavin alone.*

bilirubin, would be between 57 and 52% of the rate observed in the absence of bilirubin. In fact, the comparison of the rates shown in Fig. 6 indicates that the apparent first-order rate of riboflavin photofading in the presence of 1.44 × 10^{-5} *M* bilirubin is approximately 64% of the rate observed in the absence of bilirubin. Within experimental limitations, it appears that the inhibition of riboflavin photofading by bilirubin may be attributed solely to an inner filter effect. Since the observed rate is slightly greater than, if not equal to, the predicted rate in the presence of bilirubin, the possibility of an energy transfer (triplet-triplet or singlet-singlet) from riboflavin to bilirubin, existence of which would be expected to depopulate the riboflavin triplet state and further slow riboflavin photofading, can be ignored.

It is also evident from these studies that serum albumin-bound bilirubin must be capable of participation in photochemical reaction in the bound state. For singlet oxygen, with a lifetime of the order of 10^{-5} sec, to collide with a reactive substrate, the concentration of that substrate in solution should be above or in the range of 10^{-4}–10^{-5} *M* (41). Since the saturation solubility of unbound bilirubin at pH 7.4 is reported as 1.71 × 10^{-6} *M* (45), it is evident that *in vitro*, and perhaps *in vivo*, photooxidation of bilirubin *via* singlet oxygen can involve attack on bilirubin in the bound state as well as on free bilirubin.

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Homogeneity of Multicomponent Powder Mixtures

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Abstract □ A new concept of homogeneity enables a numerical value to be attributed to the homogeneity, or "mixedness," of a given system. This concept was used to calculate the homogeneity of single ingredients in multicomponent, or compound, tablets and to trace the degree of mixing in two multicomponent mixtures. The relationship between homogeneity and a mixing index is mathematically derived.

Keyphrases □ Homogeneity of multicomponent powder mixtures —numerical calculation of homogeneity and degree of mixing, particle-size considerations □ Powder mixtures, multicomponent—numerical calculation of homogeneity and degree of mixing, particle-size considerations □ Mixing and homogeneity of multicomponent powders—calculation of homogeneity and mixing indexes, particle-size considerations

The operation of powder mixing is common to the manufacture of many formulations in the pharmaceutical industry. While the problems involved with powder mixtures were elucidated previously (1), further investigations have been concerned with the relatively simple system of binary mixtures (2, 3). In practice, such binary mixtures are of academic importance only, since the mixing of either a single drug or a number of drugs with a number of excipients is a more frequent occurrence in pharmacy. Two such multicomponent powder mixing operations were recently investigated (4, 5).

DISCUSSION

The criteria for adequate mixing of binary systems were reviewed (6). Indexes of powder mixing were described utilizing the standard deviation of the theoretically randomized mixture (σ_R) originally described by Lacey (7); for simple binary mixtures of homosized particles: