

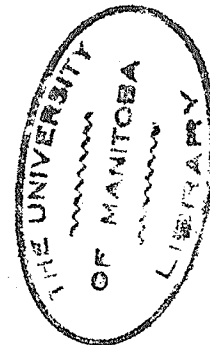
A CHROMATOGRAPHIC STUDY OF BILIRUBIN

A THESIS
PRESENTED TO
THE FACULTY OF GRADUATE STUDIES
UNIVERSITY OF MANITOBA

IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE
MASTER OF SCIENCE

BY
MURIEL BETH GOURLEY

APRIL 1956



ACKNOWLEDGMENTS

I wish to acknowledge, with gratitude and appreciation, my indebtedness to Dr. F. D. White and Dr. G. E. Delory for their helpful suggestions and criticisms, and for their continued encouragement throughout the course of this work.

ABSTRACT

The bilirubin pigments found in human blood serum have been studied by means of the reverse phase chromatographic technique. The technique itself was found to be effective only in the qualitative separation of the pigments. From the evidence of this study, it can be stated that there are three distinct bilirubin pigments, only one of which can be properly called bilirubin, the other two being of a "direct" acting nature as judged by their coupling reaction with diazo reagent. Evidence is given in support of the theory that direct acting bilirubin is attached to protein in vivo, and that this complex increases the ability of the pigment to resist oxidation by exposure to air. It has been demonstrated that greatly increased levels of bilirubin in the body are not alone responsible for the development of kernicterus in the newborn rat, and that other unknown factors are the toxic agents in this condition.

TABLE OF CONTENTS

SECTION	Page
PART I: INTRODUCTION	
REVIEW OF LITERATURE	1
PART II: EXPERIMENTAL	
Details of the Method	12
I. A STUDY OF THE LIMITATIONS OF THE TECHNIQUE . . .	16
1. The separation of a solution of pure bilirubin in chloroform solution	16
2. The effect of adding pure bilirubin to normal serum	19
3. The effect of pH on the chromatographic column	20
4. An attempt to obtain Pigment II in large quantity for study	22
5. The effect of change of pH of material before deproteinization	24
6. Partition of serum from a case of erythro- blastosis	28
7. Undissolved pigment residue treated with ether	28
8. Undissolved pigment residue treated with chloroform	30

SECTION	Page
9. The adsorption of dry pigment on kieselguhr .	33
10. The direct bilirubin-protein complex treated with trypsin	34
II. THE STUDY OF PROPOSED METHODS OF CONVERTING ONE TYPE OF BILIRUBIN TO ANOTHER	36
11. Fowweather's alkaline salt method	36
12. Najjar's pyrophosphate method	38
13. Najjar's chelating agent method	42
14. The attempt to form a metal-bilirubin complex	43
15. Incubation of bilirubin with fresh rat liver slices	44
III. THE POSSIBLE SUBSTANCES IN THE BLOOD WHICH MAY ACT IN THE TRANSPORT OF BILIRUBIN IN BLOOD	46
16. The attachment of lipid to pure bilirubin pigment	46
17. The addition of bile salt to normal serum and pure bilirubin	49
IV. AN ATTEMPT TO SEPARATE ADULT AND FETAL BILIRUBIN .	50
18. Separation of pigments from the serum from a case of obstructive jaundice	52
19. Separation of a mixture of pigments from ery- throblastosis and obstructive jaundice . .	54
V. 20. Separation of three bilirubin pigments on a butanol-water phase system	55
VI. THE ATTEMPTED PRODUCTION OF KERNICTERUS IN NEWBORN RATS	58

SECTION

Page

PART III

CONCLUSIONS	63
SUMMARY	65
BIBLIOGRAPHY	66

LIST OF FIGURES

Figure		Page
1.	The Chemical Structure of Bilirubin	3
2.	The Kieselguhr Column	14
3.	The Separation into Two Fractions of Pure Hoffman-La Roche Bilirubin	18
4.	Addition of Pure Bilirubin to a Normal Non- icteric Blood Serum	21
5.	The Effect of Overloading the Column with Excess Bilirubin	23
6.	Material for Chromatography Prepared at pH 7.0 and at pH 8.0	26
7.	The Absorption Spectrum of the Fast Moving Band of Pigment from Material Prepared at pH 8.0	27
8.	Blood Serum from an Erythroblastotic Infant, the Undissolved Residue Taken up in Ether . .	29
9.	Blood Serum from an Erythroblastotic Infant, the Undissolved Residue Taken up in Chloroform	32
10.	Blood Serum from a Case of Infectious Hepatitis Showing Three Distinct Bands of Pigment . . .	35
11.	An Attempt to Convert Indirect Bilirubin to Direct Through the Formation of its Alkaline Salt	39
12.	Crystalline Bilirubin Treated with Najjar's Phosphate Buffer at pH 10.0	41
13.	Indirect Acting Bilirubin Precipitated Using Alcoholic Sodium Cyanide	45
14.	The Effect of Adding Lecithin to a Solution of Pure Bilirubin	48

Figure	Page
15. Na Glychocholate Is Used to Bring Bilirubin into Solution in Normal Non-icteric Serum . .	51
16. Blood Serum from a Case of Obstructive Jaundice	53
17. The Simultaneous Chromatographic Separation of Blood Serum from Cases of Obstructive Jaundice and of Hemolytic Jaundice	56

PART I

INTRODUCTION

Since the year 1916, when Hijman van den Bergh and Muller (1916) discovered that the diazotization reaction could be used to indicate the presence of two different states of bilirubin in blood serum, there has been a constant search to discover the nature of these two or more fractions. The development of any new technique gives further impetus to this search, and accordingly Cole and Lathe (1953) adapted the chromatographic technique introduced by Howard and Martin (1950) in an attempt to separate serum bilirubin into its component fractions.

The solubility of bilirubin in serum at pH 7.35 has posed a major problem, since in vitro pure bilirubin is only soluble at pH 8.0 or higher. The nature of the diazo reaction in water and in alcohol, and the ability of bilirubin to remain in solution at physiological pH values seem to be closely connected. The present study was carried out with a view to discovering if the chromatographic technique was useful for separating bilirubin fractions and if it could be used to elucidate the vexed question as to how bilirubin is carried in the blood stream.

REVIEW OF THE LITERATURE

The determination of the structure of the bilirubin

molecule has been established within the last twenty years, due chiefly to the work of Fischer (1937) and of Lemberg (1949). Each has approached the problem from a different angle. Lemberg was able to synthesize bilirubin from haemin in 1935 and Fischer synthesized both bilirubin and biliverdin from simple pyrrole compounds in 1942.

The accepted formula for bilirubin as synthesized by Fischer is a tetra-pyrrole di-carboxylic acid having an open chain structure. The degradation of bilirubin by resorcinol fusion by Fischer (1931) has led to a further understanding of the mechanism by which the molecule is able to enter into coupling reactions with diazonium chloride.

This coupling reaction is of particular importance in biological chemistry because it has been used to measure the quantity of bilirubin occurring in blood. The bilirubin is capable of being split at the central carbon atom and because of its asymmetrical nature, the two resulting dipyrroles will be different. Thus one half of the tetrapyrrole forms a dipyrrole having a free alpha position, while the other half has no such free position, it being filled by a methoxy group. Because of the asymmetry of the bilirubin molecule, the molecule can split at either side of the central CH_2 group, and therefore for each of the two active dipyrroles formed, there will be an inactive form. The two possible active forms are shown in Fig. 1. These are neo-xanthobilirubinic acid and isoneoxanthobilirubinic acid. The phenyl diazonium chloride can couple at either of two