SPECIALIA

On the Stability of Bilirubin[1]

When bilirubin is treated with sodium etoxide, verdinoid pigments are produced. These pigments are the usual products of the oxidation of bilirubin and are also found in solutions of bilirubin after long exposure to visible light. A mechanism proposed for this transformation, in the absence of oxidizing agents, is a shift of π electrons from one vinyl group to the central bridge[2,3].

The aim of this research was to study the behaviour of alkaline solutions of bilirubin allowed to stand for varying periods of time, with or without zinc acetate or complexing agents such as ethylenediamine tetraacetic acid (EDTA), in order to verify whether in this case also the reaction is of prototropic nature[4]. Since under these conditions bilirubin (a) has not been oxidized, we believe that the transformation to dihydrobiliverdin (b) occurs according to the reaction presented in Figure 1.

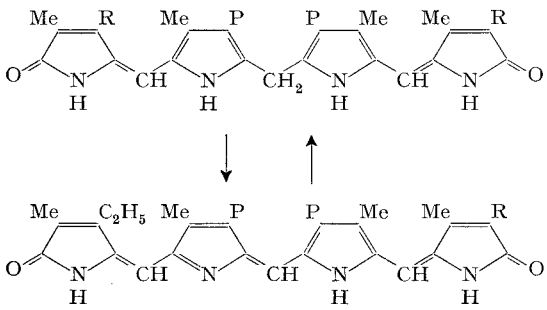

Fig. 1

The rate constants for this reaction, under different conditions, have been calculated.

Bilirubin obtained from Fisher Co. was used. We followed the reaction by measuring the change in optical density at 450 nm (the maximum for bilirubin) in a Beckman DK-2 spectrophotometer, provided with a time drive attachment. The initial and final components were examined by IR-spectroscopy which did not reveal the appearance of any new functional groups, confirming the proposed prototropic mechanism.

The results are presented in Figure 2. We have plotted the log $[(a - x)/a]$ against time, where a is the initial optical density at 450 nm and $(a - x)$ is the optical density at successive times. Working under experimental conditions designed to minimize the catalytic action of light, we established that the reaction, in alkaline solutions with (k_1) and without (k_2) EDTA, is of the first order, with the following rate constants (min^{-1}):

$$k_1 = 3.4 \cdot 10^{-6} \qquad k_2 = 2.0 \cdot 10^{-5}$$

On the other hand, when zinc acetate is present in the solution, the slope of the reaction curve seems to indicate that several steps occur. As a first approximation, this curve can be represented by two lines with angular coefficients, one higher, the other lower, than those previously calculated for solutions without zinc acetate. This result could signify that there is a fast initial reaction in which zinc acetate acts as a catalyst (at pH 10–11 the ratio of bilirubin to Zn^{2+} concentrations is of the order of 10^3–10^4).

Since in alkaline solutions the neutralization of the carboxyl group prevents the formation of hydrogen bridges with the pyrrolic ring[5], it is reasonable to suppose that this is the main reason for the decrease in the optical density even in solutions kept in the dark. The slopes of the curves corresponding to these solutions depend on the concentrations of sodium carbonate, since the stability of bilirubin varies with the basicity[4]. Furthermore, the addition of small quantities of EDTA slows down the rate of the transformation of bilirubin, probably due to the formation of metallic chelates in the alkaline solution[6].

The interpretation of our observations is further confirmed by the results obtained with irradiated solutions of bilirubin and presented in Figure 3. The rate constants (min^{-1}) obtained in this case are the following:

Alkaline solution (pH 10–11)	$k_1' = 1.7 \cdot 10^{-3}$
Alkaline solution + EDTA	$k_2' = 1.2 \cdot 10^{-3}$
Chloroform solution	$k_3' = 5.5 \cdot 10^{-3}$

The highest value for the rate constant is that of the reaction which takes place in a chloroform solution. This is probably connected with the effect that light produces on chloroform. In fact, when a chloroform solution of bilirubin is exposed to UV-light, phosgene and hydrochloric acid are produced (after 12 h of irradiation the

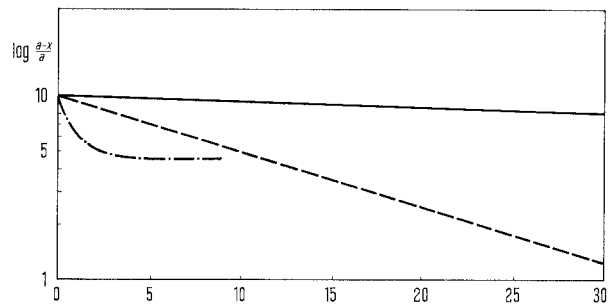

Fig. 2. Changes in relative optical density, at 450 nm, of solutions of bilirubin, plotted as a function of time measured in hours. ——— = alkaline solution with EDTA (traces). - - - - = alkaline solution (5 g/dm³). · – · – · = alkaline solution with $Zn(Ac)_2$ (traces).

[1] Research carried out with CNR financial support. A report on this investigation, with more detailed experimental data, has been published in Acta medica romana (Annali della Facoltà di Medicina e Chirurgia dell'Università Cattolica del Sacro Cuore in Roma) *2*, 192 (1964).

[2] C. H. GRAY, A. KULCZYCKA, and D. C. NICHOLSON, J. chem. Soc. *1961*, 2268.

[3] C. H. GRAY, *Bile Pigments in Health and Disease* (Charles C. Thomas Publ., Springfield 1961), p. 16.

[4] J. FOG and B. BUGGE-ASPERHEIM, Nature *203*, 756 (1964).

[5] J. FOG and E. JELLUM, Nature *198*, 88 (1963).

[6] A. E. MARTELL and M. CALVIN, *Chemistry of the Metal Chelate Compounds* (Prentice-Hall, Englewood Cliffs 1959).

pH falls to 1). Furthermore, the capacity of the products of the prototropic reaction to add halogeno-compounds to the double bond[7] may explain the rapid decrease in the optical density. Owing to the high stability of bilirubin in chloroform, we did not examine this reaction without irradiation. This stability could be explained by the presence of hydrogen bridges in the pyrrolic ring.

Conclusions. The results presented seem to indicate that a first order reaction of a prototropic nature can occur in the transformation of bilirubin. However, it is also clear that intramolecular hydrogen bonds play an important role in the stability of this pigment.

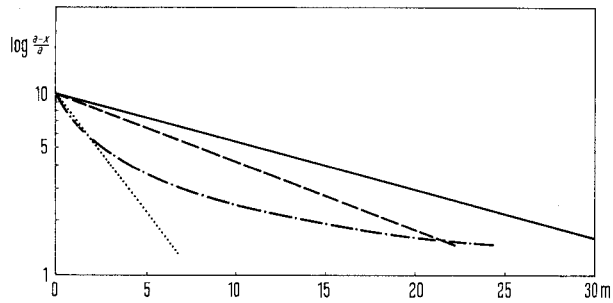

Fig. 3. Changes in relative optical density, at 450 nm, of irradiated solutions of bilirubin, plotted as a function of time measured in min. ——— = alkaline solution with EDTA (traces). - - - - = alkaline solution (5 g/dm³). – · – · – = alkaline solution with $Zn(Ac)_2$ (traces). ····· = chloroform solution.

Riassunto. Gli autori hanno studiato l'azione della luce sulla stabilità della bilirubina in soluzione alcalina e in soluzione cloroformica. Viene proposto, in via generale, un meccanismo del primo ordine per la trasformazione della bilirubina in pigmenti verdinoidi, sia per le soluzioni irradiate, sia per quelle conservate al buio.

I. W. DE EWENSON[8], F. A. GIANTURCO, and P. GRAMACCIONI[9]

Istituto di Fisica, Facoltà di Medicina e Chirurgia dell'Università Cattolica del Sacro Cuore, Roma (Italy), June 29, 1965.

[7] C. H. GRAY, *The Bile Pigments* (Methuen, London 1953), p. 18.

[8] Permanent address: Universidad de la República, Facultad de Ingegnería y Agrimensura, Instituto de Física, Montevideo (Uruguay).

[9] We are indebted to Prof. P. FASELLA, University of Rome, and to Prof. A. VACIAGO, Sacred Heart Catholic University Medical School, for their helpful suggestions and constructive criticisms. One of us (I. W. DE E.) is also grateful to Prof. Ing. W. S. HILL, University of Montevideo, for his suggestion to her to investigate bilirubin spectroscopically.

Structure Activity Relationships of some Centrally Active Dialkyl Substituted Propanediol Sulfites

During preliminary toxicity studies in male albino mice (Swiss Webster) of several dialkyl propanediol cyclic esters of sulfurous acid synthesized by one of us (E.R.B.) in 1957, it was noted that slight changes in the length of the alkyl chain (Table) brought about opposing effects on the central nervous system. A study of the data suggests that Compounds I (dimethyl), IV (diethyl) and XI were quite potent convulsants. In addition, Compounds II (methyl, propyl) and V (ethyl, propyl) were less potent but still effective central nervous system stimulants. Conversely, Compound VI (ethyl, *n*-butyl) was found to be a moderately potent central nervous system depressant. The activity was essentially lost when the substitution was di-*n*-butyl (Compound IX) or methyl-hexyl (Compound III).

The ability of these compounds (10 mice/dose), when administered intragastrically, to alter hexobarbital sleep times[1] was not consistent (Table). Generally, the stimulant type compounds produced a decrease in the duration of sleep (antagonism) at low doses and a lengthening (enhancement) or no effect at the upper dose tested. This type of response has not been an unusual finding in our laboratory for the central nervous system stimulant type of compound. However, the absence of an enhanced effect by the depressant type compound (VI) was unexpected.

Antagonists to central nervous system depressants such as the barbiturates might have therapeutic use. Thus, Compound IV was tested for its ability to reverse pentobarbital anesthesia in the dog[2]. Four dogs were tested and the predominant effects seen were shaking, trembling and jerks progressing to clonic-type convulsions. Two of these dogs tried unsuccessfully to get to their feet. The pattern observed resembled that observed in our laboratories for pentylenetetrazol[2,3].

The depressant compound (VI) was tested for its anticonvulsant effects in mice[4]. At relative high doses it was effective against pentylenetetrazole seizures (0.8 g/kg) and electrically induced seizures (1.6 g/kg). The other possible depressant compounds were ineffective at 0.8 g/kg by either test.

Several of the compounds (VI, VIII, IX) were evaluated for possible analgetic and antipyretic activity[5] without success. In routine cardiovascular studies[6] in the

[1] L. C. WEAVER, J. W. NEWBERNE, and T. L. KERLEY, Arch. int. Pharmacodyn. *131*, 716 (1961).

[2] L. C. WEAVER, W. M. ALEXANDER, B. E. ABREU, and G. R. BURCH, J. Pharmacol. exp. Therap. *116*, 268 (1956).

[3] W. M. ALEXANDER, B. E. ABREU, L. C. WEAVER, H. E. FAITH, and J. W. NEWBERNE, Arch. int. Pharmacodyn. *119*, 423 (1959).

[4] L. C. WEAVER and W. R. JONES, J. pharm. Sci. *52*, 508 (1963).

[5] L. C. WEAVER and B. E. ABREU, J. Am. pharm. Ass., Scient. Ed. *49*, 298 (1960).

[6] B. E. ABREU, A. B. RICHARDS, L. C. WEAVER, G. R. BURCH, C. A. BUNDE, E. R. BOCKSTAHLER, and D. L. WRIGHT, J. Pharmacol. exp. Therap. *115*, 419 (1955).