CANADIAN HEPATIC FOUNDATION

HEPATOLOGY—Research and Clinical Issues • Volume 2

JAUNDICE

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PLENUM PRESS • NEW YORK AND LONDON

Library of Congress Cataloging in Publication Data

Main entry under title:

Jaundice: [proceedings of the second international symposium of the Canadian Hepatic Foundation, held in Montreal, May 31 and June 1, 1974]

(Hepatology–research and clinical issues; v. 2)
Includes bibliographical references and index.
1. Jaundice–Congresses. I. Goresky, C. A., 1932- II. Fisher, Murray M., 1934- III. Canadian Hepatic Foundation. [DNLM: 1. Jaundice–Congresses. W1 HE913 v. 2 / WI703 J41 1974]
RC851.J38 616.3'625 75-8782
ISBN 0-306-34802-0

Proceedings of the Second International Symposium of the Canadian Hepatic Foundation, held in Montreal, May 31 and June 1, 1974

A Division of Plenum Publishing Corporation
227 West 17th Street, New York, N.Y. 10011

United Kingdom edition published by Plenum Press, London
A Division of Plenum Publishing Company, Ltd.
Davis House (4th Floor), 8 Scrubs Lane, Harlesden, London, NW10 6SE, England

Printed in the United States of America

JAUNDICE

HEPATOLOGY

Research and Clinical Issues

Volume 1 • Viral Hepatitis

Edited by M. M. Fisher and J. W. Steiner
Canadian Medical Association Journal
(Vol. 106, Special Issue, pp. 417–528, 1972)

Volume 2 • Jaundice

Edited by C. A. Goresky and M. M. Fisher

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Preface

Jaundice is much more than a clinical sign of liver disease. It is also a pathophysiological disorder. Through studying it we have come to a much better understanding of how the liver functions under normal and abnormal circumstances. In spite of several important advances in this field, it has not recently been the subject of a comprehensive and interdisciplinary review.

This Symposium was held in Montreal on May 31 and June 1, 1974, and the experts who participated in it came together for the purpose of reviewing the current status of Jaundice. The Editors sincerely appreciate the outstanding contribution which these experts made to the Second International Symposium of the Canadian Hepatic Foundation. They are also particularly indebted to Valerie M. Price, Executive Director, Canadian Hepatic Foundation, for her invaluable role in the preparation of this publication.

Carl A. Goresky
Murray M. Fisher

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AN OVERVIEW OF BILIRUBIN CHEMISTRY

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"From gall disease, that is from the yellow jaundice cometh great evil; it is of all disease most powerful, when there wax within a man, unmeasured humors; these are the tokens; that the patient's body all becometh bitter and as yellow as good silk; and under the root of his tongue there be swart veins and pernicious, and his urine is yellow"(1). The yellow pigment referred to in this vivid eleventh-century Anglo-Saxon description of cirrhosis was first isolated in crystalline form by Virchow in 1847(2), and named bilirubin by Stadeler in 1864(3). Its structure was determined by Siedel and Fischer in 1933(4) and confirmed by total synthesis by Fischer and Plieninger in 1943(5) and more recently by Plieninger et al(6). Much of the basic chemistry of the pigment was elucidated more than two decades ago, principally by the Fischer school. And today, despite the fact that bilirubin is a metabolic waste-product of no practical utility other than its diagnostic value, scientific interest in the pigment continues unabated. In the past two to three years alone bilirubin has been mentioned in over 1600 publications, and of these about 400 specifically with some aspect of the chemistry or biochemistry of the molecule. The areas of bilirubin chemistry and biochemistry which appear to have received the most attention in recent years are the mechanism of bilirubin formation, the mechanism of bilirubin conjugation and excretion, the nature of bilirubin conjugates, bilirubin-protein complexes, and the photochemistry of bilirubin. In addition there has been a steady flow of new, or improved, methods for the estimation of bilirubin and its conjugates in biological fluids.

In the brief account of bilirubin chemistry which follows, no attempt has been made to provide a comprehensive review of the subject. Instead, some of the basic chemical properties of the molecule are outlined with particular emphasis on recent work.

GENERAL PROPERTIES

The chemical formula of bilirubin is shown in Figure 1. Inspection of this structure shows that by interchanging the substituents on the pyrrole rings a number of structural isomers of bilirubin would be formed which could, in theory, exist. Few of these bilirubin isomers have in fact been made, and the only one which occurs naturally is the isomer shown. This is designated bilirubin IX-α because it is derived from the natural IX isomer of ferriprotoporphyrin by cleavage of the porphyrin ring at the α bridge position.

Bilirubin IX-α is a stable crystalline solid which crystallizes readily from chloroform-methanol solutions. Commercial preparations of the pigment, which are obtained from animal bile or gallstones, may contain isomers of bilirubin (see below)(7) or non-bilirubin material as impurities(8). Samples which are isomerically homogeneous are easy to purify(9,10), but the removal of unwanted isomers can only be accomplished on a small scale using thin-layer chromatography(7,10). The pure pigment is soluble in a number of organic solvents (e.g. chloroform, methylene chloride, pyridine, dimethyl sulfoxide), but is essentially insoluble in petroleum ether, methanol, or water. Its solubility in water is very low at pH 7.4 but increases with increasing pH (11,12), giving solutions which tend to be unstable in the presence of air even in the dark(13). Metastable supersaturated solutions of bilirubin in water at physiological pH containing about 9×10^{-5} moles/l (5 mg%) of the pigment are easy to prepare by addition of a concentrated solution in 0.1M NaOH or dimethyl sulfoxide to excess pH 7.4 buffer. However, the pigment slowly precipitates from these solutions on standing or on agitation.

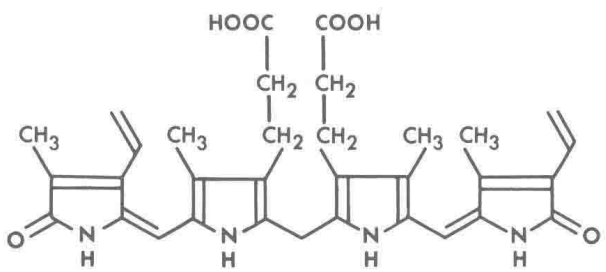

Fig. 1 The chemical structure of bilirubin IX-α

By the use of cationic or neutral detergents such as cetyltrimethylammonium bromide or Tween 20, bilirubin can be solubilized in water over a wide range of acidic and basic pH values(14,15).

Much of the chemistry of bilirubin is readily predictable from its structure. As shown in Figure 2, the molecule contains two carboxylic acid side-chains which should readily form esters and are weakly acidic, and on the lactam positions of the end rings there is a further pair of very weakly acidic protons which should also ionize in strong alkali. The central pyrrole rings, on the other hand, would be expected to be weakly basic and become protonated in strong acids. The molecule has a number of double bonds which should be reducible, especially those in the side-chains and the end rings. However, the more stable double bonds of the aromatic central pyrrole rings would be expected to be more resistant to reduction.

If bilirubin is drawn in the form of the hydroxypyrrole tautomer rather than the lactam tautomer shown in Figure 1, it can be seen that its structure is a hybrid of a pair of dipyrrylmethenes and a dipyrrylmethane (Fig. 3). The dipyrrylmethene portions, with their extended system of conjugated double bonds, are responsible for the yellow color of the molecule and are relatively stable. But the dipyrrylmethane segment is much less stable and may be regarded as the Achilles heel of the molecule. Dipyrrylmethanes are attacked by electrophiles and tend to undergo cleavage about the central $-CH_2-$ bridge in the presence of strong acids. They are also prone to oxidative dehydrogenation to the corresponding, more fully conjugated, dipyrrylmethenes. It is not surprising, therefore, to find that bilirubin undergoes similar reactions.

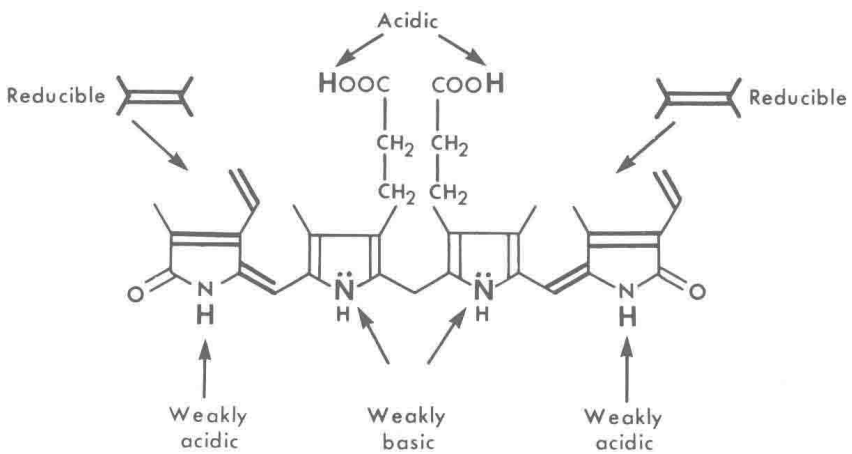

Fig. 2 Bilirubin IX-α -- functional groups

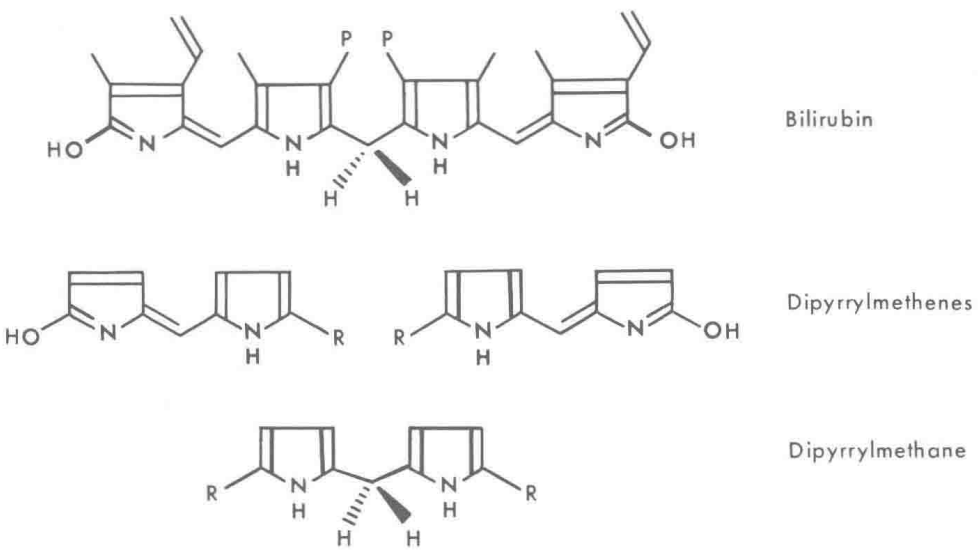

Fig. 3 Bilirubin IX-α -- a hybrid structure

SPECIFIC REACTIONS

1. Esterification

In mammals the water-solubility of bilirubin is enhanced and its excretion facilitated by enzymatic esterification with the polar sugar glucuronic acid. Non-enzymatic esterification of bilirubin with glucuronic acid has not been achieved, but methyl, ethyl and other alkyl esters of the molecule are easily prepared by treating bilirubin with the corresponding commercially-available 1-alkyl-3-p-tolyltriazene (Fig.4)(16). Diazomethane has also been used to prepare bilirubin dimethyl ester, but with this reagent methylation of the lactam oxygen atoms also occurs and several products are obtained(17). Acid-catalyzed esterification with alcohols cannot be used because of the instability of the molecule towards acid (see below). The esters of bilirubin, including bilirubin diglucuronide, are more susceptible to autoxidation than bilirubin itself. This has been attributed to a stabilizing effect of intermolecular hydrogen-bonds in the free acid which is destroyed by esterification(18).

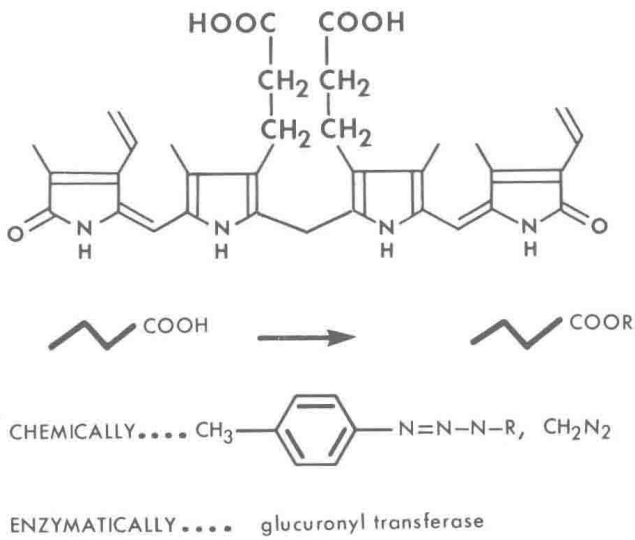

Fig. 4 Bilirubin IX-α -- esterification

2. Reduction

Bilirubin can be readily reduced using sodium amalgam or catalytically hydrogenated using palladium on charcoal (Fig. 5) (19). The hydrogens add on two by two, first at the exo-vinyl group (site 1) and then at the endo-vinyl group (site 2) to give mesobilirubin. Further reduction (sites 3) yields the colorless urobilinogen and finally (sites 4) stercobilinogen. A similar series of reactions takes place in the gut, catalyzed by bacterial enzymes of the gut flora (19).

3. Oxidation

Bilirubin undergoes a variety of oxidative reactions, some of which are summarized in Figure 6. On treatment with strong oxidizing agents such as chromic acid or potassium permanganate the molecule is rapidly cleaved to monopyrrolic units (20).

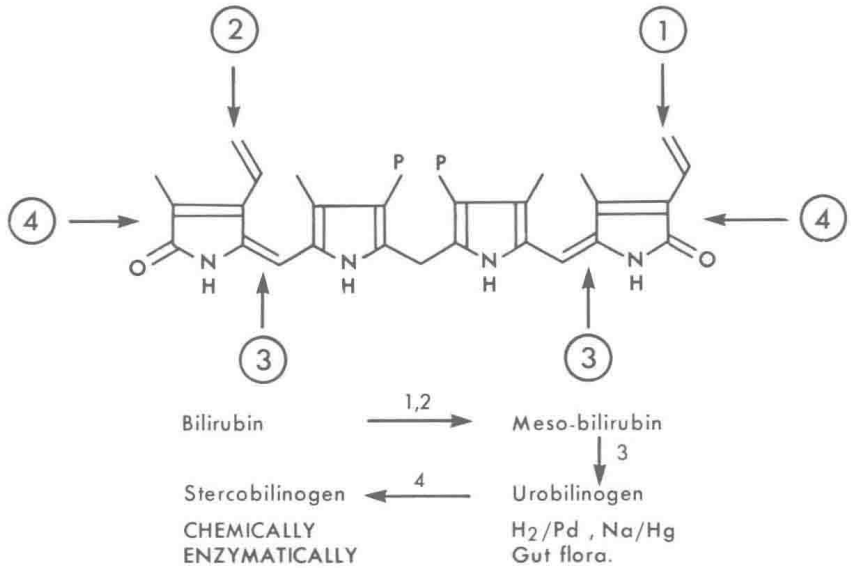

Fig. 5 Reduction (hydrogenation) of bilirubin IX-α

Less powerfull oxidizing agents, for example ferric chloride (21), smoothly dehydrogenate the pigment at the central bridge to give the fully conjugated biliverdin IX-α. This reaction has practical utility for preparing biliverdin, and perhaps the best reagent to use is benzoquinone in the presence of acetic acid with dimethyl sulfoxide as solvent (22).

Even in the dark and in the absence of adding oxidizing agents, bilirubin undergoes spontaneous oxidation in solution by reaction with atmospheric oxygen (23). This auto-oxidation occurs at a negligible rate in chloroform, but is significant in alkaline aqueous solutions, particularly if the concentration of bilirubin is low (15) and transition metal ions are present as trace contaminants (13). The nature of the auto-oxidation products has not been determined but they are probably the colorless water-soluble dipyrrolic compounds called water-propentdyopents (23,24). The autoxidation, which is frequently a nuisance in practical work, can be inhibited by addition of EDTA or ascorbic acid(25), but is most easily obviated by purging the solvent with an inert gas such as argon.

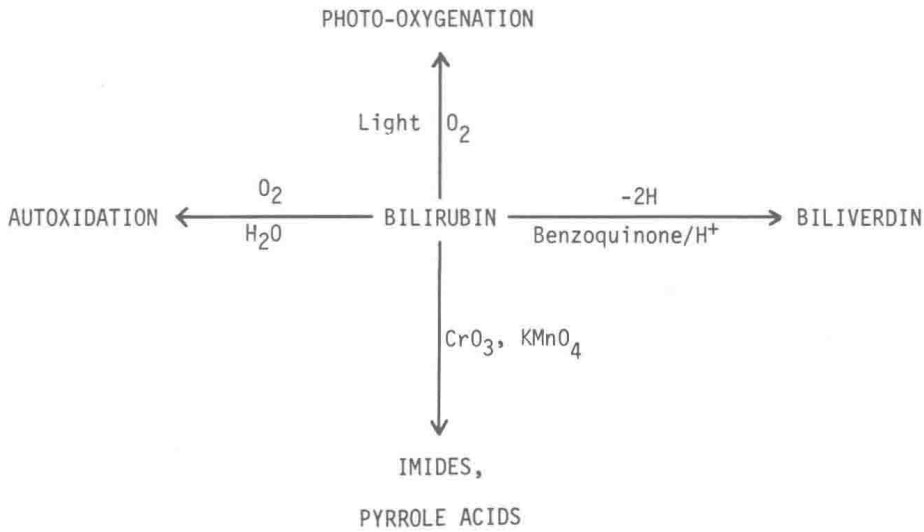

Fig. 6 Some oxidative reactions of bilirubin IX-α

The oxidative reaction of bilirubin which has drawn most attention recently because of its probable significance in phototherapy is photo-oxidation. This reaction is responsible for the familiar photo-degradation of the pigment which occurs when solutions of it are exposed to visible light with wavelengths of about 420-450 nm. The mechanism and products of bilirubin photo-oxidation vary somewhat with the nature of the solvent, but the predominant reaction in most solvents seems to be a photo-oxygenation process in which bilirubin acts as a photosensitizer of its own destruction(26-28). The overall mechanism is as follows:

$$\text{Bilirubin} + \text{Light} \longrightarrow \text{Bilirubin}^*$$

$$\text{Bilirubin}^* + O_2 \longrightarrow \text{Bilirubin} + O_2^*$$

$$\text{Bilirubin} + O_2^* \longrightarrow [\text{Bilirubin} \,.O_2] \longrightarrow \text{Products}$$

Absorption of light by the pigment generates an excited-state molecule which can transfer its excitation energy to molecular oxygen dissolved in the solution. This gives a reactive high-energy form of molecular oxygen called singlet oxygen. Singlet oxygen reacts avidly with certain types of double bonds and attacks bilirubin predominantly by addition to the bridge double bonds or across the central pyrrole rings as indicated in Figure 7. This generates unstable oxygen addition products which decompose thermally or, in hydroxylic solvents, undergo secondary reactions with the solvent. The main products which are formed on photo-oxygenation of bilirubin in ammoniacal methanol are shown in Figure 8(28-32). These products are all soluble in water and are colorless. A competing reaction which also occurs on irradiation of bilirubin solutions and may be marked in some solvents, is photo-oxidation of the pigment to biliverdin(31,33,34). It is currently thought that photo-oxygenation of bilirubin by a singlet oxygen mechanism occurs in the skin of jaundiced infants during phototherapy(35,36).

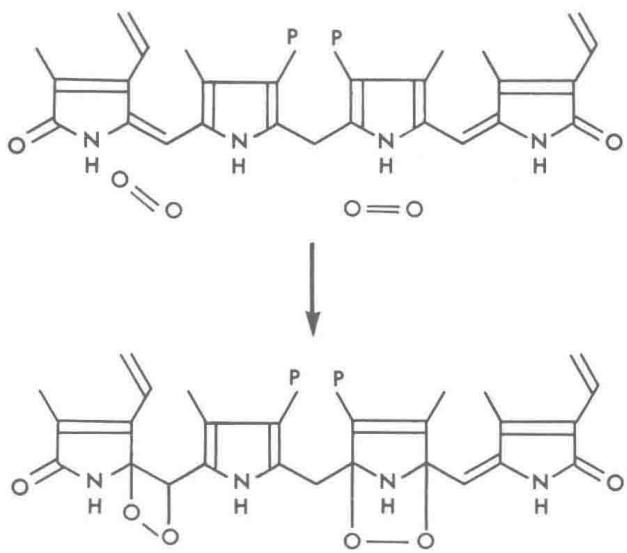

Fig. 7 Predominant modes of addition of singlet oxygen to bilirubin IX-α