ON THE AUTOXIDATION OF BILIRUBIN

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SUMMARY The instability of oxygenated aqueous solutions of bilirubin in the dark is due to several distinguishable processes: autoxidation, surface phenomena and precipitation-aggregation. Autoxidation occurs in aqueous solutions over a pH range 7.4-13.2 in the presence of even traces of oxygen. Several autoxidation products have been isolated and identified. At pH 7.4-8.8 bilirubin precipitates from 2.5 x 10^{-5} M solutions and adsorbs to the walls of the glass container. In ammoniacal methanol, chloroform and dimethyl sulfoxide aggregation phenomena do not occur and autoxidation is very slow.

INTRODUCTION

The instability of bilirubin IX-α (BR)* in solution, especially alkaline aqueous solution, is a well-known, yet confusing facet of bile pigment chemistry (1,2). And although the photodecomposition of BR is currently receiving wide attention because of its importance in the phototherapy of jaundiced infants (3), the decompositon of Br in the dark has still not been completely characterized. At high pH values, for example in aerated solutions of 0.1 N NaOH, NH_4OH or Na_2CO_3, BR deteriorates at rates of 2-10% per hour when measured by the loss in visible absorbance or diazo reactivity (4,5). The reaction is inhibited by ascorbic acid (6), cysteine (7) and EDTA (8) and can be accelerated by the addition of metal ions (8,9); little else is known regarding its mechanism. The products of the reaction have not been fully identified. Küster observed that biliverdin (BV), hematinic acid and methyl-hydroxyethyl-maleic acid were formed during the air oxidation of BR in alkaline solutions

* Abbreviations used, BR: bilirubin, BV: biliverdin, EDTA: ethylenediaminetetraacetic acid.

together with insoluble brown pigments (10). Several authors have noted that strongly alkaline solutions of BR develop a strong pentdyopent test on standing, which indicates the formation of dipyrroles (4,11,12). Ostrow *et al.* (4) and Kimura (13) observed that BR is transformed into a new yellow pigment on standing in 0.1 N NaOH, and Takeuchi (14) suggested that in strongly alkaline solutions BR is converted to bilifuscins with BV and pentdyopents as intermediates. More recently, Ostrow *et al.* (15) have presented evidence that BR exposed to 0.1 N NaOH for 20 hrs yields a large number of products including BV and the bis-retro-aldol product, a diformyldipyrromethane. Decomposition of BR also occurs at less alkaline pH (12,16) where the pigment may exist largely as a dimer of its dianion (17). In addition, at pH values from about 7.4 - 9.0 other factors appear to contribute to the instability of BR solutions. The pigment undergoes an oxygen-dependent isomerization to a mixture of its spectroscopically similar IIIα, IXα and XIIIα isomers, probably *via* a radical mechanism (18), and has a tendency to form colloidal or insoluble aggregates and surface films that may become adsorbed to solid surfaces (2, 19-22). Alkaline solutions of BR can be stabilized by the addition of proteins (1), especially serum albumin, or detergents*, and colloid formation may be prevented by addition of ethanol, acetone or pyridine (2). In non-hydroxylic organic solvents such as benzene or chloroform, BR is stable in the dark for at least 2 hr, but solutions in the hydroxylic solvent chloroform:methanol-1:7 are unstable (4). This instability, which was thought to be due to decomposition of the pigment (4), has subsequently been shown to be due to formation of colloidal BR (5) and adsorption of BR to the glass container*.

To date there has been no comprehensive treatment of the dark reactions of BR. This is surprising in view of the need for stable BR preparations in clinical analyses and experimental studies and the current interest in photochemical reactions of BR. Such knowledge could also prove useful in under-

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* McDonagh, A.F., unpublished observations.

standing BR metabolism and lead to an alternative method to phototherapy for destroying BR *in vivo*. In the following we describe a preliminary survey of BR stability in solution in which an effort was made to distinguish between chemical and physical processes.

MATERIALS AND METHODS

Buffered aqueous solutions of BR (Koch-Light or Matheson, Coleman and Bell) were made up by dissolving 1.5 mg (2.5 μmole) of BR in a few drops of conc. NH_4OH (or 6 N NaOH) and diluting at once to 100 ml with 0.1 M phosphate buffer (pH 7.2 before addition and 7.4 after) or borate buffer (pH 7.4 before addition and 8.0 after, pH 8.4 before addition and 8.8 after) which contained, in some experiments, human serum albumin (HSA, Sigma, 255 mg/100ml). For solutions of BR in 0.1 M carbonate buffer (pH 11.0, 13.0) and organic solvents, the pigment (2.5 μmole) was mixed directly with the solvent. The pH 8.9 NaOH solution was prepared by dissolving BR in 6 N NaOH and lowering the pH by careful titration with 1 N HCl. Buffers were prepared by established procedures (23). Chloroform was distilled from P_2O_5 then passed through a column of activity I basic alumina to remove traces of ethanol. Reagent grade dimethylsulfoxide (Aldrich) was distilled from NaOH pellets. Methanol was anhydrous reagent grade (Baker Analyzed). In some experiments glassware was "silanized" by brief exposure to hexamethyldisilazane (PCR, Inc.). Nitrogen (Matheson, ultra-high purity, 99.999%) was routinely deoxygenated by passage through aqueous pyrogallol.

For oxygenation experiments, solutions were divided into three parts. One part (~10 ml) was purged with argon (Matheson, ultra-high purity, 99.999%) for 5 - 10 min and kept stoppered in the dark as one control. Another part (~45 ml) was oxygenated with a slow stream (0.05 - 0.1 l/min) of small bubbles of O_2 in a 125 ml Erlenmeyer flask in the dark, and the remainder (~45 ml) was similarly bubbled with N_2 as a second control. Volumes were held constant by the periodic addition of solvent to compensate losses due to evaporation during bubbling. This correction was especially important with methanol and chloroform solvents. For more rigorous controls, oxygen-free BR solutions were prepared and kept under Ar in a glove bag using doubly-distilled, deionized water that had been boiled and purged with Ar. The pH of each solution was measured before, during and after each autoxidation experiment. Changes of pH were observed only with dilute NH_4OH solutions, where the pH decreased during the bubbling experiments from 11.0 to 10.7, presumably due to loss of NH_3 by entrainment.

BR disappearance was monitored by recording the visible spectrum of each solution at 1 hr intervals and following the decrease of the absorbance maximum of BR near 440 nm using a Cary 17 or 14 spectrophotometer. To distinguish precipitation and aggregation from other losses, several drops of 6 N NaOH were added to aliquots (~3 ml) of the test solutions in the cuvette to re-dissolve BR, and the spectra were run again as soon as mixing was complete.

RESULTS AND DISCUSSION

Although the spectral changes exhibited by BR undergoing autoxidation (Figure 1) are clearly reminiscent of those observed during BR photo-oxygenation (18,24,25), study of the autoxidation is complicated by isomerization (18) and other losses apparently unrelated to oxygen (2). The data of Table 1

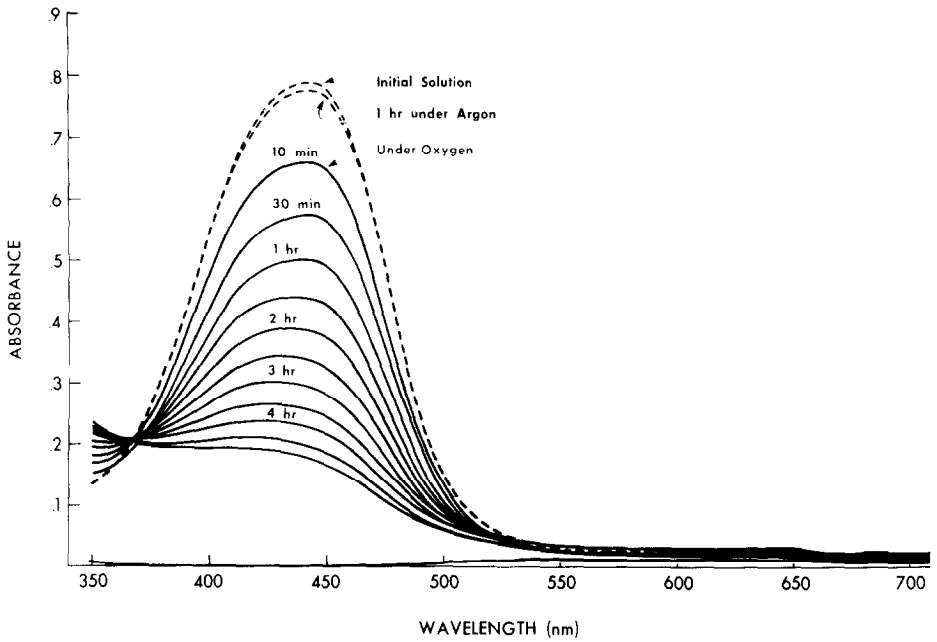

Fig.1 Time dependent study of the dark autoxidation of 15 μ*M* bilirubin in 0.05 *M* Tris buffer, pH 8.5. Reaction times are indicated on the curves. All solvents were initially degassed by Ar bubbling and placed in an Ar filled glove bag. In the glove bag, Br (0.9 mg) was dissolved in 0.3 ml of 0.1 *N* NaOH and diluted with 0.05 *M* Tris buffer, pH 8.5. The solution was transferred to an anaerobic cuvette [Hodgson, E. K., McCord, J. M; and Fridovich, I., (1973) *Anal. Bioch.*, 5, 470], purged with Ar for 5 min and the cuvette closed. The spectrum was run (time 0), and after 1 hr in the dark the spectrum was rerun. Oxygen was then bubbled through the solution for 10 min and the cuvette resealed. Spectra were run at the various indicated time intervals.

clearly indicate losses due to precipitation or aggregation and adsorption and also reveal the sensitivity of BR to O_2 in the dark. Thus, BR precipitates to some extent from aqueous solutions at pH 7.4 - 8.9 during bubbling of O_2 or N_2, or even on standing under Ar gas. These apparent losses vary somewhat with the rate of bubbling, especially during the first few hours and can also be induced by mechanical agitation as previously noted by Broderson and Theilgaard (2).

TABLE 1 Losses of Bilirubin in 2.5×10^{-5} Molar Solutions

	Solvent	λ_{max} (nm)	ϵ l./mole/cm	% Decrease in the Long Wavelength Absorbance Maximum of Bilirubin After 24 Hrs.†					
				N_2 Bubbling		O_2 Bubbling		Ar Standing	
				Total Loss	Aggregation††	Total Loss	Aggregation††	Total Loss	Aggregation††
pH 7.4	Phosphate Buffer	435	43,000	45	23	59	12	34	12
				43*	31*	60*	15*	37*	20*
								5**	0
pH 8.0	Borate Buffer	438	40,000	39	14	64	1	34	3
						72*	0*	23*	9*
								2**	2**
pH 8.8	Borate Buffer	435	44,000	40	14	82	0	12	0
								12**	0**
pH 10.5	Carbonate Buffer	435	47,000	15	0	90	0	17	0
pH 8.9	aq. NaOH	435	43,000	35	9	81	5	25	20
pH 11.0	aq. NaOH	435	47,000	12	0	68	0	26	0
								28*	0*
pH 11.0	aq. NH_4OH	435	46,000	7	0	67	0	15	0
pH 13.0	aq. NaOH	420	46,000	12	0	81	0	27-49	0
				9*	0*	89*	0*	9*	0*
pH 13.2	aq. NH_4OH	442	59,000			62	0	50	0
				21*	0*	94*	0*	12*	0*

†The data are an average of several runs with only small deviations from the mean, except where noted.

††Precipitation was observed where cited values are >5%.

*Silanized flask.

**Doubly distilled deionized water further deaerated by Ar purging at reflux, cooled in Ar filled glove bag, and buffer solution prepared in glove bag. Control reaction run in glove bag. Containers were not silanized.

Therefore, *aqueous* solutions of BR at pH 7.4 - 8.9, which are generally prepared by diluting a solution of higher pH and not by direct dissolution, are *metastable* at 2.5×10^{-5} M, and given sufficient encouragement, BR tends to aggregate in precipitable or colloidal form. This indicates that the true solubility of BR at values close to physiological pH is considerably lower than previously suggested (20,31), at least at the ionic strengths used in this study, and thus confirms earlier studies (2).

To distinguish between real losses of BR due to autoxidation and apparent losses due to aggregation and precipitation, a relatively small volume of 6 N NaOH was added to solutions at the time of spectral measurement. This method would not account, however, for apparent losses due to adsorption of BR onto the walls of the flask. Comparative measurements made using silanized flasks indicated that adsorption was responsible for a small but significant part of the total BR loss and that this was reduced by silanization (Table 1). Even after accounting for losses due to aggregation and adsorption, some loss of BR remained unaccounted for in the 24 hr N_2 and Ar controls. This was presumably due to traces of O_2, since these losses became negligible when the Ar controls were prepared with more rigorous exclusion of O_2 (Table 1).

Data on the autoxidation of BR by O_2 bubbling are summarized in Table 2. The values given are minimal values since they do not include the amount of autoxidation that occurred in the N_2 controls due to traces of O_2. It is clear that a significant, but variable, autoxidation of BR occurs in all aqueous solvents, and the protective influence of serum albumin (26) is confirmed. No unusual buffer effects were observed (*cf.* pH 8.8 borate buffer and pH 8.9 NaOH, pH 10.5 carbonate buffer and pH 11.0 NaOH and NH_4OH), but there was a trend toward a greater degree of autoxidation with increasing pH. This trend may be more apparent than real since at the lower pH values the amount of BR truly in solution and available for autoxidation is progressively reduced by aggregation Certainly the autoxidation in hydroxylic media does not seem to be related to pH alone since basic methanol solutions of BR are surprising more stable than

Solvent	λ_{max} (nm)	ε (l. mole^{-1} cm^{-1})	Oxygen Bubbling 1 Hr	5 Hr	24 Hr
pH 7.4 Phosphate Buffer†	435	43,000	8	17	25
pH 8.0 Borate Buffer	438	40,000	13	26	38
pH 8.8 Borate Buffer	435	44,000	16	33	56
pH 10.5 Carbonate Buffer	435	47,000	9	48	75
pH 7.4 Phosphate Buffer + HSA	450	47,000	2	7	21
pH 8.0 Borate Buffer + HSA	455	52,000	2	6	21
pH 8.8 Borate Buffer + HSA	461	38,000	1	7	29
pH 8.9 NaOH (aq)	435	43,000	18	49	65
pH 11.0 NH_4OH (0.1 N, aq)**	435	46,000	3	15	60
pH 11.0 NaOH (0.001 N, aq)	435	46,000	5	22	56
pH 13.2 NH_4OH (conc., aq)†	442	59,000	10	46	73
pH 13.0 NaOH (0.1 N, aq)	420	46,000	51	80	80
pH 8.8 NaOH (CH_3OH)	450	48,000	0	0	1
pH 10.6 NH_4OH (0.1 N, CH_3OH)	450	59,000	2	2	5
Chloroform	453	60,500	1	4	6
Dimethyl Sulfoxide	458	64,000	5	19	20

*Losses were calculated by subtracting non-aggregated losses of BR incurred during N_2 bubbling from non-aggregated losses incurred during O_2 bubbling in the dark.

†Green coloration (BV?) appears near 24 hrs of autoxidation in phosphate buffer and near 5 hrs in conc. NH_4OH. Green coloration did not appear in any other solvent systems, even 0.1 N aq NaOH.

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their aqueous counterparts as are other solutions with organic solvents, e.g. $CHCl_3$ and DMSO. This difference is probably related to different states of aggregation and solvation of BR in aqueous and organic solvents as suggested by the collagation experiments of Brodersen (17) and the isomerization experiments of McDonagh (18). The former indicated that BR exists as a dimeric divalent anion at pH 8.25, $K_d = 2.7 \times 10^{-4}$ M, and probably at pH 7.5 - 9.0, but as a monomeric acid in an organic solvent such as methyl isobutyl ketone. The latter showed that BR IXα (8.5×10^{-5} M) undergoes an oxygen-promoted isomerization to a mixture of its IXα, IIIα and XIIIα isomers at 37° in aqueous base (pH < 11) but not in organic solvents. We believe that the isomerization mechanism involves radical formation in a BR dimer or aggregate and that dimers or aggregates form in aqueous solutions whereas the monomer is preferred in organic solvents. The isomerization, which must involve at least two BR molecules, is a much slower and less probable process with monomeric BR than in aggregated BR, as indeed even radical formation may be. Since under prolonged aqueous isomerization conditions, especially with very dilute solutions, BR and its isomers become decomposed, we propose that any BR (or half-Br) radicals formed by the action of O_2 may be lost by reaction with solvent and/or O_2 in addition to the cited isomerization mode. This rationale serves to explain the sensitivity of BR to even traces of O_2 and suggests that *at least two* routes may be involved in its autoxidation: O_2 induced radical dissociation of BR leading eventually to its destruction, and direct attack of O_2 leading immediately to irreversible losses.

At the termination of the autoxidation experiments, all solutions were colorless to yellow except for those in pH 7.4 phosphate buffer and pH 13 aq. NH_4OH (but not pH 13 NaOH) which were green (BV?). We have isolated several autoxidation products from pH 11 aq. NH_4OH. These include methylvinylmaleimide (27), three isomeric propentdyopents (28), hematinic acid (25), bilifuscins (29), a dipyrromethane dialdehyde (15), and five as yet unidentified compounds*. Further work on the mechanistic aspects of the autoxidation reac-

* Norris, R. D., unpublished observations.

tions with special emphasis on product identification is underway in our laboratories.

CONCLUSION

These studies, which are in part confirmatory of earlier work by others (2,17) show that the instability of aqueous solutions of BR in the dark is due to autoxidation, aggregation and surface adsorption. Autoxidation occurs over the pH range 7.4 - 13.2 and may be significant even in the presence of trace amounts of O_2 in the dark. We stress, therefore, the need to deoxygenate the water used in preparing aqueous solutions if decomposition of BR is to be avoided, but add the caveat that deoxygenation of BR solutions at lower pH values by bubbling inert gases may also lead to loss of pigment due to physical processes.

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REFERENCES

1. With, T. K. (1968) Bile Pigments, Academic Press, New York.
2. Brodersen, R. and Theilgaard, J. (1969) *Scand. J. Clin. Lab. Invest.*, 24, 395-398.
3. Odell, G. B., Schaffer, R. and Simopoulos, A. P. (1974) Phototherapy in the Newborn: *An Overview*, Natl. Acad. Sci. (US).
4. Ostrow, J. D., Hammaker, L., and Schmid, R. (1961) *J. Clin. Invest.*, 40, 1442 - 1452.
5. Clarke, J. T. (1965) *Clin. Chem.*, 11, 681-690.
6. Barac, G. (1939) *Bull. Soc. Chim. Biol.*, 21, 1163- 1170.
7. Barac, G. (1946) *Bull. Soc. Chim. Biol.*, 28, 632-633.
8. Fog, J., and Bugge-Asperheim, B. (1964) *Nature*, 203, 756-757.
9. Velapoldi, R. A., and Menis, O. (1971) *Clin. Chem.*, 17, 1165-1170.
10. Küster, W. (1922) Hoppe-Seyler's *Z. Physiol. Chem.*, 121, 94-109.
11. Bingold, K. (1935) *Klin. Wochenschr.*, 14, 1287- 1289.
12. Kono, H. (1959) *Okayama Igakkai Zasshi*, 71, 7203.
13. Kimura, T. (1955) *Igaku Kenkyu*, 25, 93.
14. Takeuchi, R. (1959) *Okayama Igakkai Zasshi*, 71, 7653.
15. Ostrow, J. D., Nicholson, D. C. and Stoll, M. S. (1971) *Gastroenterology*, 60, 186 (Abstract); Ostrow, J. D. (1972) *Seminars in Hematology*, 9, 113-125
16. Chen, R. F. (1973) The Fluorescence of Bilirubin-Albumin Complexes in *Fluorescence Techniques in Cell Biology*. Eds., A. A. Thaer and M. Sernetz. Springer-Verlag, New York, 273-282.

17. Brodersen, R. (1966) *Acta. Chem. Scand.*, 20, 2895-2896.
18. McDonagh, A. F. (1975) *Ann. N. Y. Acad. Sci.*, 244, 553-568.
19. Stenhagen, E. and Rideal, E. K. (1939) *Biochem J.*, 33, 1591-1598.
20. Overbeek, J. Th. G., Vink, C. L. J. and Deenstra, H. (1955) *Rec. Trav. Chim.*, 74, 81-84.
21. Brodersen, R., Funding, L., Pedersen, A. O. and Röigaard-Petersen, H. (1972) *Scand. J. Clin. Lab. Invest.*, 29, 433-446.
22. Lee, J. J., and Cowger, M. L. (1974) *Res. Commun. Chem. Path. Pharm.*, 8, 327-339.
23. Dawson, R. M. C., Elliott, D. C., Elliott, W. H. and Jones, K. M., eds. (1959) *Data for Biochemical Research*, Oxford Univ. Press.
24. McDonagh, A. F. (1971) *Biochem. Biophys. Res. Commun.*, 44, 1306-1311.
25. Lightner, D. A. (1974) In Vitro Photooxidation Products of Bilirubin in reference 3, 34-55.
26. Ostrow, J. D., and Branham, R. V. (1970) *Gastroenterology*, 58, 15-25.
27. Bonnett, R., and McDonagh, A. F. (1969) *Chemistry and Industry (London)*, 107.
28. Bonnett, R, and Stewart, J. C. M. (1975) *J. C. S. Perkin Trans. I*, 222-231.
29. Siedel, W., Stich, W., and Eisenreich, F. (1948) *Naturwiss.*, 35, 316-317.
30. Siedel, W. and Möller, H. (1939) Hoppe-Seyler's *Z. Physiol. Chem.*, 259, 113-136.
31. Burnstine, R. C. and Schmid, R. (1962) *Proc. Soc. Exp. Biol. Med.*, 109, 356-358.