0031-3998/84/1804-0378$02.00/0
PEDIATRIC RESEARCH

Vol. 18, No. 4, 1984
Printed in U.S.A.

pH Dependence of the Water Solubility of Bilirubin Photo-Derivatives and its Relevance to Phototherapy

PAOLO MANITTO,[28] DIEGO MONTI, AND ERSILIA GARBAGNATI

Institute of Organic Chemistry, Faculty of Science and CNR Center for the Study of Natural Products, University of Milan, Milan, Italy and Division of Pediatrics, L. Sacco Hospital, Milan, Italy

Summary

Deoxygenated chloroform solutions of bilirubin were irradiated with visible light and continuously extracted with aqueous solutions at different pHs in the range of 7.20–8.20. The aqueous solutions became yellow rapidly and progressively: the higher their pH, the more intense their coloration. The water soluble E-isomers of bilirubin may not represent the only photoproducts transferred into water. It clearly appears from visible absorption measurements that the photopigment formed in our experiments can be partitioned from chloroform into water at pH as low as 7.2–7.4. Although the water solubility of the photopigment cannot be exactly calculated from experimental data, a direct relationship between water partitioning and water solubility can be reasonably assumed. The fact that the water solubility of the photopigment sharply increases in inverse proportion to the hydrogen ion concentration can be of great relevance to the treatment of jaundiced infants with phototherapy.

Abbreviations

HPLC, high pressure liquid chromatography
MS, mass spectrometry
TLC, thin layer chromatography

It is now well recognized that bilirubin, when irradiated in solution with blue light, undergoes rapid transformation into a mixture of yellow products absorbing in the range 400–500 nm. Among them, the three geometric isomers of bilirubin with different configurations about the 5, 15 *meso* double bond (Fig. 1) are likely predominant, as supported by chemical and spectroscopic evidence (9, 16). Their formation precedes or accompanies other photoreactions of bilirubin, such as photo-oxidation (8), photo-addition of nucleophilic molecules to the 18-vinyl group (4, 12, 13), photoscrambling to IIIα and XIIα isomers (1), and production of certain poorly characterized yellow products, e.g., photobilirubin II (27), "430 pigment" (6), "415 pigment" (21), and "peak 1" (5). The configurational photoisomers of bilirubin, commonly known as photobilirubin (9), appear to be more polar, less lipophilic, and probably more acidic than the natural pigment (10). These properties are a consequence of the reduction in the number of intramolecular hydrogen bonds involving the carboxy groups of bilirubin (14) (Fig. 1).

Since 1974 bilirubin photoisomerization has been suggested as the key reaction in phototherapy of the neonatal jaundice (18), in that it could explain the enhanced excretion of pigment in bile during phototherapy (11, 24), the almost instantaneous biliary pigment excretion that occurs when Gunn rats are exposed to light (15), and the rapid excretion of injected photobilirubin in the Gunn rat kept in the dark (19). Finally, convincing evidence that photobilirubin is actually formed *in vivo* has recently been given by Onishi *et al.* (22, 23) and Lamola *et al.* (7), who detected a significant amount of it in sera of hyperbilirubinemic newborns during phototherapy by HPLC and fluorimetric methods, respectively.

These considerations prompted the present study on the solubility of photobilirubin at different pHs in the range 7.20–8.20. To our knowledge no such study has been reported despite its relevance to optimizing phototherapy.

MATERIALS AND METHODS

Bilirubin (Merck) was purified as described (17) and checked for purity by elemental analysis and by HPLC (23). Chloroform was prepared ethanol free by percolation through a column of alumina.

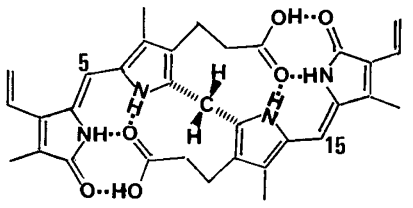
Visible absorbances of aqueous solutions were measured in 3-ml cuvettes using a Cary-188 dual beam spectrophotometer. The light source was a 20 W white fluorescent tube (Osram). Analogous results were obtained with a 20 W blue fluorescent tube.

Irradiation of chloroform solutions of bilirubin (9.4×10^{-5} M) and extraction of the resulting photopigment by aqueous solutions at different pHs (0.005 M phosphate buffers ± 0.02 pH unit) were performed as follows. A cylindrical reactor surrounding the fluorescent tube was connected to a liquid-liquid extractor with a tap for the discontinuous sampling of the light phase. The reactor containing the chloroform solution of bilirubin (200 ml) was in turn surrounded by a copper jacket with a sealed coil for circulating water. This allowed the temperature inside the reactor to be constant (20°C) and the solution to be screened from external light. The irradiated chloroform solution of bilirubin dripped into the buffered solution at different pHs (60 ml) contained in the extractor burette (screened with an aluminum foil) and was recycled to the reactor by a pump; thus, a continuous enrichment of the buffer in water-soluble photoproducts was achieved. Samples of the yellow aqueous solution were taken at intervals, transferred into the spectrophotometer cuvette and read (within 30 sec) for their absorbance in the range 400–500 nm using the buffer at zero time as reference.

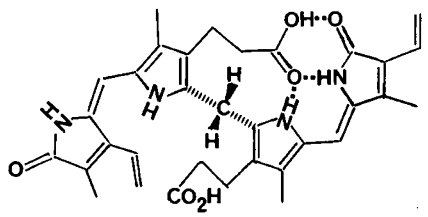
Before switching on the light, both chloroform and aqueous solution were completely deoxygenated by a slow flow of pure (99.999%) argon (30 min) and all the apparatus was kept under inert atmosphere during irradiation.

RESULTS AND DISCUSSION

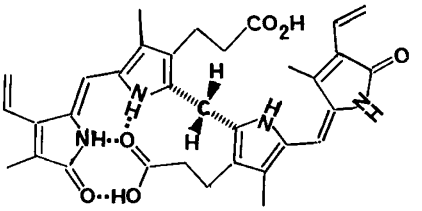
Deoxygenated chloroform solutions of bilirubin were irradiated with visible light and continuously extracted with aqueous



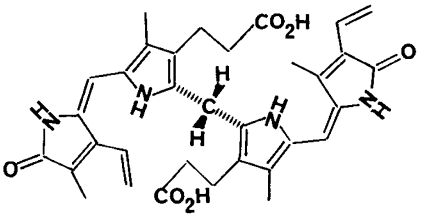
5Z,15Z-BILIRUBIN (NATURAL)



5E,15Z-BILIRUBIN



5Z,15E-BILIRUBIN



5E,15E-BILIRUBIN

Fig. 1. Stereoisomeric bilirubins (of the IXα series).

solutions at different pHs in the range 7.20–8.20. The aqueous solutions became yellow rapidly and progressively: the higher their pH, the more intense their coloration. Their visible absorption spectra are shown in Figure 2. When the extraction of bilirubin solutions was carried out in the absence of the fluorescent light, the aqueous buffers remained uncoloured or, if they were yellow from a previous irradiation, did not increase its absorbance at 422 nm. That irradiation is the only cause for the yellow colouring of the aqueous buffers is in agreement with the well-known very low solubility of the natural pigment in neutral or lightly alkaline solutions (2).

The pigment extracted from chloroform, which we call here "photopigment," for the reason given below, probably includes the water-soluble E-isomers of bilirubin, *i.e.*, the "photobilirubin" described by Lightner *et al.* (9). These arise from photoisomerization of bilirubin in the chloroform solutions undergoing irradiation-extraction, as shown by absorption difference spectra and TLC analyses (9). In addition, varying amounts of bilirubin were recovered when aqueous solutions of the photopigment were acidified or kept in the dark at room temperature for a few hours. The recovered amounts were checked for identity by TLC (17), MS (7) and HPLC (23). Photobilirubin is known to revert easy to bilirubin both thermally and by acid catalysis (9).

The water-soluble E-isomers of bilirubin may not represent the only photoproducts transferred into water. It clearly appears from Figures 2 and 3 that the photopigment formed in our experiments can be partitioned from chloroform into water at pH as low as 7.2–7.4. On the other hand, previous reports indicate that photobilirubin can be extracted by pH 8.5–9.0 tris buffer, but not by 0.1 M phosphate buffer at pH 7.4 (10). The discrepancy in solvent partitioning of the bilirubin photoderivatives between us and other authors (10) could be explained taking into account the higher efficacy of the our continuous extraction, or assuming that, in addition to E-isomers, significant amounts of other photoproducts are formed that are soluble even in water with pH at near neutral values. The latter explanation appears more likely if we consider recent results on the chemical composition of the bilirubin photopigment present *in vitro* under photostationary conditions (20).

A saturation level was observed after 2 h of irradiation in experiments with buffer pH 7.4 (Fig. 3). At present, a rigorous comparison between the water-solubility of bilirubin and its photopigment at pH 7.4 is not possible because the extinction coefficient of the latter is still unknown. Experiments performed to isolate and characterize the photopigment were unsuccessful; bilirubin being recovered in all cases. Nonetheless, similar molar extinction coefficients for bilirubin (ca. 50,000) (2) and its water-extracted photopigment can reasonably be assumed for approximate evaluations. A value of *ca* 2 μM can be estimated for the concentration of the photopigment in water at pH 7.4, ionic strenght 0.015 M (Fig. 2), *i.e.*, about 200 times that estimated for bilirubin in the same conditions (3).

Curves in Figure 3 indicate clearly a strong dependence on pH of the photopigment partitioning from chloroform to water. Considering that the enhancement of transport and excretion of bilirubin *in vivo* under irradiation is probably related to the

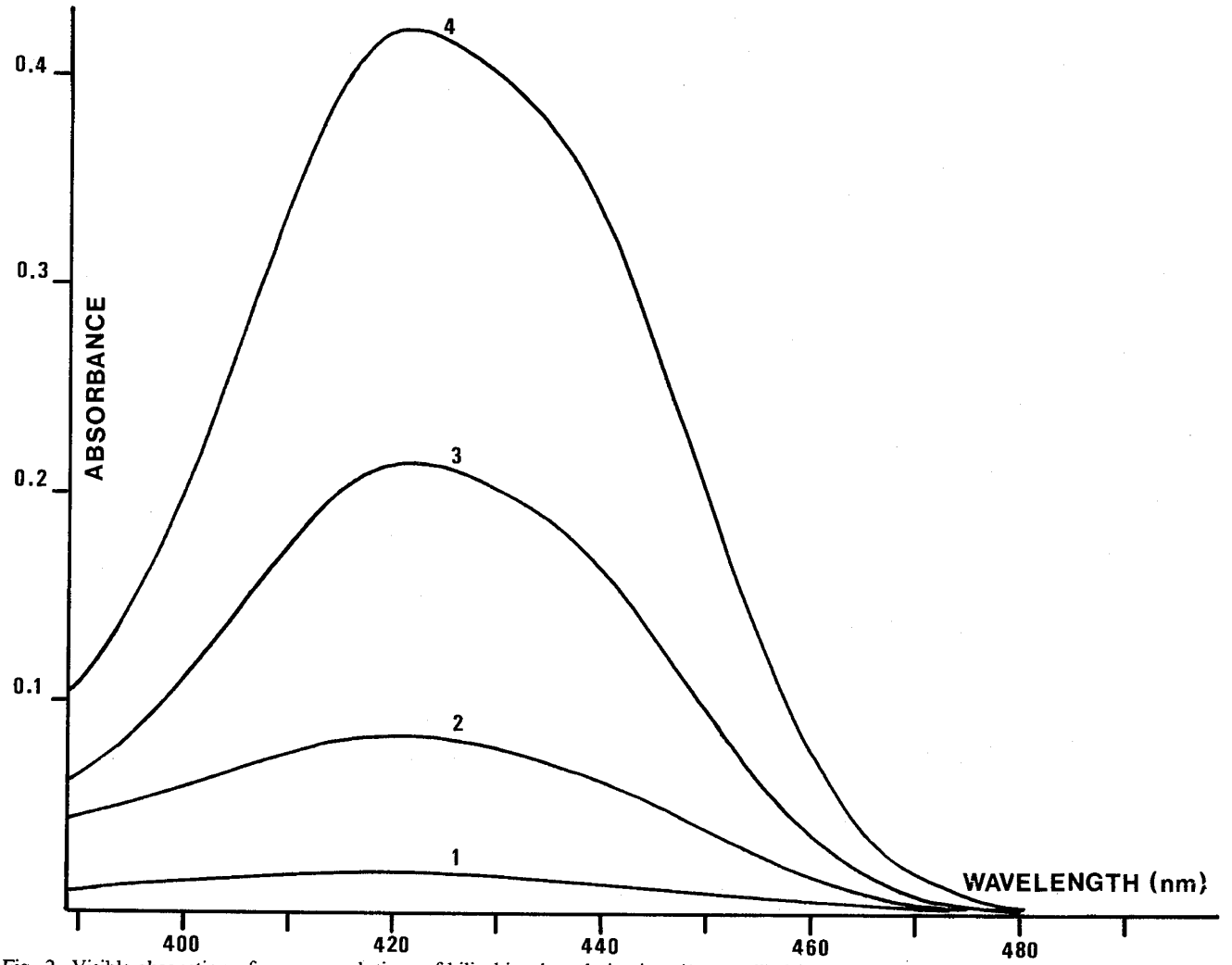


Fig. 2. Visible absorption of aqueous solutions of bilirubin photoderivatives (1-cm cell). They resulted from continuous extraction (1 h) of an irradiated chloroform solution of bilirubin. Curves: 1, pH 7.20; 2, pH 7.40; 3, pH 7.70; and 4, pH 7.90.

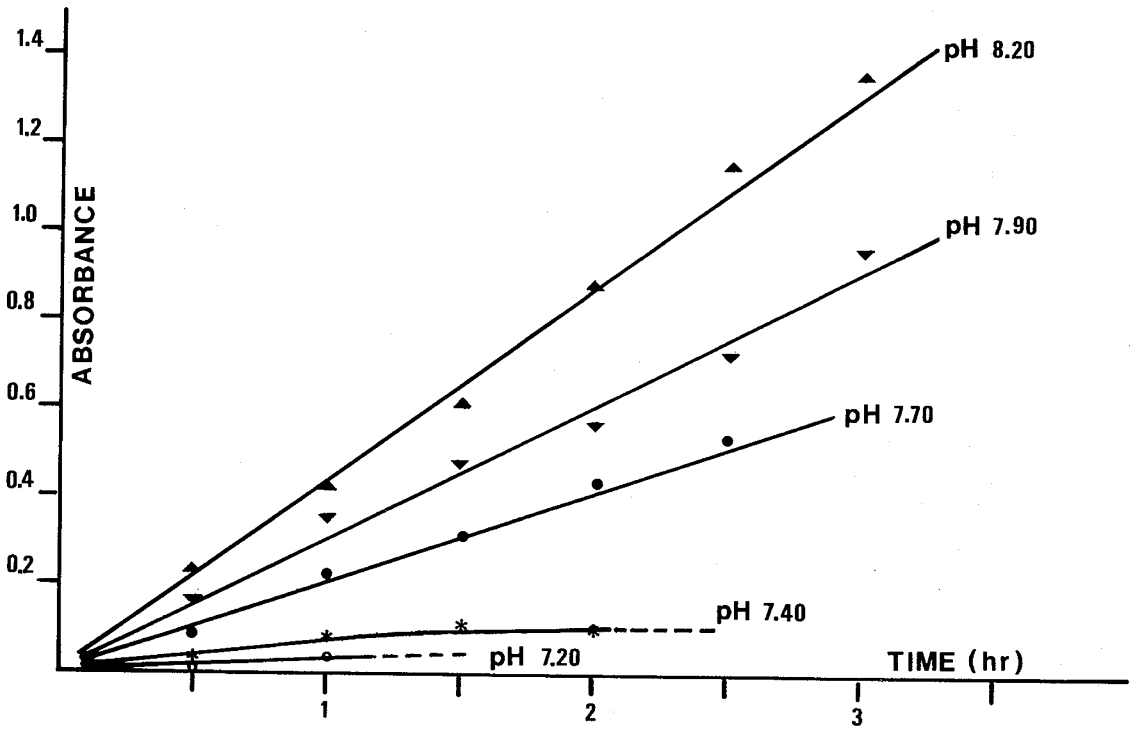


Fig. 3. Plot of the absorbances (at 420 nm) of photopigment aqueous solutions *versus* time of irradiation. *Dashed lines* indicate that aqueous solutions became green with appearance of an absorbance maximum at ca. 650 nm due to the formation of unidentified verdinoid products.

higher water solubility of photoderivatives compared with natural bilirubin (18), the fact that the water solubility of the photopigment sharply increases in inverse proportion to the hydrogen ion concentration can be of great relevance to the treatment of jaundiced infants with phototherapy.

It has been firmly established that acidosis increases the risk of kernicterus (25, 26). The generally accepted explanation is that an increase of hydrogen ion concentration tends to shift bilirubin from plasma albumin into the tissues (17). According to our results, a low hydrogen ion concentration could also improve the detoxifing effect of phototherapy by increasing the partitioning of bilirubin photoproducts from tissues, where they are reversibly formed, into plasma. In addition, taking into account the effect of hydrogen ion concentration on the behavior of photopigment *in vitro*, we caution against the indiscriminate use of light in babies with acidosis. Under this condition, significant amounts of the circulating photopigment and of bilirubin itself, arising from acid catalyzed photopigment reversion, could pass back into tissues with obvious risks for irradiated subjects.

REFERENCES AND NOTES

1. Au, Y. N. and Hutchinson, D. W.: The photoinduced isomerisation of bilirubin in cationic detergent solutions. Biochem. J., *191:* 657 (1980).
2. Brodersen, R.: Binding of bilirubin to albumin. Implications for prevention of bilirubin encephalopathy in the newborn. C. R. C. Critical Reviews. Cli. Lab. Sci., *11:* 305 (1979).
3. Brodersen, R.: Bilirubin solubility and interaction with albumin and phospholipid. J. Biol. Chem., *254:* 2364 (1979).
4. Garbagnati, E. and Manitto, P.: A new class of bilirubin photoderivatives obtained *in vitro* and their possible formation in jaundiced infants. J. Pediatr., *83:* 109 (1973).
5. Isobe, K. and Onishi, S.: Kinetics of the photochemical interconversion among geometric photoisomers of bilirubin. Biochem. J., *193:* 1029 (1981).
6. Kapitulnik, J., Kaufmann, N. A., Goitein, K., Cividalli, G., and Blondheim, S. H.: A pigment found in Crigler-Najjar syndrome and its similarity to an ultrafiltrable photoderivative of bilirubin. Clin. Chim. Acta, *57:* 231 (1974).
7. Lamola, A. A., Blumberg, W. E., McClead, R., and Fanaroff, A.: Photoisomerized bilirubin in blood from infants receiving phototherapy. Proc. Natl. Acad. Sci. USA, *78:* 1882 (1981).
8. Lightner, D. A.: The photoreactivity of bilirubin and related pyrroles. Photochem. Photobiol., *26:* 427 (1977).
9. Lightner, D. A., Wooldridge, T. A., and McDonagh, A. F.: Photobilirubin: An early bilirubin photoproduct detected by absorbance difference spectroscopy. Proc. Natl. Acad. USA, *76:* 29 (1979).
10. Lightner, D. A., Wooldridge, T. A., and McDonagh, A. F.: Configurational isomerisation of bilirubin and the mechanism of jaundice phototherapy. Biochem. Biophys. Res. Comm., *86:* 235 (1979).
11. Lund, H. T. and Jacobsen, J.: Influence of phototherapy on the biliary bilirubin excretion pattern in newborn infants with hyperbilirubinemia. J. Pediatr., *85:* 262 (1974).
12. Manitto, P.: Photochemistry of bilirubin. Experientia, *27:* 1147 (1971).
13. Manitto, P. and Monti, D.: Photoaddition of sulphydryl groups to bilirubin in vitro. Experientia, *28:* 379 (1972).
14. Manitto, P. and Monti, D.: Free-energy barrier of conformational inversion in bilirubin. J. C. S. Chem. Comm., p. 122 (1976).
15. McDonagh, A. F. and Ramonas, L. M.: Jaundice phototherapy: micro flow-cell photometry reveals rapid biliary response of Gunn rats to light. Science, *201:* 829 (1978).
16. McDonagh, A. F., Lightner, D. A., and Wooldridge, T. A.: Geometric isomerisation of bilirubin-IXα and its dimethyl ester. J. C. S. Chem. Comm., p. 110 (1979).
17. McDonagh, A. F.: Bile pigments: bilatrienes and 5,15-biladienes. In: D. Dolphin, Ed.,: The Porphyrins, Vol 6. p. 421–445 (Academic Press, New York, 1979).
18. McDonagh, A. F., Palma, L. A., and Lightner, D. A.: Blue light and bilirubin excretion. Science, *208:* 145 (1980).
19. McDonagh, A. F. and Palma, L. A.: Hepatic excretion of circulating bilirubin photoproducts in the Gunn rat. J. Clin. Invest., *66:* 1182 (1980).
20. Monti, D. and Manitto, P.: Results to be published.
21. Onishi, S., Yamakawa, T., and Ogawa, J.: Photochemical and photobiological studies on light-treated newborn infants. Proc. XIII Intern. Congr. Paediatr., *1:* 373 (1971).
22. Onishi, S., Isobe, K., Itoh, S., Kawade, N., and Sugiyama, S.: Demonstration of a geometric isomer of bilirubin-IXα in the serum of a hyperbilirubinaemic newborn infant and the mechanism of jaundice phototherapy. Biochem. J., *190:* 533 (1980).
23. Onishi, S., Kawade, N., Itoh, S., Isobe, K., and Sugiyama, S.: High-pressure liquid chromatographic analysis of anaerobic photoproducts of bilirubin-IXα *in vitro* and its comparison with photoproducts *in vivo*. Biochem. J., *190:* 527 (1980).
24. Ostrow, J. D.: Photocatabolism of labelled bilirubin in the congenitally jaundiced (Gunn) rat. J. Clin. Invest., *50:* 707 (1971).
25. Perlman, M., Kapitulnik, J., Blondheim, S. H., Alayoff, A., and Russell, A.: Bilirubin binding and neonatal acidosis. Clin. Chem., *27:* 1872 (1981).
26. Stern, L. and Denton, R. L.: Kernicterus in small premature infants. Pediatrics, *35:* 483 (1965).
27. Stoll, M. S., Zenone, E. A., Ostrow, J. D., and Zarembo, J. E.: Preparation and properties of bilirubin photoisomers. Biochem. J., *183:* 139 (1979).

28. Requests for reprints should be addressed to: Prof. Paolo Manitto, Istituto di Chimica Organica, Via Venezian, 21, 20133 Milano, Italy.
29. Received for publication December 21, 1982.
30. Accepted for publication June 6, 1983.