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Laser Investigation of Bilirubin-Photobilirubin Photoconversion

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ABSTRACT. The reversibility of the configurational photoisomerization process of bilirubin (BR) with laser lines in the blue-green spectral region is investigated. Photoisomerization efficiency of BR is found to depend strongly on wavelength, and to decrease when the excitation wavelength is increased from blue to green. Reversion of BR photoisomers (≡ photobilirubin, PBR) back to native BR is demonstrated for several laser lines by irradiating PBR/BR mixtures with wavelengths greater than the excitation wavelengths. Green lines turn out to be very efficient for PBR → BR reversion. The PBR concentrations at photoequilibrium, obtained from the spectrophotometric data, are in close agreement with the corresponding values measured with the high performance liquid chromatography technique in the case of 10 nm bandwidth filtered light reported in the literature. The 457 nm blue laser line produces 32% PBR concentration at photoequilibrium; only 14, 7, and 3% PBR concentrations are produced by the blue-green lines at 488, 501, 514 nm, respectively. The effect on the photostationary PBR/BR mixture of successive irradiations with different wavelengths, and the influence of the wavelength sequence are reported. In the case of blue lines our results support the assumption of the first-order kinetics for the BR ⇌ PBR photoreaction. Departures are observed with green-lines (501, 514 nm). The present results, together with the i) good clinical efficiency reported for fluorescent green lamps; and ii) slow elimination of configurational photoisomers in infants, tend to confirm the lumirubin-pathway as the main mechanism for phototherapy, and call for clinical investigation of narrow-spectrum lamps with peak emission wavelength in the (biologically safer) 480 ÷ 530 nm range. (*Pediatr Res* 19: 166–171, 1985)

Abbreviations

BR, bilirubin
HBR, hyperbilirubinemia
PBRs, photobilirubin
LR, lumirubin
PE, photoequilibrium
HSA, human serum albumin
DAS, difference absorption spectra

The investigation of the photophysical and photochemical properties of BR is of great relevance with a view to the application of phototherapeutical techniques to the treatment of HBR of infants (18). Light-induced conversion of BR into a mixture of conformational and structural isomers less lipophilic than BR is considered today the main mechanism responsible for the lowering of BR concentration in skin tissues and blood (11). The conformational photoisomers, called collectively PBRs, are known to be unstable geometric configurations of BR, which may revert into BR or go into more stable isomeric forms upon thermal or optical excitation (12). A new structural, nonreversible, isomer of BR has been recently discovered, and called LR (11). In the following scheme the "fast" anaerobic photochemistry of BR as presently accepted is illustrated (11):
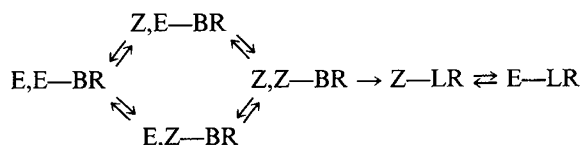
$$\text{Z,E—BR} \quad \text{Z,Z—BR} \rightarrow \text{Z—LR} \rightleftharpoons \text{E—LR}$$
$$\text{E,E—BR} \qquad \text{E,Z—BR}$$

On the time scale of the Z–E conversion, LR formation is slow and irreversible. Any progress in the understanding of BR photochemistry may lead to a better knowledge of the mechanism responsible for phototherapy and to an improvement in phototherapeutical procedures.

From the clinical point of view the most effective spectral region of BR degradation in the organism is still considered that matching the visible absorption spectrum maximum of BR *in vitro* (~450 nm). However, recent investigation of BR photodegradation *in vitro* and *in vivo* made with monochromatic optical sources (lasers) shows that other spectral regions at longer wavelengths may be more suitable for phototherapy (5, 17). The use of lasers for the analysis of the photophysical and photochemical properties of BR and of its photoproducts has been found to be particularly suitable due to their monochromaticity and frequency tunability, which make it possible to show up small differences in the physical and chemical behavior of PBRs. Moreover, ultra-short laser pulses can be very conveniently used to investigate the BR → PBR photoreaction on a very short time scale (4).

Herein we report the laser investigation of the chromatic dependence of PBR/BR PE and of PBR → BR photoreversion. Connections with clinical application are discussed.

MATERIALS AND METHODS

Light source. Monochromatic light was provided by a continuous wave Argon laser (model 165–008 Spectra Physics) at 457.9, 465.8, 488.0, 501.7, 514.5 nm. The laser beam was expanded to provide essentially uniform energy fluence across the cuvette face. For all the experiments, irradiance was monitored with a laser power meter (EG & G model 460).

Bilirubin solution. BR and HSA Cohn fraction V were supplied by SERVA. Each was used as supplied. Solution of BR (10 μM/liter) and HSA (115 μM/liter) contained 0.15 M NaCl and 0.01 M phosphate buffer (purged with Ar), pH = 7.4. [The HSA concentration was calculated assuming the molecular weight to be equal to 69000, while the BR concentration was determined spectrophotometrically, assuming the molar absorptivity at 460 nm to be equal to (4.7 ± 0.13) 10^4 cm^{-1} M^{-1}. No attempt was done to determine the degree of purity of both compounds.] The HSA-phosphate buffered saline solution, which was prepared 24 h before each experiment, was gently and magnetically stirred for 30 min in pure Ar. Two hours before each experiment, BR was dissolved in 0.05 M sodium hydroxide without shaking and rapidly added to the HSA-buffered solution. Solutions containing BR were manipulated in dim light and were kept under an atmosphere of Ar to avoid the photooxygenation of BR. Spectral measurements were carried out at 23 ± 2° C.

Absorbance difference spectroscopy. A standard experimental set-up has been used to measure DAS of irradiated BR solutions, and is reported elsewhere (15).

At small photon fluence rate, configurational isomers (PBRs) are the main photoproducts (7); structural, nonreversible photoproducts (such as lumirubin), and photooxidative products are produced at much smaller rates (11), but, as they accumulate in time, their concentration may increase to a significant level when the irradiation time necessary to reach PBR PE is long.

RESULTS

Wavelength dependence of PBR formation. The formation of PBRs and the achievement of the photostationary state of the reaction BR ≤ hν ≥ PBR have been investigated using the 457, 465, 488, 501, 514 nm laser lines at excitation irradiances of 25, 50, 500 μW/cm^2, and 15 mW/cm^2. The laser output powers at the various wavelengths were adjusted to give the same value of photon fluence rate.

Figure 1*A* shows the DAS in the 430 ÷ 520 nm range taken at irradiance of 500 μW/cm^2, for several values of the excitation wavelength. An isosbestic point is present at $\lambda \simeq 465$ nm, and the DAS maximum occurs at $\lambda \simeq 490$ nm. Similar spectral patterns are obtained at the lower and higher irradiances used. As can be seen, the 457-line produces the highest value for the PBR/BR mixture, while the 514-line is quite ineffective for PBR formation. At high irradiance the PE is reached in quite a short time: further irradiation of the solution produces slow degradation of the PBR/BR mixture, as demonstrated by the corresponding lowering of the entire DAS.

These results are in qualitative agreement with the recently reported measurements of PBR concentration at PE using 10 nm bandwidth filtered light at different peak wavelengths (2).

Spectral dependence of PBR → BR reversion. PBRs have been observed to revert to original BR upon irradiation with $\lambda = 510$ nm radiation (2, 10). We have checked PBR reversion in greater detail using laser lines at 488, 501, 514 nm, and at several values of laser irradiance. Figure 1*B* shows the formation of a new PE value of PBR/BR mixture, obtained upon irradiation of BR at 457 nm, using the three lines at 488, 501, 514 nm. It therefore appears that i) the complete reversion of PBR to BR cannot be achieved with these lines; ii) greater reversion efficiency is associated with the 514-line, followed in descending order by the 501 and 488 lines.

A comparison of the new PE values obtained with the irradiation sequence 457/λ_i (λ_i = 488, 501, 514 nm) with those generated by direct excitation with the λ_i lines (Fig. 1) shows that the DAS values for the two-step 457/488 and one-step 488 excitation are equal within the experimental errors. On the contrary the green line excitation produces DAS values that depend on the irradiation sequence, the effect being more pronounced with the 514-line. Moreover, the reverted DAS of the two-step irradiation are now asymmetrical, with a red-shifted maximum and a larger absorption in the long-wave wing. This effect is much less evident with the 488-line (Fig. 1 *B*, *a*).

Effect of the irradiation sequence order. As a further step, we have checked the reversibility of the new PE mixtures back again to the initial PE mixture by irradiating them with the 457-line. Figure 2*A* shows schematically the behavior of the DAS at $\lambda = 490$ nm as a function of energy fluence for the sequence 457/λ_i/457 (λ_i = 488, 501, 514 nm); for $\lambda_i = 501$ nm the full DAS are shown below each time cycle (Fig. 2 *B*). The initial PE values formed with direct excitation were in all cases recovered with a good degree of accuracy. However the DAS shapes revealed a larger absorption at longer wavelengths, that is more pronounced for the green than for the blue lines (Fig. 2 *A*, *3*, and *1*).

Finally we have examined the dependence of the one-step intermediate and two-step final PE values on the order of irradiation sequence. BR solutions have been irradiated with the 488, 501, 514 lines to give three different PE values, and then irradiated with the 457-line up to the final PE. The DAS at 490 nm for these λ_i/457 sequences are shown in Figure 2 *C*. As can be seen the final PE values of the λ_i/457 sequence equal with good degree of accuracy the corresponding 457/λ_i/457 PE values. Again the $\lambda_i = 488$ nm intermediate PE values for the two sequences are nearly equal. On the contrary, the green-line intermediate PE values are different and the difference is greater for the 514-line. Moreover, the DAS obtained with direct excitation with any line are symmetrical and similar in shape, while they differ from the DAS of the intermediate PE (the effect with the 488-line is much smaller than with the green lines) [in Fig. 2 *B*, *4* only the DAS for $\lambda_i = 501$ nm is shown (1)]. Furthermore, the comparison of DAS shapes of the three and two-step cases shows that i) the absorption in the long-wave region after the 457/λ_i sequence is higher than the corresponding value after the λ_i/457 sequence; ii) this effect increases with increasing λ_i.

Configurational isomerization of BR is considered a first-order reversible photoreaction (7). The fractional PBR concentration at PE, $n_{PBR}(\lambda_p)$, can be obtained from the difference absorbance
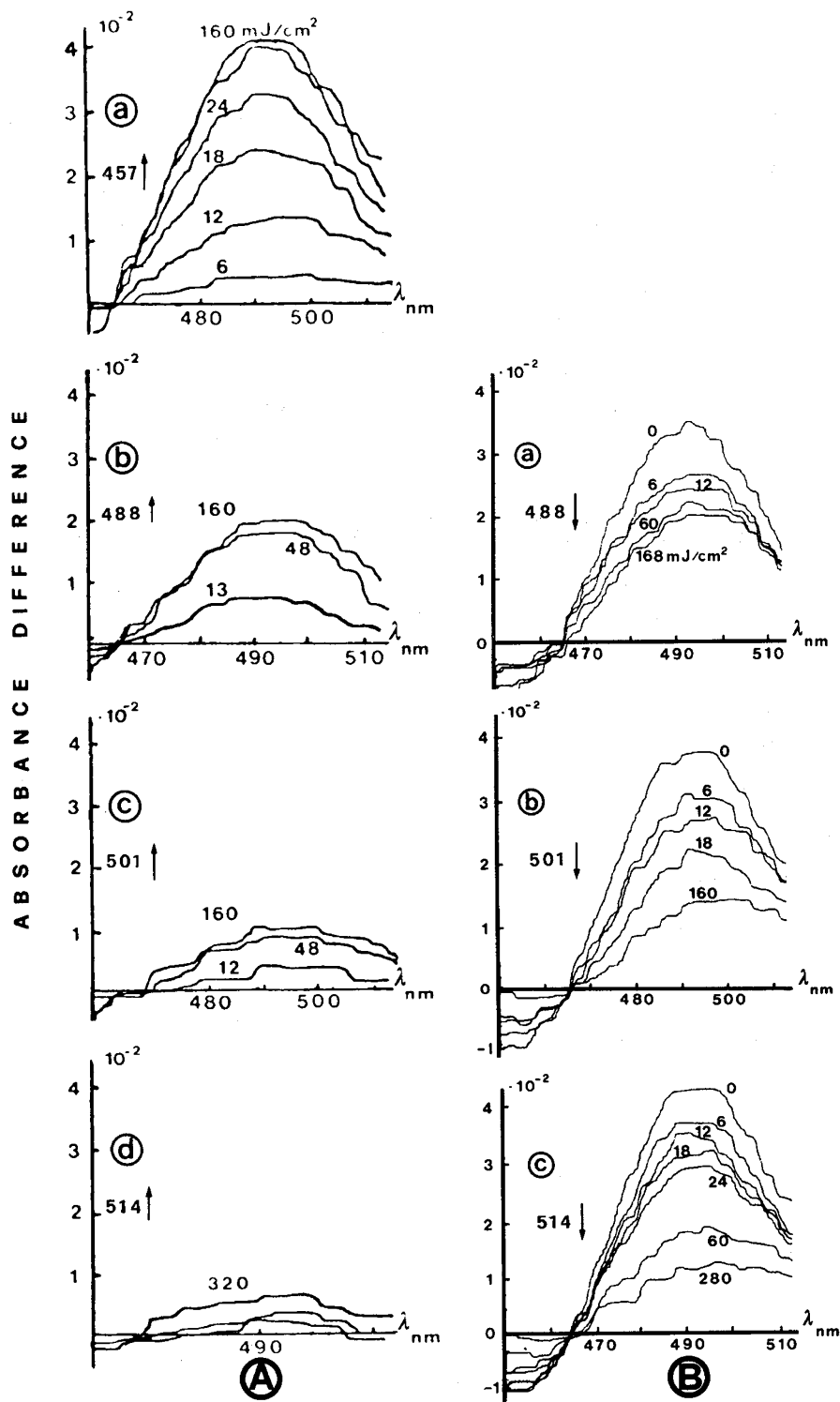
Fig. 1. *A*, DAS at increasing energy fluence up to photoequilibrium for various irradiation wavelengths: *a*, 457, *b*, 488, *c*, 501, *d*, 514. $P_{laser} \simeq 0.5$ mW/cm². *B*, DAS obtained by irradiating at different wavelengths λ_i the PE mixture, generated by the 457-line, up to new photoequilibrium values. $\lambda_i = a$, 488.0 nm; *b*, 501.7 nm; *c*, 514.5 nm.

spectra:

$$\Delta\alpha(\lambda, \lambda_p) = -\alpha_{10}(\lambda)n_{PBR}(\lambda_p)[1 - \sigma_2(\lambda)/\sigma_1(\lambda)] \qquad (1)$$

where $\alpha_{10}(\lambda)$ denotes the initial absorption coefficient of the BR solution; σ_1, (λ), $\sigma_2(\lambda)$ the absorption cross-section of BR, and PBR, respectively; The analytical expression of $n_{PBR}(\lambda_p)$ for mon-

ochromatic light excitation at wavelength λ_p is given by (14):

$$n_{PBR}(\lambda_p) = \left[1 + \frac{\phi_2\sigma_2(\lambda_p)}{\phi_1\sigma_1(\lambda_p)}\right]^{-1} \qquad (2)$$

here $\phi_1(\phi_2)$ denotes the quantum yield for BR (PBR) conversion to PBR (BR). According to equation (1), the shape of DAS

depends only on σ_1 and σ_2, and on their values at the excitation wavelength λ_p (and quantum yields). Moreover, in case of multi-step irradiation with different λ_p, it turns out that the final PE value depends only on the last λ_p used, and thus it equals the PE value produced by direct excitation at that λ_p.

While the quantum yield ratio ϕ_2/ϕ_1 has been found to be ~4 by Lamola et al. (7) using fluorescence spectroscopy, the PBR cross-section $\sigma_2(\lambda)$ is not yet available in the literature. We have
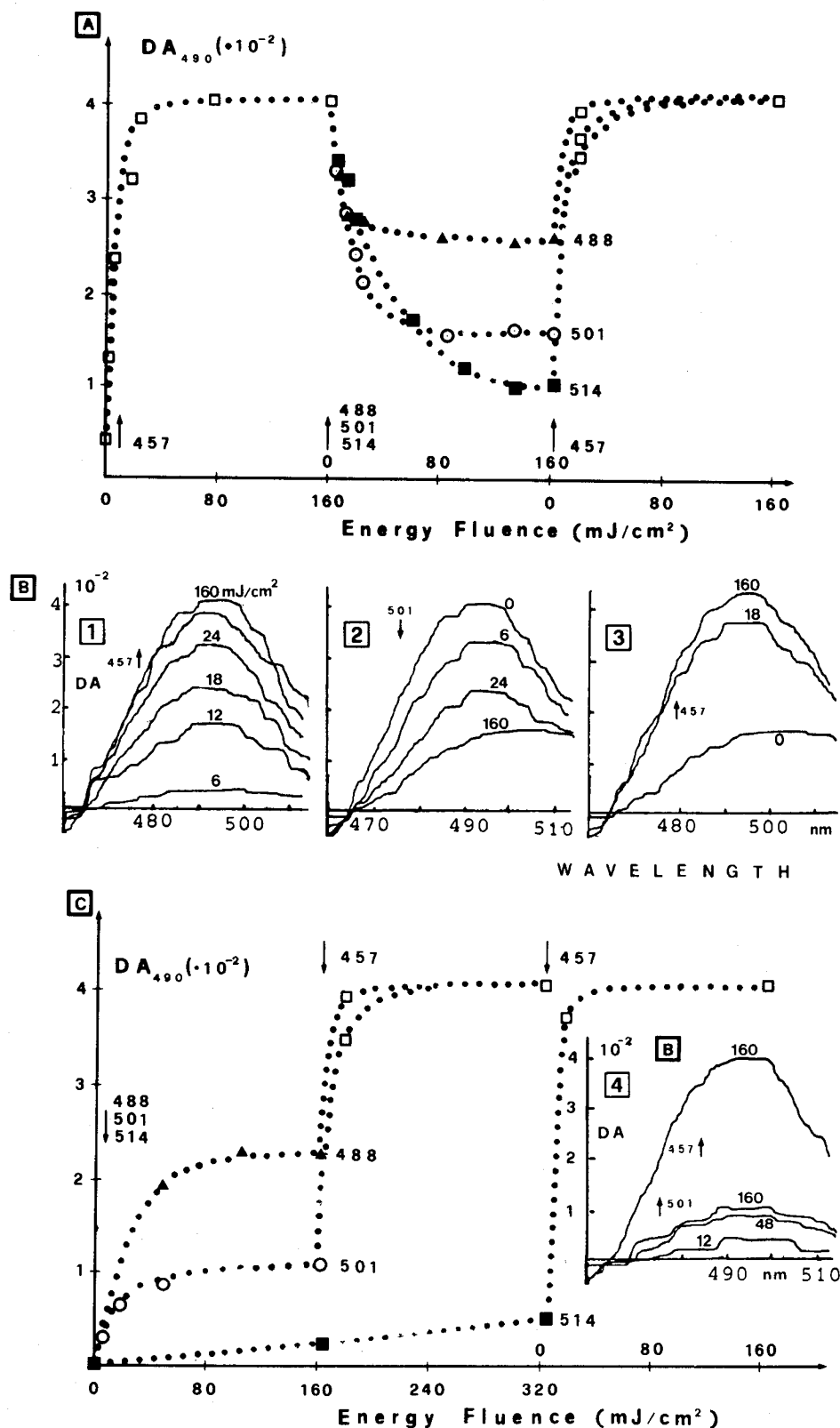

Fig. 2. Temporal behavior of 490 nm-absorbance during irradiation at different wavelength sequences. A, the BR solution is irradiated up to PE, first with the 457-line, then with the λ_i-line up to the new PE; the 457-line is finally used to recover the initial PE value. C, in the second sequence the λ_i-line is used first to generate the PBR/BR PE mixture, followed by irradiation with the 457-line. The corresponding DAS at PE are shown below each time cycle for $\lambda_i = 501$ nm. (B 1, 2, 3 for the 457/λ_i/457 sequence; and B 4 for the λ_i/457 sequence.)

Table 1. *Experimental values of n_{PBR} obtained from equation (1)*

	$n_{PBR}(\%)$	
λ_p (nm)	Experimental	Theory
457	32.3	31
488	14.5	18.7
501	7.6	14.5
514	3.4	12.0

indirectly obtained σ_2 from the differential absorption spectrum published by Ennever et al. (2). Moreover, by fitting their PE concentrations of PBR with equation (2) we have found ϕ_2/ϕ_1 ~2.3 in the spectral range 390 ÷490 nm (14).

In Table 1 we present the experimental values of n_{PBR} obtained from equation (1). The n_{PBR} values of equation (2) with ϕ_2/ϕ_1 = 2.3 are also shown for comparison purposes. A close agreement is found with blue lines; green lines, however, produce PBR densities much smaller than those predicted by equation (2). It is worth noting the large difference between PBR densities produced by blue and green lights.

Most of our results with blue lines (*i.e.* complete reversibility of the photoreaction, equivalence of irradiation sequences) support the first-order kinetics for BR photoisomerization. However, departures of the DAS values and shapes from those expected have been observed when the green lines (501, 514 nm) are used. The observed deviation from the expected reversibility of the Z-E reaction may be due to the presence of free BR, which is quite possible at high BR/HSA ratio. Owing to the long-wave narrowing of the absorption spectrum of free BR in acqueous solutions, the 488, 501, and 514-lines will have increasingly longer PE rise-times, and the PE state will not be reached at the intermediate steps λ_i. Therefore, in the $457/\lambda_i/457$ sequence only a small fraction of free Z,E-BR reverts to native free Z,Z-BR, while direct irradiation at λ_i produces only a small amount of Z,E-BR during irradiation times suitable to obtain the PE of BR/HSA.

DISCUSSION

The present results show that green light at 514 nm is quite ineffective in producing photoisomerization of BR to PBR, and very efficient for causing PBR to revert to native BR. This depends on a larger cross-section of PBR than BR in this spectral region and/or on a larger quantum yield for the PBR → BR than for the BR → PBR process.

Since our clinical results clearly indicate that green lamps ($\lambda_p \cong 525$ nm, $\Delta\lambda \cong 35$ nm) are more effective than white lamps (19, 20) or as efficient as special-blue lamps (1), the present data would seem to suggest that BR configurational isomerization is not the main process responsible for BR elimination in the organism. Our *in vitro* and clinical observations support the findings of Ennever and Speck (3), in whose work large PE concentrations of PBR during PT and very long decay times after cessation of PT were measured in infant sera. LR on the other hand reached low PE concentration and quickly disappeared after PT interruption.

If LR i) is the main PT pathway and ii) originates only from BR, the formation of PBR is no longer a useful process for PT. Good PT efficiency could, then, be achieved by using a light source producing sufficient absorption by BR to form LR and, at the same time, much larger absorption by PBR to inhibit the photoisomerization process (14). The clinical success of fluorescent green lamps may have been due to this mechanism.

In conclusion we have presented the detailed evidence of wavelength-dependence of the photoisomerization process of BR and of its configurational isomers PBRs using laser light excitation. The present measurements further tend to confirm the simple mechanism for the photoisomerization reaction BR ⇌

PBR (7), and are in general agreement with the results of the rate equation theory of BR photoisomerization (14). The relative efficiency of the blue-green lines to form PBR/BR mixtures has been evidenced. The 514-line of the Ar laser turned out to produce very small steady-state PBR concentration and to revert PBR to BR very efficiently. The detailed knowledge of the photophysical processes of BR and PBRs is particularly important with a view to the possible improvements of the phototherapeutical procedure. The present results, connected with the unexpected good clinical efficiency of narrow-spectrum fluorescent green lamps and with the measurements of the production and elimination of PBR and LR in infants exposed to PT, i) support the new idea that configurational isomerization of BR is not the main process in PT of HBR in infants; ii) indicate that fluorescent green lamps may produce sufficient LR and at the same time inhibit PBR formation to ensure good clinical efficacy; iii) stimulate the determination of a clinical action spectrum in the spectral interval 480 ÷ 530 nm, that could lead to an improvement in PT efficiency. The above wavelength range would, moreover, ensure a reduction in the amount of DNA damage [strand breakage, whose action spectrum exhibits a secondary maximum at 450 nm (21)], and in the filtering action of skin (16).

Before concluding, it is worth reporting that recent measurements carried out by our group show that more than 50% of the PBR concentration produced by fluorescent green lamps (Sylvania F12F20/G) is due to the intense Hg-lines (15). These lamps seem to work as a "two-light" source (13), where the long-wave component efficiently quenches the Z→E counterproductive process. However, final conclusions can be drawn for the clinical procedure only from accurate clinical studies. If the "twist-back" to low-quantum-yield processes is confirmed by further *in vitro* and clinical studies, the role of the photooxidative process has to be reestablished (9), since its quantum yield is only five times smaller than that of LR (6).

Added in proof. The degree of BR purity was determined by TLC analysis. BR isomers IIIα + XIIIα concentration was <3%.

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Serum Neutral α-D-Glucosidase from Patients with Cystic Fibrosis and Chronic Pulmonary Disease

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ABSTRACT. Previous studies have indicated that α-D-glucosidase activity is increased and exhibits abnormal properties in tissues from patients with cystic fibrosis (CF). In the present investigation serum α-D-glucosidase from patients with CF and from patients with chronic pulmonary disease (e.g. asthma and bronchiectasis) has been studied to determine the specificity of the α-D-glucosidase alteration(s) in CF sera. Both groups of patients have elevated α-D-glucosidase activity levels and similarly abnormal isoelectric focusing profiles with significantly less activity associated with acidic enzymatic forms (i.e. having isoelectric points below 4.8). These results suggest that the abnormalities of CF serum α-D-glucosidase may be secondary to chronic pulmonary disease and emphasize the importance of including appropriate pathological controls in biochemical studies on CF. (Pediatr Res 19: 171–174, 1985)

Abbreviations

CF, cystic fibrosis
pI, isoelectric point

CF is one of the most common genetic diseases among Caucasian children and young adults. It manifests itself as a gener-

alized disorder primarily affecting exocrine glands and the pulmonary and gastrointestinal systems (9). The major clinical findings include abnormally high sodium and chloride concentrations in sweat, pancreatic exocrine insufficiency, and chronic progressive obstructive and destructive pulmonary disease. The prognosis is poor and death usually occurs before or in early adulthood, most often as a result of respiratory failure (9). The primary biochemical defect in this lethal disease is unknown and no adequate biochemical marker for detecting heterozygous carriers is yet available.

Many biochemical studies have investigated the presence of factors, abnormal proteins and/or altered enzymes in tissues derived from patients with CF (1, 7, 9). Numerous studies have been done on lysosomal glycosidases but few consistent findings have been reported. Among the most reproducible of the findings on glycosidases is the significant elevation of α-D-glucosidase activity in CF tissues (2–7). In a previous study we confirmed the fact that neutral α-D-glucosidase activity is significantly elevated in CF sera (2). In addition, we demonstrated that this increased activity was not due to 1) increased stability upon storage at −20° C, 2) the presence of activators in CF sera or inhibitors in control sera, or 3) kinetic differences of the α-D-glucosidases. The major apparent difference between CF and control sera α-D-glucosidases was revealed in isoelectric focusing studies which indicated that significantly less CF α-D-glucosidase activity was associated with enzymatic forms with pI's below 4.8.

In the present investigation additional studies have been performed on serum α-D-glucosidase from patients with CF and from patients with chronic pulmonary disease (without CF) to determine the specificity of the α-D-glucosidase alteration(s). The results indicate that both groups of patients have elevated serum α-D-glucosidase activity levels and similarly abnormal isoelectric focusing profiles, suggesting that the abnormalities may be secondary to chronic pulmonary disease.