THE INFLUENCE OF DRUG TREATMENT ON THE SUSCEPTIBILITY TO JAUNDICE AND KERNICTERUS IN THE HUMAN NEONATE

by

Louise Antoinette Friedman BSc (Hons)

Being a thesis submitted for the
degree of Doctor of Philosophy
in the University of London

Department of Clinical Pharmacology
Institute of Obstetrics and Gynaecology
Queen Charlotte's Hospital
London W6 0XG

ABSTRACT

Louise Antoinette Friedman

THE INFLUENCE OF DRUG TREATMENT ON THE SUSCEPTIBILITY TO JAUNDICE AND KERNICTERUS IN THE HUMAN NEONATE

This thesis is concerned with the way in which perinatal drug treatments can influence neonatal jaundice and the binding of bilirubin to serum albumin.

Neonatal jaundice is thought to have increased in recent years. However, an international survey undertaken in Europe and North America did not confirm this clinical impression.

The factors influencing the incidence of neonatal jaundice were investigated in one obstetric hospital. Gestational age had the greatest effect. Maternal oxytocin administration and epidural anaesthesia increased the frequency of jaundice but their effect was small.

Novobiocin, oxytocin, bupivacaine, para-amino benzoic acid and pethidine were tested in vitro for their effect on bilirubin conjugation by rat liver microsomes. Only novobiocin and pethidine showed inhibitory activity.

Three semi-synthetic penicillins and sulphafurazole, a documented displacer of bilirubin, were investigated for their ability to displace bilirubin from albumin binding. Ampicillin had no effect. Supra-therapeutic concentrations of flucloxacillin and cloxacillin displaced bilirubin from human cord serum albumin but not from bovine albumin. Therapeutic sulphafurazole concentrations displaced bilirubin from both albumins. The significance of these results for kernicterogenic potential was tested in vivo using neonatal guinea pigs. Sulphafurazole but not cloxacillin or flucloxacillin increased relative brain to plasma concentrations of bilirubin. These results could have been predicted from Sephadex Gel filtration results on guinea pig serum.

Finally, a double blind therapeutic trial of antipyrine as prophylaxis for neonatal jaundice was carried out. Pregnant women at 38 weeks gestation were treated with either antipyrine or placebo. Serum bilirubin concentrations on the fourth day after birth were significantly lower in the group receiving the inducing agent.

TABLE OF CONTENTS

	PAGE
TITLE PAGE	1
ABSTRACT	2
TABLE OF CONTENTS	3
LIST OF TABLES AND ILLUSTRATIVE MATERIAL	12
ACKNOWLEDGEMENT	17
ABBREVIATIONS	18
CHAPTER 1 INTRODUCTION	21
CHAPTER 2 METHODS	29
Measurement of unconjugated and conjugated bilirubin	30
a) In plasma or serum	30
b) In microsomal incubate	31
Measurement of free bilirubin	32
a) In guinea pig brain and fat	32
Extraction	32
Biochemistry	33
Brain bilirubin	33
Fat bilirubin	34
Standard curve	34
b) In artificially jaundiced pooled human cord serum (PCS), neonatal guinea pig - pooled serum (GP-PS) and bovine serum albumin (BSA)	35
Preparation of columns	35
Evaluation of method	37

PAGE

1) Capacity of Sephadex for free bilirubin 37

Method 37

Results 37

2) To check efficiency of separation of bound from free bilirubin 39

i) Bound bilirubin 39

Method 39

Results 39

ii) Free bilirubin 41

Method 41

Results 41

3) To check that no protein remains on the column after elution with phosphate buffer 41

Method 41

Results 43

Conclusion 43

Experimental procedure 43

Preparation of rat liver microsomes 45

Preparation of guinea pigs for infusion and withdrawal of blood samples 45

Preparation of venous cannulae 46

Preparation of arterial cannulae 46

CHAPTER 3 SURVEY ON THE INCIDENCE OF JAUNDICE IN NEONATES 49

SUMMARY 50

INTRODUCTION 50

METHODS 54

PAGE

RESULTS 54

Observations on the incidence of jaundice 54

Date of possible increase 57

Actual incidence of jaundice in 1975 58

Reliability of response 58

Sample size from which the actual incidence was derived 58

Length of stay in clinic 60

Prophylaxis of neonatal jaundice 60

Factors associated with a changing incidence of neonatal jaundice 60

DISCUSSION 62

CHAPTER 4 FACTORS INFLUENCING THE INCIDENCE OF JAUNDICE IN NEONATES 66

SUMMARY 67

INTRODUCTION 67

METHODS 69

RESULTS 72

Serial figures 72

Individual factors 73

Small-for-dates infants 78

Breast feeding and neonatal jaundice 81

DISCUSSION 84

Serial figures 84

Oxytocin 88

Breast feeding 89

Race 90

Small-for-dates 90

PAGE

CHAPTER 5 EFFECT OF DRUGS ON BILIRUBIN CONJUGATION IN VITRO 92

SUMMARY 93

INTRODUCTION 93

Bilirubin metabolism 93

Placental transfer of drugs 94

Icterogenic drugs 96

METHODS 97

Estimation of apparent Km for bilirubin 99

Estimation of apparent Km for UDPGA 100

Effect of enzyme concentration on glucuronide formation rate 100

Drug studies 100

Calculation of the bilirubin glucuronidation rate 101

RESULTS 101

DISCUSSION 105

Bupivacaine 108

Pethidine 108

Oxytocin 109

Para-amino benzoic acid 109

Novobiocin 110

Multiple forms of UDP-glucuronyl transferase 110

CHAPTER 6 EFFECT OF DRUGS ON BILIRUBIN BINDING BY NEONATAL SERUM AND BOVINE SERUM ALBUMIN IN VITRO 112

SUMMARY 113

PAGE

INTRODUCTION 114

Binding capacity of albumin 114

Total bilirubin levels 115

Free bilirubin levels 116

Relationship between total bilirubin, free bilirubin and kernicterus 116

Drugs and kernicterus 117

Kernicterogenic drugs - in vivo studies 118

Kernicterogenic drugs - in vitro studies 119

Equilibrium dialysis 119

Ultrafiltration 120

Peroxidase method 120

Erythrocyte binding 120

Mitochondrial binding 121

HBABA dye method 121

Fluorescent dye binding 121

Spectral curves 121

Fluorescence spectroscopy 122

Salicylate method 122

Electrophoresis 122

Dextran coated charcoal method 123

Sephadex Gel filtration 123

METHODS 125

Measurement of bilirubin binding by BSA 126

Effect of drugs on binding by BSA 126

Measurement of bilirubin binding by human pooled cord serum 127

Effect of drugs on bilirubin binding by PCS 128

PAGE

RESULTS 127

Statistics 127

a) Conventional plot 128

b) Scatchard plot 128

Effect of drugs on bilirubin binding by BSA 131

Effect of drugs on bilirubin binding by PCS 136

DISCUSSION 149

CHAPTER 7 EFFECT OF DRUGS ON BILIRUBIN DISPOSITION IN NEONATAL GUINEA PIGS 155

SUMMARY 156

INTRODUCTION 156

Kernicterus 156

Follow-up studies of infants with neonatal jaundice 158

Displacement of bilirubin from albumin by drugs 159

Animal models of neonatal jaundice 160

Choice of animal for disposition studies 161

METHODS 163

In vitro studies 163

The effect of sulphafurazole, cloxacillin and flucloxacillin on bilirubin binding by guinea pig serum using Sephadex Gel filtration 163

In vivo studies 164

Preliminary investigations 164

a) Determination of bilirubin half-life in neonatal guinea pigs 164

Method 164

Results 165

PAGE

b) To see if a steady-state had been reached after 3 hours constant bilirubin infusion 167

Method 167

Results 167

Drug studies 169

a) In guinea pigs infused with bilirubin only 169

Controls 169

Animals receiving sulphafurazole 170

Animals receiving cloxacillin or flucloxacillin 170

b) In guinea pigs infused with bilirubin and pooled cord serum (PCS) 171

Controls 171

Animals receiving sulphafurazole 171

Animals receiving cloxacillin or flucloxacillin 171

RESULTS 172

In vitro studies 173

Statistics 172

In vivo studies 176

Statistics 176

Guinea pigs infused with bilirubin and saline 180

Guinea pigs infused with bilirubin and PCS 184

DISCUSSION 184

Blood-brain barrier 192

PAGE

CHAPTER 8 TRIAL OF ANTIPYRINE AS PROPHYLAXIS FOR JAUNDICE IN HUMAN NEONATES 194

SUMMARY 195

INTRODUCTION 195

Background 195

Clinical trials with phenobarbitone as prophylaxis for neonatal jaundice 196

Toxicity of phenobarbitone 197

Other prophylactic treatments 198

Antipyrine - an inducing agent 199

Pharmacology of antipyrine 200

METHODS 201

Drug dose 202

Patient selection 202

Patient enrolment 203

Tablet counting 203

Bilirubin measurement 204

RESULTS 204

Serum bilirubin levels 204

Birth weight 206

Number of tablets taken 206

Sex ratios 206

Reason for refusal after enrolment 206

Side effects in mothers 209

Side effects in infants 209

PAGE

DISCUSSION 209

Refusal to participate in trial subsequent to agreement 211

Antipyrine toxicity 212

Kernicterogenic potential of antipyrine 213

Transplacental enzyme induction 213

a) Animal studies 214

b) Human studies 214

CHAPTER 9 CONCLUSION: INFLUENCE OF PERINATAL DRUG USAGE ON JAUNDICE IN THE NEONATE 217

APPENDIX 222

REFERENCES 223

PUBLICATION (1) Oxytocin and neonatal jaundice 241

PUBLICATION (2) Factors influencing the incidence of neonatal jaundice 242

LIST OF TABLES AND ILLUSTRATIVE MATERIAL

		PAGE
Figure 1:1	Normal baby	24
Figure 1:2	Jaundiced baby	24
Figure 1:3	Jaundiced baby under phototherapy	25
Figure 2:1	Standard curve for evaluation of bilirubin content of guinea pig brain and fat	36
Figure 2:2	Effect of Sephadex column height on the absorbance of free bilirubin in phosphate buffer eluate	38
Figure 2:3	Absorbance of protein in serial samples of eluate after treatment of column with BSA and subsequent elution with phosphate buffer and absorbance of protein-bound bilirubin after treatment with artificially jaundiced BSA and subsequent elution with buffer.	40
Figure 2:4	Effect of NaOH volume used to elute free bilirubin from Sephadex column on the percentage of bilirubin recovered.	42
Figure 2:5	a) Cannulated neonatal guinea pig infused by Palmer pump	48
	b) Close-up of guinea pig during infusion	
Table 3-1a	Questionnaire on the incidence of neonatal jaundice	56
Table 3-1b	Questionnaire on the incidence of neonatal jaundice	56
Table 3-2	Response to questions about the incidence of neonatal jaundice from a questionnaire mailed to paediatricians in Europe and North America	59
Table 3-3	Incidence of neonatal serum bilirubin concentrations in various published series	64
Figure 4:1	Percentage of neonates with serum bilirubin concentration of $\geq$ 205 µmol/l and $\geq$ 291 µmol/l month by month over five years	71

		PAGE
Table 4-1	Changing incidence of jaundice during 1971-75 (excluding all cases of Rh disease, ABO incompatibility, G-6-PD and those infants who did not remain in hospital for at least 7 days)	74
Table 4-2	Proportion of neonates with jaundice (peak bilirubin $\geqslant$ 205 µmol/l) according to various factors	75
Table 4-3	Mean peak neonatal serum bilirubin concentrations according to various factors	76
Table 4-4	Influence of various factors on the incidence of neonatal jaundice $\geqslant$ 205 µmol/l calculated by linear discriminant function analysis	77
Table 4-5	Influence of various factors on peak neonatal serum bilirubin concentrations calculated by multiple regression analysis	79
Figure 4:2	Mean peak neonatal serum bilirubin values according to gestational age for groups of infants with different birth weights	80
Figure 4:3	Mean peak neonatal serum bilirubin values according to birth weight for groups of infants with different gestational ages	82
Table 4-6	Influence of various factors on the incidence of neonatal jaundice $\geqslant$ 205 µmol/l calculated by linear discriminant function analysis	83
Table 4-7	Influence of various factors on peak neonatal serum bilirubin concentrations calculated by multiple regression analysis.	85
Figure 5:1	Bilirubin metabolism	95
Figure 5:2	Lineweaver-Burk plots showing the effect of novobiocin on bilirubin conjugation	102
Figure 5:3	Lineweaver-Burk plot showing the effect of UDPGA concentration on bilirubin glucuronidation	103
Table 5-1	Effect of novobiocin on bilirubin glucuronidation rate at variable bilirubin concentrations	104
Figure 5:4	Effect of liver concentration on bilirubin glucuronidation rate	106

PAGE

Table 5-2	Effect of drugs on bilirubin conjugation by rat liver microsomes	107
Figure 6:1	Two methods of plotting bilirubin binding data	129
Figure 6:2	Effect of sulphafurazole concentration on bilirubin binding by bovine serum albumin	132
Figure 6:3	Scatchard plots showing the effect of sulphafurazole concentration on bilirubin binding by bovine serum albumin	133
Table 6-1	Relationship between sulphafurazole concentration and bilirubin binding by bovine serum albumin as calculated by Scatchard analysis	134
Table 6-2	Comparison between values for BBC of primary binding site on BSA obtained by classical and conventional methods at various sulphafurazole concentrations	135
Figure 6:4	Effect of cloxacillin concentration on bilirubin binding by bovine serum albumin	137
Figure 6:5	Effect of flucloxacillin concentration on bilirubin binding by bovine serum albumin	138
Figure 6:6	Effect of ampicillin concentration on bilirubin binding by bovine serum albumin	139
Figure 6:7	Effect of sulphafurazole concentration on bilirubin binding by human pooled cord serum	140
Figure 6:8	Scatchard plots showing the effect of sulphafurazole concentration on bilirubin binding by human pooled cord serum	141
Figure 6:9	Effect of cloxacillin concentration on bilirubin binding by human pooled cord serum	143
Figure 6:10	Effect of flucloxacillin concentration on bilirubin binding by human pooled cord serum	143
Figure 6:11	Scatchard plots showing the effect of cloxacillin concentration on bilirubin	145
Figure 6:12	Scatchard plots showing the effect of flucloxacillin concentration on bilirubin binding by human pooled cord serum	146

PAGE

Figure 6:13	Effect of 16 mM ampicillin on bilirubin binding by human pooled cord serum	147
Table 6-3	Relationship between drug concentration and bilirubin binding by human pooled cord serum as calculated by Scatchard analysis	148
Table 7-1	Bilirubin half-life, plasma concentration at zero time and dose in four neonatal guinea pigs	166
Figure 7:1	Plasma bilirubin levels during bilirubin infusion in three neonatal guinea pigs	168
Figure 7:2	Effect of total bilirubin concentration on unbound bilirubin concentration in guinea pig pooled serum containing 1 mM sulphafurazole	173
Figure 7:3	Effect of total bilirubin concentration on unbound bilirubin concentration in guinea pig pooled serum containing either 2 mM flucloxacillin or cloxacillin	174
Table 7-2	Unbound/total bilirubin ratios for artificially jaundiced guinea pig sera before and after treatment with 1 mM sulphafurazole	175
Table 7-3	Brain/plasma, fat/plasma and fat/brain ratios in guinea pigs treated with bilirubin and sulphafurazole, flucloxacillin or cloxacillin	177
Table 7-4	Brain/plasma, fat/plasma and fat/brain ratios in guinea pigs treated with bilirubin and saline or pooled cord serum	178
Table 7-5	Brain/plasma, fat/plasma and fat/brain ratios in guinea pigs treated with bilirubin, pooled cord serum and sulphafurazole, flucloxacillin or cloxacillin	179
Figure 7:4	Relationship between unconjugated plasma bilirubin levels and brain bilirubin levels in neonatal guinea pigs receiving saline or sulphafurazole	181
Figure 7:5	Relationship between unconjugated plasma bilirubin levels and brain bilirubin levels in neonatal guinea pigs receiving saline or cloxacillin	182
Figure 7:6	Relationship between unconjugated plasma bilirubin levels and brain bilirubin levels in neonatal guinea pigs receiving saline or flucloxacillin	183

		PAGE
Figure 7:7	Relationship between unconjugated plasma bilirubin levels and brain bilirubin levels in neonatal guinea pigs receiving saline or PCS	185
Figure 7:8	Relationship between unconjugated plasma bilirubin levels and brain bilirubin levels in neonatal guinea pigs receiving PCS only or PCS and sulphafurazole	186
Figure 7:9	Relationship between unconjugated plasma bilirubin levels and brain bilirubin levels in neonatal guinea pigs receiving PCS only or PCS and either flucloxacillin or cloxacillin	187
Figure 8:1	Hydroxylation of antipyrine to 4-hydroxyantipyrine	201
Table 8-1	Enrolment of women into antipyrine trial and reasons for non-inclusion into the final analysis	205
Figure 8:2	Fourth day neonatal serum bilirubin levels in infants of mothers treated with placebo or antipyrine	207
Figure 8:3	Birth weights of infants whose mothers received either placebo or antipyrine	207

ACKNOWLEDGEMENT

My sincere thanks go to my supervisor Dr Peter Lewis for his constant direction, encouragement and patience throughout my research.

I am grateful to Professor Merton Sandler who made it possible for me to come and work at Queen Charlotte's Hospital and to Dr David Harvey for kindling my interest in neonatal jaundice.

Helpful discussions were had with Dr Colin Ruthven, QCH; Dr Donald Davies, Royal Postgraduate Medical School; Dr Michael Orme, Liverpool University Medical School; Dr Susan Boobis, St Bartholomew's Hospital Medical School and Dr John Caldwell, St Mary's Hospital Medical School.

I am also indebted to Professor Abraham Greenbaum, University College London for his practical guidance and advice when I first started my research.

Acknowledgements are also due to Mrs Evelyn Moniram, Research Nurse and Mr Frank Moore, Pharmacy QCH; for their cooperation particularly during the clinical trial, to Mr Richard Scrivener and Mrs Ros Brodie for their photographic assistance and to the medical staff at QCH.

ABBREVIATIONS

BBC	Bilirubin binding capacity
BSA	Bovine serum albumin
c	concentration
^{o}C	Temperature in degrees celsius
cm	Centimetre
CNS	Central nervous system
FFA	Free fatty acid
g	Gram
G	Relative centrifugal force
GP-PS	Guinea pig - pooled serum
i.v.	Intra-venous
Kl	Kernicterus
kg	Kilogram
l	Litre
µ	Micro
mg	Milligram
ml	Millilitre
mm	Millimetre
mM	Millimolar
M	Molar
mol	Moles
nm	Nanometre
N	Normal
n	Number
O.D.	Optical density

PABA	Para-amino benzoic acid
%	Percent
PCS	Pooled cord serum
rpm	Revolutions per minute
S.D.	Standard deviation
S.E.M.	Standard error of the mean
SGF	Sephadex Gel filtration
UDPGA	Uridine diphosphoglucuronic acid
v	Volume
w	Weight

Those drugs thou hast, and their adoption tried,
Grapple them to thy soul with hoops of steel;
But do not dull thy palm with entertainment
Of each new-hatch'd, unfledged remedy.

after Shakespeare

CHAPTER ONE

INTRODUCTION

This thesis is concerned with several aspects of drugs and neonatal jaundice. In the early 1970's an apparent increase in the incidence of non-haemolytic neonatal jaundice was noted in Britain (Campbell, Harvey and Norman, 1975) and elsewhere (John, 1975). In several centres this came to the attention of the obstetricians because the planned discharges from hospital of mother and baby was delayed pending the resolution of unexpected hyperbilirubinaemia in the infant (Chalmers, Campbell and Turnbull, 1975). Although an association between neonatal jaundice and oxytocin administration had been noted earlier by German workers in both preterm (Mentzel and Wolf, 1963) and term infants (Mast, Quakernack, Lenfers and Hagan, 1971) a letter (Ghosh and Hudson, 1972) to the Lancet in 1972 drawing attention to this association stimulated a flurry of research activity.

As a result of the Campbell, Harvey and Norman (1975) study which was carried out at Queen Charlotte's Hospital, it was suggested to me on my arrival that the increase in jaundice might be due to the increased use of oxytocin for induction of labour and of bupivacaine for epidural anaesthesia, changes which both occurred in the early 1970's.

Phenolic disinfectants have also been associated with two isolated outbreaks of neonatal jaundice (Wysowski, Flynt, Goldfield, Altman and Davis, 1978). However, at Queen Charlotte's Hospital no change in cleaning policy occurred at the time of rise in incidence of jaundice

and only diluted phenolic disinfectants are used to clean cot mattresses which are well aired before use.

I therefore started to carry out my investigation into neonatal jaundice with respect to drug treatment during the perinatal period.

Initially I experienced some doubt as to whether a 'real' increase in neonatal jaundice had occurred since its diagnosis is rather subjective (figures 1:1 and 1:2) except in severe cases (figure 1:3) and the incidence was likely to rise in proportion to the amount of interest taken in the condition. Furthermore verification of this increase was difficult, due to the paucity of prospective studies in the literature.

Moreover, if there had been a drug related increase in jaundice, one would expect it to be widespread. In order to verify this hypothesis, an international survey on the incidence and trends in neonatal jaundice was carried out among paediatricians in other centres. To keep the centres included in the study as homogeneous as possible in terms of medical practice, only paediatricians in Europe and North America were circularised.

In spite of doubts that the incidence of neonatal jaundice had increased it remained nevertheless an important problem at Queen Charlotte's Hospital in terms of the time and money spent dealing with it, by both clinical and laboratory staff. Furthermore the long-term consequences



Figure 1:1 Normal baby



Figure 1:2 Jaundiced baby



Figure 1:3 Jaundiced baby under phototherapy

of its management with phototherapy or exchange transfusion are difficult to evaluate especially at a time when mother-infant bonding is important.

I therefore decided to investigate suggestions that changing obstetric practices were responsible for the 'increase' in neonatal jaundice using multiple regression analysis to examine independently the influence of various factors on neonatal jaundice. This is the first time to my knowledge that this type of statistical analysis has been applied to the problem of neonatal jaundice.

The ideal study would of course have been prospective and every child would have had a bilirubin measurement carried out. However due to lack of time and resources, a retrospective study was carried out over the period 1971-75 at Queen Charlotte's Hospital. In the final analysis this involved over 12,000 deliveries. Various obstetric factors and sex, race, birth weight and gestational age of the infant were examined for their effect on jaundice rates and mean serum bilirubin levels.

Perinatal drug treatments which inhibit the conjugation of bilirubin are likely to be icterogenic, especially at a time when enzyme activities are depressed. Novobiocin, the antibiotic, is one such inhibitory drug which has been shown to result in raised serum bilirubin levels in neonates (Sutherland and Keller, 1961) and is now never used during the neonatal period.

Oxytocin, bupivacaine, pethidine, para-amino benzoic acid and novobiocin were therefore examined for their effect on bilirubin conjugation by rat liver microsomes as a source of the enzyme glucuronyl transferase. Of course, negative in vitro results cannot definitely rule out the absence of icterogenicity as their metabolites may be glucuronated.

Follow-up studies have shown that low neurological scores are related to elevated neonatal bilirubin levels (Ounsted, Boyd, Hendrick, Mutch, Simons and Good, 1978). The risk of brain damage resulting from hyperbilirubinaemia is due to the free 'unbound' bilirubin fraction passing into the brain and resulting in kernicterus. Since the sulfonamide, sulphafurazole, was found to precipitate kernicterus in preterm infants (Silverman, Andersen, Blanc and Crozier, 1956) many other highly protein-bound drugs have been shown to displace bilirubin from albumin in vitro (Schiff, Chan and Stern, 1971; Odell, 1973) and in vivo (Yeary and Davis, 1974).

Among these drugs were flucloxacillin and cloxacillin, two semi-synthetic penicillins commonly used during the neonatal period. It was therefore decided to investigate the effect of amplicillin, flucloxacillin, cloxacillin and sulphafurazole on bilirubin binding by human cord serum and bovine serum albumin in vitro using Sephadex Gel chromatography to quantify free bilirubin (Jirsova, Jirsa, Heringova, Koldovsky and Weirichova, 1967).

In order to establish whether Sephadex Gel filtration changes reflect disposition changes in vivo, artificially hyperbilirubinaemic neonatal guinea pigs were used as animal models of human neonatal jaundice. By drug infusion and measurement of brain, fat and plasma bilirubin ratios, the effect of drugs on bilirubin disposition could be evaluated.

In the first chapters of this thesis I have dealt with drugs which may inhibit bilirubin glucuronyl transferase and with drugs which may precipitate kernicterus by competing with bilirubin for its albumin binding sites. Certain drugs can induce their own metabolism and the metabolism of other drugs and toxic substances. By treating pregnant women with these drugs, transplacental induction of glucuronyl transferase may occur. Phenobarbitone is an inducing agent which has been used as prophylaxis for neonatal jaundice (Trolle, 1968). However it is infrequently used, perhaps due to its sedative side effects or maybe because paediatricians do not consider neonatal jaundice to be a serious problem.

Antipyrine, a pyrazolone derivative is another inducing drug of a different type which has recently been shown to induce glucuronyl transferase in man and animal (Orme, Davies and Breckenridge, 1974). However it has not previously been used as prophylaxis for neonatal jaundice. A double-blind trial was therefore carried out using antipyrine to treat pregnant women for at least 9 days prior to delivery, in order to establish whether bilirubin levels in the neonates of drug treated mothers were lower than those in untreated neonates.

CHAPTER TWO

METHODS

In this section the bulk of the methodology associated with laboratory investigation is described in detail, although it will be briefly mentioned in subsequent chapters.

MEASUREMENT OF UNCONJUGATED AND CONJUGATED BILIRUBIN

a) In plasma or serum

Bilirubin estimation was based on the coupling of diazotised sulfanilic acid and bilirubin to produce a reddish-purple azo compound. The ultramicro modification of the micro method of Lathe and Ruthven (1958) was used so that bilirubin could be measured in small samples of plasma or serum.

Working diazo reagent was made freshly just before use by thoroughly mixing 5.0 ml diazo reagent A (1% sulfanilic acid in 0.2 N HCl) with 0.15 ml diazo reagent B (0.5% sodium nitrite). 0.1 ml of plasma or serum was mixed well with 3.1 ml of water in a test tube marked BLANK (B), 1.6 ml of the diluted serum were pipetted into a second test tube marked TEST (T). 0.4 ml diazo blank (0.25 N HCl) and 0.4 ml working diazo reagent were added to the BLANK and TEST respectively.

The solutions were mixed well and left to stand for five minutes. Then, using an automated microlitre pipette (Eppendorf) exactly 1.0 ml was transferred from the BLANK to a macro cuvette (1 cm lightpath) avoiding bubble formation. The optical density (O.D.) was read in a Vitatron colorimeter using a 540 nm filter. The cuvette was rinsed

with water and the O.D. of exactly 1.0 ml from the TEST was measured in the same way. These measurements gave the conjugated pigment. 1.0 ml of methanol was then added to the remaining 1.0 ml of BLANK and TEST respectively. They were mixed and left to stand for 8-10 minutes and then read in the Vitatron. These measurements gave the total pigment.

STANDARD (S)

The grey neutral density filter gave an O.D. reading (approx. 0.500) equivalent to 355.7 µmol conjugated bilirubin/l serum and 684.1 µmol total bilirubin/l serum under the conditions of the test.

CALCULATIONS

$$\text{Conjugated bilirubin} = \frac{T-B}{S} \times 355.7 \text{ µmol/l}$$

$$\text{Total bilirubin} = \frac{T-B}{S} \times 684.1 \text{ µmol/l}$$

Total bilirubin - conjugated bilirubin = unconjugated bilirubin

b) In microsomal incubate

The ethyl anthranilate method (Van Roy and Heirwegh, 1968; Black, Billing and Heirwegh, 1970) with minor modifications was used to measure the conjugated bilirubin present after bilirubin and uridine disphosphoglucuronic acid had been incubated with rat liver microsomes

as the error caused by reaction of unconjugated bilirubin is negligible when this method is used.

The protocol was as follows.

After terminating the incubation, 0.2 ml of freshly prepared diazo reagent (see P.33) was added to each tube. The tubes were then incubated at 37° for 30 minutes in a shaking incubator. The reaction was terminated by the addition of 0.15 ml 0.8% (w/v) freshly prepared ascorbic acid solution. The tubes were left to stand for 5 minutes and 2.5 ml of a mixture of methyl-n-propyl ketone and n-butyl acetate (17/3 v/v) were then added to each tube and mixed for 30 minutes on a vortex mixer. The tubes were centrifuged for 5 minutes at 5°C at 2000 rpm and the optical density in the upper organic layer measured against a solvent mixture blank at 530 nm in a Pye Unicam SP 1800 spectrophotometer. The absorbance of the sample minus the blank value was used to calculate the amount of bilirubin conjugated.

MEASUREMENT OF FREE BILIRUBIN

a) In guinea pig brain and fat

Extraction

Bilirubin was extracted from brain and fat by the following procedure which was adapted from the method of Diamond and Schmid (1966). Adherent blood, meninges, and the choroid plexus were carefully wiped off the brain with cotton buds. Any superficial blood vessels were

removed from the fat specimens.

The brain and fat specimens were weighed carefully and homogenised separately at 900 rpm for 1 minute with 1 v/w glacial acetic acid and 3 v/w chloroform by means of an electric drill adapted to drive a teflon homogeniser. A further 3 v/w chloroform were added and homogenisation carried out for a further minute. The chloroform phase was separated and centrifuged for 5 minutes at 2000 rpm in order to clear any turbidity created by particles of brain or fat.

Biochemistry

The bilirubin in the chloroform phase was measured by coupling with the diazonium salt of ethyl anthranilate in the presence of alcohol with produces a reddish-purple azo-pigment. The method was adapted from Van Roy and Heirwegh (1968) and Black, Billing and Heirwegh (1970). The diazo reagent was freshly prepared before use as follows:- 0.1 ml ethyl anthranilate (Eastman Kodak Ltd) was finely dispersed in 10 ml 0.15 M HCl: to this was added, with shaking, 0.3 ml 0.5% sodium nitrite.

Brain bilirubin

2 ml of the chloroform phase were added to 3 ml propan-1-ol and 0.5 ml diazo reagent in a stoppered test tube. Blanks contained 0.5 ml 0.15 N HCl instead of diazo reagent. The tubes were left in the dark at room temperature for 30 minutes. Approximately 1 ml from each reaction vessel was transferred to semi-micro glass cuvettes (1 cm lightpath) and the absorbance of the azo pigment was read at 530 nm against a solvent mixture blank in a Pye Unicam SP 1800 spectrophotometer.

By injecting a tracer dose of ^{131}I-labelled human albumin or ^{125}I-labelled guinea pig albumin into 12 newborn guinea pigs infused with bilirubin containing solutions and kept in humidified room air for 30 minutes, Diamond and Schmid (1966) estimated the volume of the plasma compartment of the brain to be 0.64 ml (S.D. ± 0.07) per 100 g brain wet weight. This value was used to correct for the bilirubin in the plasma compartment of the brain of each guinea pig.

Fat bilirubin

Since only small amounts of fat could be obtained, 1 ml of the chloroform phase was added to 4 ml propan-1-ol and 0.5 ml diazo reagent. However, the sensitivity of the method was not decreased as fat contains approximately 5 times the concentration of bilirubin as that in brain. The same procedure was carried out as described for brain.

Correction was not made for bilirubin contained in the plasma compartment, as fat contains minimal vasculature compared to the brain.

Standard Curve

10 mg bilirubin were dissolved in 5 ml 0.1 N NaOH in the dark, 5 ml of 0.45% Na_2CO_3 0.45% NaCl buffer were then added, so that 1 µl ≡ 1 µg. 3 ml propanol, 2 ml $CHCl_3$/Acetic acid mixture (6:1 v/v) and 0.5 ml diazo reagent were put into stoppered test tubes and 2-20 µg of the bilirubin solution were added to the test tubes using a microlitre syringe, with two test tubes at each concentration. The blank contained 0.5 ml 0.15 NHCl instead of diazo reagent. After shaking to ensure

thorough mixing, the tubes were incubated for half an hour in the dark before reading the colour in a Pye Unicam SP 1800 spectrophotometer at 530 nm against a solvent mixture blank. Figure 2:1 shows the absorbance of each sample (mean of two) plotted against the amount of bilirubin in each test tube.

Reading off the standard curve, the concentration of bilirubin in the brain and fat of each animal (µg/g wet wt) can be calculated by multiplying the value on the abscissa by 4 for brain samples and by 8 for fat samples since brain and fat were diluted by 4 and 8 times respectively. These values can then be converted into µmol bilirubin/kg wet weight of brain or fat.

b) In artificially jaundiced pooled human cord serum (PCS), neonatal guinea pig - pooled serum (GP-PS) bovine serum albumin (BSA)

Sephadex Gel filtration was used to separate the free from bound bilirubin in serum or albumin. The procedure was adapted from Kauffman, Kapitulnik and Blondheim (1969).

Preparation of columns

Sephadex G-25 M (Pharmacia Fine Chemicals) was soaked for 24 hours in 0.066 M phosphate buffer (KH_2PO_4 - 1.743 g, Na_2HPO_4 - 9.596 g/litre pH 7.4), 5 ml plastipak hypodermic syringes (1 cm int. diameter) were plugged with cotton wool filled with the prepared Sephadex and used as filtration columns. Two-way luer locks were used to prevent

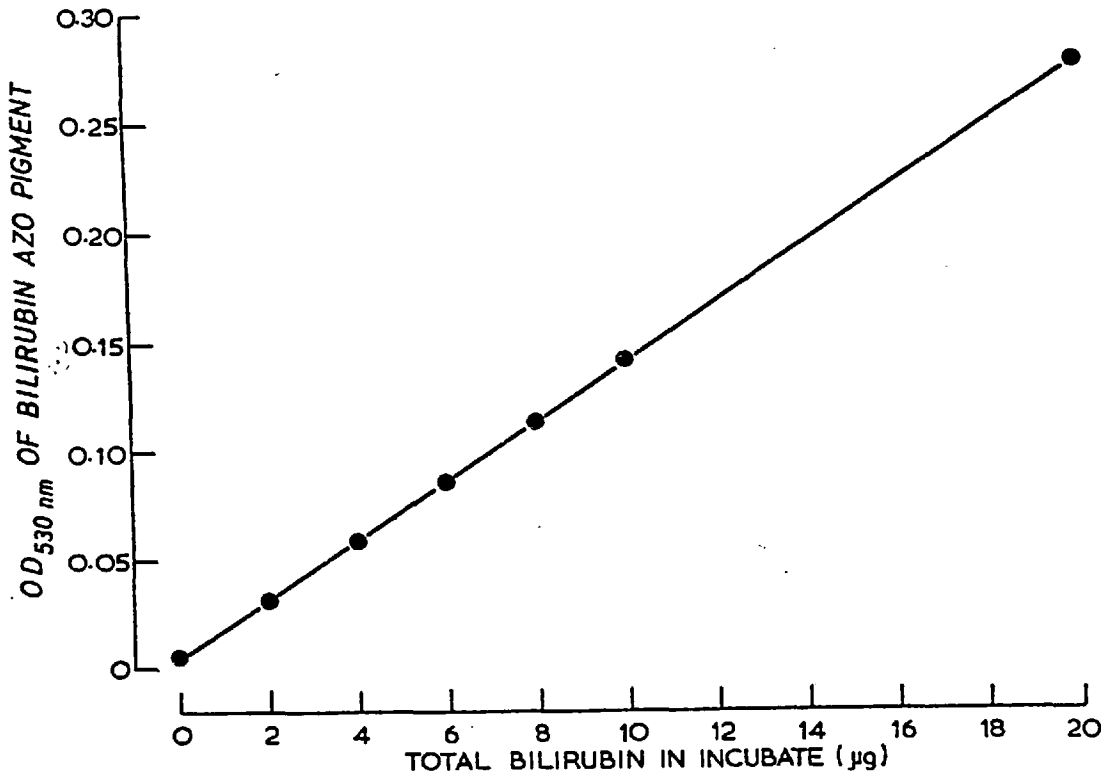


Figure 2:1 Standard curve for evaluation of bilirubin content of guinea pig brain and fat. Each point is the mean of two samples.

the buffer passing through the plugs until the start of the experiment.

Evaluation of method

(1) Capacity of Sephadex for free bilirubin

Method

Six syringes without taps were filled with Sephadex to heights of 0.5 - 3.0 cm. 10 mg anhydrous bilirubin (Sigma Chemical Co.) were weighed out and dissolved in 1 ml 0.1 N NaOH. When the bilirubin was completely in solution, 1 ml buffer (0.45% $Na_2 CO_3$ and 0.45% NaCl pH 7.8) was added and mixed well. This preparation was used immediately. 50 µg of the bilirubin solution (10 µl) were layered onto each of the columns and allowed to sink in. 5 ml phosphate buffer were poured carefully onto each column taking care not to disturb the Sephadex and the eluate from each column was collected. The absorbance of the eluates was read against a phosphate buffer blank at 450 nm in a Pye Unicam SP 1800 spectrophotometer and plotted against the corresponding column heights.

Results

Figure 2:2 shows the effect of column height on the absorbance of free bilirubin in the phosphate buffer eluate. No bilirubin could be detected in the eluates from the columns containing 2 cm of Sephadex or greater. Since 50 µg free bilirubin are greatly in excess of the amount of free bilirubin that would be present in the binding experiments, a height of 2 cm of Sephadex was chosen for further experiments.

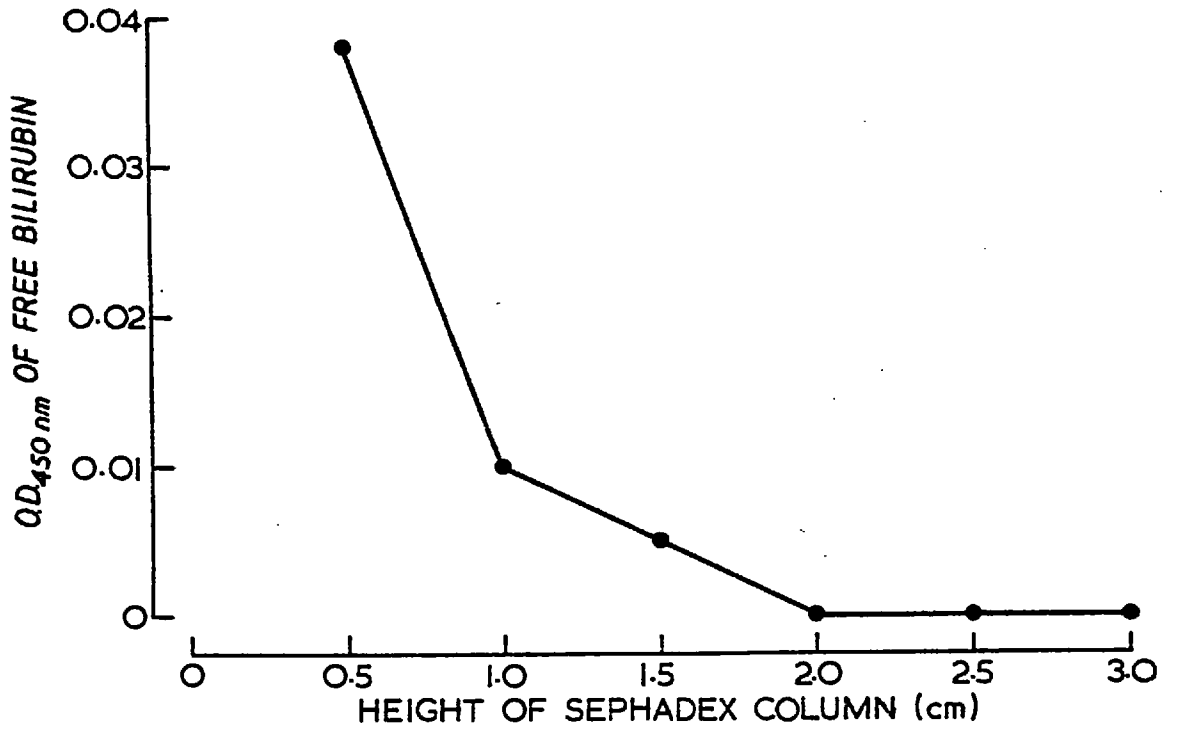


Figure 2:2 Effect of Sephadex column height on the absorbance of free bilirubin in phosphate buffer eluate

(2) To check efficiency of separation of bound from free bilirubin

(i) Bound bilirubin

Method

100 µl of bovine serum albumin (30 g/l) (Sigma Chemical Co.) were layered onto a drained Sephadex column and eluted with 5 ml phosphate buffer. Five drops of eluate were collected in disposable plastic cuvettes (Sarstedt), and the volume was made up to 1 ml with buffer. The absorbance of the protein in each cuvette was read at 280 nm against buffer in a Pye Unicam SP1800 spectrophotometer.

100 µl BSA containing 1.37 mmol/l bilirubin were layered onto a second column containing drained Sephadex. 5 ml phosphate buffer were used to elute the bilirubin bound to protein. 5 drops of eluate were collected into a series of plastic cuvettes and the volume made up to 1 ml with buffer. The absorbance of the bound bilirubin in each cuvette was read at 460 nm.

Results

The absorbances of protein only and protein-bound bilirubin in the samples of eluate were plotted against the total volume of eluate collected (figure 2:3). The almost simultaneous presence of the two discrete peaks which appear in the first ml eluate indicates that the eluted bilirubin was bound to the protein. The unbound fraction remained yellow staining on the column after 5 ml buffer had drained through.

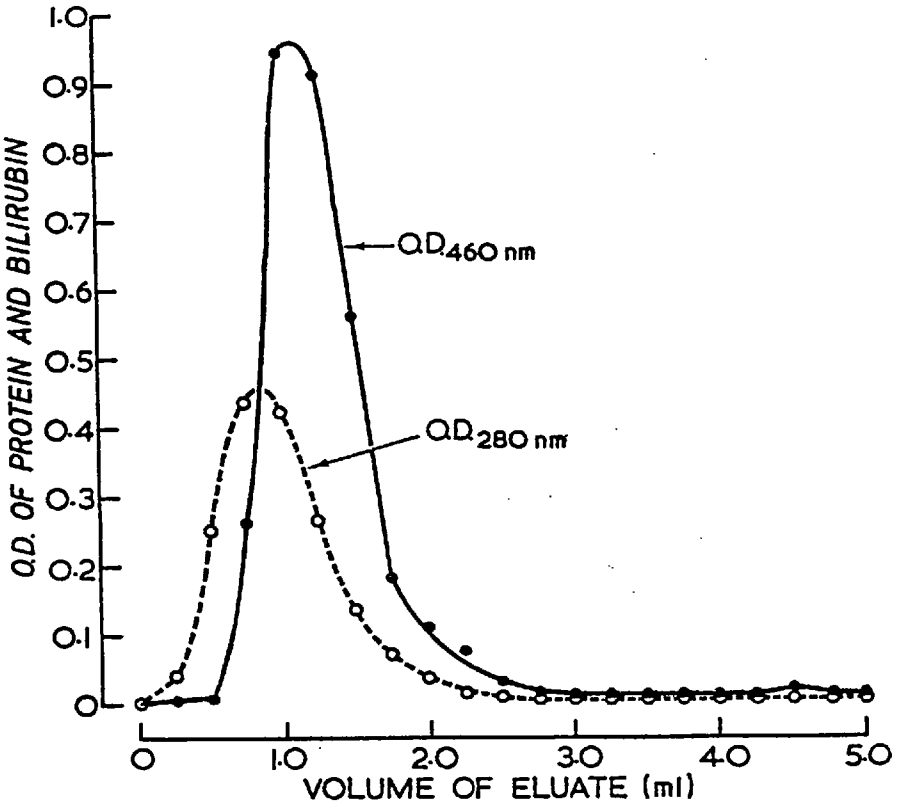


Figure 2:3 Absorbance of protein in serial samples of eluate after treatment of column with BSA and subsequent elution with phosphate buffer, open circles and absorbance of protein-bound bilirubin after treatment with artificially jaundiced BSA and subsequent elution with buffer, closed circles.

(ii) Free bilirubin

Method

100 µl of PCS containing 855 µmol/l bilirubin solution were layered onto a fresh column and washed through with 5 ml phosphate buffer. A 25 gauge hypodermic needle (Gillette) was attached to the nozzle of the syringe to reduce the flow rate and the free bilirubin was recovered by eluting with 10 ml 0.1 N NaOH. ½ ml serial samples of eluate were collected in cuvettes and the absorbance measured at 425 nm (peak for bilirubin in NaOH) against NaOH.

Results

The cumulative absorbance of free bilirubin plotted as a percentage of the total was plotted against the volume of eluate collected (figure 2:4). No bilirubin could be detected in the eluate after 8 ml NaOH had drained through the column. 10 ml 0.1 N NaOH were therefore used in further experiments to elute the free bilirubin.

(3) To check that no protein remains on the column after elution with phosphate buffer

Method

100 µl PCS were layered onto a fresh Sephadex column and eluted with 5 ml phosphate buffer. A 20 µl sample of the total eluate collected was tested for the presence of protein using the Lowry protein estimation (Lowry, 1951). The absorbance of the protein in this sample was then compared with the absorbance of the protein in 20 µl of a solution containing 5 ml buffer and 100 µl PCS.

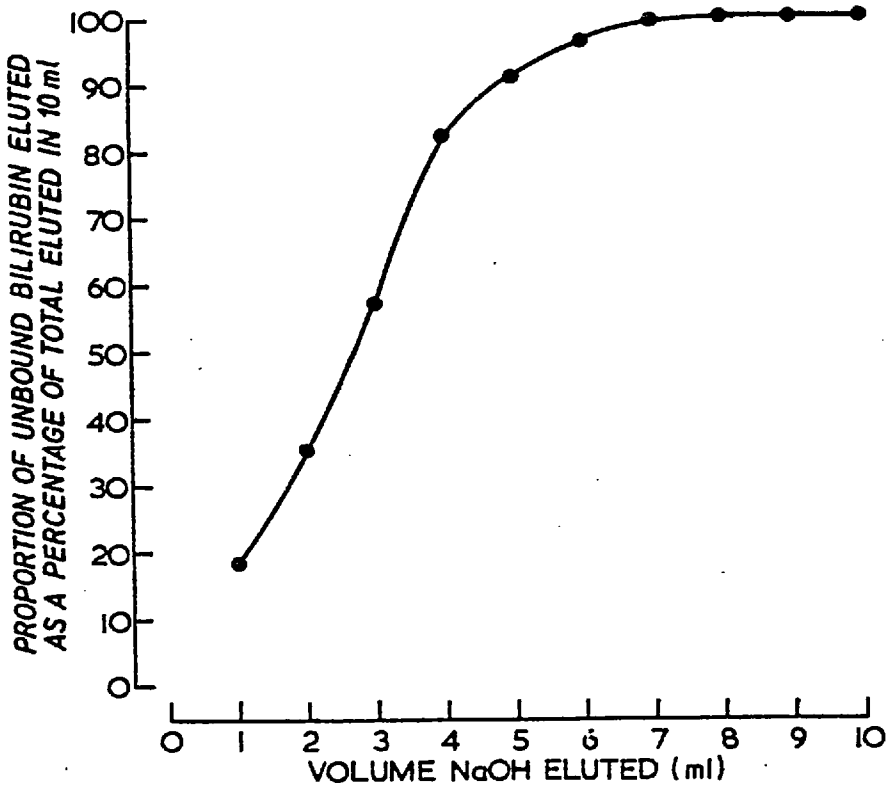


Figure 2:4 Effect of NaOH volume used to elute free bilirubin from Sephadex column on the percentage of bilirubin recovered

To estimate protein, 50 ml reagent A (2% $Na_2 CO_3$ in 0.1 N NaOH) were added to 1 ml reagent B (0.5% $CuSO_4$ 5 H_2O in 1% sodium or potassium tartrate) and then 1 ml of this solution was added to the two samples to be tested and left to stand for 10 minutes. 0.1 ml Folin reagent was then added and mixed immediately. The samples were left for 15 minutes and read at 700 nm in a Unicam SP 1800 spectrophotometer against distilled water.

Results

The absorbance of the two samples was identical, indicating that all the serum had been eluted from the column by 5 ml phosphate buffer.

Conclusion

As a result of the control studies, the following parameters were used in the binding experiments; a column height of 2 cm, elution of bound bilirubin with 5 ml phosphate buffer, elution of free bilirubin with 10 ml of 0.1 N NaOH and a maximum free bilirubin concentration with respect to serum or albumin of 855 µmol/l.

Experimental Procedure

On the basis of results obtained from the control studies the following protocol was performed in the binding studies.

10 mg anhydrous bilirubin (Sigma Chemical Co.) were weighed out and dissolved in 1 ml 0.1 N NaOH (cord serum and BSA experiments) or 2 ml NaOH (guinea pig experiments). 1 ml 0.45% $Na_2 CO_3$ 0.45% NaCl pH 7.8 buffer or 2 ml phosphate buffer (guinea pig experiments) were added and mixed well. This preparation was used immediately. Bilirubin was added to 100 µl of either BSA, PCS or GP-PS so that the bilirubin concentration

in the PCS or BSA increased by 85.5 μmol l^{-1} μl^{-1} of bilirubin solution added, and in the GP-PS by 42.75 μmol l^{-1} μl^{-1} of bilirubin solution added.

The buffer on the Sephadex columns was drained off by removal of the taps and the treated serum was layered onto the Sephadex Gel and allowed to absorb into the gel matrix. The bound bilirubin was washed down with 5 ml phosphate buffer. The free bilirubin if present, yellow staining on the Sephadex, was recovered by eluting with 10 ml of 0.1 N NaOH after attachment of Gillette 25 gauge hypodermic needles to the nozzles of the syringes to reduce the flow rate.

The eluate was collected in 15 ml glass-stoppered centrifuge tubes containing 0.2 ml 1% ascorbic acid (freshly prepared) acidified with 1 ml of 1 N HCL and the bilirubin was extracted into 2 ml chloroform (Analar) by mixing in a vortex for 60 seconds.

Complete separation was effected by centrifuging for 5 minutes at 2000 rpm. The absorbance of free bilirubin was read at 450 nm in a Unicam SP 1800 spectrophotometer with chloroform as blank. The absolute concentration of bilirubin in the serum was calculated from the extinction coefficient of bilirubin in chloroform. (E450 nm for bilirubin in chloroform = $60.7 \pm 0.8 \times 10^3$ M^{-1} cm^{-1} (Merck index)).

PREPARATION OF RAT LIVER MICROSOMES

The microsomal preparation was prepared by differential centrifugation. The method was adapted from Davies, Gigon and Gillette (1968). Three male Wistar rats were killed by stunning. The livers were removed immediately and weighed. They were homogenized in glass at 900 rpm in 4 volumes ice cold 1.15% w/v KCl solution using an electric drill adapted to drive a teflon homogeniser, taking care to keep the homogenate cool. The livers weighed 39.6 g. The homogenate was divided up and spun at 9000 G in a refrigerated high speed centrifuge (MSE 18) for 20 minutes at $4^{o}C$. The supernatants were carefully decanted and spun at 78,000 G for 60 minutes in an evacuated MSE ultracentrifuge at $4^{o}C$. All G values were calculated for the centre of the centrifuge tube. The 78,000 G supernatants were decanted and the microsomal pellets were resuspended in 25 ml 1.15% KCl containing 0.02 M Tris buffer (ph 7.4) using a ground glass to glass homogeniser. Care was taken not to dislodge the glycogen from the bottom of the centrifuge tube when removing the microsomal pellet.

The microsomal suspension was divided up and kept frozen at $-20^{o}C$ for a maximum of two weeks before use.

PREPARATION OF GUINEA PIGS FOR INFUSION AND WITHDRAWAL OF BLOOD SAMPLES

Although the basic idea for the preparation of the animals was taken from Diamondand Schmid (1966), the following protocol was found to be as satisfactory and manifested fewer technical difficulties.

A 7-14 day Dunkin Hartley guinea pig was removed from its mother and weighed. It was placed in a dessicator containing cotton wool impregnated with anaesthetic ether. As soon as the animal was semi-conscious it was removed and the thoracic region was shaved. The guinea pig was replaced in the ether until fully anaesthetised. Anaesthesia was maintained during dissection by applying an ether soaked anaesthetic mask when required.

Preparation of venous cannulae

In order to allow free movement of the animal in its cage, a length of approximately 20 cm of PE-10 polyethylene tubing (Clay Adams, Inc; New York) was taken. It was filled with sterile saline (0.9%) via a 1 ml disposable syringe connected to a 30 gauge hypodermic needle (B-D and Co) taking care to remove all air bubbles.

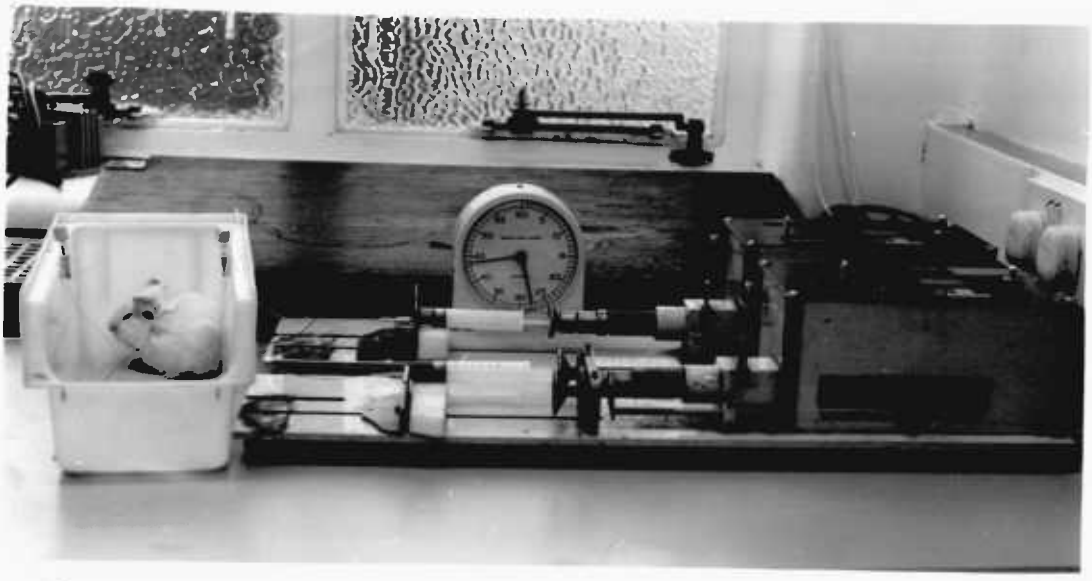
Preparation of arterial cannulae

Approximately 20 cm of polyethylene tubing (I.D 0.025 inches, O.D. 0.045 inches B-D and Co.) was stretched about 1 cm at one end, in order to narrow it for insertion into the artery yet have sufficient width to maintain patency of the cannula. The bevel of a 21 gauge hypodermic needle (Gillette) was removed with a file and the needle was inserted into the wide end of the cannula. A 1 ml disposable syringe was used to fill the cannula with heparinised saline.

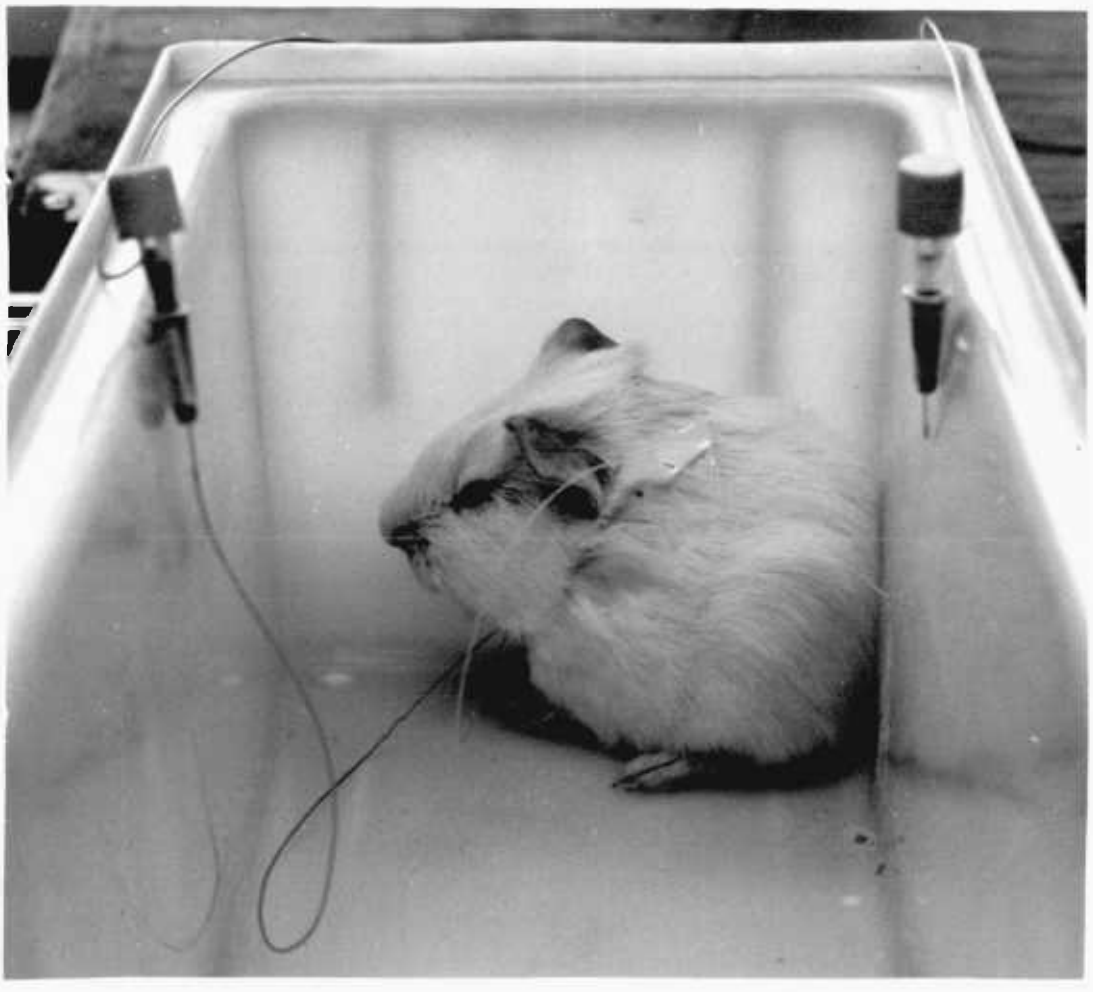
The left and right internal jugular veins were exposed at the level of the thyroid and the venous cannulae were inserted to a depth of 2-4 mm. Blood was withdrawn from the vein to ascertain correct insertion.

Either common carotid artery was exposed at the same level and cannulated so that blood samples could be withdrawn for calculation of bilirubin half-life. The incision was stitched with cotton. Hypoglycaemia and dehydration were prevented by subcutaneous administration of a single dose of 2 ml isotonic dextrose made up in sterile saline. The cannulae were attached to the back of the animal with cellophane tape.

The animal was placed unrestrained in a small cage. Infusion was carried out using two Palmer pumps fitted with 10 ml plastipak syringes (B-D & Co.) connected to remote injection catheters (Portex Ltd) which were ultimately connected to the cannulae via the hypodermic needles (figure 2:5). The infusion was not started until the animal was fully conscious and standing.



a)



b)

Figure 2:5 a) Cannulated neonatal guinea pig infused by Palmer pump
b) Close-up of guinea pig during infusion

CHAPTER THREE

SURVEY ON THE INCIDENCE OF JAUNDICE IN NEONATES

SUMMARY

Ninety paediatricians answered a questionnaire on the incidence of neonatal jaundice, thirty nine Europeans and fifty one North Americans. Less than one quarter reported an increase in jaundice since 1965 or since 1970.

The majority of respondents could only give a rough estimate of the incidence of jaundice in 1975. However, the mean estimates were similar to recent published incidences. Phenobarbitone prophylactic treatment was used by 10% of the respondents, but only in a few selected cases. Oxytocics and increased premature survival were most frequently associated with any increase in the incidence of jaundice and phototherapy with any decrease.

There was considerable similarity in the replies of paediatricians from Europe and North America, although neonates from Europe stayed in the clinics after birth for longer than those born in North America.

INTRODUCTION

Several UK centres have reported an increase in neonatal jaundice in recent years (Ghosh and Hudson, 1972; Eden, 1976). Four centres have cited serial figures in support of their claims.

Campbell, Harvey and Norman (1975) reported a 5 year retrospective analysis of the incidence of neonatal jaundice over a period 1969-1973 at Queen Charlotte's Hospital. They documented a sharp increase in the incidence of serum bilirubin levels > 206 µmol/l in 1972. Their sample excluded babies with rhesus iso-immunisation and the percentage incidence of neonates with serum bilirubin levels > 206 µmol/l was 6.2, 7.1, 8.1, 12.1 and 15.5 respectively in the years 1969-1973. Similarly, the percentage incidence of neonates with serum bilirubin > 290 µmol/l was 1.5, 1.8, 1.7, 2.8 and 4.0.

A 5 year retrospective analysis of the incidence of neonatal jaundice over the period 1971-5 has been reported from the same hospital (Chapter 4). The study population was selected to omit all cases of multiple birth, rhesus iso-immunisation, ABO incompatibility and G-6-PD deficiency, and babies who did not remain in the hospital for at least 7 days after delivery. The percentage incidence of neonates with serum bilirubin levels $\geq$ 205 µmol/l was 8.0, 12.7, 14.8, 14.2 and 14.4 respectively in the years 1971-5. Similarly, the percentage incidence of neonates with serum bilirubin $\geq$ 291 µmol/l was 1.7, 2.5, 3.9, 2.6 and 2.8. Sims and Neligan (1975) merely state that the incidence in neonates of a serum bilirubin level of $\geq$ 256 µmol/l rose from 2.2% in the first 3 months of 1973 to 5.5% in 1974.

Smith and Wilson (1978) reported a 7 year retrospective analysis of the incidence of neonates with serum bilirubin > 250 µmol/l. The infants were divided by weight. Those weighing over 2.5 kg and those weighing

2.5 kg and under. One can calculate from these figures the incidence of neonates with serum bilirubin > 250 μmol/l. The percentage incidence was 0.2, 0.35, 0.04, 0.13, 0.5, 2.1, 2.9, 3.9 and 4.6 respectively between 1968 and 1976. As these studies are all retrospective, it could be argued that the increase in incidence of jaundice might be due to an increased awareness of jaundice leading to more bilirubin estimations being carried out. One reason why this is unlikely is that the serial changes in incidences of neonates with jaundice of 290 μmol/l or greater follows closely the serial changes in incidence of lesser degrees of jaundice ⩾ 205 μmol/l (Campbell, Harvey and Norman, 1975; Friedman, Lewis, Clifton and Bulpitt, 1978).

Another means of deciding if a real increase in neonatal jaundice has occurred is to try and find in the older literature any figures on the incidence of jaundice. Unfortunately there are few figures for the period before the wide adoption of oxytocin induction of labour which can be used to establish a normal incidence for neonatal jaundice.

One such early study is that of Billing, Cole and Lathe (1954). In this report 49 neonates were selected according to body weight, a preponderance of low birthweight infants being studied. A clear association between low birthweight and jaundice was shown. No babies studied with a birthweight over 2.5 kg had a serum bilirubin exceeding 205 μmol/l in the first week after birth. By contrast, in a recent large retrospective study (Chapter 4), data was gathered on 2376 infants weighing

between 2.5 and 3.0 kg at birth. 17% of this group had a serum bilirubin $\geq$ 205 µmol/l.

However, in a study carried out in 1959 (Sutherland and Keller, 1961) in which novobiocin treatment of term neonates was observed to result in a higher incidence of neonatal jaundice, the incidence of jaundice > 15 mg % (256 µmol/l) in a control group of 2044 infants was 2.4%. This value is very similar to an incidence of jaundice $\geq$ 291 µmol/l of 2.7% in a group of 12,461 infants born at Queen Charlotte's Hospital between 1971-5 (Chapter 4).

As a result, therefore, of reports of an increase in neonatal jaundice, changes in obstetric practice have been implicated. The most frequently cited single causative factor has been the introduction of oxytocin for induction or acceleration of labour (Davies, Gomersall, Robertson, Gray and Turnbull, 1973; Beazley and Alderman, 1975; Chew and Swann, 1977). Among other factors implicated are operative delivery (Friedman and Sachtleben, 1976) and epidural anaesthesia (Sims and Neligan, 1975; Campbell, Harvey and Norman, 1975).

In order to determine whether any increase in neonatal jaundice has been noted by paediatricians in other centres, an international postal survey was carried out (Friedman, Lewis and Harvey, 1977). Since it could be presumed that if a change in obstetric practice associated with an increase in drug use has caused an increase in jaundice, then this would have been observed in centres outside the UK.

METHODS

The geographical area selected for the survey was Europe and North America. This was to obtain as great a degree of homogeneity as possible with regard to medical practice, since any innovation in obstetric practice would need to have occurred around the same time for information obtained from the survey to be meaningful.

A short, easy to answer, questionnaire in booklet form (see Table 3-1a and 1 b) was circularised with an explanatory letter and a self-addressed envelope to 65 senior members of the European Society for Paediatric Research and a random 1 in 5 sample of the American Paediatric Society, total 135. Of the 200 paediatricians mailed, 152 replied. Having excluded the 62 responders who were not practicing neonatal paediatrics at present, 90 replies were analysed:- 39 European paediatricians (E) and 51 North Americans (A). The results are presented with the questions as actually asked on the questionnaire.

RESULTS

Observations on the incidence of jaundice (Table 3-2)

(1) Is it your impression that the number of newborn babies who develop significant jaundice (arbitrarily defined as a plasma bilirubin concentration equal to or greater than 12 mg/100 ml or 205 µmol/l in the first 7 days after birth) in your clinic or practice is greater now

Institute of Obstetrics and Gynæcology
University of London

Queen Charlotte's Maternity Hospital
Goldhawk Road, London, W6 0XG
01-746 6601

In Confidence

Survey on Neonatal Jaundice

Dear Doctor,

This short questionnaire on the incidence of neonatal jaundice and related factors should take only a few minutes of your time.

All information will be used only in statistical tables and anonymous presentations.

Yours sincerely,

D.R. Harvey — Paediatrician

P.J. Lewis — Clinical Pharmacologist

(If you are not practising neonatal paediatrics at present please check here ☐ and return).

1. Is it your impression that the number of newborn babies who develop significant jaundice (arbitrarily defined as a plasma bilirubin concentration equal to or greater than 12 mg/100 ml or 206 μmol/l in the first 7 days after birth) in your clinic or practice is greater now than in:

a) 1965

Yes	
No	
Uncertain	

b) 1970

Yes	
No	
Uncertain	

2. If there has been any increase, when did this occur?

_____

3. What do you estimate is the percentage of neonates having this significant degree of jaundice in 1975? ☐ %

4. Is this estimate:

a) fairly exact ☐

or

b) a rough estimate ☐

Table 3-1a Questionnaire on the incidence of neonatal jaundice

5. What is the total number of newborn babies from which this figure is derived? ☐

6. What percentage of newborn babies stay in your clinic for 7 days or longer after birth? ☐

7. Is phenobarbitone treatment of women in late pregnancy used in your clinic with the object of reducing jaundice in the newborn?

a) Yes, routinely ☐

or

b) In most but not all cases ☐

or

c) In a few selected cases ☐

or

d) Never used ☐

8. Do you associate any increase or decrease in the incidence of neonatal jaundice with any changes in medical practice in recent years? If so, please mention these:-

a) Obstetric factors _____

b) Anaesthetic factors _____

c) Paediatric factors _____

d) Environmental or social factors _____

e) Other factors _____

9. If you are engaged in any research relevant to this problem it would be most helpful if you would mention some reference to this or give a brief note of your interests.

_____

_____

THANK YOU VERY MUCH FOR ANSWERING THESE QUESTIONS; AN ADDRESSED ENVELOPE HAS BEEN INCLUDED FOR YOUR REPLY.

Table 3-1b Questionnaire on the incidence of neonatal jaundice

than in: a) 1965 b) 1970?

Respondents could anwer Yes, No or Uncertain.

Some 38% (E) and 45% (A) (percentage of paediatricians actively practicing) thought that there had been no increase in neonatal jaundice since 1965. Only 21% (E) and 18% (A) thought that there had been an increase since 1965, the remainder were uncertain. The majority, 66% (E) and 60% (A) thought there had been no increase since 1970. Only 23% (E) and 24% (A) thought jaundice had increased since 1970. The remainder were uncertain.

Hence, approximately twice as many (E) and two and a half times as many (A) paediatricians thought there had been no increase in jaundice since 1965 compared with those who thought that there had been an increase. Similarly, approximately three times as many (E) and (A) paediatricians thought that there had been no increase in jaundice since 1970 compared to those who thought there had.

Date of possible increase

(2) If there has been any increase, when did this occur?

Only 20% (E) and 14% (A) answered this question. The majority of respondents thought that the increase had occurred since 1970, but no year was cited more frequently than any other.

Actual incidence of jaundice in 1975 (Table 3-2)

(3) What do you estimate is the percentage of neonates having this significant degree of jaundice since 1975?

Replies from the Europeans ranged from 2-30% with a mean of 14.2% (n = 31). An identical range of replies was obtained from the North Americans with a mean of 12.6% (n = 44).

Reliability of response

(4) Is this estimate exact?

36% (E) thought their estimate to be fairly exact and 44% (E) thought it to be a rough estimate. The remainder did not respond to this question. By contrast only 16% (A) thought their estimate to be fairly exact and 69% (A) thought it to be a rough estimate. The remainder did not reply.

Sample size from which the actual incidence was derived

(5) What is the total number of newborn babies from which this figure is derived?

69% (E) and 84% (A) answered this question. The range of sample size obtained from European respondents was 185-20,000 babies with a mean of 3,365. From North American respondents it varied from 120-10,000 babies with a mean of 2,662.

	EUROPE			NORTH AMERICA		
Questionnaires Mailed	65			135		
Response	52 (80%)			100 (74%)		
Actively practising	39			51		
Is the number of neonates who develop significant jaundice* greater now than in: a) 1965 b) 1970?		a	b		a	b
	Yes	21%**	23%	Yes	18%	24%
	No	38%	66%	No	45%	60%
	Uncertain	41%	11%	Uncertain	37%	16%
What do you estimate is the percentage of neonates having significant jaundice in 1975?	Mean	14.2		Mean	12.6	
	Range	2 - 30		Range	2 - 30	

*Significant jaundice was defined as a plasma bilirubin concentration ≥ 12 mg/100 ml (205 µmol/l) in the first 7 days after birth

**Percentage of those actively practising

TABLE 3-2 Response to questions about the incidence of neonatal jaundice from a questionnaire mailed to paediatricians in Europe and North America

Length of stay in clinic

Since peak serum bilirubin levels can occur up to the sixth day, it was important to know what proportion of babies stayed in hospital for a week.

(6) What percentage of newborn babies stay in your clinic for 7 days or longer after birth?

Some 67% (E) and 78% (A) answered this question. The range of replies from the Europeans varied from 5-100% babies staying in hospital for 7 days or longer after birth. North American respondents stated that 0-30% babies stayed in hospital for 7 days or more.

Prophylaxis of neonatal jaundice

(7) Is phenobarbitone treatment of women in late pregnancy used in your clinic with the object of reducing jaundice in the newborn?
The respondents could answer:

a) Yes, routinely, b) in most but not in all cases,

c) in a few selected cases, d) never used.

Only 13% (E) and 8% (A) paediatricians used this occasionally, none used it routinely.

Factors associated with a changing incidence of neonatal jaundice

(8) Do you associate any increase or decrease in the incidence of neonatal jaundice with any change in medical practice in recent years? If so, please mention these:-

a) obstetric factors b) anaesthetic factors

c) paediatric factors d) environmental or social factors

e) other factors

Most respondents did not check any of the possibilities. An increase in the incidence of jaundice was associated with the following suggested factors:-

Oxytocic agents (4 (E) and 3 (A) respondents)

Epidural anaesthesia (2 (E) and 2 (A) respondents)

Increased premature survival rate (6 (A) respondents)

Increased awareness causing more bilirubin to be measured (2 (E) and 1 (A) respondents)

Breast feeding revival (3 (E) and 1 (A) respondents)

The following factors were each suggested by one respondent:

Environmental pollution, lack of light in nurseries, use of phenolic disinfectants, increase in food additives and more teenage pregnancies.

A decrease in the incidence of jaundice was associated with the following suggested factors:-

Phototherapy was the most frequently postulated factor (6 (E) and 5 (A) respondents)

Immuno-anti-D-prophylaxis (2 (E) respondents)

Early feeding (2 (E) respondents)

The following factors were each suggested by one respondent:

Fewer traumatic deliveries, better temperature control in nurseries, fewer preterm infants and a drop in induction rate.

DISCUSSION

Before it can be claimed that the incidence of neonatal jaundice has not increased in recent years, two potential objections must be considered.

Firstly the 48 paediatricians who did not respond - nearly a quarter of those mailed - may have completely different experiences from those who did respond. The non-responding group is probably heterogeneous, including those not actively practicing, those uninterested in the subject and some perhaps who felt too strongly about it to confine their comments to the limited answers required by this kind of questionnaire. Nevertheless, the replies came from almost two-thirds of all respondents, corresponding to those who were actively practicing.

The second objection is that the conclusions are based mainly on paediatricians' impressions rather than on data obtained from prospective studies in which every neonate has a bilirubin measurement carried out. Furthermore, in very few instances did the majority of neonates in a particular clinic stay in hospital for a week or more, thus precluding the opportunity for proper assessment of the condition of the infant.

However, providing the routine in each clinic has remained constant over the time investigated, any change in "apparent" incidence of jaundice would be observed by the paediatrician.

There was a remarkable concensus of opinion between the two groups of paediatricians in their impression of whether they thought the incidence of jaundice had increased. Their estimations of the present incidence of jaundice were also very similar. These results either reflect the similarity in obstetric practice between Europe and North America, if in fact jaundice levels are associated with obstetric practice, or they reflect the similarity in gene pool between the two populations if the level of jaundice is not largely determined by obstetric and other external factors.

In spite of the large range in estimates of the present incidence of jaundice, the mean values for both European and American paediatricians are very similar to the published incidence in unselected series (Friedman and Lewis, 1977) (Table 3-3) and therefore lend additional support to the hypothesis that neonatal jaundice has not increased. The study population in each of these publications was chosen in different ways and studied with greater or less care. Nevertheless, with the exception of the British Births Survey 1970 (1975) the incidence of neonatal jaundice is remarkably similar in all these surveys, which took place between 1968 and 1975.

The authors report that the low incidence of jaundice quoted in the British Births Survey is probably due to the low proportion of babies in this population survey who had a measurement of serum bilirubin.

Reference	% Incidence of bilirubin level (µmol/l)			
	> 171	> 205	> 291	Total Sample
Trolle, 1968	22.6	15.8	2.9	1469
British Births 1970, 1975	2.6	-	-	16077
Ghosh and Hudson, 1972	-	12.1	-	197
Dahms et al, 1973	21.0	-	-	199
Beazley and Alderman, 1975	-	8.4	-	1353
Chalmers et al, 1975	9.2	-	-	10591
Campbell et al, 1975	-	9.8	2.4	18331
Thomas, 1976	16.3	-	1.3	239
Friedman et al, 1977	-	12.9	2.7	12543

TABLE 3-3 Incidence of neonatal serum bilirubin concentrations over stated figure in various published series

Only 26% of all those reported to be clinically jaundiced had such a measurement. If it were to be assumed that all clinically jaundiced babies had a similar range of serum bilirubin then the actual incidence of serum bilirubin over 171 µmol/l might rise towards 10%, very much more in line with the other series quoted.

If oxytocin or bupivacaine were icterogenic then one would expect most paediatricians to have seen an increase in jaundice. This association is made more tenuous by the fact that only 12% of respondents suggested either of these factors to be associated with an increase in jaundice.

Furthermore, if an increase in jaundice levels had occurred, this "increase" was certainly not reflected by the small proportion of respondents who prescribed phenobarbitone prenatally as prophylactic treatment for jaundice (Trolle, 1968).

CHAPTER FOUR

FACTORS INFLUENCING THE INCIDENCE OF JAUNDICE IN NEONATES

SUMMARY

A retrospective study of 12,461 single births showed an incidence of neonatal jaundice ⩾ 205 μmol/l of 12.9% between 1971-5 and confirmed an association between maternal oxytocin infusion and neonatal jaundice. The effect of oxytocin on jaundice was independent of gestational age at birth, sex, race, epidural anaesthesia, method of delivery and birthweight, each of which was significantly associated with neonatal jaundice.

The effect of oxytocin was, however, small, producing a calculated mean increase in peak plasma bilirubin concentration of 8.6 μmol/l; this excess was independent of sex and less than the effect of the baby being born one week earlier. A further study of 9,300 of the cases confirmed an association between breast feeding at discharge and neonatal jaundice. However, the effect of breast feeding was less than seven of the other factors including oxytocin infusion, epidural anaesthesia, sex of infant and gestation.

INTRODUCTION

Although mild jaundice is accepted as normal or physiological, severe or prolonged jaundice is thought by some workers to be a potential cause of mental or motor impairment (Scheidt, Mellits, Hardy, Drage and Boggs, 1977). They found that low mean eight month motor scores and delayed one year motor development are associated with serum bilirubin levels

in the range of 171-239 μmol/l and above. This relationship was strongest for low-birth-weight/short gestational period infants. A persistent association of developmental outcome with hyperbilirubinaemia was also found over and above the variation of maturity within the birth weight/gestational age categories.

Other workers have found that a reduction in intelligence does not occur in non-haemolytic jaundiced babies with serum bilirubin below about 342 μmol/l (Culley, Powell, Waterhouse and Wood, 1970).

In recent years there have been several suggestions that the incidence of significant jaundice has increased (Sims and Neligan, 1975; Campbell, Harvey and Norman, 1975; Smith and Wilson, 1978). Changes in obstetric practice have frequently been blamed. The increase in epidural anaesthesia (Sims and Neligan, 1975; Campbell, Harvey and Norman, 1975) has been suggested, as has forceps delivery (Eden, Revolta, and Adjei, 1974; Friedman and Sachtleben, 1976), ventouse extraction (Campbell, Harvey and Norman, 1975), breech delivery (Friedman and Sachtleben, 1976), breast feeding (Eden, Revolta and Adjei, 1974; Jeffares, 1977) oxytocin for induction of labour (Ghosh and Hudson, 1972; Davies, Gomersall, Roberston, Gray and Turnbull, 1973; Chalmers, Campbell and Turnbull, 1975; Chew and Swann, 1977), and oxytocin for acceleration of labour (Ghosh and Hudson, 1972). Use of prostaglandin E_2 for induction of labour (Calder, Moar, Ounsted and Turnbull, 1974), weight loss during the first week of life (Sims and Neligan, 1975) and

phenolic disinfectants (Wysowski, Flynt, Goldfield, Altman and Davis, 1978) are also thought to be icterogenic.

However, the increased incidence of neonatal jaundice is largely based on clinical impression. The role of oxytocin in the increase is difficult to confirm since oxytocin usage is associated with other icterogenic factors such as preterm delivery, instrumental delivery and epidural anaesthesia.

Neonatal jaundice rates at Queen Charlotte's Hospital were, therefore, analysed over five years. The rates were examined for the factors associated with neonatal jaundice and the relative contribution of the icterogenic factors identified was quantified.

METHODS

The study population was drawn from the 15,932 live births at Queen Charlotte's Hospital in the five years 1971-5. Cases of multiple birth, rhesus incompatibility, ABO incompatibility and G-6-PD deficiency were excluded as were those where the infant did not remain in hospital for seven days or more post delivery (in this case the jaundice may have developed after discharge). The study group thus comprised 12,461 live births without haematological predisposition to neonatal jaundice who were observed in hospital for the first week after birth.

Date of delivery, sex, race, birthweight, gestational age and method of delivery were recorded as were presence or absence of amniotomy, oxytocin infusion (whether for induction or acceleration of labour), epidural anaesthesia or general anaesthesia. When serum bilirubin was measured, the peak serum bilirubin in the first seven days after delivery was recorded. Serum bilirubin was only measured when the neonate appeared clinically jaundiced.

The proportion of neonates having significant jaundice, a peak serum bilirubin of 205 μmol/l or greater was calculated for each variable. Statistical differences between groups were calculated using the Chi-squared test. The mean peak serum bilirubin level was also calculated for each variable. In order to do this, a 'normal' peak serum bilirubin value of 77 μmol/l was assigned to those infants where no measurement had been carried out as they had no clinical sign of jaundice. Statistical differences between groups were calculated using the Student t-test.

Each factor showing a relationship with jaundice was also studied using a linear discriminant function (0 = no jaundice, 1 = bilirubin $\geq$ 205 μmol/l) to determine whether the factor had an influence dependent on or independent of other factors. A multiple regression analysis was carried out to determine the influence of each independent variable on the incidence of significant jaundice.

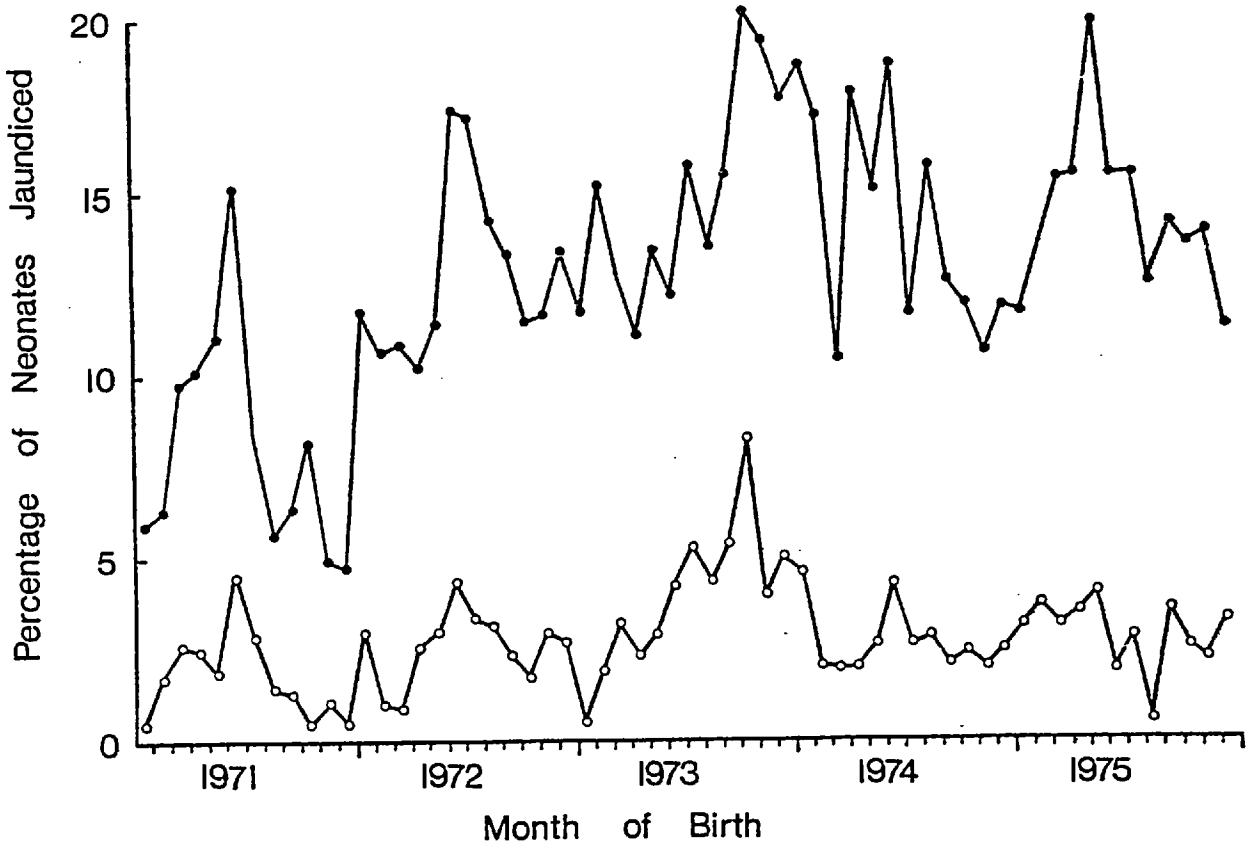


Figure 4:1 Percentage of neonates with serum bilirubin concentration of ⩾ 205 µmol/l closed circles, and ⩾ 291 µmol/l, open circles month by month over five years

A second multiple regression analysis was carried out to determine the numerical influence of each independent variable on the peak neonatal bilirubin concentration. In this regression, the actual serum bilirubin values were used and where the infant had not had a bilirubin measurement carried out, a 'normal' value of 77 µmol/l was assigned.

A second study population from the 12,461 cases previously described was subsequently analysed. In this population, the effect of breast feeding as an independent factor was studied. Details of these patients were obtained from the ward admissions book, unfortunately some of these books had been discarded so that only 9,300 of the original cases could be further analysed. In these books, type of feeding at discharge was recorded.

Only those babies who were receiving no breast milk at all on discharge were counted as not being breast fed. The incidence of significant jaundice and the mean peak bilirubin level associated with this factor were calculated. The two types of multiple regression analysis were also performed.

RESULTS

Serial Figures

The proportion of neonates having peak serum bilirubin of 205 µmol/l or 291 µmol or greater over the sixty month period are shown in figure 4:1. The incidence of jaundice was lowest in 1971 and remained

steady thereafter. The incidence of both mild and severe jaundice appear to be related.

The incidence of both mild and severe jaundice by year are shown in Table 4-1.

Individual Factors

Seven factors were associated with a significantly difference incidence of jaundice (Table 4-2). Four other factors investigated, caesarean section, forceps delivery, general anaesthesia and amniotomy were not associated with signficantly different rates of jaundice.

Nine factors were associated with a significantly different mean peak serum bilirubin level (Table 4-3). Two other factors investigated, caesarean section and forceps delivery were not associated with significantly different mean peak serum bilirubin levels.

Linear discriminant function analysis, where the numerical factors birth weight in grams and gestational age in weeks are also included, gave slightly different results (Table 4-4). The analysis confirmed an independent influence of six of the factors previously noted; asiatic race, ventouse extraction, oxytocin, male sex, epidural anaesthesia and negro race but did not definitely confirm breech delivery ($p > 0.09$) and introduced general anaesthesia as an independent factor ($p < 0.005$).

Year	Total No. of live births	Infants with peak serum bilirubin ⩾ 205 µmol/l	Infants with peak serum bilirubin ⩾ 291 µmol/l
1971	2434	8.02	1.72
1972	2655	12.74	2.46
1973	2495	14.79	3.91
1974	2444	14.26	2.65
1975	2435	14.41	2.84

Table 4-1

Changing incidence (%) of jaundice during 1971-75 (excluding all cases of Rh disease, ABO incompatibility, G-6-PD and those infants who did not remain in hospital for at least 7 days).

Factor	Neonates with factor		Neonates without factor		p value (χ^2 test)
	Total No.	% with jaundice	Total No.	% with jaundice	
Asiatic race*	220	24.1	12,241	12.6	< 0.001
Breech delivery	265	22.6	12,196	12.6	< 0.001
Ventouse extraction	502	21.3	11,959	12.5	< 0.001
Oxytocin	2820	15.3	9,641	12.1	< 0.001
Epidural anaesthesia	5915	15.0	6,546	10.8	< 0.001
Male sex	6140	14.9	6,296	10.6	< 0.001
Negro race	911	7.0	11,550	13.3	< 0.001

*Asians of Chinese or Japanese origin excluding Indian race

Table 4-2

Proportion of neonates with jaundice (peak bilirubin $\geq$ 205 µmol/l) according to various factors

Factor	Neonates with factor		Neonates without factor		p value (Student 't' test)
	Total No.	Mean bilirubin (µmol/l)	Total No.	Mean bilirubin (µmol/l)	
Asiatic race*	224	123.1	12,295	99.1	< 0.001
Breech delivery	268	118.0	12,251	99.1	< 0.001
Ventouse extraction	507	118.0	12,012	99.1	< 0.001
Oxytocin	2833	104.3	9,686	99.1	< 0.001
Epidural anaesthesia	5931	104.3	6,588	95.7	< 0.001
Amniotomy	3319	102.2	9,200	99.5	< 0.05
Female sex	6168	95.7	6,326	104.3	< 0.001
General anaesthetic	1165	95.7	11,354	100.9	< 0.05
Negro race	921	88.9	11,598	100.9	< 0.001

*Asians of Chinese or Japanese origin excluding Indian race

Table 4-3

Mean peak neonatal serum bilirubin concentrations (µmol/l) according to various factors

Factor	Partial regression coefficient	Significance (F Value)	Proportion of variation explained (R^2)
Asiatic race*	+ 0.101	21.49 ($p < 0.001$)	0.074
Ventouse extraction	+ 0.074	25.16 ($p < 0.001$)	0.072
Negro race	- 0.068	37.95 ($p < 0.001$)	0.068
Oxytocin	+ 0.043	36.66 ($p < 0.001$)	0.070
Female sex	- 0.044	57.60 ($p < 0.001$)	0.065
Epidural anaesthesia	+ 0.036	36.03 ($p < 0.001$)	0.061
General anaesthesia	- 0.028	7.90 ($p < 0.01$)	0.076
Gestational age (weeks)	- 0.047	543.54 ($p < 0.001$)	0.054
Birth weight (kg)	- 0.019	8.50 ($p < 0.01$)	0.075

* Asians of Chinese or Japanese origin excluding Indian race

Table 4-4

Influence of various factors on the incidence of neonatal jaundice ≥ 205 µmol/l calculated by linear discriminant function analysis (0 = no jaundice, 1 = bilirubin ≥ 205 µmol/l)

Gestational age in weeks was the most important of all factors considered. Birth weight was also significantly related to neonatal jaundice as a factor independent of gestational age. The proportion of variation in the incidence of jaundice explicable in terms of the 9 factors totalled 7.6%. The multiple regression analysis where the value of 77 µmol/l bilirubin was assigned to non-jaundiced infants gave nearly identical results. The partial regression coefficients of this equation are shown in Table 4-5, these values can be equated to the mean increase in peak bilirubin level in µmol/l attributable to each of the independent variables or a unit increase in that variable. The proportion of variation in the mean peak serum bilirubin levels explicable in terms of the 9 factors totalled 8.2%.

Figure 4:2 shows mean peak bilirubin levels plotted against gestational age for varying birthweight groups. It can be seen that increasing gestational age causes a significant fall in peak bilirubin levels for all birthweight groups.

An extremely good correlation can be seen between the partial regression coefficients obtained from the multiple regression analyses and the incidence of jaundice and mean peak bilirubin levels obtained from the raw data.

Small-for-dates infants

An unexpected and interesting incidental finding was found in relation to small-for-dates infants. Although the expected negative correlation

Factor	Partial regression coefficient	Significance (F value)	Proportion of variation explained (R^2)
Asiatic race*	+ 20.5	25.36 ($p < 0.001$)	0.080
Ventouse extraction	+ 15.4	32.01 ($p < 0.001$)	0.078
Negro race	- 13.0	38.48 ($p < 0.001$)	0.073
Oxytocin	+ 8.6	39.72 ($p < 0.001$)	0.076
Female sex	- 8.6	60.43 ($p < 0.001$)	0.070
Epidural anaesthesia	+ 6.8	35.66 ($p < 0.001$)	0.066
General anaesthesia	- 6.2	10.87 ($p < 0.01$)	0.081
Gestational age (weeks)	- 9.4	603.79 ($p < 0.001$)	0.059
Birth weight (kg)	- 3.4	8.00 ($p < 0.01$)	0.082

* Asians of Chinese or Japanese origin excluding Indian race

Table 4-5

Influence of various factors on peak neonatal serum bilirubin concentrations (µmol/l) calculated by multiple regression analysis in which the bilirubin concentration of 77 µmol/l was assigned to non-jaundiced infants

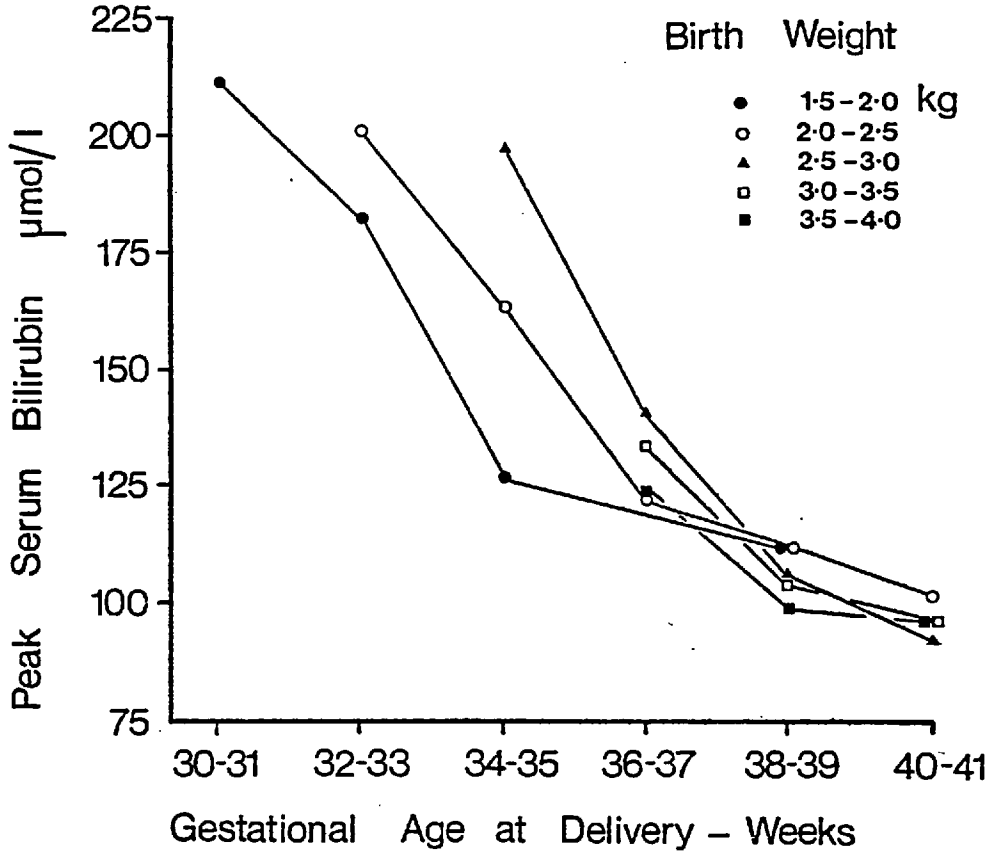


Figure 4:2 Mean peak neonatal serum bilirubin values according to gestational age for groups of infants with different birth weights. Points have been omitted where fewer than 20 infants fitted the category.

was seen between peak bilirubin and gestational age when each birthweight group was considered separately, if only the infants of less than 36-37 weeks gestational age were considered, the lighter infants had a lower mean bilirubin level than those of heavier weight (figure 4:2). Figure 4:3 shows the mean peak bilirubin levels plotted against birthweight for differing gestational ages and the same unexpected finding can be seen for low gestational age groups.

This finding is contrary to what might have been expected and may represent a second example of accelerated physiological maturity in 'small-for-dates' infants.

Breast feeding and neonatal jaundice

The proportion of infants having a peak serum bilirubin of 205 µmol/l or greater associated with breast feeding was 13.2% compared with 11.4% amongst infants who had been fed artificially ($p < 0.01$, x^2 test).

The mean peak serum bilirubin concentration associated with breast feeding was 100.9 µmol/l against 97.1 µmol/l for those infants who were artifically fed. ($p < 0.005$, Student's 't' test). Multiple regression analysis using the linear discriminant function (0 = no jaundice, 1 = bilirubin $\geq$ 205 µmol/l) gave slightly different results to those seen when the 12,461 cases were analysed (Table 4-6). Gestational age was still the most important factor, however breech delivery was significantly related to the incidence of neonatal jaundice

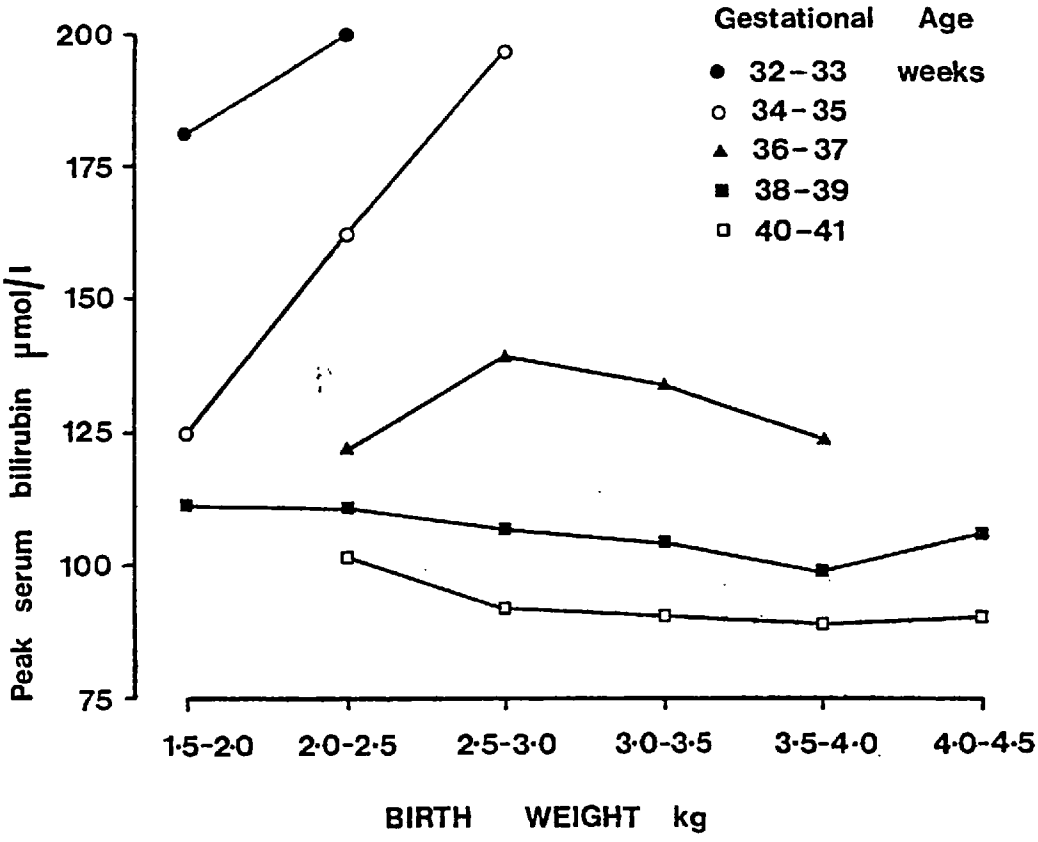


Figure 4:3 Mean peak neonatal serum bilirubin values according to birth weight for groups of infants with different gestational ages. Points have been omitted where fewer than 20 infants fitted the category.

Factor	Partial regression coefficient	Significance (F value)
Asiatic race*	+ 0.135	29.84 ($p < 0.001$)
Ventouse extraction	+ 0.079	22.99 ($p < 0.001$)
Negro race	- 0.078	33.89 ($p < 0.001$)
Breech delivery	+ 0.054	4.80 ($p < 0.05$)
Oxytocin	+ 0.053	43.42 ($p < 0.001$)
Female sex	- 0.041	37.50 ($p < 0.001$)
Epidural anaesthesia	+ 0.032	21.72 ($p < 0.001$)
Breast Feeding	+ 0.025	13.65 ($p < 0.001$)
Gestational age (weeks)	- 0.043	293.49 ($p < 0.001$)

*Asians of Chinese or Japanese origin excluding Indian race

Table 4-6

Influence of various factors on the incidence of neonatal jaundice $\geq$ 205 µmol/l calculated by linear discriminant function analysis (0 = no jaundice; 1 = bilirubin $\geq$ 205 µmol/l)

as an independent factor but birthweight ($p > 0.05$) and general anaesthesia ($p > 0.1$) were not.

Breast feeding was less important than seven of the other factors. The multiple regression analysis where the bilirubin value of 77 µmol/l was assigned to non-jaundiced infants gave nearly identical results (Table 4-7).

DISCUSSION

Serial Figures

The serial figures for the incidence of neonatal jaundice show a possible increase in 1971 and a stable rate thereafter. Among several reports in the literature quoting a recent increase in neonatal jaundice, three include serial incidence rates. Sims and Neligan (1975) quote an increase from 2.2% to 5.5% as the proportion of bilirubin values of 15 mg % (250 µmol/l) or greater between 1973 and 1974. Campbell, Harvey and Norman (1975) quoted a progressive increase in yearly jaundice rates between 1969 and 1973. Data from the same hospital as the present survey were used but their method of sampling was not as rigorous, since all infants born in the hospital were included except for rhesus cases.

Smith and Wilson (1978) reported a 7 year retrospective analysis of the incidence of neonates with serum bilirubin > 250 µmol/l. The infants were divided by weight, those weighing over 2.5 kg and those weighing 2.5 kg and under. One can calculate from these figures the incidence of neonates with serum bilirubin > 250 µmol/l. The

Factor	Partial regression coefficient	Significance (F value)
Asiatic race*	+ 26.64	33.64 ($p < 0.001$)
Ventouse extraction	+ 16.53	28.86 ($p < 0.001$)
Negro race	- 14.16	32.45 ($p < 0.001$)
Oxytocin	+ 10.72	51.29 ($p < 0.001$)
Breech delivery	+ 10.22	5.01 ($p < 0.01$)
Female sex	- 8.03	42.78 ($p < 0.001$)
Epidural anaesthesia	+ 6.24	23.45 ($p < 0.001$)
Breast feeding	+ 5.02	16.15 ($p < 0.001$)
Gestational age (weeks)	- 8.38	320.40 ($p < 0.001$)

*Asians of Chinese or Japanese origin excluding Indian race

Table 4-7

Influence of various factors on peak neonatal serum bilirubin concentrations (µmol/l) calculated by multiple regression analysis in which the bilirubin concentration of 77 µmol/l was assigned to non-jaundiced infants

percentage incidence was 0.2, 0.35, 0.04, 0.13, 0.5, 2.1, 2.9, 3.9 and 4.6 respectively between 1968-1976.

If a genuine increase in neonatal jaundice had occurred in recent years as a result of some obstetric innovation then the present incidence (approximately 12.9% of neonates have a peak bilirubin of 205 µmol/l or greater) should be greater than figures quoted in earlier literature.

These surveys are few, but Trolle (1968) measured plasma bilirubin in a sample of 1,469 untreated infants. The incidence of plasma bilirubin of 205 µmol/l or more was 15.8%. The postal survey of neonatologists in Europe and the USA (Chapter 3) confirmed that most had seen no evidence of an increase in neonatal jaundice either since 1970 or since 1965 (Friedman, Lewis and Harvey, 1977).

One can speculate that jaundice rates fluctuate according to the interest taken in the condition and hence the measurements made of bilirubin. However, since the change in incidence of severe jaundice at this hospital seems to follow closely the change in incidence of mild jaundice this hypothesis seems unlikely to account for the increased incidence of jaundice observed.

In this study certain factors suggested to have been associated with this 'increase' in neonatal jaundice, notably oxytocin (Jeffares, 1977) epidural anaesthesia (Sims and Neligan, 1975) and breast feeding (Eden, Revolta and Adjei, 1974) have been analysed. The technique of

multiple regression analysis enables these factors to be examined independently of their frequent association with one another and with other known icterogenic factors such as prematurity and instrumental delivery. There appears to be no literature in which this type of statistical treatment has been used to examine the influence of various factors on neonatal jaundice.

Two types of multiple regression analysis were performed in order to test the validity of allocating a 'normal' bilirubin level of 77 µmol/l to those infants who were not 'clinically jaundiced'. Both methods gave substantially the same answers.

The results show that both oxytocin and epidural anaesthesia have a small effect, probably explaining why some authors have found these factors to be associated with jaundice (Davies, Gomersall, Robertson, Gray and Turnbull, 1973; Sims and Neligan, 1975; Beazley and Alderman, 1975; Chew and Swann, 1977) while others have not (Boylan, 1976; Friedman and Sachtleben, 1977).

Incorporation of breast feeding into the analysis showed that this factor also had a small effect, however it was less important than 7 of the other factors including oxytocin and epidural anaesthesia. This would explain why some workers have failed to find an association between breast feeding and increased jaundice (Dahms, Krauss, Gartner, Klain, Soodalter and Auld, 1973; Campbell, Harvey and Norman, 1975).

The mechanism of the icterogenic effect of these drug treatments or breast feeding is obscure. Neither bupivacaine, the local anaesthetic used in epidural blocks, nor oxytocin has any direct effect on glucuronidation of bilirubin in vitro (Friedman and Lewis, 1977; Chapter 5). However the metabolites of bupivacaine might inhibit glucuronidation since substantial amounts of a glucuronated metabolite are excreted in the urine (Caldwell, Notarianni and Hawes, 1978).

Oxytocin

The hypo-osmotic effect of oxytocin has been implicated (Singhi and Singh, 1977) as has the increased placental transfusion of fetal blood resulting from oxytocin (Gate, 1978). On the other hand, Ghosh (1975) suggests that fetal hypoxia which occurs as a result of a reduction in placental blood circulation in the hypertonic uterus when therapeutic oxytocin doses are exceeded, may disturb the hepatic enzyme system of conjugation resulting in neonatal hyperbilirubinaemia. It is possible that the relationship between oxytocin induction and jaundice is due to its use in chronologically mature but physiologically immature infants who fail to initiate the parturition process.

If this were true, then one would also expect the use of prostaglandin E_2 for induction of labour to result in elevated serum bilirubin levels. Calder, Moar, Ounsted and Turnbull (1974) prospectively examined the effect of oxytocin (i.v.), prostaglandin PGE_2 (i.v.) and extra-amniotic prostaglandin on neonatal mean serum bilirubin levels on day 5 and compared these with levels obtained in infants born

after spontaneous labour. The oxytocin (i.v.) group and the PGE_2 (i.v.) group had significantly higher mean bilirubins than the spontaneous group, whereas the extra-amniotic PGE_2 group did not have significantly different levels. However, this group had a lower mean inducibility rating.

Other workers failed to find any association between PGE_2 for induction of labour and higher bilirubin levels (Beazley and Weekes, 1976). They carried out a prospective study on 447 labours and the resulting newborn and failed to reveal any significant difference between the incidence of neonatal hyperbilirubinaemia ($\geq$ 205 µmol/l) or mean serum bilirubin levels, following spontaneous labour and after labour induced or accelerated with PGE_2.

Breast Feeding

Breast milk is thought to be icterogenic due to the presence of conjugation inhibitors which inhibit hepatic glucuronyl transferase. The steroid pregnane-3α20β diol was suggested to be inhibitory but was not subsequently found to be present in inhibitory milk (Ramos, Silverberg and Stern, 1966) and theoretical objections to this steroid being the cause of prolonged neonatal jaundice have been put forward (Hargreaves and Piper, 1971). Free fatty acids also inhibit glucuronyl transferase activity in rat liver slices (Bevan and Holton, 1972) and it is thought that even though free fatty acid concentrations in breast milk samples increase on storage which questions the validity of in vitro measurements, it is possible for free fatty acids to accumulate

in the infant and inhibit conjugation in the liver.

RACE

The effect of race on bilirubin levels has been shown by other workers. Horiguchi and Bauer (1975) compared the incidence of neonatal jaundice in 75 Japanese and 50 Caucasian infants born between January 1970 and August 1972 in a New York hospital. They found that 23% of the Japanese group and none of the Caucasian group developed hyperbilirubinaemia exceeding 15 mg% (256 µmol/l). These results, together with the findings from this hospital suggest that since these elevated bilirubin levels were found in infants who were not born in their country of origin, they are probably due to genetic differences between orientals and Caucasians.

Small-for-dates

The finding that preterm, small-for-dates infants were protected from jaundice by their low weight suggests that hepatic glucuronyl transferase is prematurely developed in small-for-dates infants. This situation recalls the accelerated surfactant production by the lung in small-for-dates infants which has been attributed to intra-uterine stress (Gluck and Kulovich, 1973). A teleological explanation of this would be that early maturation of liver and lung enzymes may prepare small-for-dates infants for preterm delivery.

There is little doubt that neonatal jaundice is a clinical problem which takes up much paediatric and biochemical time. Severe neonatal

jaundice is associated with mild degrees of brain damage (Boggs, Hardy and Frazier, 1967; Fenwick, 1975; Scheidt, Mellits, Hardy, Drage and Boggs, 1977), although classical kernicterus is rare.

The results of this survey indicate that the problem is unlikely to be resolved by changes in obstetric practice, for even elimination of epidural anaesthesia and oxytocin use would have little impact. These considerations may suggest to paediatricians that they should look again at prophylactic prenatal drug treatment for neonatal jaundice. Phenobarbitone is known to be effective (Trolle, 1968) but it is not often used, maybe because of its adverse side effects such as sedation, which may be considered greater than the morbidity and medical costs of a significant rate of neonatal jaundice, or because obstetricians think that neonatal jaundice is too trivial a condition to worry about. Other enzyme inducing agents might be found with fewer side effects.

CHAPTER FIVE

EFFECT OF DRUGS ON BILIRUBIN CONJUGATION IN VITRO

SUMMARY

There have been suggestions that various compounds administered to the mother or baby are icterogenic, due to their ability to inhibit the conjugation of bilirubin by hepatic glucuronyl transferase. This has been investigated in rat liver microsomes for novobiocin, an antibiotic which is a well documented inhibitor; bupivacaine, an epidural anaesthetic; oxytocin, a hormone which induces labour; pethidine, an analgesic agent administered during labour and para-amino benzoic acid; a solubilizer and preservative used in diazepam injections.

Novobiocin significantly reduced the conjugation of bilirubin by UDP glucuronyl transferase, pethidine exerted a small inhibitory effect at a high concentration and bupivacaine, oxytocin and PABA had no effect. However, since most drugs are not directly conjugated and their metabolites are not likely to be formed in vitro using a pure microsomal preparation the ability of this system to detect potentially icterogenic drugs is questioned.

INTRODUCTION

Bilirubin Metabolism

Bilirubin is a by product of haemoglobin catabolism. When haemoglobin is destroyed, the protein portion, globin, may be re-utilized, either as such or in the form of its constituent amino acids, and the iron enters the iron 'pool' also for re-use. However, the porphyrin

portion, haem, is broken down to form first biliverdin and then bilirubin.

Bilirubin is a dicarboxylic acid with a pKa of 8. It is therefore remarkably insoluble at neutral or acid pH and is transported in the plasma bound to albumin. In order for it to be excreted, it is conjugated principally in the liver to form the more polar diglucuronide. The conjugation of bilirubin is dependent upon a series of enzymatic steps, culminating in the transfer of an active glucuronide moiety from the donor substance, uridine diphosphoglucuronic acid (UDPGA) to bilirubin. The transfer is catalysed by an enzyme 'glucuronyl transferase' located primarily in the microsomes of hepatic parenchymal cells and also in the kidney and gastrointestinal tract. About 10-15% of conjugated bilirubin forms conjugates other than the glucuronide e.g. sulphates.

Reaching the intestine in the bile, the bilirubin is reduced to urobilinogens by the action of intestinal bacteria. The urobilinogens may in part be absorbed from the intestine and returned to the liver for re-excretion, or may be excreted in the urine. The great majority of the bile pigments are however, excreted with the faeces (see Figure 5:1).

Several types of drugs are metabolised by glucuronidation. Alcohols and phenols which form 'ether type' glucuronides are the most common examples. Many aromatic and some aliphatic carboxylic acids form 'ester type' glucuronides. Various amines form N-glucuronides and

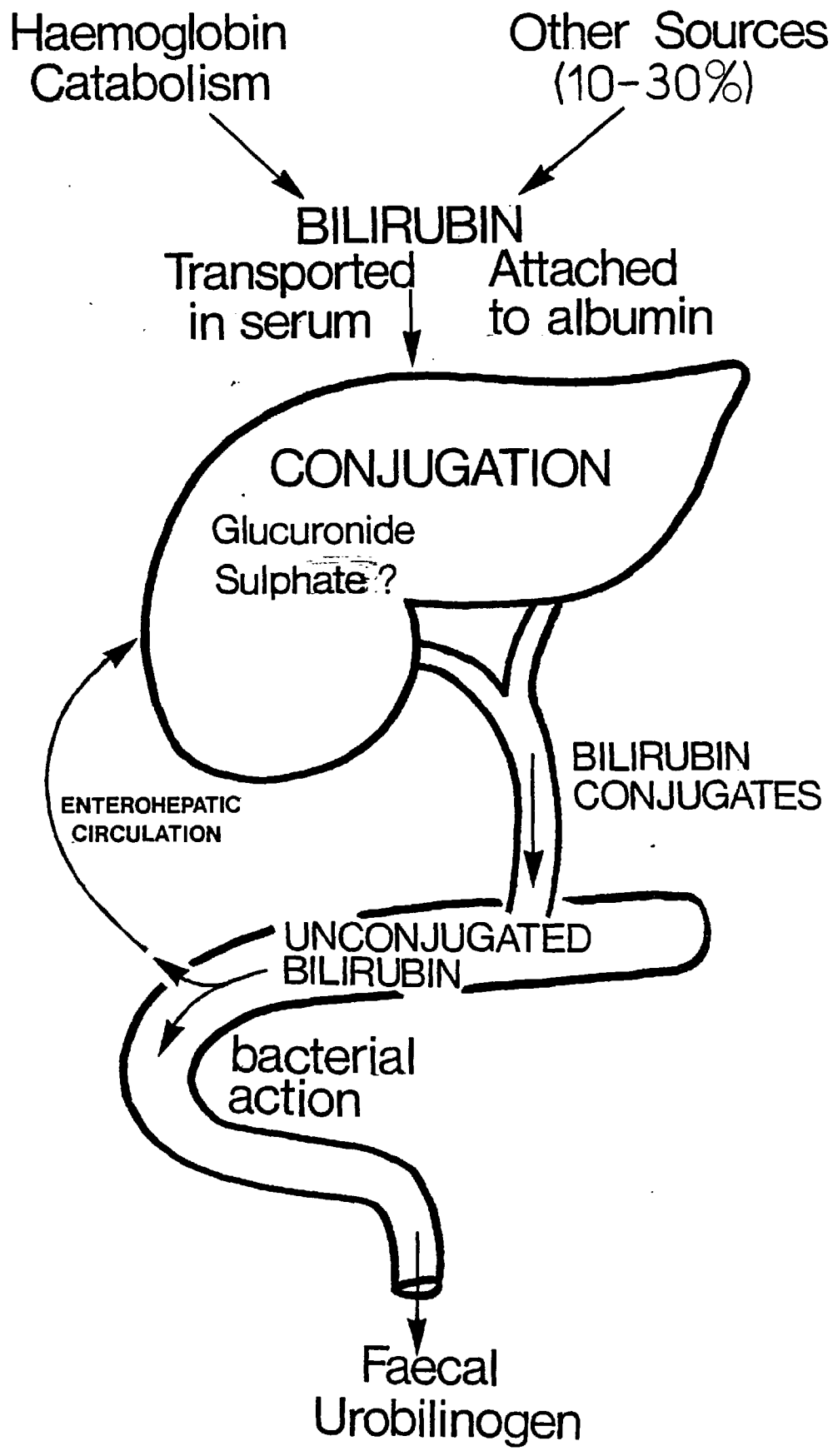


Figure 5:1: Bilirubin metabolism

certain thiol compounds form S-glucuronides. In addition, other normally occurring substrates apart from bilirubin such as steroids and thyroxine also conjugate with glucuronic acid.

Placental transfer of drugs

Every pharmacological substance given to the mother can and does cross the placenta by a process of simple diffusion. Substances with a molecular weight of less than 500 generally cross the placenta easily; those with a molecular weight around 1000 or higher, with difficulty. It is thus theoretically possible for drugs given to the mother or baby to influence the capacity of the neonatal liver to conjugate bilirubin especially at a time when enzyme activity is low due to hepatic immaturity.

Icterogenic drugs

Vitamin K analogues e.g. Menadiol Sodium diphosphate (Synkavite) which are used to treat haemorrhagic disease in the newborn and the antibiotic novobiocin are two such drugs observed to be icterogenic. Allison (1955) noted a connection between kernicterus and haemolytic anaemia and Vitamin K administration to premature infants. Waters, Dunham and Bowen (1958) showed that Synkavite inhibited the conjugation of bilirubin in vitro by rat liver microsomes.

Sutherland and Keller (1961) observed that the incidence of serum bilirubin > 256 μmol/l was increased three fold when a group of newborn infants were treated with novobiocin to control a staphylococcal

infection. A similar though less intense jaundice could also be produced in newborn rats that were injected with novobiocin.

Hargreaves and Holton (1962) found that novobiocin inhibited the conjugation of both bilirubin and O-aminophenol by rat liver slices. Hsia, Dowben and Riabov (1964) showed novobiocin to be a potent inhibitor of glucuronyl transferase in vitro using O-aminophenol, P-nitrophenol and 4-methylumbelliferone as substrates and rat liver homogenate as a source of enzyme. Chloramphenicol and streptomycin have also been shown to inhibit bilirubin conjugation in vitro (Waters, Dunham and Bowen, 1958).

A study was therefore carried out on the effect of some drugs on glucuronidation of bilirubin by uridine diphosphoglucuronic acid (UDPGA) using rat liver microsomes in vitro. The drugs investigated were novobiocin, as a positive control; oxytocin, a hormone used to induce labour; bupivacaine, an epidural anaesthetic; pethidine, an analgesic given to relieve pain during labour and para-amino benzoic acid (PABA); a solubilizer or buffer preservative in injectable diazepam which is used as an anticonvulsant or relaxant in infants.

METHODS

Rat liver microsomes were prepared by differential centrifugation (see Chapter 2 for details). The assay of the bilirubin-UDP glucuronyl transferase activity was adapted from the methods of Black, Billing and Heirwegh (1970) and Orme, Davies and Breckenridge (1974).

Incubations were carried out in 10 ml glass centrifuge tubes. The final volume of all incubates was 198 µl. Each incubate contained 70 µl of microsomes suspended in Tris (0.02 M), 30 µl drug dissolved in Tris, 30 µl of UDPGA (Sigma Chemical Co.) 60 µl bilirubin (Sigma Chemical Co.) 8 µl $MgCl_2$ (125 mM). Controls contained ethylene diamine tetra-acetic acid (EDTA) (125 mM) instead of $MgCl_2$ and water instead of the bilirubin.

UDPGA was freeze-dried in 8 mg aliquots and was dissolved just before use to the required dilution in triethanolamine buffer (pH 7.4) which was prepared by adding 2 M triethanolamine to 50 ml 1 M HCL and bringing the volume to 100 ml with water. Bilirubin (5 mg) was dissolved in the dark in 0.1 N NaOH (containing 0.001 M EDTA) and 9 ml of a 2.4% (w/v) bovine serum albumin solution (Sigma Chemical Co.) were then added. The pH of the final solution was 7.4.

The initial incubations were performed at 37°C in a shaking incubator for 30 minutes and the incubation was terminated by transferring the tube to ice and adding 0.2 ml of a glycine-HCl buffer (prepared by adding 0.4 M HCl to 2 M glycine buffer to give a final pH of 2.8).

The incubation time of 30 minutes was chosen because the reaction has been shown to be linear with respect to time for 60 minutes (Black, Billing and Heirwegh, 1970; Potrepka and Spratt, 1971), whereas hydroxylation reactions are linear only for the first 15 minutes. A

final concentration of $MgCl_2$ of 5.0 mM has been shown to give maximal glucuronidation (Black, Billing and Heirwegh, 1970). After stopping the initial incubation, conjugated bilirubin was measured colorimetrically by the method of Black, Billing and Heirwegh (1970) with minor modifications (see Chapter 2).

A preliminary series of experiments were carried out in order to establish the apparent Km for bilirubin and UDPGA and to establish the ideal microsomal enzyme concentration for further studies.

Estimation of apparent Km for bilirubin

In this experiment the UDPGA concentration was kept constant at 7.29 mM and the microsomal enzyme concentration at 0.73 g liver/ml incubate, while the concentration of bilirubin was varied to give final concentrations of 32, 64, 128, 170.6, 192 and 256 µm. It was assumed that the concentration of UDPGA and liver were optimal. If, on further experimentation, this had not been the case, then the experiment to establish the Km for bilirubin would have been repeated. A Lineweaver Burk plot of $\frac{1}{V}$ against $\frac{1}{S}$ where V = the bilirubin glucuronidation rate expressed as nmoles bilirubin conjugated per gram liver per hour and S = bilirubin concentration, was used to establish the V max and Km for bilirubin, since the intercept on the y axis gives $\frac{1}{Vmax}$ and the intercept on the x axis = $\frac{-1}{Km}$.

Estimation of apparent Km for UDPGA

In this experiment the bilirubin concentration was kept constant at 256 µM and the liver concentration at 0.73 g liver/ml incubate, while the concentration of UDPGA was varied to give final concentrations of 0.45, 0.91, 1.82, 3.64, 7.29, 14.59 and 29.19 mM. A Lineweaver Burk plot of $\frac{1}{V}$ against $\frac{1}{S}$ was used to establish the V max and Km for UDPGA.

Effect of enzyme concentration on glucuronide formation rate

In this experiment the bilirubin concentration was kept constant at 256 µM and the UDPGA concentration at 7.29 mM, while the liver concentration varied from 0.09 g to 0.73 g liver/ml incubate. The relationship between liver concentration and glucuronidation rate was examined.

Drug studies

In all further experiments a UDPGA concentration of 7.29 mM and a liver concentration of 0.5 g/ml incubate were kept constant.

The effect of 0.16 and 0.32 mM novobiocin (Upjohn Ltd) on the glucuronidation rate of 32, 64 and 256 µM bilirubin were studied. Other drugs that were tested for their ability to inhibit glucuronyl transferase at a bilirubin concentration of 64 µM were bupivacaine (Duncan Flockhart and Co. Ltd) (0.01 and 1 mM), oxytocin (Sandoz Products Ltd) (0.015 and 1.5 units/ml), pethidine (Roche Products Ltd) (0.01 mM and 1 mM) and PABA (Eastman Kodak Co) (0.01 and 1 mM). In this series of experiments, 0.08 and 0.16 mM novobiocin provided the positive controls.

Calculation of the bilirubin glucuronidation rate

Let O.D. 530 nm of conjugated pigment - control value = Z, and the E_{530} value for the azo pigment = 44.36×10^3 $M^{-1}cm^{-1}$ (Van Roy and Heirwegh, 1968) then the bilirubin glucuronidation rate in nmoles bilirubin conjugated per g liver per h

$$= \frac{Z \times 2.5 \times 10^3 \times 20}{44.36}$$

where 2.5 = volume of organic solvent (ml).

RESULTS

Figure 5:2 shows Lineweaver-Burk plots of kinetic studies at variable bilirubin concentrations without inhibitor and in the presence of 0.16 and 0.32 mM novobiocin. Table 5-1 gives values obtained from these plots for the apparent V max and apparent Km with and without inhibitor.

The apparent V max for bilirubin was 333 nmoles bilirubin conjugated per g liver per hour and the apparent Km was 0.119 mM. 0.16 mM novobiocin reduced both the V max and Km for bilirubin and 0.32 mM further reduced them. Novobiocin therefore appears to behave as a 'mixed function' inhibitor, giving rise to both competitive and non-competitive effects.

Figure 5:3 shows a Lineweaver-Burk plot of kinetic studies in which the UDPGA concentration was varied and the bilirubin concentration kept constant. The apparent V max for UDPGA was 270 nmoles bilirubin

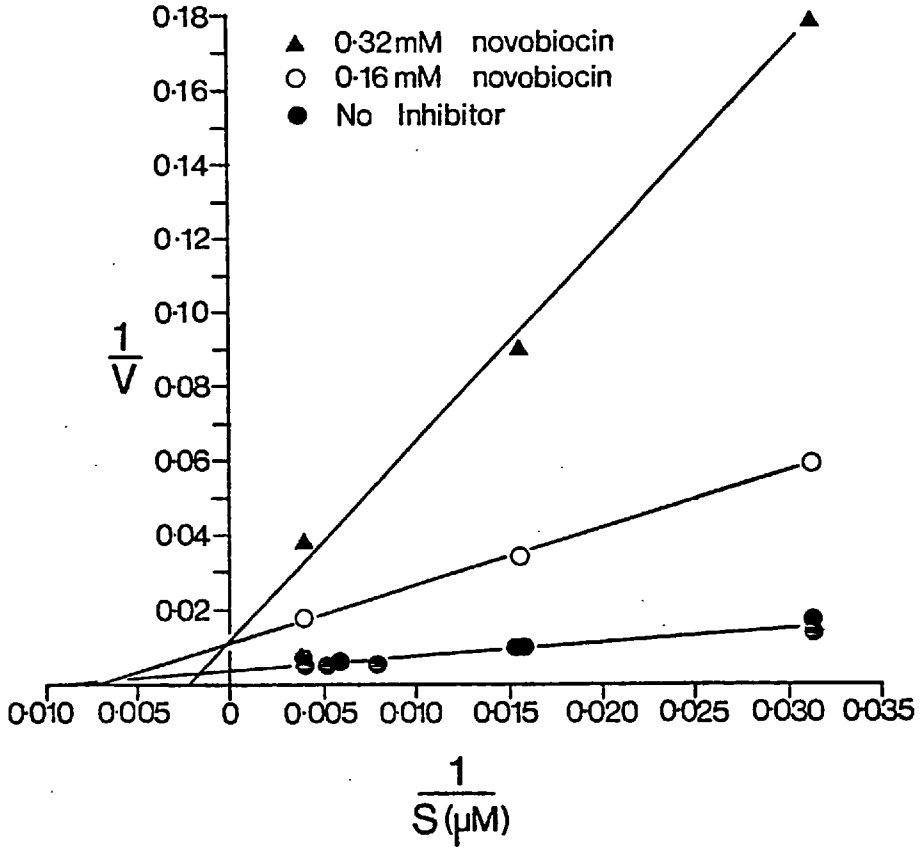


Figure 5;2 Lineweaver-Burk plots of kinetic studies where S = bilirubin concentration, showing values without inhibitor and with two concentrations of novobiocin.

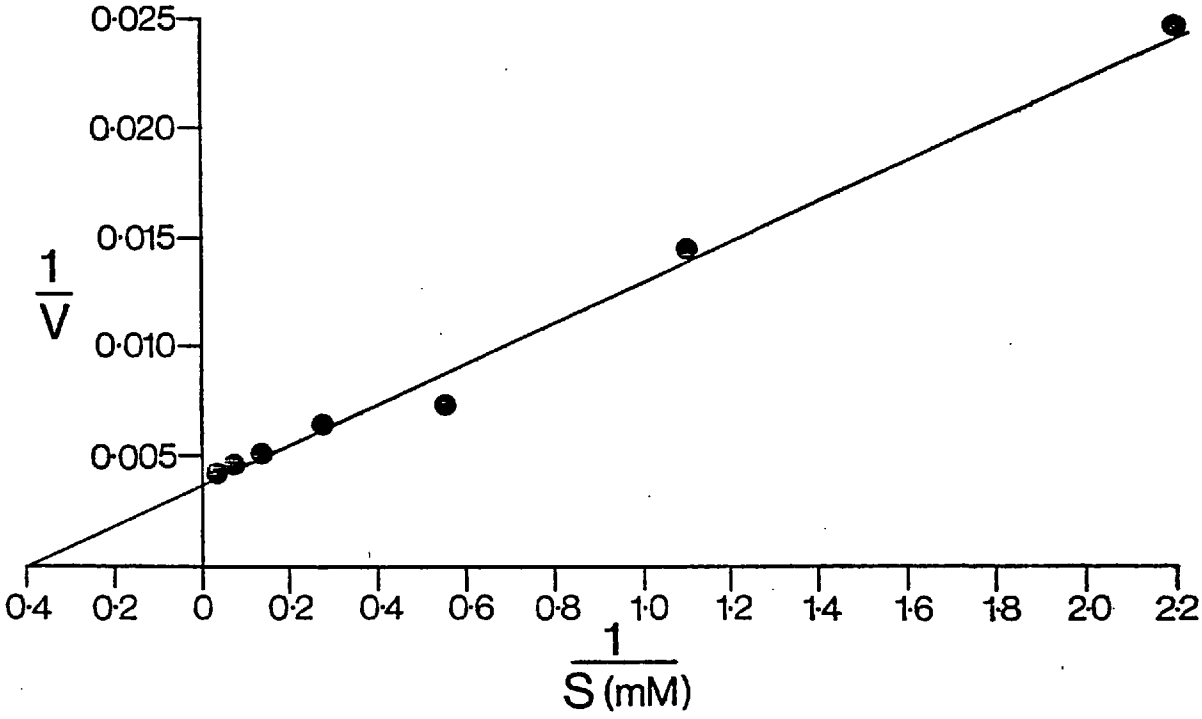


Figure 5:3 Lineweaver-Burk plot of kinetic studies where S = UDPGA concentration

Novobiocin concentration (mM)	Vmax*	Km (mM)
Control	333.3	0.119
0.16	90.9	0.143
0.32	83.3	0.454

*Rate is expressed as nmoles bilirubin conjugated per g liver per hr.

Table 5-1

Effect of novobiocin on bilirubin glucuronidation rate at variable bilirubin concentrations

conjugated per gram liver per hour and the apparent Km was 2.51 mM.

Figure 5:4 shows the effect of increasing liver concentration in the incubate on the bilirubin glucuronidation rate. There is a linear relationship between concentration and rate from 0.2 to 0.7 g liver/ml incubate. Table 5-2 shows the effect of the drugs studied on bilirubin conjugation.

Novobiocin, the positive control, caused 70% inhibition of glucuronidation at 0.08 mM and 95% inhibition at 0.16 mM. 1 mM pethidine caused 28% inhibition, however, 1 mM bupivacaine, 1.5 U/ml oxytocin and 1 mM PABA exerted no effect.

DISCUSSION

The apparent Kms of UDPGA and bilirubin were similar to values obtained by Orme, Davies and Breckenridge (1974). They found the apparent Kms of UDPGA and bilirubin to be 2.43 ± 0.29 mM and 0.067 ± 0.01 mM respectively.

The results obtained with bupivacaine, oxytocin and PABA were essentially negative. None of them had any effect on glucuronidation in vitro. Pethidine, however, diminished the bilirubin glucuronidation rate at a high concentration. Nevertheless, these results do not rule out the possiblity that metabolites formed in vivo may be icterogenic.

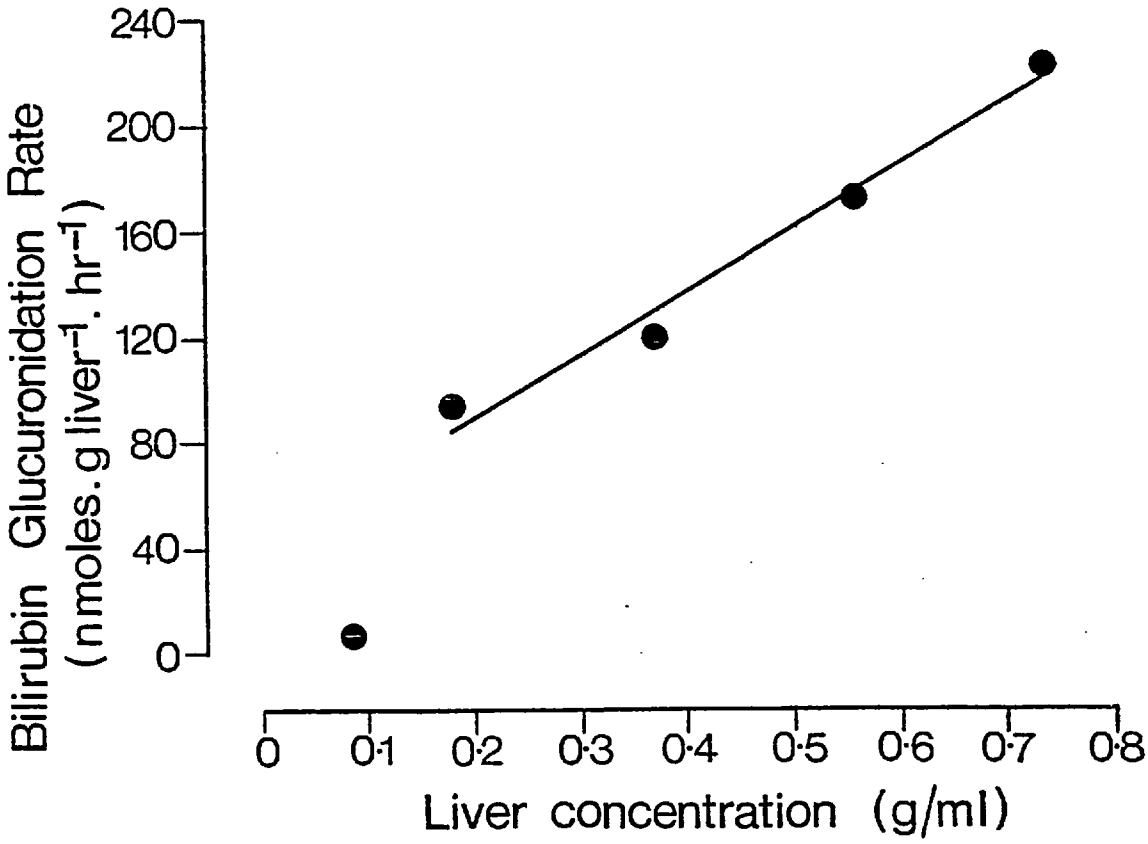


Figure 5: 4 Effect of liver concentration on bilirubin glucuronidation rate

Drug	Drug concentration	Bilurubin glucuronidation rate*
Control	-	117.2
Novobiocin	0.08 mM	36.0
Novobiocin	0.16 mM	6.7
Bupivacaine	0.01 mM	110.4
Bupivacaine	1.00 mM	108.2
Oxytocin	0.015 U/ml	119.5
Oxytocin	1.50 U/ml	119.5
Pethidine	0.01 mM	108.2
Pethidine	1.00 mM	84.5
PABA	0.01 mM	102.5
PABA	1.00 mM	126.2

*Rate is expressed as nmoles bilirubin conjugated per gram liver per hour

Table 5-2

Effect of drugs on bilirubin conjugation by rat liver microsomes

Bupivacaine

Caldwell, Moffat, Smith, Lieberman, Cawston and Beard (1976) have shown that the rate of disappearance of bupivacaine from the blood of newborn infants was far slower than that found in adults. Furthermore, Caldwell, Notarianni and Hawes (1978) found that in an adult that had excreted 50% of the dose of bupivacaine after 72 hours only 0.5% of the dose was recovered unchanged indicating that the decline in blood concentration was due to its metabolism.

Bupivacaine is metabolized by dealkylation, hydroxylation, amide hydrolysis and conjugation. The conjugates of bupivacaine are excreted as glucuronic acid conjugates in substantial amounts in the urine (Caldwell, Notarianni and Hawes, 1978). However, since the conjugation step is subsequent to NADPH dependent reactions which are extremely unlikely to occur in a pure microsomal preparation, the negative results obtained in vitro are predictable.

Pethidine

There is also impaired elimination of pethidine in newborn infants and this has been found to be due to defective metabolism (Caldwell, Notarianni and Smith, 1978). These workers showed that pethidine underwent 3 separate metabolic fates which may overlap. These were ester hydrolysis to the corresponding acid, N-demethylation to nor-pethidine and N-oxidation to pethidine N-oxide. These acids were excreted in part as their glucuronic acid conjugates.

The inhibition observed with 1 mM pethidine in vitro was therefore probably due to esterase activity in the hepatic preparation converting the pethidine to pethidinic acid which competed with bilirubin for conjugation by glucuronyl transferase.

Since the half-life of bupivacaine in neonates is 25 hours ($\beta t_{0.5}$) (Caldwell, Moffat, Smith, Lieberman, Cawston and Beard, 1976) and that of pethidine in neonates is 22.7 hours (Caldwell, Wakile, Notarianni, Smith, Lieberman, Jeffs, Coy and Beard, 1977) it is likely that the metabolites of these drugs do in fact compete with bilirubin for glucuronyl transferase in vivo resulting in raised serum bilirubin levels.

Oxytocin

Oxytocin, a nonapeptide is broken down by the enzyme oxytocinase which is produced in large quantitities by the placenta during pregnancy into its constituent amino acids. It is thus theoretically possible for tyrosine to conjugate with glucuronic acid and inhibit bilirubin conjugation.

Para-amino benzoic acid

PABA conjugates principally with glycine and is also acetylated. Metabolites include P-aminohippuric acid, P-acetamidohippuric acid and P-acetamidobenzoic acid. At high concentrations it will form small amounts of the glucuronide. The lack of inhibition observedwith oxytocin and PABA is therefore predictable when one considers their metabolic fates.

Novobiocin

By contrast, novobiocin is conjugated with glucuronic acid in the liver, passes out in the bile and is excreted principally in the faeces, small quantities appearing in the urine. Since this drug has a similar metabolic rate to that of bilirubin, competition with bilirubin for glucuronyl transferase is likely.

Multiple forms of UPD-glucuronyl transferase

Although it is true to say that compounds or their metabolites that do not conjugate with glucuronic acid will not compete with bilirubin for glucuronyl transferase, the reverse is not necessarily the case.

A considerable body of evidence has accumulated which suggests that multiple forms of UDP glucuronyl transferase exist. An example of this, is the lack of inhibition of glucuronyl transferase in vitro with bilirubin as substrate in the presence of sulphafurazole, a drug which is known to be excreted principally as the glucuronide (Waters, Dunham and Bowen, 1958).

Neither can positive in vitro results be conclusive evidence of inhibition in vivo. Mulder (1974) studied the inhibition of UDP-glucuronyl transferase activity by a number of monoamine oxidase inhibitors of the hydrazine type and concluded that most of the effects were caused by the action of the compounds on microsomal membrane structure and therefore that the physiological relevance of inhibition of the enzyme in vitro is questionable.

Anomalous results obtained with Synkavite lend additional support to Mulder's conclusions. Synkavite inhibits the conjugations of O-aminophenol, P-nitrophenol and 4-methylumbelliferone (Hsia, Dowben and Riabov, 1964) and bilirubin (Waters, Dunham and Bowen, 1958) in vitro. However, Synkavite unlike novobiocin injected subcutaneously into newborn Wistar rats did not raise serum bilirubin levels measured at various time intervals after injection compared to controls, neither did it inhibit the conjugation of O-aminophenol using the livers from the treated animals (Hsia, Dowben and Riabov, 1969).

The icterogenic effect of high doses of Vitamin K seen in neonates is therefore unlikely to be due to enzyme inhibition but to increased haemolysis of the red blood cells as was originally suggested (Allison, 1955).

It appears therefore that a compound can only be stated conclusively to interfere with bilirubin conjugation in the human neonate if it satisfies the following criteria. It must be icterogenic both in the human neonate and in the animal used to carry out in vivo studies, it must inhibit conjugation of bilirubin in vitro by both microsomes and liver slices and it must not be associated with haemolysis or any other icterogenic mechanism. Novobiocin appears to be the only compound at present which satisfies these criteria.

CHAPTER SIX

EFFECT OF DRUGS ON BILIRUBIN BINDING BY NEONATAL SERUM AND BOVINE SERUM ALBUMIN IN VITRO

SUMMARY

The effect of drugs on bilirubin binding by bovine serum albumin and human pooled cord serum was studied using Sephadex Gel filtration. Sulphafurazole, a well documented bilirubin displacer, and three semi-synthetic penicillins commonly used during the neonatal period were tested. The primary and secondary affinities and primary capacity of bovine and human albumin for bilirubin were calculated by Scatchard analysis.

Ampicillin, a poorly protein$_{\lambda}^{\text{bound}}$drug, did not displace bilirubin from either bovine or human albumin. Sulphafurazole but not cloxacillin or flucloxacillin displaced bilirubin from both primary and secondary sites on bovine albumin and also reduced the primary capacity. Equimolar sulphafurazole, cloxacillin and flucloxacillin displaced bilirubin from primary and secondary sites on human albumin, however the capacity of the primary site remained unchanged.

The minimum cloxacillin and flucloxacillin concentrations which displaced bilirubin were 5 and 20 times therapeutic neonatal serum concentrations respectively whereas therapeutic sulphafurazole concentrations resulted in displacement. It therefore appears unlikely that these penicillins are kernicterogenic.

INTRODUCTION

Bilirubin is a dicarboxylic acid with a single pKa of 8.0, and is therefore remarkably insoluble at neutral or acid pH. It is carried in the plasma attached to albumin in equilibrium with very small amounts of highly lipid soluble, neurotoxic, free bilirubin. Under normal circumstances, the binding capacity of the albumin is not exceeded. However, bilirubin levels are frequently elevated during the neonatal period due to the immaturity of the neonatal liver. The albumin may then become saturated resulting in a dramatic increase in free bilirubin which can then enter the CNS and cause kernicterus (KI).

Binding capacity of albumin

Not only do low plasma albumin concentrations in neonates cause reduced binding of bilirubin, but Wallace (1976) has found that the binding capacity for some drugs in neonates is lower than might be expected from their serum albumin concentrations. This may be due to the presence of a competing ligand in the neonatal ultrafiltrate and non-esterified FFA may be responsible (Chignell, Vesell, Starkweather and Berlin, 1971). In a later study, Wallace (1977) showed that human neonatal albumin had a different amino acid composition to that of adult plasma albumin and this may intrinsically have a lower affinity for drugs than does adult albumin.

Cord albumin fractionated from pooled cord serum is said to bind 1 mole bilirubin/mole albumin to the high affinity primary site (Krasner, Giacoia and Yaffe, 1973). This corresponds to a plasma bilirubin level of approximately 580 µmol/l below which bilirubin should be tightly bound to albumin. Above this level some bilirubin would be bound weakly to the low affinity high capacity secondary sites.

However, Kapitulnik, Blondheim and Kaufmann (1972) found that the bilirubin binding capacity (BBC) in a group of 12 neonates ranged from 234-422 µmol/l and these values were independent of albumin concentration. The mean bilirubin/albumin ratio at saturation was 0.66 with a range of 0.45-0.77. Furthermore, preterm or sick neonates have lower BBC's than their term counterparts (Cashore, Horwich, Laterra and Oh, 1977).

Total bilirubin levels

Since mean peak neonatal serum bilirubin levels are about 97 µmol/l (Maurer, Wolff, Finster, Poppers, Pantuck, Kuntzman and Conney, 1968), most neonates will not attain a serum bilirubin level high enough to saturate the BBC. However, in one maternity hospital 12.9% of infants born between 1971-5 were found to have peak serum bilirubin levels of $\geq$ 205 µmol/l (Friedman, Lewis, Clifton and Bulpitt, 1978). Nevertheless, the most important single factor when assessing the risk of KI to an infant is the level of free bilirubin.

Free bilirubin levels

Zamet, Nakamura, Perez-Robles, Larroche and Minkowski (1975) measured free bilirubin concentrations in 112 jaundiced newborns, 57 cases were found to have free bilirubin $\geq$ 1.7 µmol/l and all those with KI fell into this category. Gestational age is also an important factor. Free bilirubin levels were measured in a group of 26 healthy newborns with ABO incompatibility and 15 of them were found to have free bilirubin ranging from 1.7-6.8 µmol/l however no KI was observed during the neonatal period. On the contrary, in a group of 51 pre-term infants without haemolysis, a little more than half of the cases had free bilirubins ranging from 1.7-6.8 µmol whereas 4 macroscopic KI cases were observed.

Relationship between total bilirubin, free bilirubin and kernicterus

Total bilirubin levels are not generally a good indication of free bilirubin levels. Arkans and Cassady (1978) found a significant relation between free and total bilirubin but the scatter of values was wide. However Frenzel, Sander and Schramm (1973) failed to find any significant correlation. In spite of this, a total bilirubin concentration of 342 µmol/l is generally accepted to be the level at which exchange blood transfusions are carried out, since this value corresponds to a 'normal' binding capacity.

Clinical evidence has indeed shown in some studies that at total bilirubin levels below 342 µmol/l, the neonate is not at risk.

Polacek (1966) found that in a series of 67 erythroblastotic newborns with elevated levels of conjugated bilirubin, there were 10 cases of KI, all in infants in whom the concentration of unconjugated bilirubin exceeded 342 µmol/l.

However, Zamet, Nakamura, Perez-Robles, Larroche and Minkowski (1975) measured the free bilirubin and total bilirubin in 112 jaundiced newborns and found that in seven cases of KI only 3 occurred in the total bilirubin group above 342 µmol/l. In a second series in which 40 cases of KI in preterms were evaluated as a function of birth weight 13 out of the 40 cases were observed for a peak total bilirubin below 205 µmol/l.

Therefore not only are preterm infants more susceptible to free bilirubin than term infants but they are at risk of KI at lower total bilirubin concentrations. Furthermore it seems likely that KI can occur at total bilirubin levels below the BBC especially when there are circulating highly protein bound drugs which may displace bilirubin from its binding sites on albumin.

Drugs and kernicterus

Silverman, Andersen, Blanc and Crozier (1956) were the first to observe the connection between the administration of a highly protein bound drug used to treat preterm newborns and subsequent KI and death. They looked at 2 groups of preterm infants who were allotted 2 different prophylactic antibacterial regimens. They received either penicillin

and sulphafurazole, or oxytetracycline. Some 36% of those infants in the penicillin/sulphafurazole group who died during the trial period (≤ 120 hours) were found to have KI at necropsy compared to 6% in the oxytetracycline group. Penicillin was known not to cause KI. Therefore sulphafurazole was implicated. Subsequently Odell (1959a; 1959b) proposed that certain anions including sulfonamides were capable of displacing protein-bound bilirubin from albumin and other studies have shown sulphafurazole to displace bilirubin from hyperbilirubinaemic sera in vitro (Loria, Echeverria and Smith, 1976) and to result in KI in Gunn rats (Schenker, McCandless and Zollman, 1966).

Kernicterogenic drugs - in vivo studies

Since then, in vivo studies have implicated other highly protein bound drugs. Satge, Colin, Voyer, Crumiere and Charlas (1974) measured the free bilirubin levels in preterm infants with clinical jaundice. They found that neonates treated with cloxacillin had elevated free bilirubin levels including those infants with a low total bilirubin.

Hanefeld and Ballowitz (1976) showed that administration of flucloxacillin to young homozygous Gunn rats resulted in a striking loss of Purkinje cells, compared with mild damage to these neurones in untreated littermates.

Kernicterogenic drugs - in vitro studies

The effect of drugs and endogenous substances on bilirubin binding is frequently investigated in vitro. Many different sources of albumin have been used in these studies. Among these are whole human adult or neonatal serum, purified human adult albumin and neonatal albumin and bovine serum albumin. The large number of methods that exist to measure BBC or free bilirubin are an indication of the difficulties involved. These are principally due to the instability of free bilirubin and the very small percentage of free bilirubin normally present compared with the bound faction.

Equilibrium dialysis

Equilibrium dialysis is probably the technique which is most often employed to study the binding of drugs and other ligands to protein. It is not specific for bilirubin. The protein solution is placed in a short length of dialysis tubing which is tied at each end and then immersed in buffer. At equilibrium, the activity of the free ligand is the same on both sides of the membrane. The concentration of the ligand on the protein free side can be measured by means of any suitable analytical technique. The main disadvantage of this technique is that it is rather slow and tedious and it requires fairly large amounts of protein. However, it is relatively simple and inexpensive.

Ultrafiltration

Ultrafiltration differs from equilibrium dialysis in that the protein-free phase is separated from the protein containing phase by being forced through the semipermeable membrane, either by ultracentrifugation or by gas pressure. Ultrafiltration has the advantage over equilibrium dialysis in that measurements generally only take a short period of time.

Peroxidase method

A sensitive enzymatic method for measuring low concentration of unbound bilirubin was developed by Jacobsen (1969), based on the observation that bilirubin can be oxidised by hydrogen peroxide in the presence of peroxidase. Excess albumin greatly inhibits oxidation, because of albumin bilirubin complex formation. Bilirubin and albumin equilibrate very rapidly compared with the rate at which unbound bilirubin is oxidised. Thus the concentration of unbound bilirubin is determined from the initial oxidation rate of total bilirubin.

More recently Nakamura and Lee (1977) have adapted Jacobsen's method by replacing H_2O_2 by glucose and glucose oxidase as a source of peroxide. This adaptation has the advantage that bilirubin is not significantly degraded before the addition of peroxidase. The oxidation rate is determined by spectrophotometry and the chloroform extraction in Jacobsen's method is eliminated.

Erythrocyte binding

Bilirubin may bind to erythrocytes and it has been shown that this binding is markedly increased if there is a reduction in the capacity of albumin

to bind bilirubin (Bratlid, 1972). However this method measures changes in capacity indirectly.

Mitochondrial binding

Odell (1966) used the ability of bilirubin to bind to mitochondria to study bilirubin albumin binding.

HBABA dye method

The HBABA dye 2 - (4^1 - hydroxybenzeneazo) benzoic acid method (Porter and Waters, 1966) measures the binding capacity of serum albumin and gives results in terms of reserve albumin binding capacity. It is assumed that the dye binds competitively at the same site as bilirubin but with a lesser affinity so that the dye does not displace bilirubin already bound.

Fluorescent dye binding

The fluorescent dye binding technique (Lee, Gartner and Zarafu, 1975) utilises the fluorescent dye, Direct Yellow 7, for quantitative measurement of serum albumin binding capacity. The dye binds exclusively to serum albumin at sites which are normally occupied by bilirubin with enhancement of fluorescence.

Spectral curves

Analysis of spectral curves (Odell, 1959b) is based on the fact that free bilirubin has a maximal absorbance at 420-440 nm whereas albumin bound bilirubin has a peak at 460-465 nm. The introduction of a competing anion, which displaces bilirubin from the albumin molecule, thus

leading to an increase in the free bilirubin component, results in an increase in the peak absorbance at 420 nm and a corresponding decrease at 465 nm.

Fluorescence spectroscopy

Fluorescence spectroscopy (Krasner, 1973) uses the ability of bilirubin in association with albumin to fluoresce at 530 nm when activated at 435 nm to study the effect of drugs on bilirubin albumin interaction.

Salicylate method

The salicylate method (Odell, 1969) permits the indirect determination of the relative concentration of unbound bilirubin in serum by measurement of saturation of the carrier albumin for bilirubin. It is determined by a change in optical density at 460 nm of the diluted serum after addition of a standardised amount of salicylate. This method requires highly sophisticated spectrophotometric equipment and great precision in the performance of the test.

Electrophoresis

The electrophoretic method has been developed for detecting unbound bilirubin and BBC in newborn sera (Athanassiadis, Chopra, Fisher and McKenna, 1974). It is based on the principle that 'unbound' bilirubin, being a weak organic anion will migrate in an electric fluid at a different rate to the albumin-bound bilirubin. If 'unbound' bilirubin is absent, by adding increasing amounts of bilirubin to the serum one can predict the amount of bilirubin that any specific serum specimen could bind before 'unbound' bilirubin appears.

Dextran coated charcoal method

Griffiths, Diamond and Dextrase (1975) have reported a method to assess both the primary and secondary BBC of human sera based on titration of bilirubin sera with progressively increasing amounts of a special dextran coated charcoal preparation.

Sephadex Gel filtration

Schmid, Diamond, Hammaker and Gundersen (1965) were the first workers to use Sephadex Gel filtration (SGF) to separate albumin bound bilirubin from unbound pigment. The bilirubin albumin complex passes through the gel rapidly, while the smaller molecules of free bilirubin remain on the column. Jirsova, Kirsa, Heringova, Koldovsky and Weirichova (1967) used this method to separate free from bound bilirubin in sera from icteric newborn. Finally Kapitulnik, Blondheim and Kaufman (1972) employed SGF to compare the BBC of human cord serum with that of human adult serum after adding bilirubin to both.

Most of the methods described have been used to investigate the effects of drugs on bilirubin binding in vitro. Schiff, Chan and Stern (1971) found that caffeine sodium benzoate in recommended therapeutic doses (64 mg/ml) and diazepam in injectable form displaced bilirubin from albumin using SGF and spectral curves methods. Sodium benzoate was found to be the active ingredient. In a later study (1972), caffeine sodium benzoate (4 mg/ml) and sodium salicylate (0.3 mg/ml) were found to reduce the BBC of neonatal sera from 0.97 moles bilirubin/mole albumin to 0.72 and 0.82 respectively using SGF.

Similar results were found in a study by Krasner, Giacoia and Yaffe (1973), they showed that sodium benzoate (1.4 mg/ml) and sodium salicylate (2 mg/ml) caused a reduction in BBC of 30% and 20% respectively using the fluorescence method.

Bratlid (1972) showed that novobiocin, cloxacillin, sulphafurazole and salicylate were potential displacers of bilirubin from lyophilised human serum albumin, by measuring the increase in bilirubin binding by erythrocytes caused by these drugs in vitro.

Odell (1973) found that salicylate (0.3 mg/ml), oxacillin (0.2 mg/ml) and dicloxacillin (0.2 mg/ml) displaced bilirubin from jaundiced sera by measuring the adsorption of drug displaced bilirubin by cholestyramine, a synthetic polyamine resin.

Yeary and Davis (1974) looked at the effect of various drugs and chemicals on bilirubin binding in vitro using several different methods for detecting bilirubin displacement. Sodium salicylate displaced HBABA and bilirubin from bovine serum using spectral curves methods. Indomethacin, mefenamic acid, oxyphenbutazone, phenylbutazone, novobiocin and sodium salicylate produced a significant displacement of bilirubin from albumin to mitochondria. P-aminobenzoic acid, piperazine, sulfathiazole and sulfadimethoxine decreased total serum bilirubin levels in the Gunn rat by 30% or more indicating displacement of bilirubin from albumin. Sulfadimethoxine and sodium salicylate displaced bilirubin from hyperbilirubinaemic Gunn rat sera at

drug concentrations above 0.2 mg/ml and 0.4 mg/ml respectively using SGF.

As a result of findings that several drugs in addition to sulphafurazole displaced bilirubin from albumin, it was decided to study bilirubin binding in the presence of some drugs in common use during the neonatal period. 3 semi-synthetic penicillins; cloxacillin, flucloxacillin and ampicillin were chosen for investigation. Sulphafurazole was tested as a positive control. The method used was SGF and the binding characteristics of bovine serum albumin (BSA) and human pooled cord serum (PCS) were studied. The mechanism of bilirubin displacement by drugs was examined. Two methods of analyzing the data were used and compared for their ability to quantify the effect of drugs on bilirubin binding.

METHODS

The method was adapted from Kaufmann, Kapitulnik and Blondheim (1969). Preparation of Sephadex Gel filtration columns and measurement of free bilirubin is described in Chapter Two. Sodium flucloxacillin, sodium cloxacillin and ampicillin trihydrate were supplied by Beechams Research Laboratories. Sulphafurazole was obtained from Sigma Chemical Co. Human cord serum was collected from unselected deliveries. It was then pooled, divided up and kept frozen at -20°C until required. Bovine serum albumin (BSA) (Sigma Chemical Co) was diluted with phospate buffer.

Measurement of bilirubin binding by BSA

Bilirubin was dissolved and added to 100 µl BSA (30 g/l) to give a final concentration of bilirubin with respect to albumin ranging from 265-769 µmol/l. The albumin and bilirubin were mixed well and layered on to Sephadex Gel filtration columns. The bound bilirubin was washed through with phosphate buffer and the free bilirubin remaining on the column was subsequently eluted with 0.1 N NaOH and extracted into chloroform (Analar). The absorbance of the free bilirubin in each sample was read at 450 nm in a Pye Unicam SP1800 spectrophotometer. The control experiment was repeated 5 times so that the coefficient of variation of the method could be calculated.

Effect of drugs on binding by BSA

Drugs were mixed with albumin before addition of bilirubin. Sulphafurazole is insoluble at physiological pH and was dissolved in 0.05 N NaOH and brought back to pH 7.4 with phosphate buffer before being added to BSA. Cloxacillin, flucloxacillin and ampicillin were dissolved in phosphate buffer before addition to BSA. The final concentration of BSA was 30 g/l. 1, 2, 4 and 8 mM drug concentrations were studied. Bilirubin concentrations ranged from 377-879 µmol/l in the sulphafurazole studies and from 427-769 µmol/l for the other three drugs. Free bilirubin in the presence of drug was measured.

Measurement of bilirubin binding by human pooled cord serum

For all drug studies except those with sulphafurazole, 100 µl of undiluted pooled cord serum (PCS) were used in which the drug was dissolved. Sulphafurazole however, was dissolved in 0.5 ml 0.1 N NaOH and then 0.5 ml phosphate buffer was added. 100 µl of the solution were added to 1.2 ml PCS. 100 µl of this solution were used for binding studies.

PCS slightly diluted with phsophate buffer was used for the sulphafurazole control data to cater for the dilution when adding drug. Samples contained bilirubin ranging from 377-879 µmol/l. 100 µl of undiluted PCS were used for ampicillin, cloxacillin and flucloxacillin controls. Samples contained bilirubin ranging from 365-879 µmol/l. Free bilirubin was estimated in all samples.

Effect of drugs on bilirubin binding by PCS

Sulphafurazole was added to PCS to give concentration of 1, 2 or 4 mM. Bilirubin concentrations ranged from 374-879 µmol/l. 16 mM ampicillin and 1, 2, 4, 8 mM cloxacillin and flucloxacillin were studied. Bilirubin concentrations ranged from 374-879 µmol/l.

RESULTS

Statistics

The data were analysed in two ways. The first involved the estimation of the BBC of neonatal serum or BSA by plotting the data in the conventional way. This method is commonly used when measuring bilirubin

binding by the SGF method (Schiff, Chan and Stern, 1972). The second involved the use of Scatchard plot (Scatchard, 1949) which is a classical method of studying drug-protein interactions. Figure 6:1 illustrates both methods.

a) Conventional plot

The absorbance of the free bilirubin is plotted against the total bilirubin in each sample. A tangent is drawn to the straight part of the curve. This line is extrapolated to the x-axis. The point at which the line crosses is taken as the BBC of the primary bilirubin binding site. Beyond this bilirubin concentration there is a dramatic increase in free bilirubin. However below this point there is still free bilirubin present in equilibrium with the bound fraction. Estimation of the BBC by this method of plotting the data therefore involves the use of data points obtained at bilirubin concentrations above the binding capacity. However, the observed displacement of bilirubin by drugs cannot be quantified.

b) Scatchard plot

This method of analysis is used to quantify in terms of affinity and capacity the interaction between highly protein bound ligand and protein. It enables one to analyse data points obtained at total bilirubin concentrations below the 'binding capacity', in other words where the bilirubin is tightly bound to albumin. Data points obtained at total bilirubin concentrations above the 'binding capacity' can also be analysed but the affinity of albumin for bilirubin is so low at the secondary sites that any change in affinity caused by drugs is

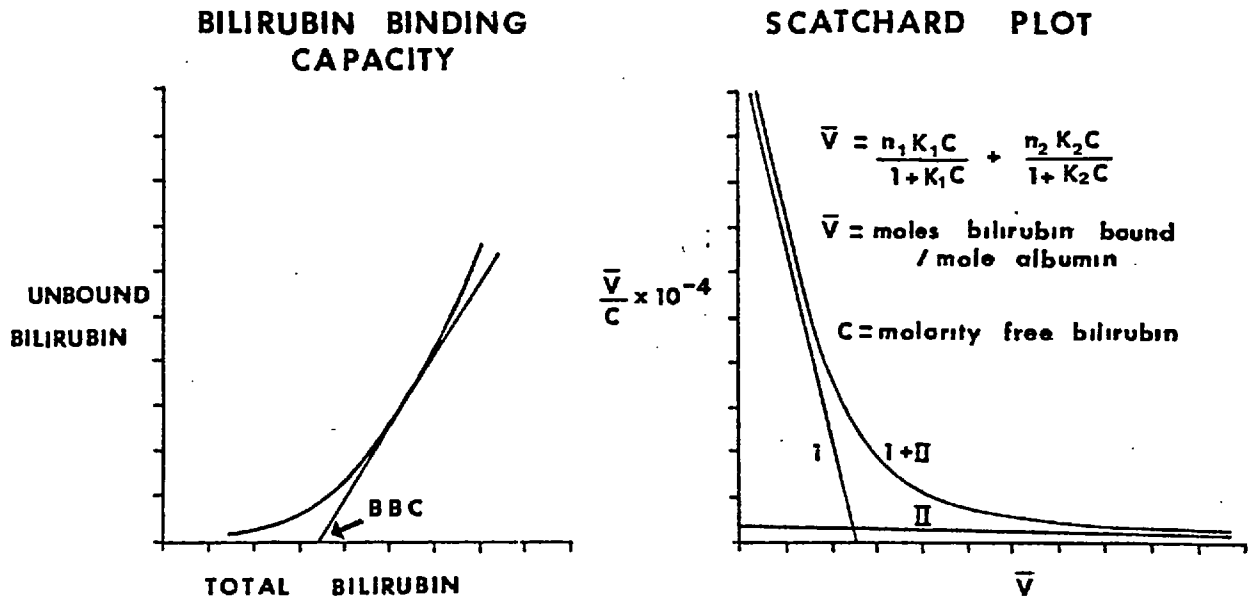


Figure 6:1 Two methods of plotting bilirubin binding data.
Left, conventional plot
Right, classical method for plotting drug-protein interaction

unimportant so far as the clinical situation is concerned.

$\frac{(V)}{(C)}$ was therefore plotted against V where V = no. of moles of bilirubin bound/mole of albumin and C = the molar concentration of free bilirubin. The points were found to lie on a curve. The Scatchard equation for two heterogeneous binding sites was consequently used to compute the 'best fit' through the experimental data points using the method of least squares.

$$V = \frac{n_1 K_1 (C)}{1 + K_1 (C)} + \frac{n_2 K_2 (C)}{1 + K_2 (C)}$$

where n_1 and n_2 = capacities of the primary and secondary sites on albumin.

where K_1 and K_2 = the apparent association constants or affinities of the primary and secondary sites on albumin.

The association constants are defined as 'apparent' since it has been reported that Sephadex partially binds bilirubin in competition with albumin and cannot therefore be expected to reflect accurately the concentration of unbound pigment (Meuwissen and Heirwegh, 1970). This was confirmed by similar findings in this study. It was found that the free bilirubin retained on the column when measured as a percentage of the total in the sample was indirectly proportional to the volume of serum filtrated.

K_1 is the gradient of the tangent to the steep part of the curve and n_1 is the intercept on the x-axis of this tangent. K_2 is the gradient

of the tangent to the shallow part of the curve and n_2 is the corresponding intercept on the x-axis. No limits for values of n_2 were incorporated into the programme. As a result unrealistic values of n_2 were obtained and have not been quoted.

Effect of drugs on bilirubin binding by BSA

Using BSA the coefficient of variation of the method was 5%.

Figure 6:2 shows the effect of sulphafurazole on bilirubin binding by BSA (0.444 mM). The data is plotted in the conventional way. Increasing drug concentration causes the curves to be displaced to the left indicating displacement of bilirubin. By extrapolating the lines to the x-axis, it can be seen that the BBC is also decreased.

Figure 6:3 shows the same data plotted as Scatchard plots. Increasing drug concentrations cause the gradients of both parts of the curve to decrease, the affinity of albumin for bilirubin at both sites is therefore decreased and can be quantified. The binding capacity of the primary site is also reduced. Table 6-1 gives computed values for n_1, K_1 and K_2 for the control data and in the presence of 1, 2 and 4 mM sulphafurazole.

Table 6-2 compares values for the BBC obtained by the conventional method with values derived from Scatchard plots from the same data points. Although the estimated BBC is given in term of 'total' bilirubin, whereas the calculated BBC is given in terms of 'bound' bilirubin,

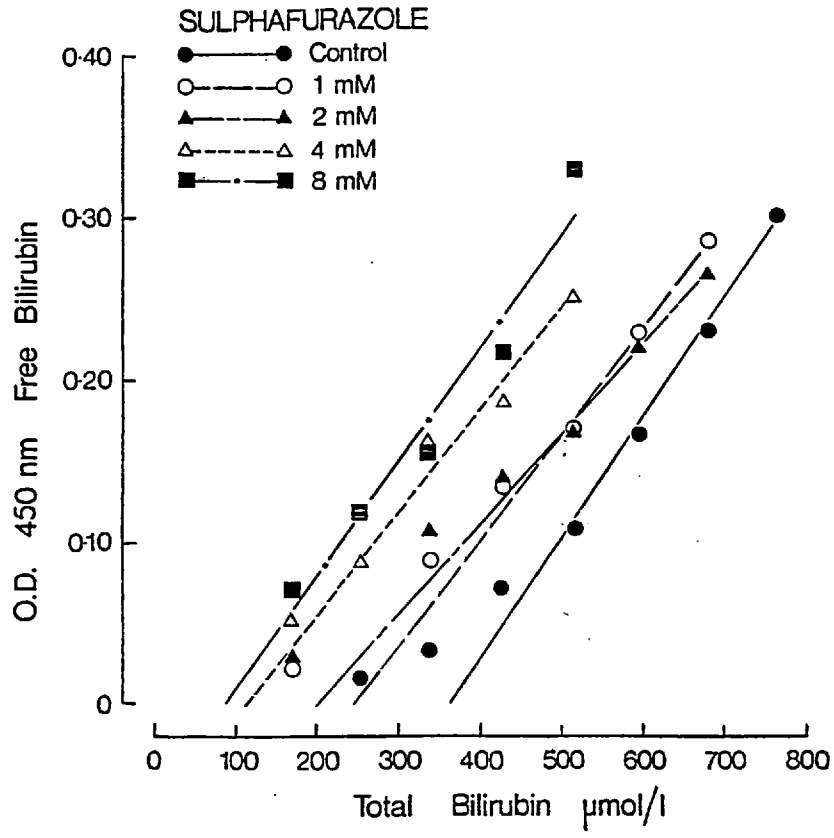


Figure 6:2 The effect sulphafurazole concentration on bilirubin binding by bovine serum albumin

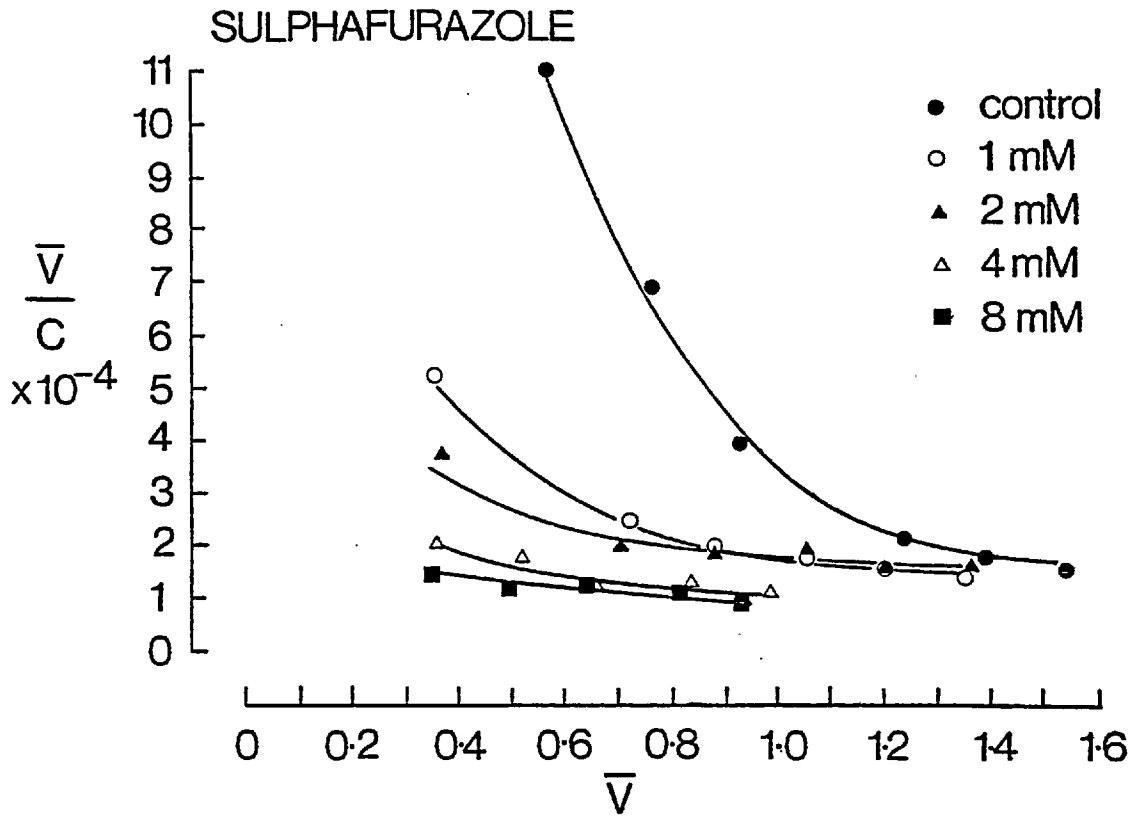


Figure 6:3 Scatchard plots showing the effect of sulphafurazole concentration on bilirubin binding by bovine serum albumin

c (mM)	n_1	K_1*	K_2*
0	0.84	31.59	0.28
1	0.51	17.64	0.17
2	0.35	17.35	0.13
4	0.33	10.25	0.05

* $(10^4\ M^{-1})$

Table 6-1

Relationship between sulphafurazole concentration and bilirubin binding by bovine serum albumin (0.444 mM) as calculated by Scatchard analysis

c (mM)	Calculated BBC (μM)	Estimated BBC (μM)
0	372	366
1	226	245
2	155	200
4	146	112

Table 6-2

Comparison between values for BBC of primary binding site on BSA obtained by classical and conventional methods at various sulphafurazole concentrations

these values can be compared since the percentage of free bilirubin at the BBC is very small compared to the total. It can be seen that when no drug is present and at 1 mM sulphafurazole, the two methods of measuring binding capacity give similar results. However, at higher drug concentrations there is a significant discrepancy between the values but this difference is bi-directional. Figures 6:4, 6:5 and 6:6 show the effect of cloxacillin, flucloxacillin and ampicillin on bilirubin binding by BSA. The data is plotted conventionally. It can be seen that none of these drugs displaced bilirubin from its binding sites on bovine albumin unlike sulphafurazole.

Effect of drugs on bilirubin binding by PCS

Figure 6:7 shows the effect of sulphafurazole on bilirubin binding by human pooled cord serum plotted conventionally. Increasing concentrations of drug cause displacement of bilirubin, however, the BBC does not appear to decrease significantly.

Figure 6:8 shows the same data plotted as Scatchard plots. The affinity of the primary site decreases, however, no change in the binding capacity is apparent.

Figures 6:9 and 6:10 show the effect of cloxacillin and flucloxacillin on bilirubin binding by PCS, plotted conventionally. Both drugs cause bilirubin displacement, yet do not appear to alter the BBC significantly. When compared to the effect of sulphafurazole, these drugs appear to cause greater displacement at the secondary sites, yet less at the primary site.

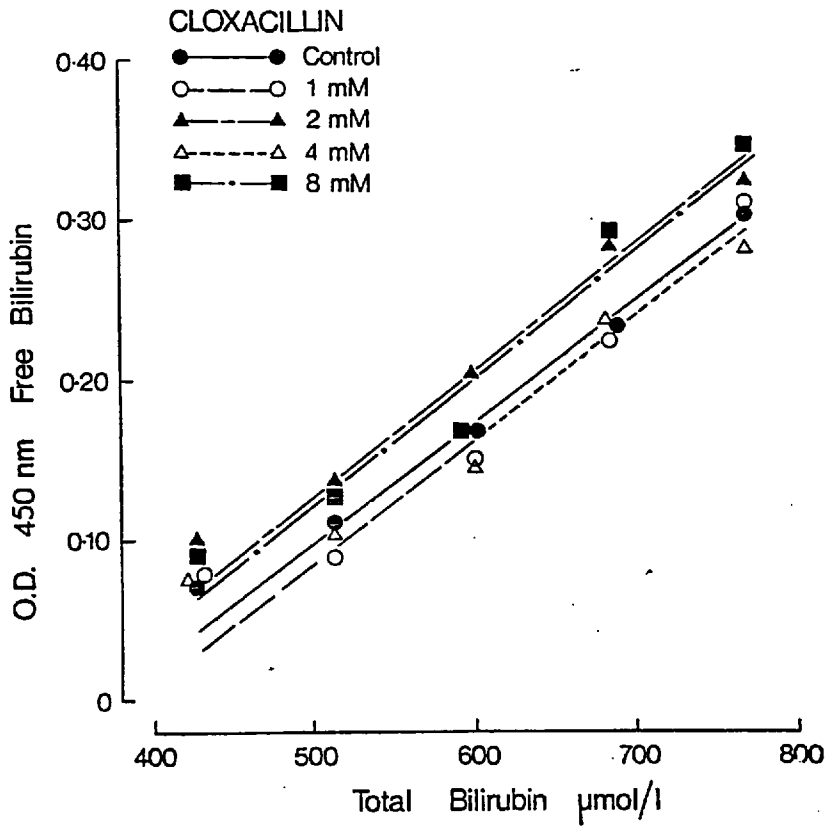


Figure 6:4 The effect of cloxacillin concentration on bilirubin binding by bovine serum albumin

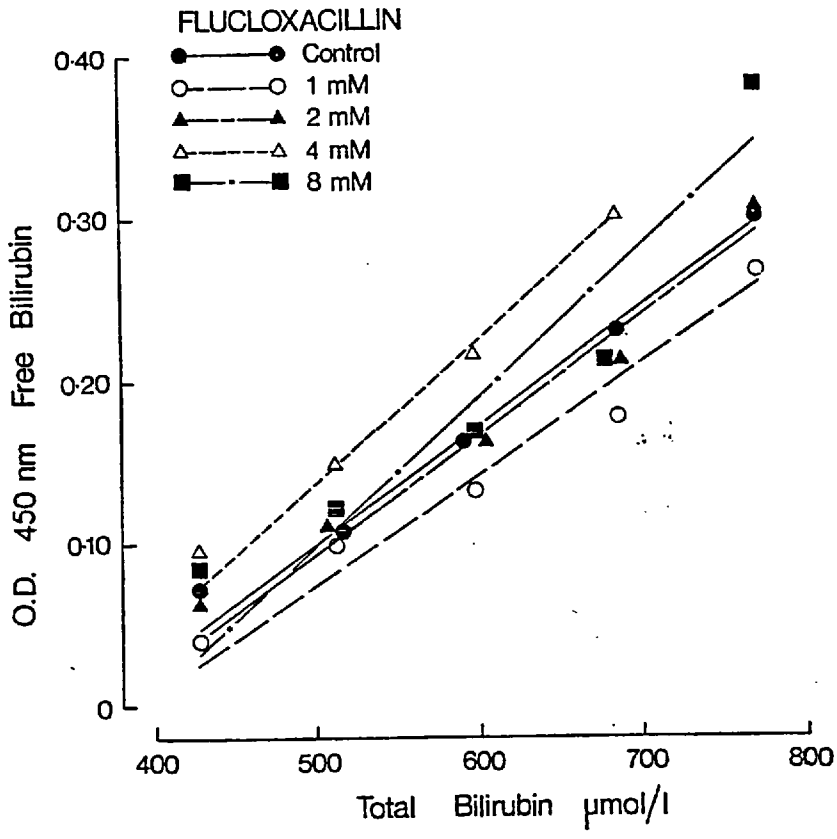


Figure 6:5 The effect of flucloxacillin concentration on bilirubin binding by bovine serum albumin

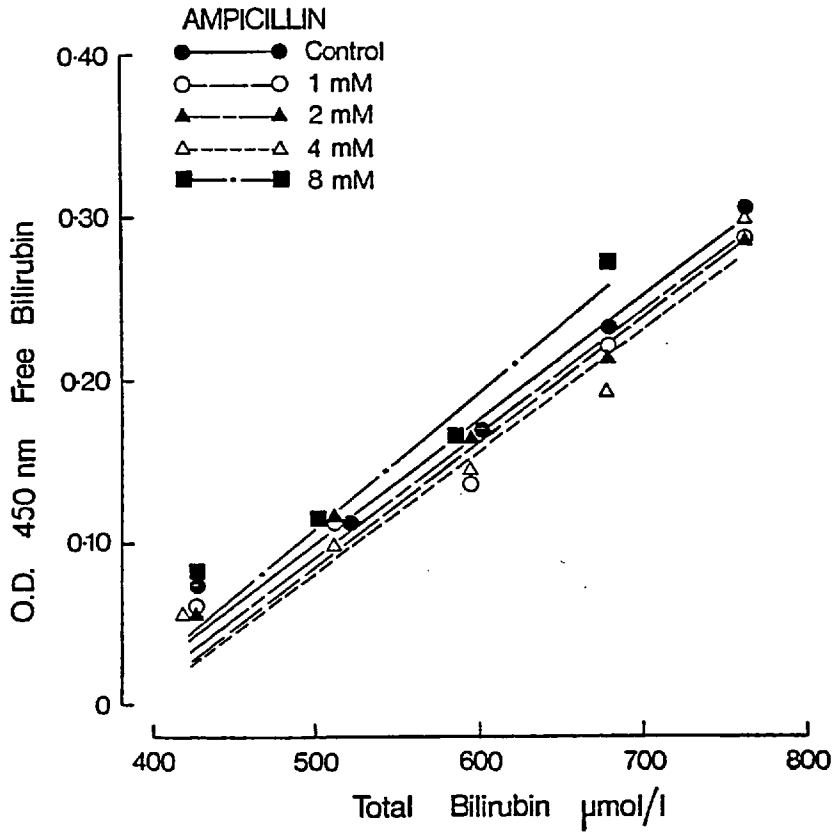


Figure 6:6 The effect of ampicillin concentration on bilirubin binding by bovine serum albumin

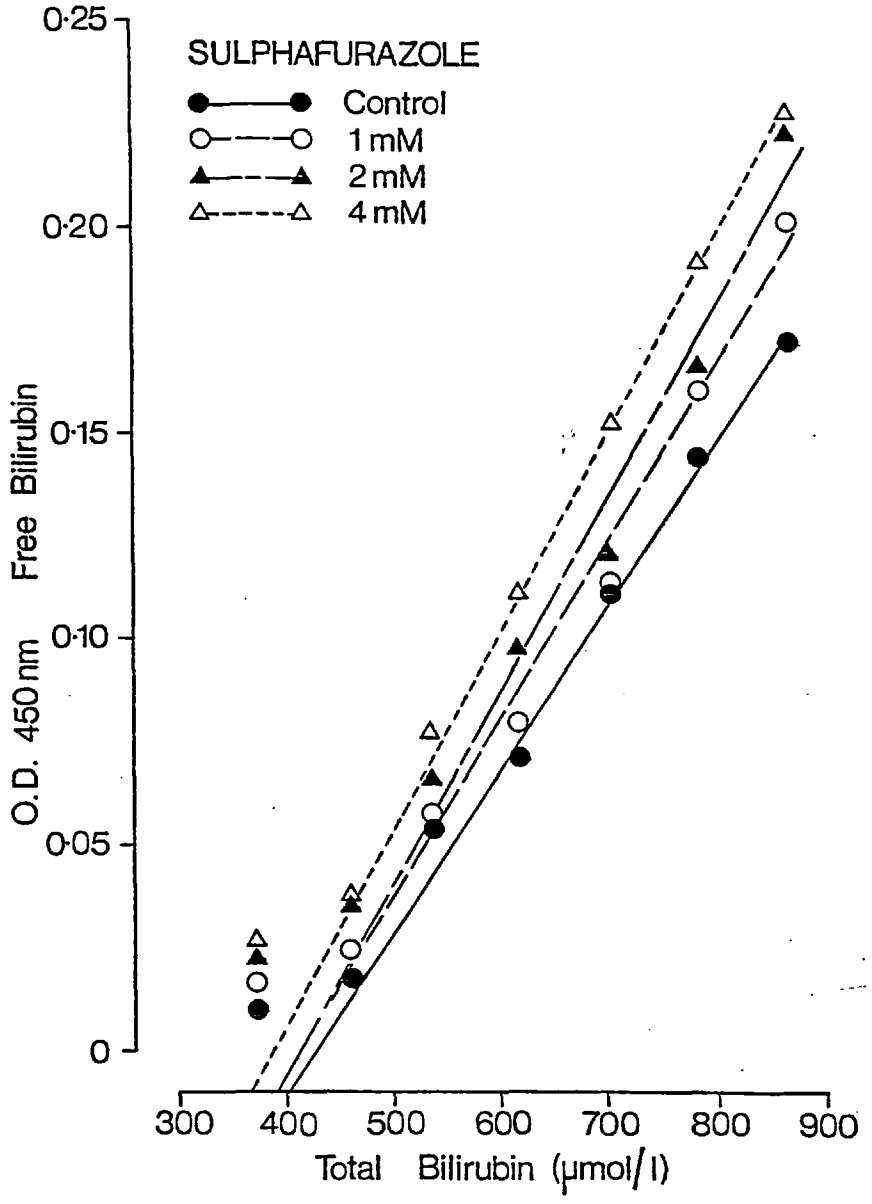


Figure 6:7 The effect of sulphafurazole concentration on bilirubin binding by human pooled cord serum.

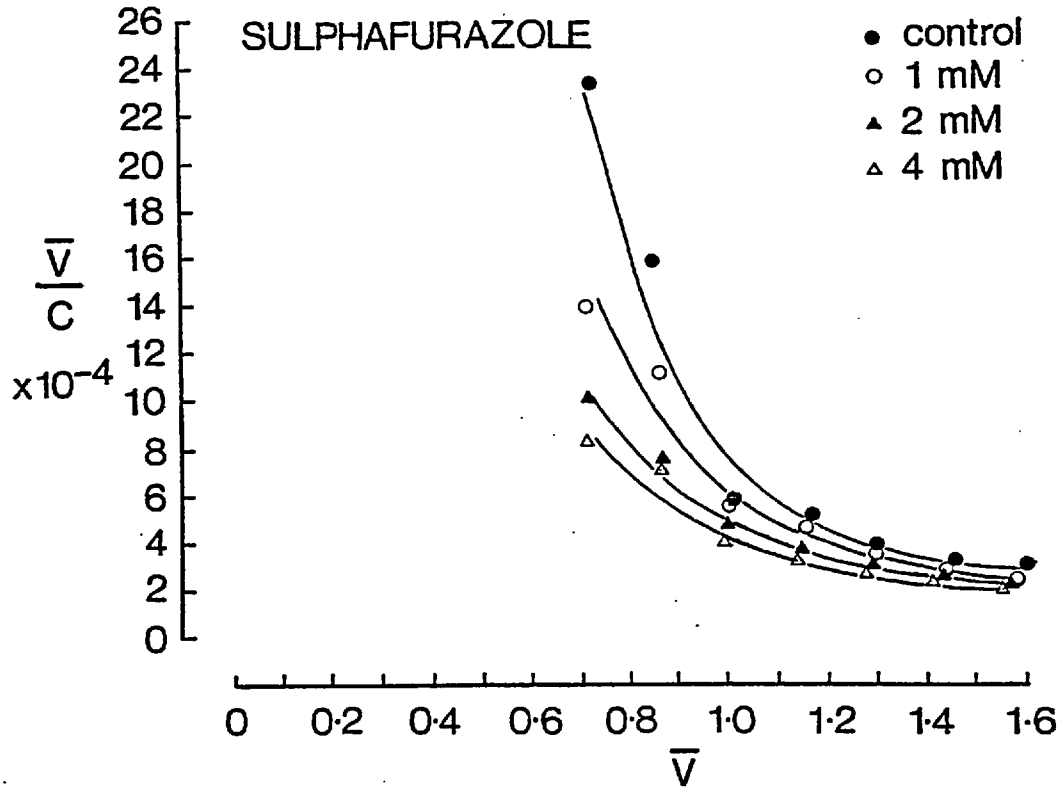


Figure 6:8 Scatchard plots showing the effect of sulphafurazole concentration on bilirubin binding by human pooled cord serum

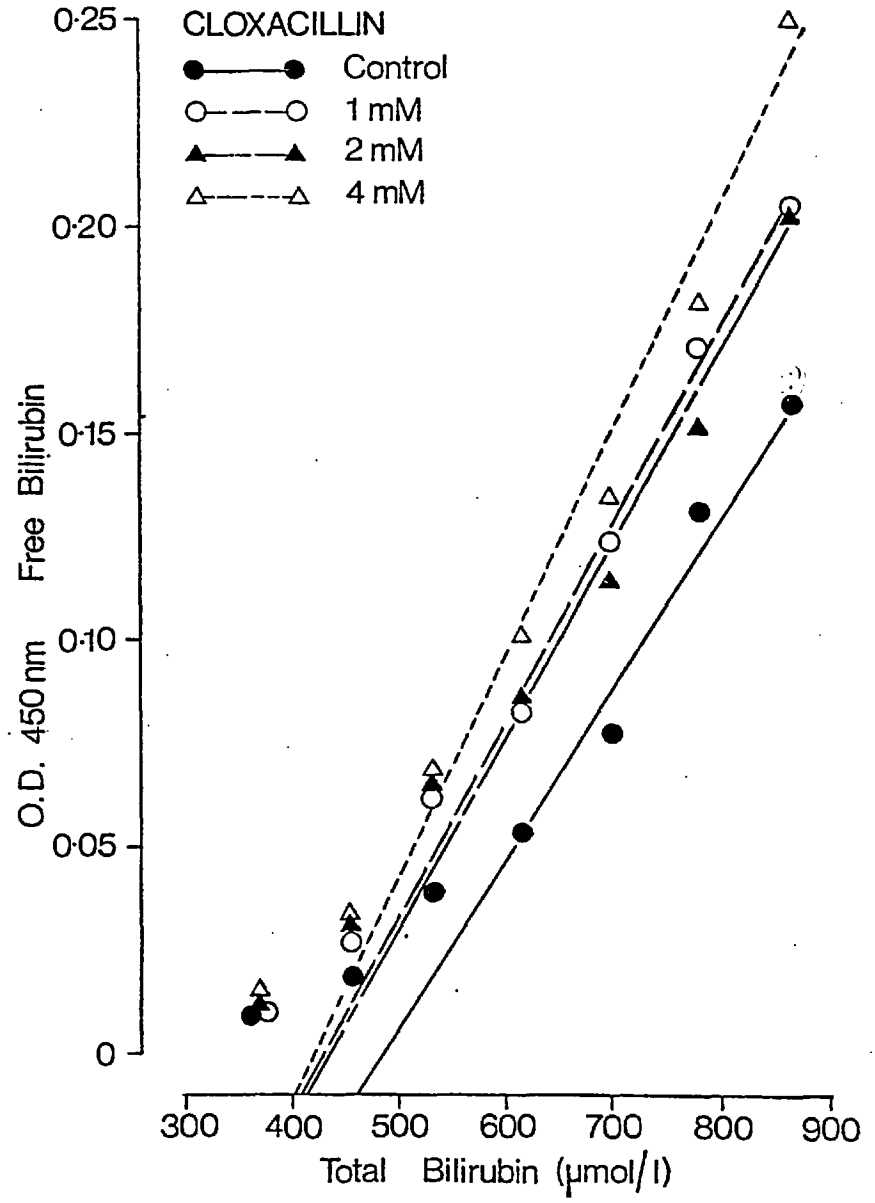


Figure 6:9 The effect of cloxacillin concentration on bilirubin binding by human pooled cord serum

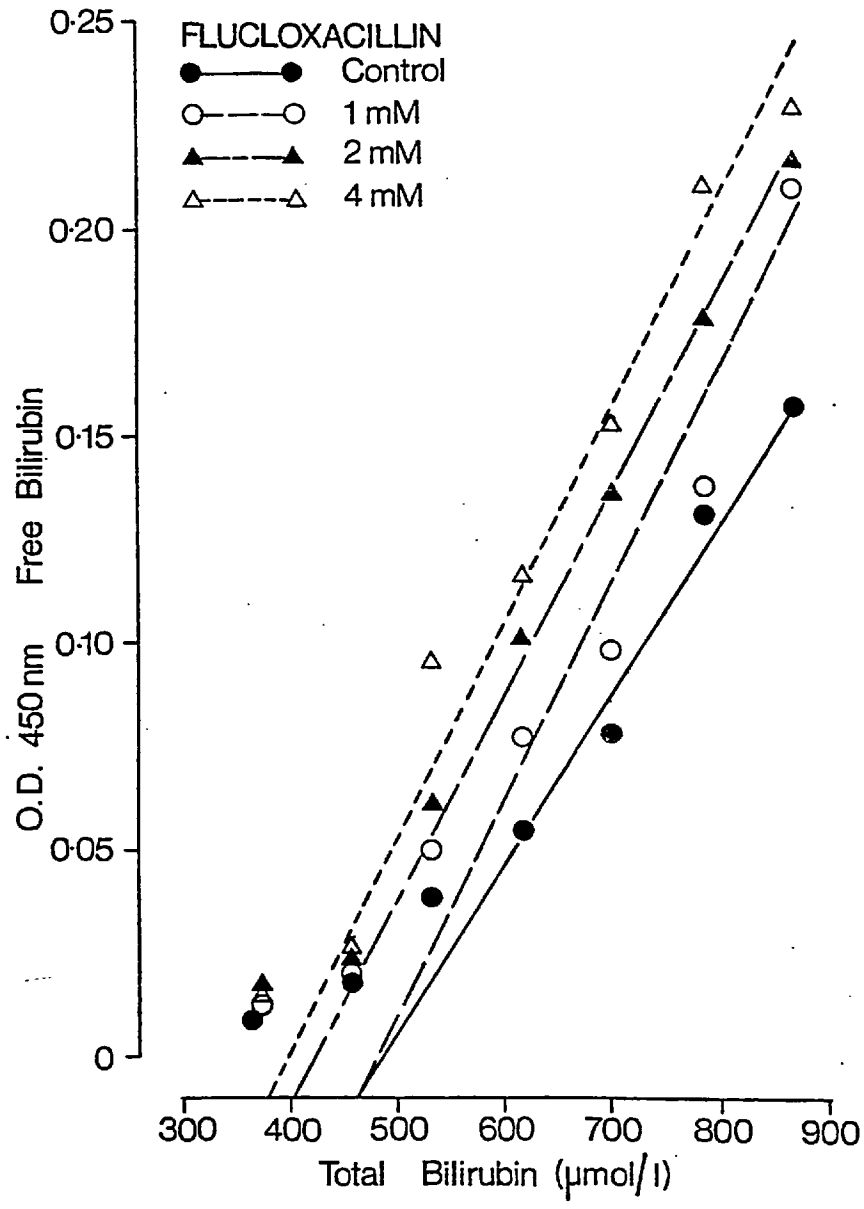


Figure 6:10 The effect of flucloxacillin concentration on bilirubin binding by human pooled cord serum

Figures 6:11 and 6:12 show the same data for cloxacillin and flucloxacillin plotted as Scatchard plots. A decrease in affinity at the primary site is seen, but no change in BBC is apparent.

Figure 6:13 shows the effect of 16 mM ampicillin on bilirubin binding by PCS, plotted conventionally. No significant displacement of bilirubin can be seen.

Table 6:3 gives values for n_1, K_1 and K_2 for PCS control data and in the presence of sulphafurazole, cloxacillin and flucloxacillin. N_1 remained unchanged at around 0.8 moles bilirubin bound/mole albumin in the presence of all three drugs even at high concentrations. This value for PCS is very similar to the value of 0.84 obtained for binding of bilirubin by BSA from control data.

K_1 decreased from around 9.6×10^5 M^{-1} with increasing drug concentration. However, sulphafurazole exerted the greatest effect.

K_2 was found to be very low compared to K_1 and decreased further with increasing concentrations of cloxacillin and flucloxacillin, however the changes in K_2 in the presence of sulphafurazole did not appear to be related to increasing drug concentrations. Since sulphafurazole can be seen to displace bilirubin from secondary site, this anomaly probably reflects the unsuitability of applyigg the Scatchard equation to data points obtained at low affinity sites especially when changes in affinity are small.

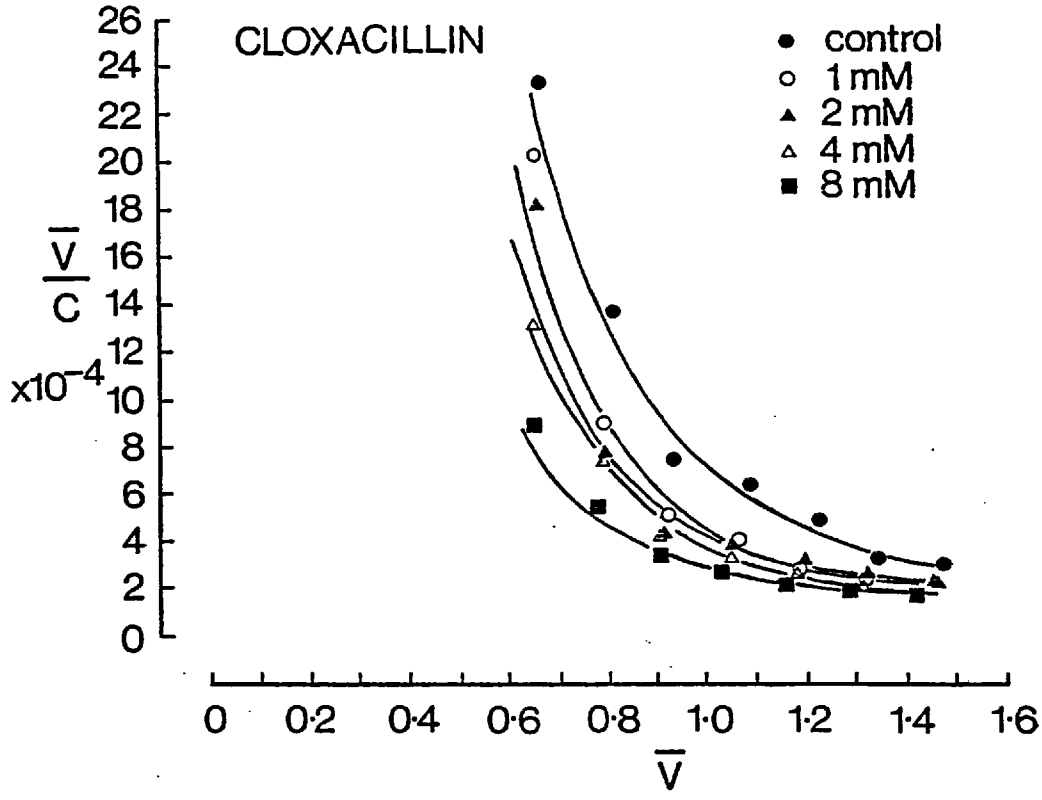


Figure 6:11 Scatchard plots showing the effect of cloxacillin concentration on bilirubin binding by human pooled cord serum

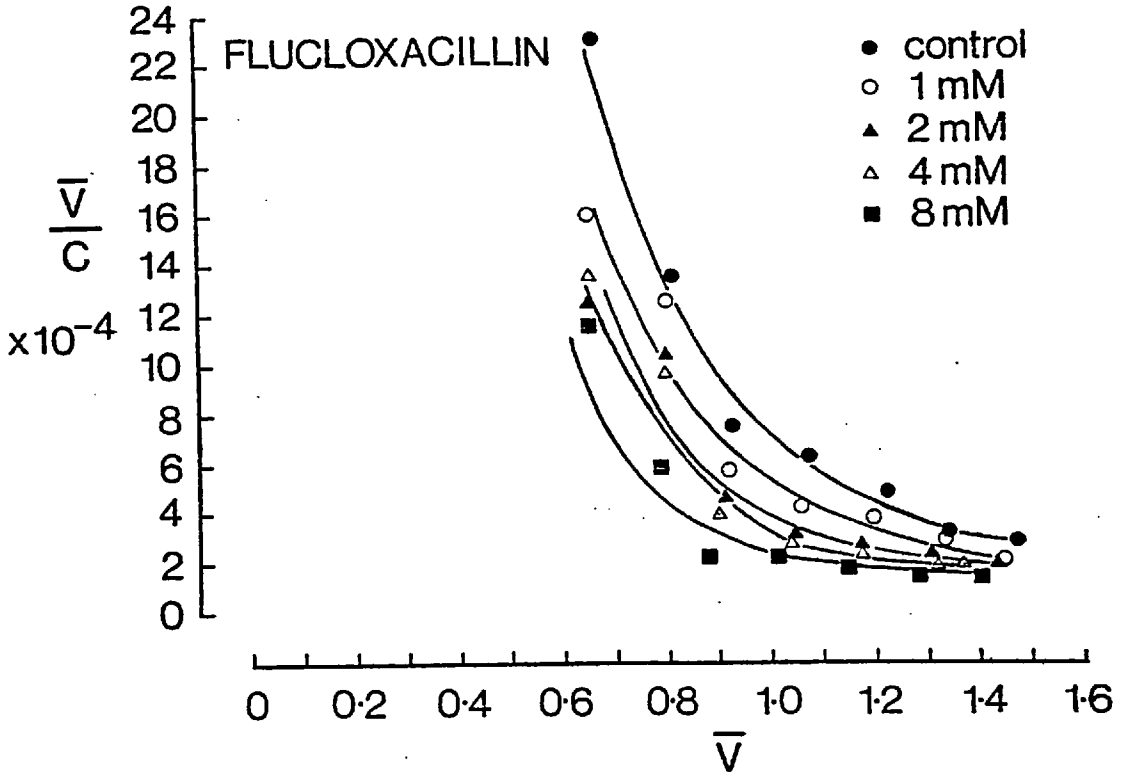


Figure 6:12 Scatchard plots showing the effect of flucloxacillin concentration on bilirubin binding by human pooled cord serum

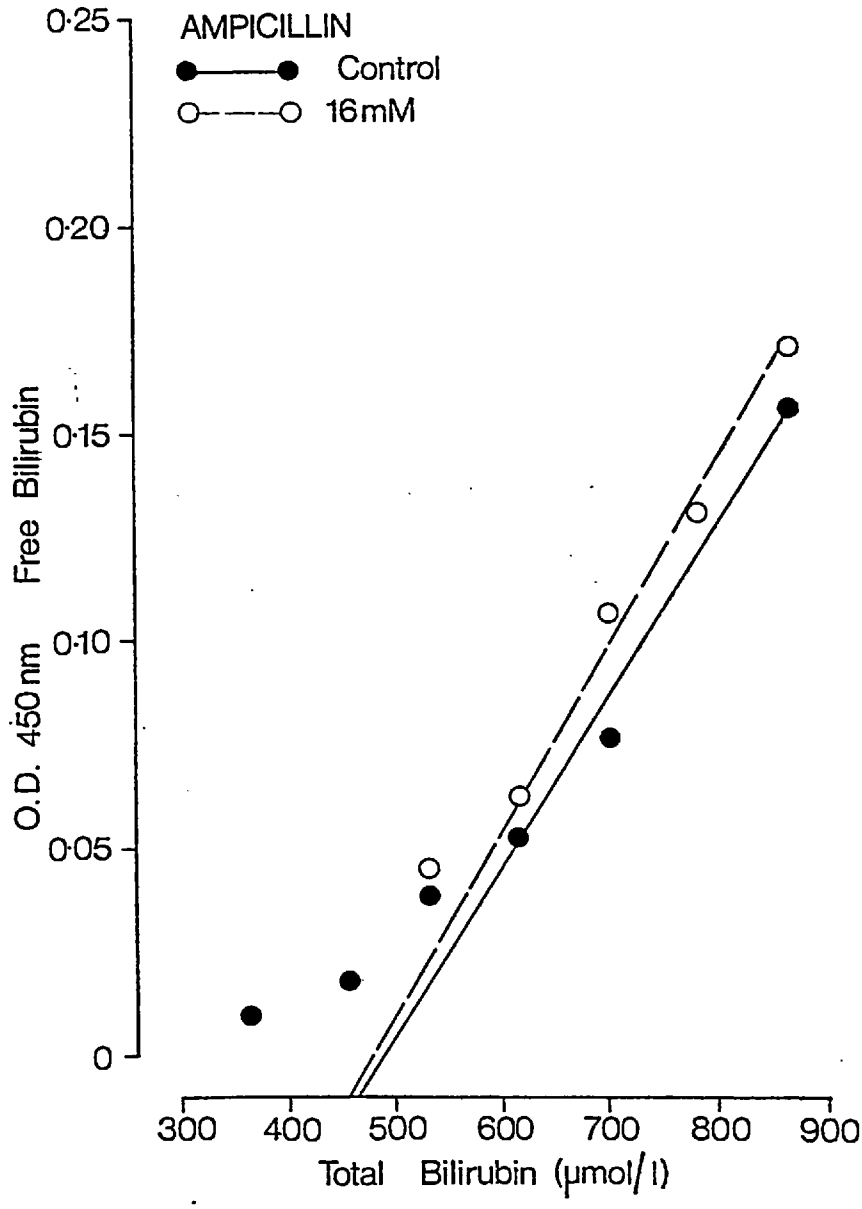


Figure 6:13 The effect of 16 mM ampicillin on bilirubin binding by human pooled cord serum

Drug	c (mM)	n_1	K_1*	K_2*
Sulphafurazole	0	0.90	95.6	0.03
	1	0.91	55.0	0.22
	2	0.79	49.9	0.49
	4	0.86	35.0	0.14
Cloxacillin	0	0.81	97.0	1.18
	1	0.82	85.7	0.03
	2	0.80	70.0	0.02
	4	0.85	50.0	0.005
	8	0.76	41.5	0.005
Flucloxacillin	0	0.81	97.0	1.18
	1	0.78	85.5	1.08
	2	0.80	76.9	0.071
	4	0.78	77.0	0.013
	8	0.72	77.8	0.005

* $(10^4\ M^{-1})$

Table 6-3

Relationship between drug concentration and bilirubin binding by human pooled cord serum as calculated by Scatchard analysis.

DISCUSSION

Bilirubin was displaced from either BSA or PCS in vitro by sulphafurazole, cloxacillin and flucloxacillin but not by ampicillin. These results could have been anticipated from work carried out by Bratlid (1972) who found that all the drugs in his investigation that were effective displacers of bilirubin were strongly protein bound. Ampicillin is less than 25% protein bound (Kunin, 1967) whereas cloxacillin, flucloxacillin (Tan, Tro, Phair and Watanakunakorn, 1972) and sulphafurazole (Anton, 1973) are over 90% bound at therapeutic plasma concentrations.

The apparent association constants K_1 of BSA and PCS for bilirubin were $3.15 \times 10^5\ M^{-1}$ and $9.56 \times 10^5\ M^{-1}$ respectively. These values are lower than the value of $5.2 \times 10^7\ M^{-1}$ obtained by Krasner, Giacoia and Yaffe (1973) by equilibrium dialysis using purified cord albumin. The discrepancy may be due to the fact that Sephadex binds bilirubin in competition with albumin (Meuwissen and Heirwegh, 1970), especially since only 100 µl of BSA or PCS were used.

BSA and PCS showed very similar primary binding capacities (n_1). Approximately 0.8 moles bilirubin were bound/mole albumin. These results are in agreement with Krasner, Giacoia and Yaffe (1973) who found that both purified cord albumin and bovine albumin show maximum fluorescence at a bilirubin/albumin molar ratio of 1. However the capacities of individual sera have been found to vary widely. Using the peroxidase method Jacobsen and Wennberg (1974) found n_1 to range

from 0.42 to 0.85 in 17 newborns most of whom were preterm.

The BBC of the PCS estimated from the conventional plots was 366 µmol/l. This value is similar to the value obtained by Kapitulnik, Blondheim and Kaufmann (1972). They found the BBC of PCS using SGF to be 393 µmol/l.

Sulphafurazole displaced bilirubin from the primary and secondary sites on BSA. It also reduced the BBC and n_1. Cloxacillin, flucloxacillin and ampicillin had no effect even at high concentrations. Sulphafurazole, cloxacillin and flucloxacillin but not ampicillin all displaced bilirubin from both primary and secondary sites on PCS however the BBC and n_1 remained unchanged.

These observations suggest that drugs which interfere with bilirubin binding do so by lowering the affinity of albumin for bilirubin, however, they do not necessarily diminish the capacity of the primary site. Sudlow (1978) carried out studies on specific binding sites on human albumin and found that the affinity of albumin for bilirubin at its primary site is so great as to make competition for binding by other ligands virtually impossible. However she also states that changes in albumin conformation as a result of ligand binding have been frequently observed. One result of this conformational mobility is that binding sites on albumin, although spatially distinct, are unlikely to be independent. Thus the affinity, specificity and number of binding sites for a given ligand may change in the presence of a

second ligand bound to a separate site.

Since bilirubin was displaced from its primary high affinity site as measured by SGF, these results can only be explained assuming Sudlow's hypothesis to be correct, if one assumes that albumin conformation has occurred. In any event, evidence for the ability of drugs to displace bilirubin from its high affinity site can be found in the literature.

Coutinho, Lucek, Cheripko and Kuntzman (1973) showed that therapeutic levels of both sulphafurazole and sulfadimethoxine displaced bilirubin from human plasma protein at bilirubin/albumin ratios of 0.25 and 1.5 using differential spectrophotometry.

A reduction in BBC by a drug also means that the affinity for bilirubin of the primary site is reduced. Schiff, Chan and Stern (1972) found that sodium benzoate and sodium salicylate reduced the BBC of neonatal sera using SGF.

It is assumed by many workers that drugs which displace bilirubin from albumin do so by reducing the BBC. However, since neither sulphafurazole, cloxacillin nor flucloxacillin reduced the BBC or n_1 of PCS it can be assumed that methods which only measure changes in BBC will not detect kernicterogenic drugs which only alter the affinity and not the capacity of albumin.

Krasner, Giacoia and Yaffe (1973) used fluorescence spectroscopy to examine the effect of drugs on bilirubin binding. This method was used to measure changes in BBC and not in free bilirubin. Consequently no effect was seen with sulphafurazole (1 mg/ml) a potent bilirubin displacer, when it was added to bilirubin and fractionated cord albumin.

Methods which can measure changes in free bilirubin and BBC or n_1, may not be suitable either. The peroxidase method (Jacobsen and Wennberg, 1974) for example, measures both free bilirubin and BBC but to perform the test conveniently it is necessary to make a 40-fold dilution of serum. Dilution has a negligible effect on bilirubin binding to the high affinity sites, but facilitates dissociation from secondary sites. Furthermore the effect of drugs which are not so strongly bound would be obscured by dilution.

The SGF method fulfils these criteria, but free bilirubin cannot be measured at total bilirubin levels greatly below the BBC and this is a disadvantage. However, by using the Scatchard equation to calculate changes in affinity the method is adequate for bilirubin/albumin molar ratios slightly below the BBC.

Although neonatal serum bilirubin was considered to have 2 heterogeneous binding sites for bilirubin and the data were subsequently analysed according to this premise, close examination of the conventional plots reveals a possible change in affinity around 540 µmol/l total bilirubin,

in addition to the dramatic change at the BBC. Insufficient data were obtained to definitely confirm the presence of a low affinity site of 120 µmol/l capacity. However, Griffiths, Diamond and Dextraze (1975) using T-20 Dextran coated charcoal for assessing the BBC of serum found the tight binding capacity of adult human serum to be 20 mg% (342 µmol/l) and the loose binding capacity to be an additional 10 mg% (171 µmol/l). This finding is of theoretical interest but of little clinical relevance since the serum albumin in most neonates will never by fully saturated.

Although cloxacillin and flucloxacillin caused displacement of bilirubin from PCS, therapeutic serum concentrations of these drugs must be taken into account when assessing for their kernicterogenic potential. Administration of 25 mg/kg oral flucloxacillin 6-hourly to neonates results in a mean 3 hour serum concentration on the second day of treatment of about 22 µg/ml (0.044 mM) (Cohen, Raeburn, Devine, Kirkwood, Elliott, Cockburn and Forfar, 1975). Administration of a single oral dose of cloxacillin (50 mg/kg) to 7 neonates results in a mean 2 hour serum concentration of 92 µg/ml (0.2 mM) (Grossman and Ticknor, 1965).

Whereas subcutaneous administration to preterm infants of 0.075 g/kg/12 hour sulphafurazole results in a blood concentration of about 120 µg/ml (0.45 mM) 12 hours after the dose. Furthermore, in one infant a peak level of 240 µg/ml (0.9 mM) occurred 3 hours after drug administration (Silverman, Andersen, Blanc and Crozier, 1956).

It is therefore unlikely that the cloxacillins are kernicterogenic in humans and there have been no reports of kernicterus resulting from administration of these drugs during the neonatal period.

CHAPTER SEVEN

EFFECT OF DRUGS ON BILIRUBIN DISPOSITION IN NEONATAL GUINEA PIGS

SUMMARY

From the literature it appears that there are several drugs which favour the passage of bilirubin into the brain in animals. This has been investigated for drugs which can be shown to displace bilirubin from pooled cord serum in vitro using Sephadex Gel filtration.

Cloxacillin, flucloxacillin and sulphafurazole were tested for their ability to alter bilirubin disposition in blood, fat and brain tissues of artificially jaundiced neonatal guinea pigs. The effect of infusing pooled cord serum into guinea pigs was also investigated. Sulphafurazole and flucloxacillin altered bilirubin disposition whereas cloxacillin did not.

When pooled cord serum was infused, none of the 3 drugs exerted an effect; pooled cord serum alone caused a decrease in free bilirubin. Guinea pigs infused with human pooled cord serum are not, therefore, a useful in vivo model for human kernicterus.

However, sulphafurazole displaced bilirubin from guinea pig serum in vitro so that Sephadex Gel filtration changes can be used to predict disposition changes in vivo.

INTRODUCTION

Kernicterus

It is over one hundred years since Orth (1875) described the 'yellow staining' of certain areas in the brain seen in children dying from

severe neonatal jaundice. Schmorl (1903) later called this kernicterus (KI).

Bilirubin is a highly lipid soluble non-polar molecule which can easily enter the central nervous system. In the blood stream it is transported principally by serum albumin, in addition, some pigment is bound to red blood cells and another fraction, the non protein bound bilirubin is in equilibrium with the interstitial fluid and the intracellular constituents, as it is diffusible and able to penetrate the cell membranes.

It is in the first week of life that the infant is most susceptible to KI due to a deficiency in hepatic glucuronyl transferase resulting in an accumulation of bilirubin in plasma, the concentration peaking around the 4th day.

It has been shown in vitro (Odell, 1970) and in vivo (Diamond and Schmid, 1966) that the toxicity of the pigment is due uniquely to this protein-free, unconjugated bilirubin fraction. Bilirubin depresses cellular respiration of various tissues and it is an uncoupler of oxidative phosphorylation in mitochondria. Its toxic effects on the metabolism of the neurones finally destroys the cells. Bilirubin also inhibits the incorporation of amino acids into proteins. It causes hypoplasia of the cerebellum and interferes with DNA synthesis and brain development from 10 days of age in Gunn rats.

In human KI, bilirubin pigmentation of the brain is seen most frequently localized in the basal ganglia, the cerebellum being less pigmented. However, the extensive communication between the basal ganglia and cerebellum and lack of definite understanding of their respective functions suggest that the neurological signs attributed in infants to lesions of basal ganglia may be due, at least partly, to cerebellar impairment.

Follow-up studies of infants with neonatal jaundice

Several follow-up studies have been carried out on human infants who suffered from varying degrees of neonatal jaundice. Fenwick (1975) looked at 18 cases of deaf children in whom the aetiology of the deafness was related to a period of neonatal jaundice. He reported that jaundice in excess of 16 mg/dl (256 µmol/l) may be associated with high tone deafness. The hearing loss was bilateral and symmetrical and increasing serum bilirubin levels were associated with more severe deafness. Prematurity and/or lower birthweight increased the degree of deafness which bore no constant relationship to the extent of the extrapyramidal lesion. Male children seemed to be more susceptible to deafness following neonatal jaundice than females.

Boggs, Hardy and Frazier (1967) observed 23,000 infants at 8 months of age. Their findings indicate a positive relationship between increasing neonatal hyperbilirubinaemia and the incidence of low motor and/or mental scores attained at 8 months. These relationships did

not begin abruptly at the 20 mg/dl (342 µmol/l) level but rose progressively and became substantial at the 16 (273 µmol/l) to 19 mg/dl (325 µmol/l) concentration.

Clinical observations make it evident that prematurity, anoxia, acidosis, sepsis or the presence of anions which compete with bilirubin for albumin binding sites make the development of neurological damage more likely. Nevertheless death or severe brain damage as a result of KI is now rare due partly to a decreasing incidence of anoxia and sepsis but also to better neonatal management of cases of established jaundice. This involves the use of phototherapy for jaundiced infants and in more severe cases, exchange transfusion.

Displacement of bilirubin from albumin by drugs

Endogenous anions such as free fatty acids and hematin present in plasma or certain highly protein bound drugs, are capable of displacing bilirubin from plasma albumin (Odell, 1959a) causing an increase in bilirubin disposition in the CNS which can lead to KI.

Silverman, Andersen, Blanc and Crozier (1956) were the first to observe that a drug treatment used in neonates could result in KI and death. They looked at two groups of premature infants who were allotted two different prophylactic antibacterial regimens. They received either penicillin and sulphafurazole or oxytetracycline. 36% of those infants in the penicillin/sulphafurazole group who died during the trial period (< 120 hours) were found to have KI at necropsy compared to 6% in the

oxytetracycline group. Penicillin was known not to cause KI, therefore sulphafurazole was held responsible.

Animal models of neonatal jaundice

Various animal models have been sought to reproduce the clinical picture seen in hyperbilirubinaemia.

A neurological disorder similar to kernicterus in neonates occurs in a mutant strain of Wistar rats (Gunn). The activity of glucuronyl transferase in vitro is very low in the livers of adult homozygous animals. It is below normal in heterozygotes, although these animals have no hyperbilirubinaemia. Gunn rats are therefore often used as an animal model of hyperbilirubinaemia (Menken, Barrett, Swarm and Berlin, 1966; Diamond and Schmid, 1966). Other possible animal models are neonatal monkeys (Lucey, Hibbard, Behrman, Esquivel de Gallardo and Windle, 1964), neonatal guinea pigs (Diamond and Schmid, 1966), neonatal kittens (Rozdilsky, 1966) and neonatal albino rats (Blanc and Johnson, 1959).

As a result of the possible role of sulphafurazole in human KI, several workers have studied the effect of this and other drugs on bilirubin disposition and toxicity in animal species.

Diamond and Schmid (1966) used newborn guinea pigs to investigate the effect of sodium salicylate on bilirubin distribution between plasma and brain. Davis and Yeary (1975) investigated the effect of sulfadimethoxine on tissue distribution of bilirubin in newborn and adult

Gunn rats. Schenker, McCandless and Zollman (1966) used sulphafurazole as a means of precipitating KI to study the effect of bilirubin on cellular toxicity in kernicteric brain of Gunn rats. They found that there was a statistically significant depletion of cerebellar ATP at all stages of neurological impairment.

Nathenson, Cohen and McNamara (1975) studied the effect of Na Benzoate on serum bilirubin levels in the Gunn rat. They reported that administration of Na Benzoate to grown and suckling Gunn rats in single doses of 7 and 35 mg/kg, failed to significantly alter serum bilirubin concentrations. However, a single dose of 100 or 200 mg/kg and repeated doses of 35 mg/kg resulted in depressed serum bilirubin concentrations.

Semba, Sato and Yamamura (1977) studied the effect of bucolome - a drug which has been used to treat or prevent hyperbilirubinaemia in infants - on Gunn rats. They found that after a subcutaneous injection of 50 mg/kg, the plasma bilirubin fell from 10.0 to 2.1 mg/dl within 10 minutes of the injection. At necropsy they found localised yellow staining of the brain including the basal ganglia. Petite, Etienne and Housset (1968) looked at the effect of a range of drugs on plasma bilirubin levels in the anaesthetised adult Gunn rat. Hanefeld and Ballowitz (1976) studied the effect of flucloxacillin on KI in infant Gunn rats.

In vitro studies on human cord serum showed that sulphafurazole, cloxacillin and flucloxacillin decreased the affinity for bilirubin of the primary and secondary binding sites on albumin, yet the capacity remained unchanged (Chapter 6). Therefore the ideal animal for investigating the effect of these drugs on bilirubin disposition, would be one in which the plasma albumin exhibited the same bilirubin binding characteristics as does neonatal serum.

Unfortunately, human albumin has a much higher affinity for bilirubin than does albumin from rats or guinea pigs (Schmid, Diamond, Hammaker and Gundersen, 1965). Teleologically this may be explained by the fact that guinea pigs do not suffer from as great a degree of physiological jaundice as do human neonates.

Choice of animal for disposition studies

The most suitable animal for studying the effect of these drugs on bilirubin disposition was found to be the neonatal guinea pig. Guinea pigs were made artificially hyperbilirubinaemic by infusion of bilirubin. Radioactive bilirubin was not used due to its high cost and low specific activity. Bilirubin was measured in blood, fat and brain biochemically after treatment of the animal with one of the three drugs. Rats were not used as the experimental animal as they were found to metabolise the infused bilirubin so rapidly that high plasma bilirubin levels could not be attained. They also need to be forcibly restrained during infusion so that removal of the cannulae does not occur, whereas guinea pigs rest fairly immobile and need not be restrained.

Gunn rats were not used as they are not commercially available in this country and their lack of glucuronyl transferase makes them inherently unsuitable.

Before carrying out in vivo studies, in vitro studies were performed using Sephadex Gel filtration to measure the effect of cloxacillin, flucloxacillin and sulphafurazole on bilirubin binding by guinea pig pooled serum (GP-PS). In this way the suitability of Sephadex Gel filtration as a method for detecting kernicterogenic drugs could be directly evaluated.

In the first series of in vivo experiments, bilirubin was infused into the animal with concurrent drug. In a second series, bilirubin was infused with concurrent human pooled cord serum (PCS) with or without drug, in order to test primarily the possibility of using a guinea pig infused with human serum as a model for investigating the effect of drugs on human KI and secondarily to test the hypothesis that drug induced KI might be due to a redistribution of free bilirubin rather than just a simple displacement of bilirubin from serum albumin.

METHOD

In vitro studies

The effect of sulphafurazole, cloxacillin and flucloxacillin on bilirubin binding by guinea pig serum using Sephadex Gel filtration

For details on measuring bilirubin binding by Sephadex Gel filtration see Chapter 2. Increasing amounts of bilirubin solution were added to 100 µl samples of guinea pig pooled serum (GP-PS) from four neonatal

guinea pigs, to give bilirubin concentrations ranging from 85.5-342 µmol/l with respect to serum. Each solution was layered onto a Sephadex Gel column. The bound bilirubin was eluted with 5 ml phosphate buffer and the unbound bilirubin remaining on the columns was measured by colorimetry after elution with 0.1 N NaOH and extraction into chloroform (see Chapter 2 for details).

The effect of 1 mM sulphafurazole, 2 mM cloxacillin and flucloxacillin was then investigated. 5.88 mg of sulphafurazole (Sigma) were weighed out and dissolved in 1 ml 0.1 N NaOH to which 1 ml phosphate buffer was then added. 10 µl of this solution were added to 100 µl samples of PGPS containing bilirubin in concentrations ranging from 85.5-342 µmol/l with respect to serum. Cloxacillin and flucloxacillin were dissolved directly in the serum. The same range of bilirubin concentrations were used. Estimation of unbound bilirubin was carried out as described for the controls.

In vivo studies

Preliminary Investigations

a) Determination of bilirubin half-life in neonatal guinea pigs

To establish the infusion time required to reach equilibrium between plasma and brain bilirubin, bilirubin half-lives were calculated in four guinea pigs after intravenous administration of varying doses of bilirubin.

Method

The guinea pigs were weighed, anaesthetised with ether, shaved and

prepared for venous infusion and arterial blood withdrawal as described in Chapter 2.

Bilirubin was dissolved in the dark, in 5 mM Sodium Taurocholate (Sigma) in isotonic saline containing 0.1 N NaOH to give a final concentration of 15-21.3 mmol/l after pH adjustment to 8 with 1 N acetic acid.

An intravenous bolus of bilirubin (1.0-1.5 ml) was administered to the guinea pig over a period of approximately one minute. 300 µl of arterial blood was withdrawn at known time intervals until 90 minutes after the bolus, and put into heparinised small glass test tubes. The withdrawn blood was replaced with an equal volume of saline. The blood samples were spun at 2000 rpm for 10 minutes in an MSE all purpose centrifuge. The plasma was drawn off and bilirubin estimations carried out using the method of Lathe and Ruthven (1958) (Chapter 2).

Results

The bilirubin concentration of each plasma sample was plotted against time after the initial bolus on semi-logarithm graph paper. The half-life ($t_{0.5}$) and the concentration at zero time (c_o) were then calculated (Table 7-1).

Values for the bilirubin half-life ranged from 28-107 minutes and increased with size of bolus. Dose dependent drug kinetics are therefore present, presumably because of the overloading of the system

	Guinea Pig (1)		Guinea Pig (2)		Guinea Pig (3)		Guinea Pig (4)	
	t(min)	Plasma bilirubin (µmol/l)	t(min)	Plasma bilirubin (µmol/l)	t(min)	Plasma bilirubin (µmol/l)	t(min)	Plasma bilirubin (µmol/l)
	2.5	424.1	2.5	763.7	2.5	925.6	2.5	1090.4
	5.0	354.3	5.0	596.5	4.5	844.2	5.0	1053.5
	10.0	240.8	10.0	550.0	10.0	833.2	11.5	1126.0
	20.0	238.1	30.0	690.9	19.5	744.3	23.0	838.7
	40.0	176.5	40.0	444.6	42.0	567.8	46.0	818.2
	60.0	76.62	60.0	301.0	60.0	344.7	59.0	834.6
	75.0	62.93	75.0	154.6	77.0	332.4	75.0	617.1
	90.0	37.62	90.0	175.1	90.0	216.1	90.0	571.9
co (µmol/l)	453		650		941		1077	
Dose ($mg.kg^{-1}.hr^{-1}$)	48		62		93		102	
t0.5 (min)	28		45		46		107	

Table 7-1

Bilirubin half-life, plasma concentration at zero time and dose in four neonatal guinea pigs

with bilirubin. First order kinetics are only seen if conjugation is not the limiting factor. As 90% Of the plateau concentration was reached at 3.3 half-lives, all further infusions were carried out for three hours.

b) To see if a steady-state had been reached after 3 hours constant bilirubin infusion

Method

Three guinea pigs were prepared as for the previous experiment. Instead of administering a bolus of bilirubin, a constant infusion of bilirubin was maintained by means of a Palmer Pump (Chapter 2) for three hours. However each guinea pig received a different dose. Blood samples were withdrawn at known times throughout the infusion and replaced with saline. Bilirubin estimations were carried out on the plasma.

Results

The level of bilirubin in each sample was plotted against the time after the start of the infusion for each animal (Figure 7:1). The maximum time required to reach a plateau was 2 hours. This corresponded to the guinea pig which received the highest dose of bilirubin (56 mg kg^{-1} hr^{-1}). For all experiments in which bilirubin was infused without PCS, maximum doses of approximately 56 mg kg^{-1} hr^{-1} were used and infusion times of 3 hours.

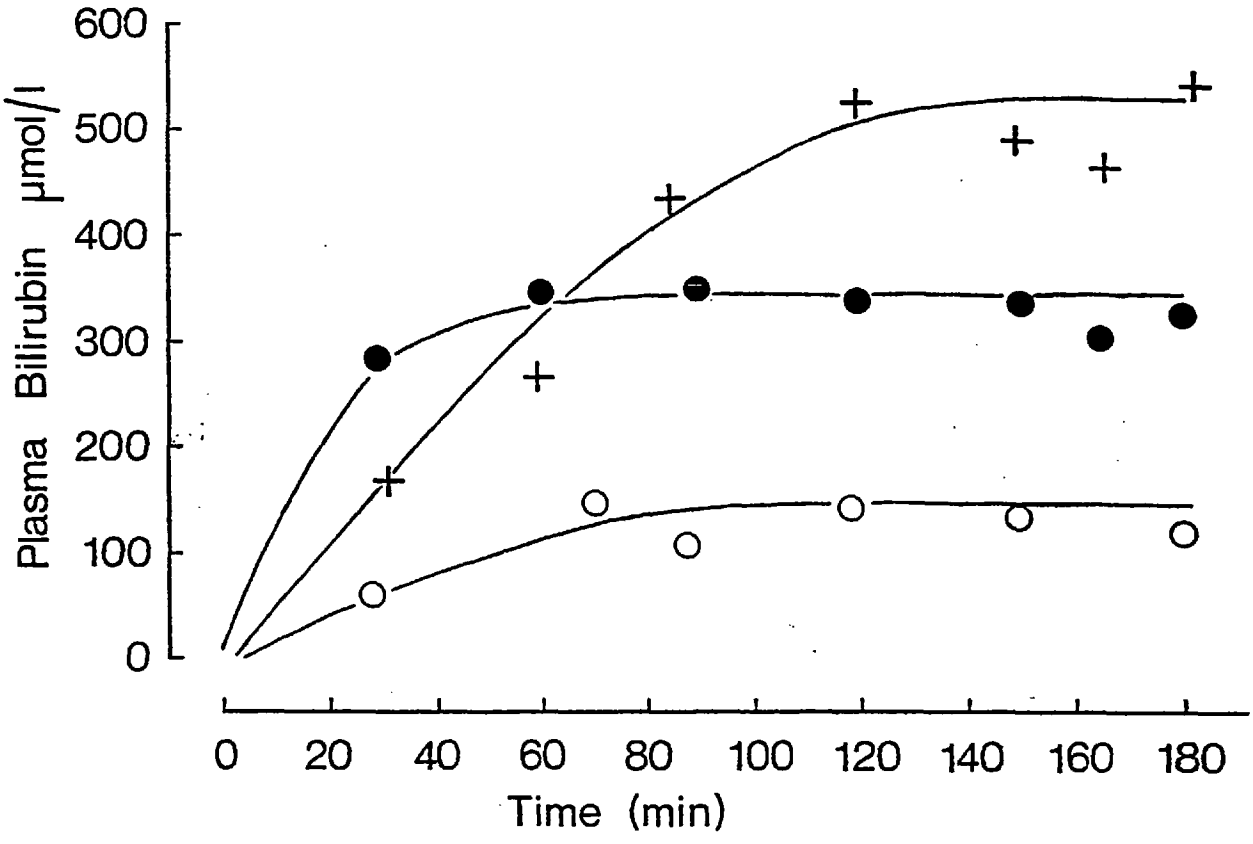


Figure 7:1 Plasma bilirubin levels during bilirubin infusion in three neonatal guinea pigs

Drug studies

a) In guinea pigs infused with bilirubin only

Controls

The animals were weighed, anaesthetised and prepared for infusion by insertion of PE-10 cannulae into both jugular veins (Chapter 2). Saline was infused into one side using a Palmer pump, set at a speed of 1"/320 minutes. Using a 10 ml Plastipak syringe, this gives an infusion rate of 0.75 ml/hr.

Bilirubin was dissolved in 5 mM sodium taurocholate in isotonic saline containing 0.1 N NaOH and the pH adjusted to 8 with 1 N acetic acid. The solution was infused concurrently into the opposite vein by a second pump loaded with a 10 ml Plastipak syringe and set at a speed of 1"/160 minutes, corresponding to an infusion rate of 1.5 ml/hr. The bilirubin in the syringe was kept covered throughout the infusion to minimise photodecomposition. At the end of the 3 hour infusion, each animal had received 6.75 ml of infusate.

Each animal received differing doses of bilirubin. Bilirubin concentrations were chosen so that the final plasma concentrations were within the physiological range for human neonates. Six animals were infused with bilirubin doses ranging from 44-55 $mg.kg^{-1}.hr^{-1}$. If the cannula became blocked due to the vein becoming twisted during the course of the infusion, the experiment was terminated.

After 3 hours bilirubin and saline infusion, the pumps were switched off and the animal quickly replaced in ether until unconscious. A blood sample for plasma bilirubin estimation was obtained by passing a heparinised 21 gauge needle attached to a 1 ml syringe into the heart via the diaphragm. Blood was withdrawn not more than 5 minutes after the end of the infusion.

The blood was placed in a heparinised container and spun at 3000 rpm for 10 minutes. The plasma was drawn off and used for the biochemical estimation of bilirubin (Chapter 2). The animal was decapitated and the brain removed. An abdominal incision was made and all the easily accessible peritoneal fat was removed. Brain and fat were either analysed for bilirubin immediately or they were kept deep frozen ($-20^{o}C$) for up to two weeks until analysed.

Animals receiving sulphafurazole

Sulphafurazole (Sigma) is not soluble at physiological pH, it was therefore dissolved in isotonic saline containing 0.1 N NaOH and adjusted to pH 8 with 1 N acetic acid. Each animal received a dose of 33.3 $mg.kg^{-1}.hr^{-1}$. The drug and saline (0.75 ml/hr) were infused into one jugular vein and the bilirubin solution was infused into the opposite vein (1.5 ml/hr). Bilirubin doses ranged from 41-54 $mg.kg^{-1}.hr^{-1}$. 6 animals were treated with sulphafurazole.

Animals receiving cloxacillin or flucloxacillin

Sodium cloxacillin and sodium flucloxacillin (Beecham Research Laboratories) were dissolved in isotonic saline so that each animal

received a drug dose of 166 $mg.kg^{-1}.hr^{-1}$. Drug and saline (0.75 ml/hr) were infused into one jugular vein and the bilirubin solution into the opposite vein (1.5 ml/hr). Bilirubin doses ranged from 40-56 $mg.kg^{-1}.hr^{-1}$. Six animals were treated with cloxacillin and 7 animals with flucloxacillin.

b) In guinea pigs infused with bilirubin and pooled cord serum (PCS) Controls

Animals received 11.53 ml $PCS.kg^{-1}.hr^{-1}$. Serum replaced the saline in the previously described series and the infusion rate was 0.75 ml/hr. PCS was also added to the bilirubin infusate (1.5 ml/hr). The PCS dose was the maximum that could be infused without altering the total volume of infusate when compared to the experiments in which the animals received bilirubin only. Six animals received bilirubin and PCS. The same infusion time (3 hrs) was used.

Animals receiving sulphafurazole

Sulphafurazole is not soluble in PCS, it was therefore dissolved in 2 ml 0.1 N NaOH and 1 ml saline. 3 ml PCS were then added and this solution was infused at 1.5 ml/hr. The drug dose was 33.3 $mg.kg^{-1}.hr^{-1}$. The bilirubin - PCS solution was infused at a rate of 1.5 ml/hr and the bilirubin doses ranged from 42-58 $mg.kg^{-1}.hr^{-1}$. Nine animals were treated with sulphafurazole.

Animals receiving cloxacillin or flucloxacillin

Both drugs are soluble in PCS. A dose of 166 $mg.kg^{-1}.hr^{-1}$ was administered, dissolved in PCS at a speed of 0.75 ml/hr. Bilirubin doses ranged from

45-56 $mg.kg^{-1}.hr^{-1}$. Five animals received cloxacillin and 6 flucloxacillin.

RESULTS

In vitro studies

Figure 7:2 shows the absorbance of the chloroform extracts (unbound bilirubin) corresponding to each bilirubin - GP-PS solution plotted against the total bilirubin concentration in each sample. Each point is the mean of two samples of equivalent bilirubin concentration. The effect of 1 mM sulphafurazole is shown.

Figure 7:3 shows the effect of 2 mM cloxacillin and flucloxacillin on bilirubin binding by GP-PS. Regression lines were calculated by computer using the method of least squares.

Table 7-2 gives the unbound/total bilirubin ratios for the artificially jaundiced sera before and after treatment with 1 mM sulphafurazole.

Statistics

The ratios of the unbound/total bilirubin for control and drug treated sera were assumed to be constant and were therefore treated statistically in the same way as the ratios brain/plasma or fat/plasma in the in vivo experiments. The mean of the ratios for the drug treated sera was compared with the mean for the controls using Student's 't' test for unpaired data.

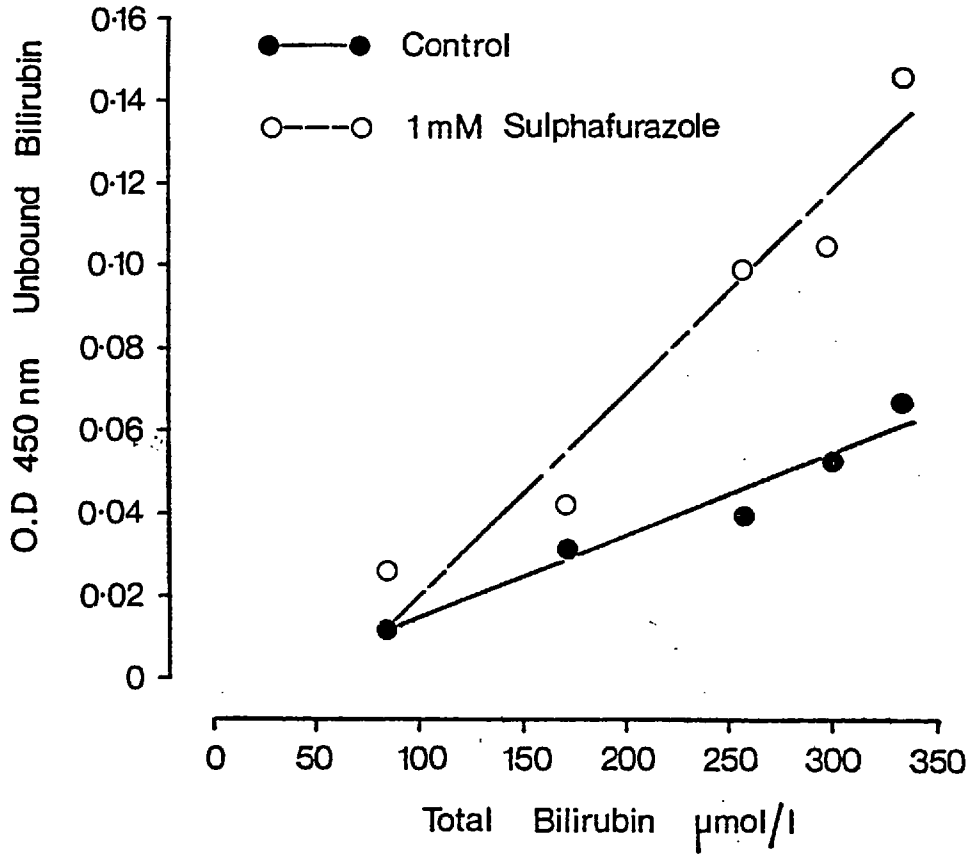


Figure 7:2 Effect of total bilirubin concentration on unbound bilirubin concentration in GP-PS, closed circles controls, open circles GP-PS containing sulphafurazole

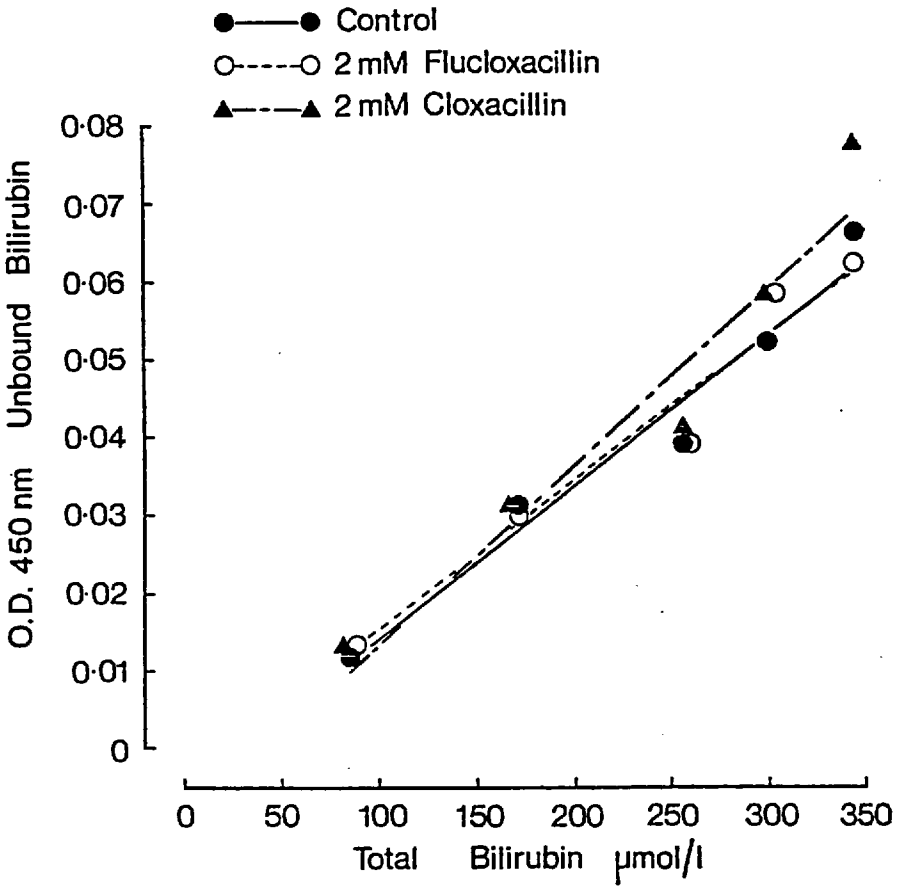


Figure 7:3 Effect of total bilirubin concentration on unbound bilirubin concentration in GP-PS, closed circles controls, open circles GP-PS containg 2 mM flucloxacillin, closed triangles GP-PS containing 2 mM cloxacillin

	Control	Sulphafurazole
Total Serum Bilirubin (μmol/l)	$\frac{\text{unbound}}{\text{total}}$ bilirubin	$\frac{\text{unbound}}{\text{total}}$ bilirubin
85.5	0.046	0.100
171	0.059	0.081
256.5	0.050	0.126
299.2	0.057	0.114
342	0.063	0.139
Mean ± SEM	$\bar{x}$ = 0.055 ± 0.003	$\bar{x}$ = 0.112* ± 0.010

*P < 0.005 when compared to control

Table 7-2

Unbound/total bilirubin ratios for artificially jaundiced guinea pig sera before and after treatment with 1 mM sulphafurazole

1 mM sulphafurazole significantly displaced bilirubin from its binding sites on neonatal guinea pig albumin, over serum bilirubin concentrations of 85.5-342 µmol/l. The mean unbound/total ratio approximately doubled when sera were treated with 1 mM sulphafurazole. Neither cloxacillin or flucloxacillin displaced bilirubin from GP-PS. In spite of these negative results, the effect of these drugs on bilirubin disposition was investigated in order to check that in vivo findings were compatible with in vitro results.

In vivo studies

Table 7-3 gives the brain/plasma, fat/plasma and fat/brain ratios with the mean ± SEM in animals that had received bilirubin and one of the three drugs, or saline.

Table 7-4 gives the same ratios in animals that had received bilirubin and either saline or PCS.

Table 7-5 gives the same ratios in animals that had received bilirubin, PCS and either one of the three drugs or no drug.

Statistics

The ratio $(\frac{y}{x})$ for any two of the measured variables; plasma, brain anf fat, were independent of the value of (x). They were therefore taken to be constants, described by the equation $y = mx + c$ where $C = 0$, $\frac{y}{x}$ = the ratio of any two of the variables and m = the mean of the group of ratios.

Brain / Plasma				Fat / Plasma				Fat / Brain			
Control	Sulf.	Fluclox.	Clox.	Control	Sulf.	Fluclox.	Clox	Control.	Sulf.	Fluclox.	Clox.
0.071	0.133	0.130	0.072	0.402	-+	0.608	0.565	5.66	-+	4.66	7.80
0.110	0.134	0.105	0.060	0.663	0.048	0.626	0.372	6.00	3.04	5.94	6.16
0.106	0.132	0.087	0.087	0.639	0.484	0.433	0.420	6.01	4.13	4.94	4.80
0.082	0.121	0.107	0.083	0.451	0.717	0.494	0.376	5.47	5.93	4.59	4.53
0.073	0.151	0.087	0.119	0.378	0.528	0.668	0.765	5.13	3.49	7.66	6.38
0.067	0.151	0.086	0.100	0.467	0.990	0.522	0.431	6.88	6.55	6.06	4.30
	0.157	0.111			0.519	0.591			3.30	5.30	
		0.132				0.533				4.01	
$\bar{x}$=0.084	$\bar{x}$=0.139*	$\bar{x}$=0.105***	$\bar{x}$=0.086	$\bar{x}$=0.500	$\bar{x}$=0.607	$\bar{x}$=0.559	$\bar{x}$=0.488	$\bar{x}$=5.85	$\bar{x}$=4.40**	$\bar{x}$=5.39	$\bar{x}$=5.66
± 0.007	± 0.005	± 0.006	± 0.008	± 0.048	± 0.086	± 0.027	± 0.062	± 0.24	± 0.60	± 0.406	± 0.550

*P < 0.005 **P < 0.025 ***P < 0.05 when compared by Student's 't' test +Fat not obtained

Table 7-3

Brain/plasma, fat/plasma and fat/brain ratios in guinea pigs treated with bilirubin and sulphafurazole, flucloxacillin or cloxacillin. Means for each group are compared with means for animals receiving saline

Brain / Plasma		Fat / Plasma		Fat / Brain	
Control	Pooled Cord Serum	Control	Pooled Cord Serum	Control	Pooled Cord Serum
0.071	0.023	0.402	0.179	5.66	6.53
0.110	0.039	0.663	0.259	6.00	6.51
0.106	0.035	0.639	0.250	6.01	6.97
0.082	0.036	0.451	0.216	5.47	5.89
0.073	0.033	0.378	0.195	5.13	5.81
0.067	0.038	0.467	0.201	6.88	5.18
$\bar{x}$=0.084	$\bar{x}$=0.034*	$\bar{x}$=0.500	$\bar{x}$=0.216*	$\bar{x}$=5.85	$\bar{x}$=6.14
± 0.007	± 0.002	± 0.048	± 0.012	± 0.24	± 0.26

*P < 0.005 when compared by Student's 't' test

Table 7-4

Brain/plasma, fat/plasma and fat/brain ratios in guinea pigs treated with bilirubin and saline or pooled cord serum. Means for each group are compared to the means for animals receiving saline.

Brain / Plasma				Fat / Plasma				Fat / Brain			
Control	Sulf.	Fluclox.	Clox.	Control	Sulf.	Fluclox.	Clox	Control.	Sulf.	Fluclox.	Clox.
0.027	0.054	0.036	0.043	0.179	0.336	0.266	0.260	6.53	5.92	7.4	9.98
0.039	0.071	0.031	0.024	0.259	0.310	0.188	0.154	6.51	4.35	5.94	6.38
0.035	0.034	0.042	0.039	0.250	0.254	0.233	0.241	6.97	7.36	5.45	6.15
0.036	0.045	0.045	0.036	0.216	0.278	0.325	0.195	5.89	6.13	7.21	5.36
0.033	0.032	0.023	0.037	0.195	0.160	0.134	0.188	5.81	5.00	5.8	5.06
0.038	0.047			0.201	0.287			5.18	6.03		
	0.030				0.216				7.22		
	0.031				0.199				6.47		
$\bar{x}$=0.034	$\bar{x}$=0.043	$\bar{x}$=0.035	$\bar{x}$=0.035	$\bar{x}$=0.216	$\bar{x}$=0.250	$\bar{x}$=0.229	$\bar{x}$=0.207	$\bar{x}$=6.14	$\bar{x}$=6.06	$\bar{x}$=6.36	$\bar{x}$=6.58
± 0.002	± 0.005	± 0.004	± 0.003	± 0.012	± 0.021	± 0.032	± 0.019	± 0.26	± 0.35	± 0.39	± 0.88

Table 7-5

Brain/plasma, fat/plasma and fat/brain ratios in guinea pigs treated with bilirubin, pooled cord serum and sulphafurazole, flucloxacillin or cloxacillin. Means for each group are compared with means for animals receiving no drug. No statistically significant differences are observed using Student's 't' test.

Student's 't' test for unpaired data was used to compare the mean ratios in animals treated with drug and/or PCS, with the mean ratios in the controls. P values > 0.05 were not accepted as significant.

Guinea pigs infused with bilirubin and saline

The mean brain/plasma ratios for animals treated with sulphafurazole and flucloxacillin were significantly higher than the mean brain/plasma ratios for animals not receiving drug. However the difference observed with flucloxacillin was only significant at the 5% level. Cloxacillin did not exert a significant effect. The mean fat/plasma ratio was not significantly altered by any of the three drugs studied.

The mean fat/brain ratio for animals treated with sulphafurazole was significantly lower than the mean fat/brain ratio of animals not receiving drug. The mean fat/brain ratios for flucloxacillin and cloxacillin were not significantly altered (Table 7-3).

Figure 7:4 shows how brain bilirubin levels vary with plasma bilirubin levels for control animals and for animals treated with sulphafurazole. All regression lines were calculated by computer by the method of least squares. It can be seen that the regression line for drug treated animals was displaced upwards compared to the control line. Figures 7:5 and 7:6 show the effect of cloxacillin and flucloxacillin on the relationship between plasma bilirubin levels and brain bilirubin levels. Regression lines have been drawn.

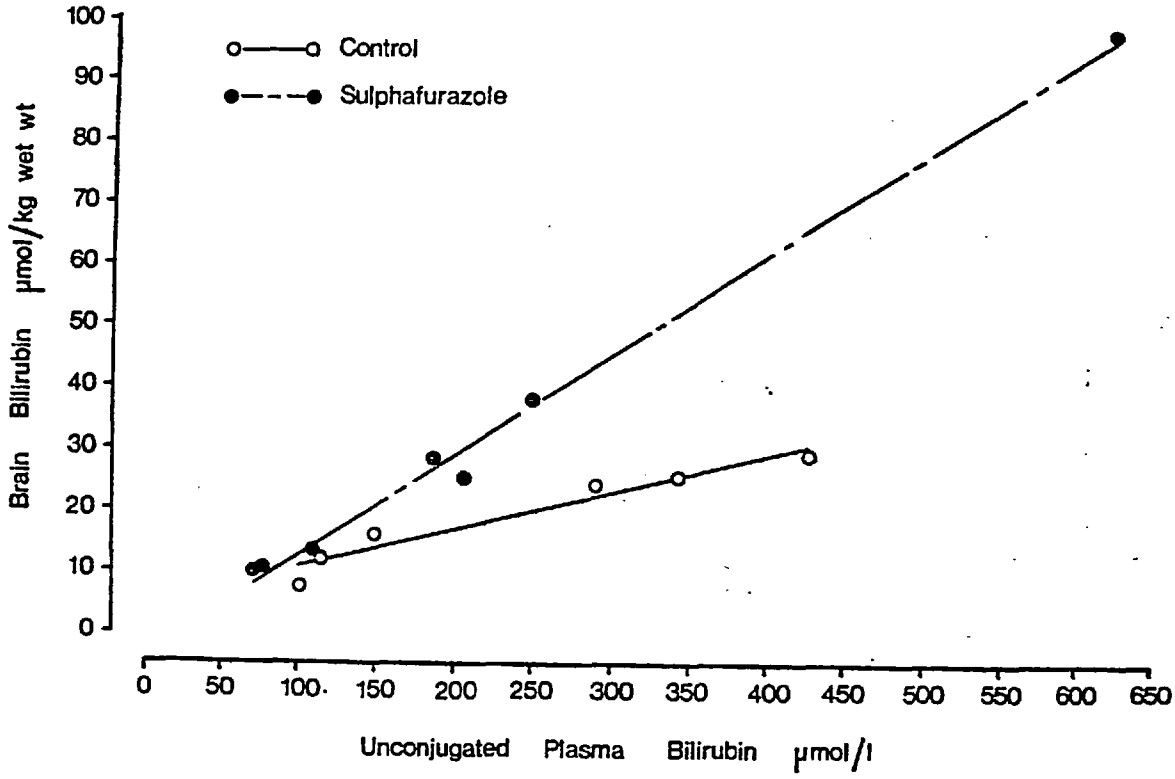


Figure 7:4 Relationship between unconjugated plasma bilirubin levels and brain bilirubin levels in neonatal guinea pigs. Open circles vehicle injected controls, closed circles guinea pigs receiving sulphafurazole.

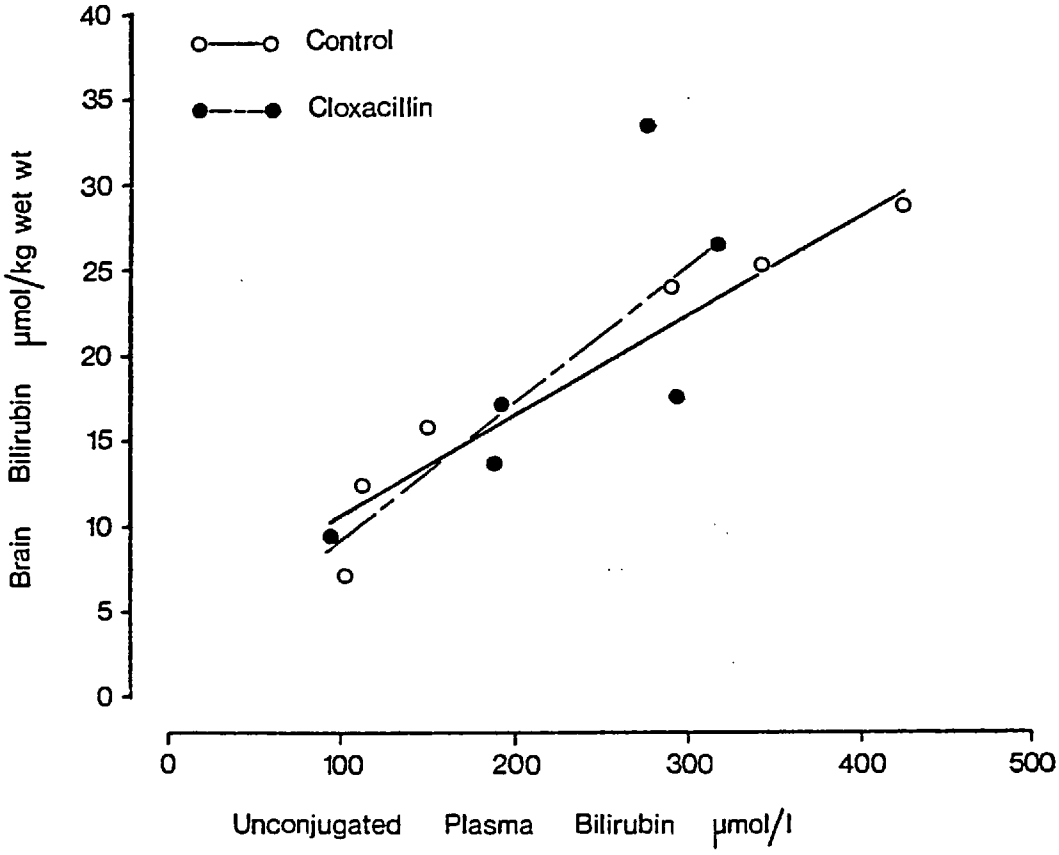


Figure 7:5 Relationship between unconjugated plasma bilirubin levels and brain bilirubin levels in neonatal guinea pigs. Open circles vehicle injected controls, closed circles, guinea pigs receiving cloxacillin

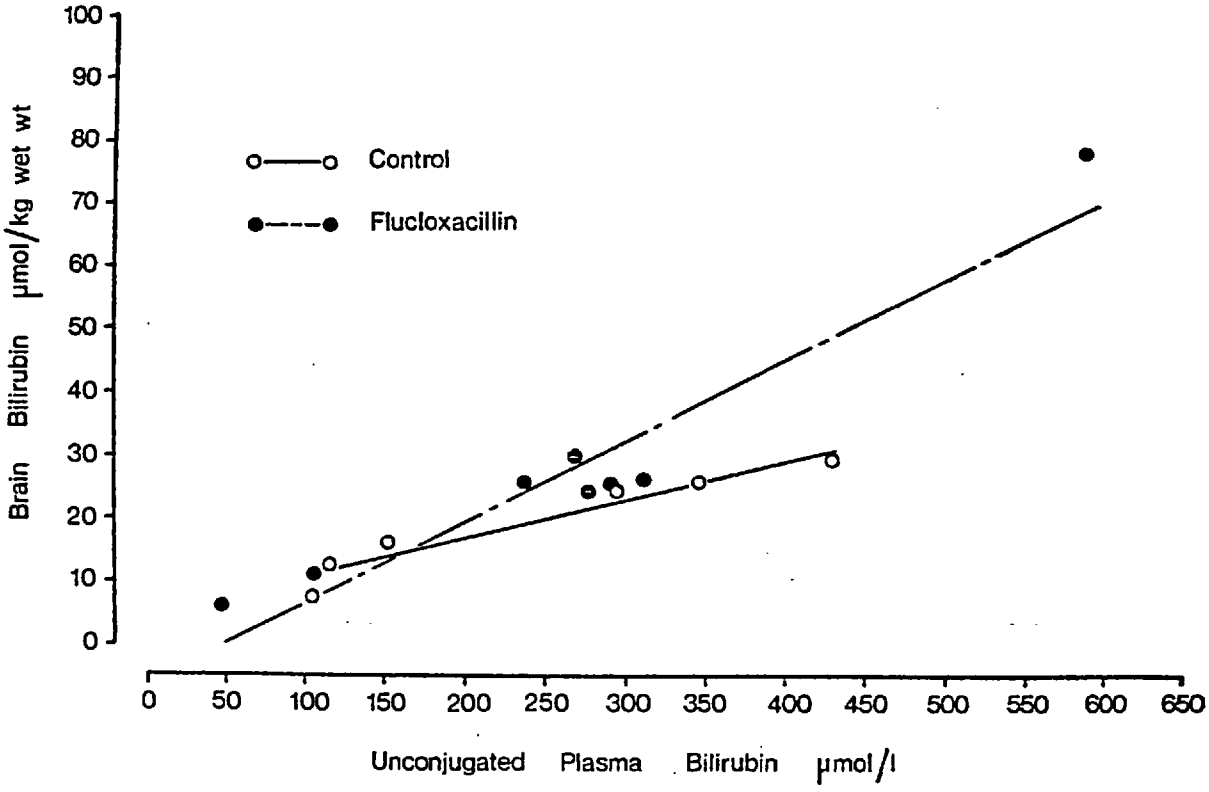


Figure 7:6 Relationship between unconjugated plasma bilirubin levels and brain bilirubin levels in neonatal guinea pigs. Open circles vehicle injected controls, closed circles guinea pigs receiving flucloxacillin

Guinea pigs infused with bilirubin and PCS

The mean brain/plasma ratio for animals infused with PCS was significantly lower than for animals infused with saline. The mean fat/plasma ratio was also significantly reduced. However the mean fat/brain ratio remained unchanged (Table 7-4).

Neither the mean brain/plasma, fat/plasma nor the fat/brain ratio were significantly changed by treatment of the guinea pigs with drugs in combination with PCS compared with animals which only received PCS (Table 7-5).

Figure 7:7 shows the effect of infusing PCS and bilirubin on the relationship between plasma bilirubin levels and brain bilirubin. It can be seen that the regression line for animals receiving PCS is displaced downwards and to the right compared to the line for animals receiving no PCS.

Figure 7:8 shows the effect of sulphafurazole on the relationship between plasma bilirubin levels and brain bilirubin. Figure 7:9 shows the effect of cloxacillin and flucloxacillin on the relationship between plasma bilirubin levels and brain bilirubin.

DISCUSSION

In vitro findings showed that 1 mM sulphafurazole displaced bilirubin from GP-PS, whereas 2 mM cloxacillin and flucloxacillin did not. These results illustrate the different binding characteristics of

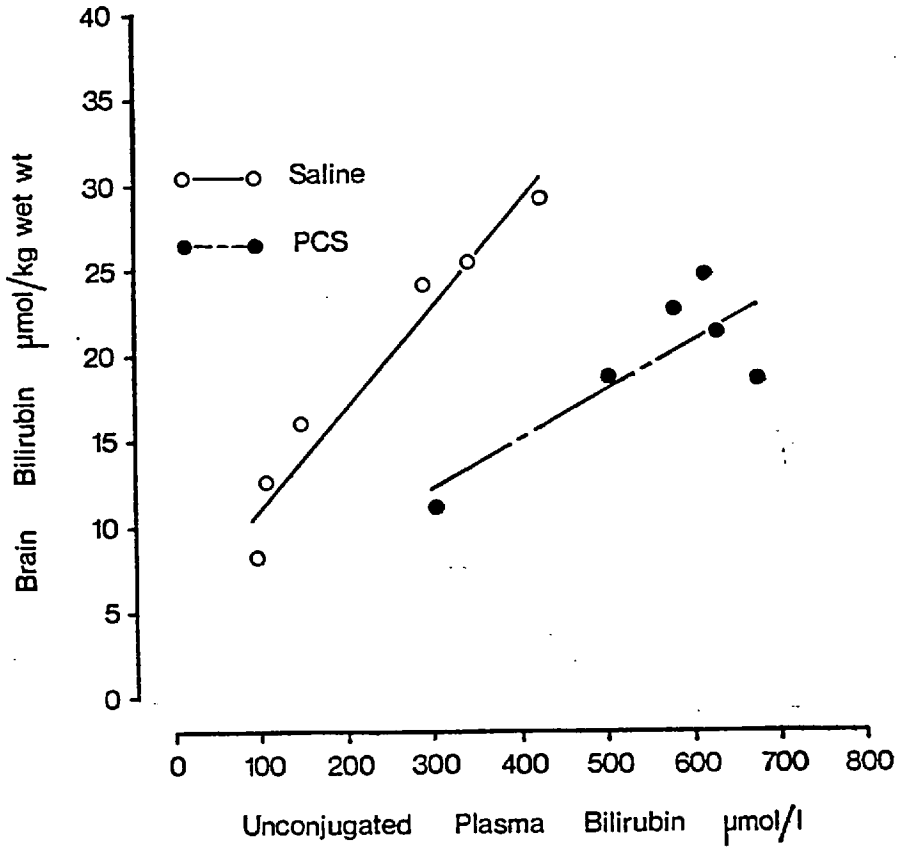


Figure 7:7 Relationship between unconjugated plasma bilirubin levels and brain bilirubin levels in neonatal guinea pigs. Open circles vehicle injected controls, closed circles guinea pigs injected with pooled cord serum

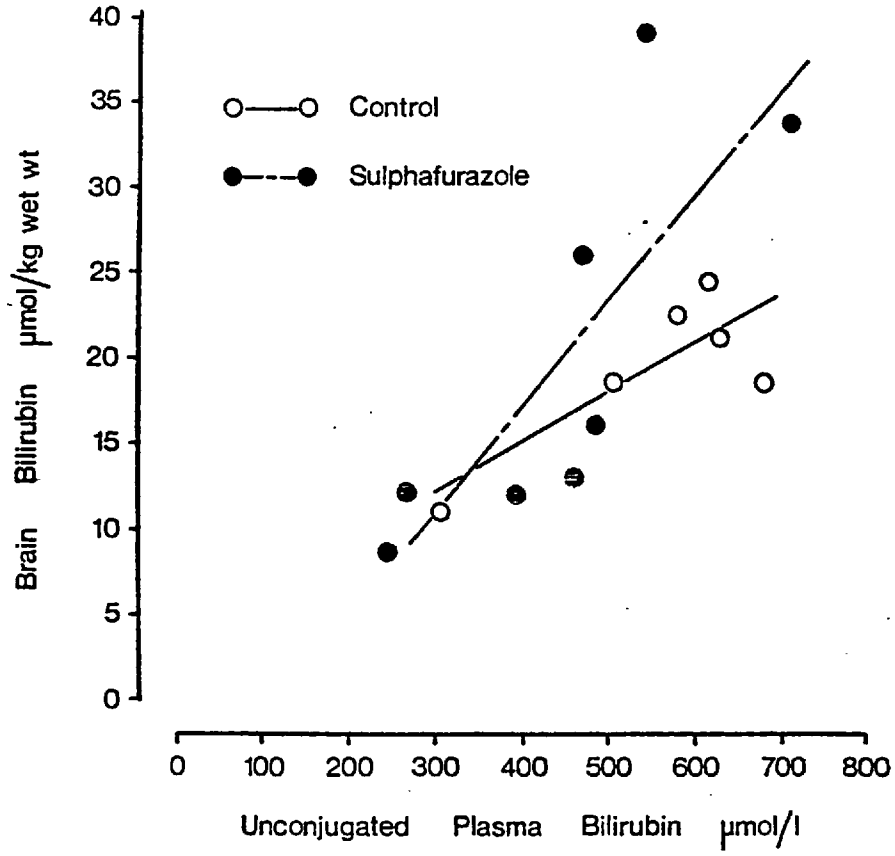


Figure 7:8 Relationship between unconjugated plasma bilirubin levels and brain bilirubin levels in neonatal guinea pigs Open circles vehicle injected controls (PCS), closed circles guinea pigs receiving sulphafurazole

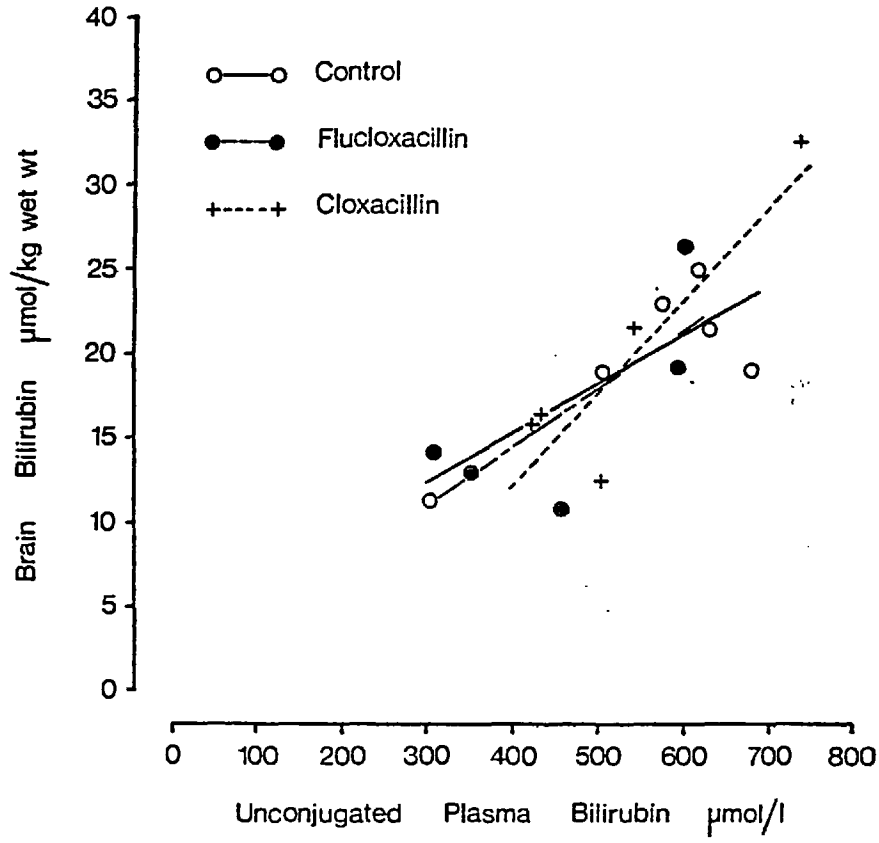


Figure 7:9 Relationship between unconjugated plasma bilirubin levels and brain bilirubin levels in neonatal guinea pigs. Open circles vehicle injected controls (PCS), closed circles guinea pigs receiving flucloxacillin, crosses guinea pigs receiving cloxacillin

albumin of different species since both these drugs failed to displace bilirubin from bovine albumin yet they displaced bilirubin from human PCS.

A linear and constant relationship was observed between plasma bilirubin levels and brain bilirubin levels in guinea pigs, indicating that the binding sites for bilirubin on guinea pig albumin are homogeneous over the range of bilirubin concentrations studied, unlike human bilirubin which has at least 2 types of site (see Chapter 6). Consequently, only the effect of highly protein bound drugs on the affinity of albumin for bilirubin can be studied in vivo using the guinea pig, information regarding possible changes in capacity cannot be obtained.

However, by studying the relationships between the bilirubin content of brain, fat and plasma in control animals and in those treated with drug, the mechanism by which drugs are kernicterogenic may be more fully elucidated.

In the control animals, the fat/plasma ratio was approximately 6 times the brain/plasma ratio. This preferential uptake by fat probably represents the greater lipid content of fat as compared to brain. Similar disparity has been shown by Davis and Yeary (1975) who found that the bilirubin ratio of renal fat/brain in adult Gunn rats was 10.33.

Sulphafurazole, the positive control, significantly increased the mean

brain/plasma bilirubin ratio. Petite, Etienne and Housset (1968) showed that 300-500 mg/kg of sulphafurazole injected subcutaneously into Gunn rats caused a fall in plasma bilirubin of 49%, 30 minutes after injection. Thus an increase in brain bilirubin is accompanied by a fall in plasma bilirubin.

Flucloxacillin, also significantly increased the mean brain/plasma bilirubin ratio but only after doses 5 times as great as the sulphafurazole dose. The present results confirm studies carried out by Hanefeld and Ballowitz (1976) on Gunn rats. They gave 2-10 injections of 500-1000 mg/kg bodyweight flucloxacillin to 18 young homozygous Gunn rats. The animals were killed at age 10-15 days. Kernicterus was studied in the cerebellum by grading the number of damaged Purkinje cells. After the injections of flucloxacillin, the serum bilirubin fell to 50-70% of the baseline concentrations of 120-154 µmol/l. All but three of the 18 homozygous infant Gunn rats showed a striking loss of Purkinje cells compared with the mild damage to these neurones in untreated littermates.

The disparity between the negative flucloxacillin in vitro results and the positive in vivo results is probably due to very high in vivo doses of flucloxacillin administered, which in fact only resulted in changes significant at the 5% level.

Cloxacillin did not alter bilirubin disposition, in agreement with in vitro results using GP-PS.

Although sulphafurazole caused an increase in the mean brain/plasma ratio, indicating an increase in the free plasma bilirubin level, this increase was not reflected by a significant increase in fat/plasma ratio, but by a decrease in fat/brain ratio. Flucloxacillin also caused a significant increase in the brain/plasma ratio, however the changes in fat/plasma and fat/brain ratios were not found to be statistically significant.

The apparent redistribution of free bilirubin favouring uptake by brain in the presence of sulphafurazole is also seen to occur with sulfadimethoxine, another highly protein bound sulfonamide which displaces bilirubin from albumin. Davis and Yeary (1975) treated adult Gunn rats with radio-labelled sulfadimethoxine (25 mg/kg IP) and subsequently measured bilirubin concentrations in various tissues. Analysis of their data reveals that the mean brain/plasma ratio (3 rats) rose from 0.005 in control rats to 0.018 in drug treated rats. The mean fat/plasma ratio rose from 0.07 to 0.10 after treatment, however the fat/brain ratio fell from 10.33 in the controls to 5.70 in drug treated animals.

Ballowitz, Hanefeld, Jerofke, Keller, Korn and Schweitzer (1978) used Sephadex Gel filtration (SGF) to measure the presence of free bilirubin in sera from animals after treatment with sulfadimethoxine. They found that when 9-10 day old Gunn rats who had previously had positive SGF

readings, were injected subcutaneously with 100 mg/kg sulfadimethoxine the SGF test carried out after 15-20 minutes on sera from the rats unexpectedly gave negative results. Drug interference with the binding capacities of the Sephadex column was excluded. Adult rats, however, who had previously had negative SGF results gave positive results 20-60 minutes after drug injection. These results perhaps also suggest a possible redistribution of free bilirubin, at least in infant animals.

PCS infused into the animals, provided an alternative means of altering circulating free bilirubin levels; by increasing the plasma albumin and subsequent binding capacity of the plasma. Examination of the mean brain/plasma, fat/plasma and fat/brain bilirubin ratios revealed that under these circumstances the brain/plasma and fat/plasma ratios were decreased, however the fat/brain ratio remained unchanged. These results further suggest that preferential uptake of bilirubin by brain only occurs in the presence of drug.

Negative results obtained with all three drugs in animals infused with PCS are rather unexpected due to the fact that sulphafurazole gave a positive result in animals not infused with PCS and all 3 drugs displace bilirubin from PCS in vitro (Chapter 6). Furthermore, Diamond and Schmid (1966) were able to show that salicylate caused an increase in bilirubin in the brain in the presence of human albumin in neonatal guinea pigs.

However, the dose of salicylate infused was approximately 1000 $mg.kg^{-1}.hr^{-1}$, 30 times the dose of sulphafurazole used in this study. Also a much higher human albumin concentration could be obtained in the guinea pig by infusing a pure human albumin solution rather than serum. Unfortunately, pure neonatal albumin is not commercially available. They were able to reduce the brain/plasma ratio approximately tenfold whereas infusion of PCS only reduces the ratio twofold.

Blood-brain barrier

The existence of a blood-brain barrier (BBB) for bilirubin, which matures gradually in the first few weeks of life, is often cited as the reason why only infants appeared susceptible to kernicterus, although there was no scientific basis for this assumption.

Experiments carried out by Diamond and Schmid (1966) in which adult Gunn rats were found to be as susceptible to KI as neonatal guinea pigs, have to some extent led to a decrease in the 'BBB' hypothesis.

In this study in which 7-14 day guinea pigs were used, a mean brain/plasma bilirubin ratio of 0.084 was obtained. This value is very close to a brain/plasma ratio of 0.080 obtained from guinea pigs born by caesarean section using radioactive bilirubin (Diamond and Schmid, 1966). If a slowly maturing BBB was present, then one would expect to see a fall in brain/plasma ratio by two weeks of age.

Furthermore, the guinea pig shows a high degree of pre-natal development; the barrier of ferrocyanide for example, being established at its adult characteristic at birth. Since the human neonate also shows a high degree of pre-natal development, unlike the rabbit, rat, mouse cat and dog which are all born with their eyes closed, there is no logical reason why adults should not be as susceptible to KI as neonates. In fact, a case of KI has been reported in a 15 year old boy who suffered from Crigler-Najjar syndrome, a condition in which bilirubin glucuronyl transferase is absent.

It seems, therefore, that for KI to occur a critical plasma bilirubin level must be obtained. This is why adults are less susceptible since increased bilirubin clearance and normal albumin levels prevent a sufficient build-up of free bilirubin.

Since sulphafurazole was found to displace bilirubin from GP-PS and PCS in vitro and was kernicterogenic in vivo, these results suggest that Sephadex Gel filtration changes do predict disposition changes in vivo and caution against the use of cloxacillins in neonates.

CHAPTER EIGHT

TRIAL OF ANTIPYRINE AS PROPHYLAXIS FOR JAUNDICE IN HUMAN NEONATES

SUMMARY

A double blind trial of antipyrine, a known inducing agent, as prophylaxis for neonatal jaundice was carried out. 35 pregnant women took part in the trial. 20 women received antipyrine (300 mg daily) and 15 women lactose placebo from the 38th week of pregnancy until delivery. Serum bilirubin levels were measured in the infants on the 4th day of life.

The mean serum bilirubin in infants of drug treated mothers was significantly lower than in the control infants. Birth weights and sex ratios in the two groups were similar and no side effects were reported.

It is suggested that antipyrine is worthy of wider trial as a prophylactic treatment for neonatal jaundice.

INTRODUCTION

This chapter is concerned with prophylaxis of neonatal jaundice using antipyrine, an inducing agent of a different type to phenobarbitone and one which has not previously been used for this purpose.

Background

Glucuronyl transferase is a microsomal enzyme that is responsible for the glucuronidation of both drugs and the natural substrate bilirubin. In 1960, Conney, Davison, Gastel and Burns showed that the activity

of the glucuronyl transferase in rat liver could be increased by pre-treatment of the rat with phenobarbitone or agents such as 3-4 benz-pyrene. Catz and Yaffe (1962) and Arias, Gartner, Furman and Wolfson (1963) also showed an increased glucuronidation of bilirubin in rat liver after pretreatment of the rat with phenobarbitone. The natural sequel was the administration of phenobarbitone to man and Yaffe, Levy, Matsuzawa and Baliah (1966) and Crigler and Gold (1966) described one infant in each report with an unconjugated hyperbilirubinaemia in whom phenobarbitone treatment lowered the plasma bilirubin for a short period.

Clinical trials with phenobarbitone as prophylaxis for neonatal jaundice

Trials with phenobarbitone have shown that administration of the drug to mothers for at least a fortnight before delivery reduced the plasma bilirubin levels in the newborn infant (Trolle, 1968; Maurer, Wolff, Finster, Poppers, Pantuck, Kuntzman and Conney, 1968) and that administration of phenobarbitone to the newborn infant only, also reduced the plasma bilirubin levels (Trolle, 1968). This has resulted in a dramatic reduction of the need to perform exchange transfusions in the newborn (Yeung and Field, 1969).

Phenobarbitone has also been used successfully to lower the plasma bilirubin in adults with severe unconjugated hyperbilirubinaemia (Kreek and Sleisenger, 1968) and has been shown to result in lowered plasma bilirubin levels in epileptics (Thompson, Eddleston and Williams, 1969).

This reduction in plasma bilirubin levels is often assumed to be due to an increase in glucuronyl transferase activity, yet in recent studies (Orme, Davies and Breckenridge, 1974) it was found that phenobarbitone does not increase the activity of hepatic glucuronyl transferase activity in rats. However, phenobarbitone has been shown to increase liver blood flow (Ohnhaus, Thorgeirsson, Davies and Breckenridge, 1971) and hepatic binding proteins (Reyes, Levi, Gatmaitan and Arias, 1969) all of which may contribute to the lowering of total serum bilirubin.

Recently, an increased rate of transfer of bilirubin from liver to bile has been implicated in the stimulatory effects of inducers on bilirubin clearance in the neonate (Klaasen, 1976). This might explain why earlier peak serum bilirubins are observed in infants whose mothers received phenobarbitone in the last two weeks of pregnancy (Maurer, Wolff, Finster, Poppers, Pantuck, Kuntzman and Conney, 1968).

Toxicity of phenobarbitone

Unfortunately, phenobarbitone has the disadvantage of being a sedative and there is some doubt in the literature as to whether phenobarbitone therapy is entirely safe, especially in children (Wilson, 1969). Schulz and Van Creveld (1958) reported an example of haemorrhagic disease in a newborn infant whose mother received large doses of phenobarbitone. A deficiency of Stuart-Prower factor was thought to be the cause.

These undesirable side effects probably explain why so few clinicians use phenobarbitone as prophylaxis for neonatal jaundice as shown in the survey discussed in Chapter Three.

Other prophylactic treatments

Uridine diphosphate-glucose (Careddu and Marini, 1968) has been successfully used to treat neonates for hyperbilirubinaemia however its use has not subsequently been adopted as prophylaxis for neonatal jaundice.

In 1969, Waltman, Bonura, Nigrin and Pipat reported that ethanol given to the mother prior to delivery can considerably lower the bilirubin level of the newborn. Contradictory findings have been reported by Jouppila, Koivisto and Suonio (1973) who did not find that bilirubin levels of the newborns whose mothers were treated with ethanol were significantly lower during the first six days of life.

Maternal cigarette smoking has also been shown to significantly depress neonatal serum bilirubin levels (Nymand, 1974). It is suggested that the cyanide originating from tobacco smoke induces glucuronyl transferase. However, cigarette smoking as prophylaxis for neonatal jaundice is not recommended!

Bucolome a non-hypnotic barbituric product has been used as prophylaxis for neonatal jaundice as a favourable alternative to phenobarbitone, Segni, Polidori and Romagnoli (1977) treated 25 preterm babies with oral bucolome (30 mg/kg per day for 5 days) and serum bilirubin levels

were measured and compared with levels in a control group. Serum bilirubin levels in treated infants from day 4 onwards were consistently lower than those of the control infants.

Bucolome has subsequently been found to precipitate kernicterus in jaundiced Gunn rats 6 hours after an i.v. injection of drug. The plasma total bilirubin fell immediately after the injection and the lowered level persisted for more than 6 hours (Semba, Sato and Yamamura, 1978). It is suggested that bucolome displaces bilirubin from albumin, transferring bilirubin from blood into tissues including the brain and that the use of bucolome in infants with hyperbilirubinaemia should be contra-indicated.

Antipyrine - an inducing agent

In 1974, Orme, Davies and Breckenridge showed that antipyrine (phenazone) was an inducer of bilirubin glucuronyl transferase in man and animals. In the clinical studies 300 mg antipyrine was prescribed twice daily for 8 weeks to patients suffering from Gilbert's disease, a condition of idiopathic unconjugated hyperbilirubinaemia. Weekly estimations of serum bilirubin over the last 6 weeks were compared with the values over the 6 weeks of the control period. After the start of the treatment the serum bilirubin fell quite rapidly and maximum reduction was achieved by 2 weeks. After this time, the serum bilirubin remained relatively constant. No adverse effects were seen.

Pharmacology of antipyrine

Antipyrine was introduced into medicine in 1884. It was originally employed for antipyresis but was later on prescribed for analgesia. Antipyrine lost favour as the salicylates became more prominent and is infrequently prescribed today.

The average therapeutic oral dose in adults is 300-600 mg 4 hourly. It is rapidly and essentially completely absorbed from the GI tract and peak plasma levels are attained in 1-2 hours. It is a pyrazolone derivative and is slowly metabolised in man, disappearing from the plasma at a rate averaging only 6% per hour. The drug is distributed in various tissues according to their water content and hence has been used for the measurement of total body water. Antipyrine is metabolised by the hepatic microsomal enzyme system to 4-hydroxyantipyrine (see figure 8:1), norantipyrine and 3-hydroxymethylantipyrine. Together, these metabolites account for more than 80% of the dose.

Antipyrine is an ingredient of dichloralphenazone which is a commonly prescribed hypnotic for women in late pregnancy and has been used for estimating total body water in low birth weight infants on the first and fourth day (Murdock, Thorgeirsson, Rossiger and Davies, 1975), i.v. doses of 25 mg/kg were used.

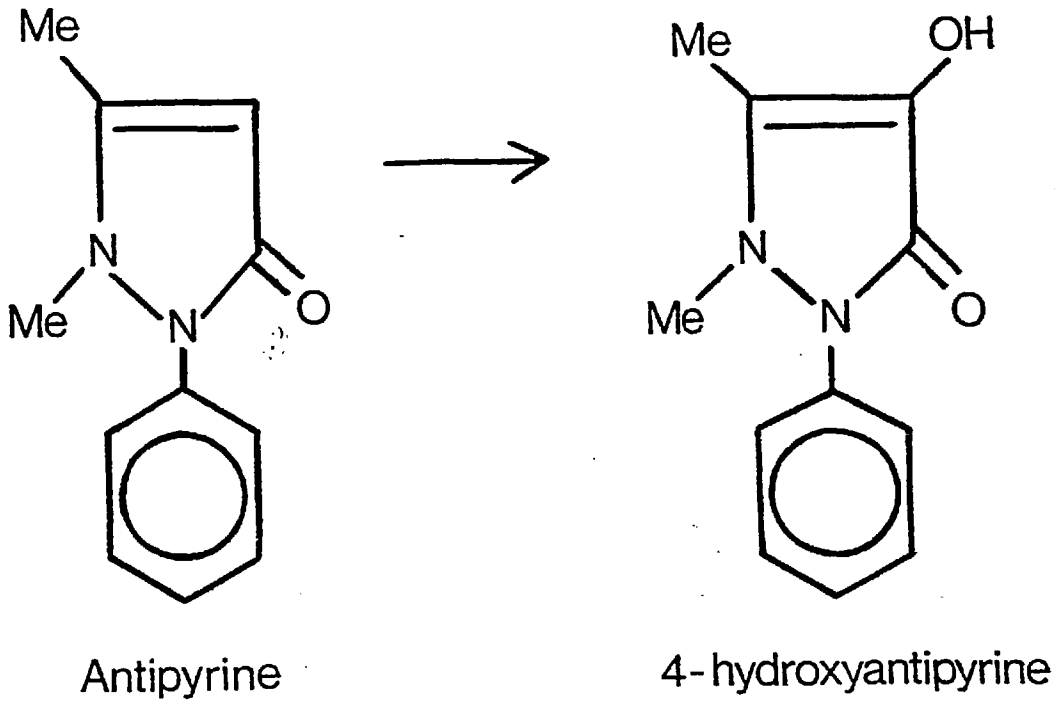


Figure 8:1 Hydroxylation of antipyrine to 4-hydroxyantipyrine

The preceding chapters in this thesis have dealt with the way in which drugs administered during the perinatal period may increase the incidence of neonatal jaundice or may precipitate kernicterus. This chpater is concerned with prophylaxis of neonatal jaundice at Queen Charlotte's Hospital with antipyrine, a known enzyme inducer not previously used for this purpose.

A double blind clinical trial was therefore carried out. Antipyrine was administered orally once daily for at least 9 days to mothers prior to delivery. Serum bilirubin levels were measured in neonates by heel prick on the fourth day of life. Serum bilirubin levels of infants of drug treated mothers were compared with levels in infants of mothers who had taken lactose placebo.

METHODS

Drug dose

The dose of antipyrine was 300 mg daily for at least 9 days prior to delivery. This dose of 300 mg is less than the antipyrine content of the usual dose of dichloralphenazone (1300 mg) of which approximately one third is antipyrine. Non-identical placebos contained lactose.

Patient selection

The notes of the patients to be approached for inclusion in the trial were identified by the research nurse prior to the 38 week ante-natal visit. They were selected according to the following criteria:-

38 weeks gestation, intended hospital stay of 7 days or more, no medical or obstetric problem, no asthma or allergies, white women only (jaundice rates are different in other races) and singleton pregnancies.

Patient enrolment

The pre-selected patients were enrolled into the study by the obstetrician seeing the patient at the clinic. The object of the trial was briefly explained to them. Antipyrine was prescribed on the patient's drug sheet and the patient was then dispensed either 21 antipyrine tablets or 21 placebos, according to a series of random numbers, by the hospital pharmacist. If the patient finished taking all 21 tablets prior to admission, a further 7 tablets were dispensed on return to the clinic.

Tablet counting

Date of admission of the patient was obtained from the hospital admissions sheet published daily by the medical records department. The patient was subsequently visited by the research nurse and the number of tablets remaining in the bottle counted. If no tablets had been taken after prescription, this was noted and the reason for refusal was obtained if possible. If the patient had taken at least 9 tablets she was re-informed that the baby's bilirubin would be measured on the fourth day after birth.

Bilirubin measurement

If the baby was born before 12.00 p.m. the date of birth was counted as day one, if it was born after 12.00 p.m. it was counted as day 0. On the morning of the fourth day, heel prick blood was collected for estimation of serum bilirubin. The status of the patient in the trial was unknown to the person who did the estimation. Serum bilirubin was measured biochemically (see Chapter 2) in the majority of cases, but occasionally had to be measured on the bilirubinometer.

RESULTS

Sixty-six women were enrolled into the trial by the obstetricians during their 38 week ante-natal visit. Thirty-five of these women were included in the final analysis. Twenty women had taken antipyrine and 15 lactose placebo.

Of the remaining 31 women, 12 refused subsequent to enrolment in the trial, 12 delivered before they could take 9 tablets, 2 gave up due to side effects, 2 were wrongly included, 1 was missed, 1 was discharged early and 1 baby was transferred to another hospital for an operation for congenital heart disease (see Table 8-1).

Serum bilirubin levels

The mean neonatal serum bilirubin level on the fourth day was 122.00 ± 17.8 µmol/l, range 27.4-243.0 µmol/l. The mean neonatal serum bilirubin in the drug treated group was 65.7 ± 13.0 µmol/l, range 8.0-222.0 µmol/l

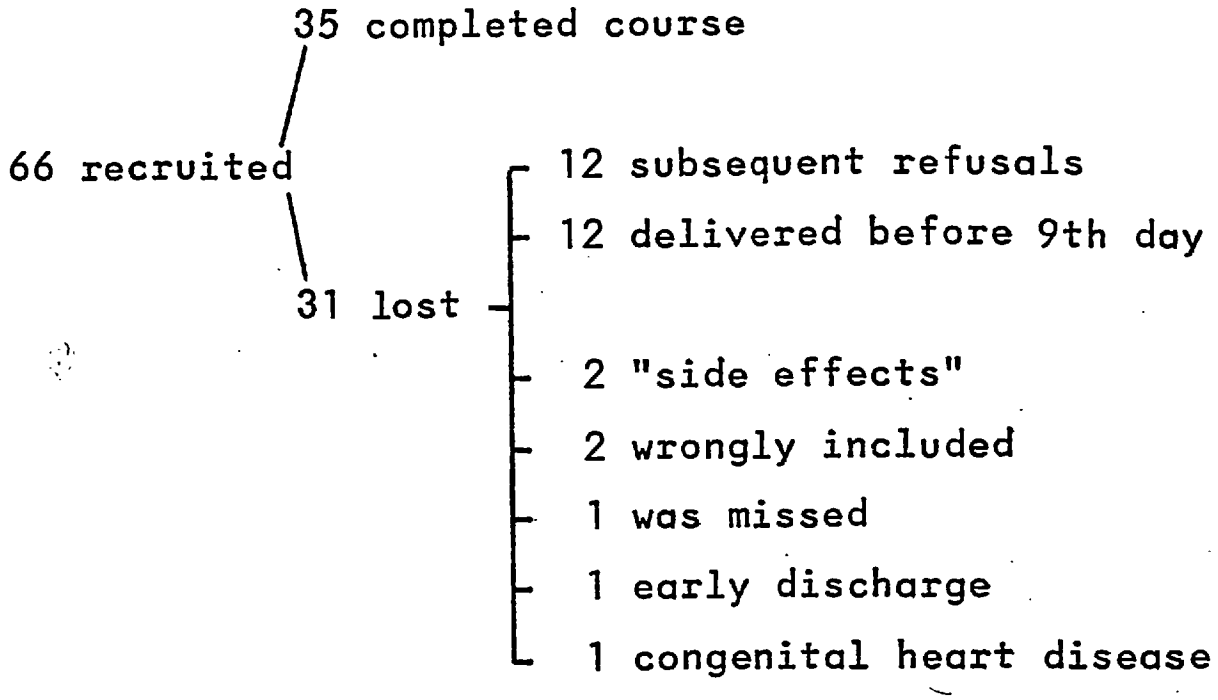


Table 8-1 Enrolment of women into antipyrine trial and reasons for non-inclusion into the final analysis.

(Figure 8:2). The two groups were compared using Student's 't' test for unpaired data. They were found to be significantly different ($p < 0.01$).

Birth weight

The mean birth weight in the placebo group was 3410 ± 134 g, range 2520-4340 g. The mean birth weight in the drug treated group was 3454 ± 117 g, range 2620-4650 g (Figure 8:3). The two groups were compared using Student's 't' test for unpaired data. They were found not to be statistically different.

Number of tablets taken

In the placebo group the mean number of tablets taken was 16.0 with a range of 9-23. In the antipyrine group it was 15.4 with a range of 9-24.

Sex ratios

In the placebo group there were 8 female infants and 7 male infants. In the antipyrine treated group there were 10 female infants and 10 male infants.

Reason for refusal after enrolment

The majority of patients who refused stated that they changed their minds after enrolment and the remainder stated that their husbands objected to them taking unnecessary drugs.

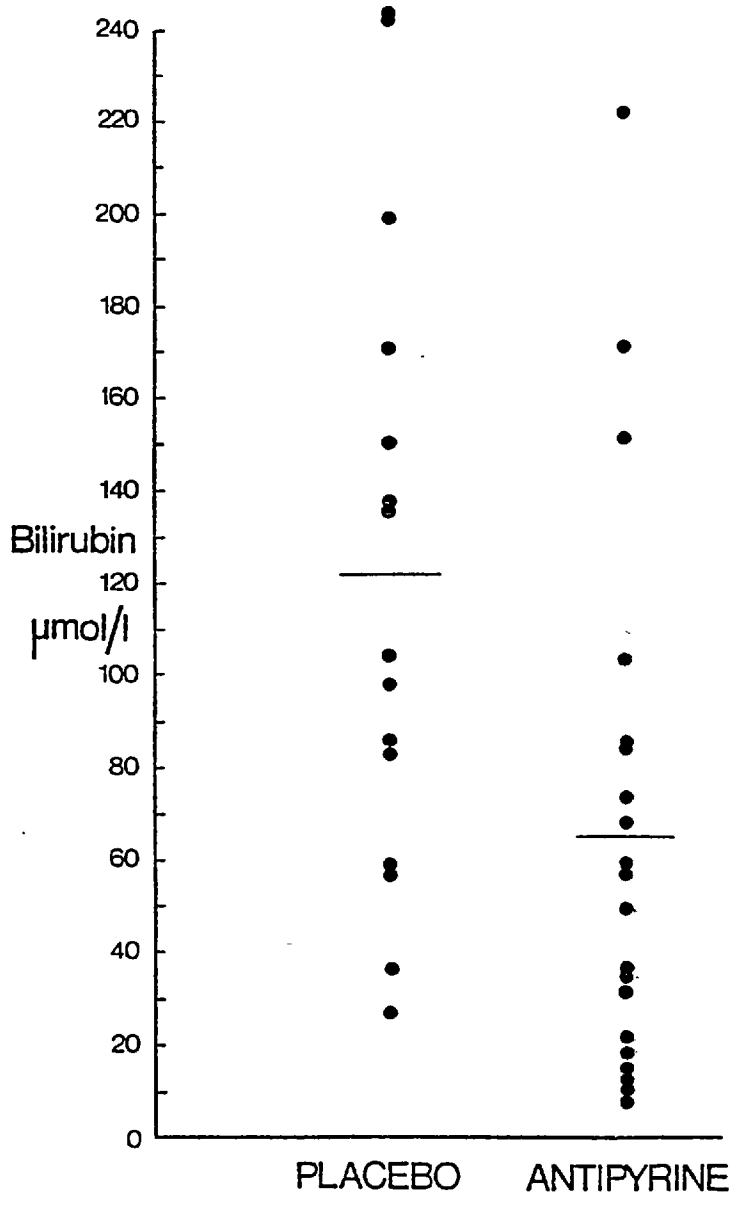


Figure 8:2 Fourth day neonatal serum bilirubin levels with means in infants of mothers treated with placebo or antipyrine

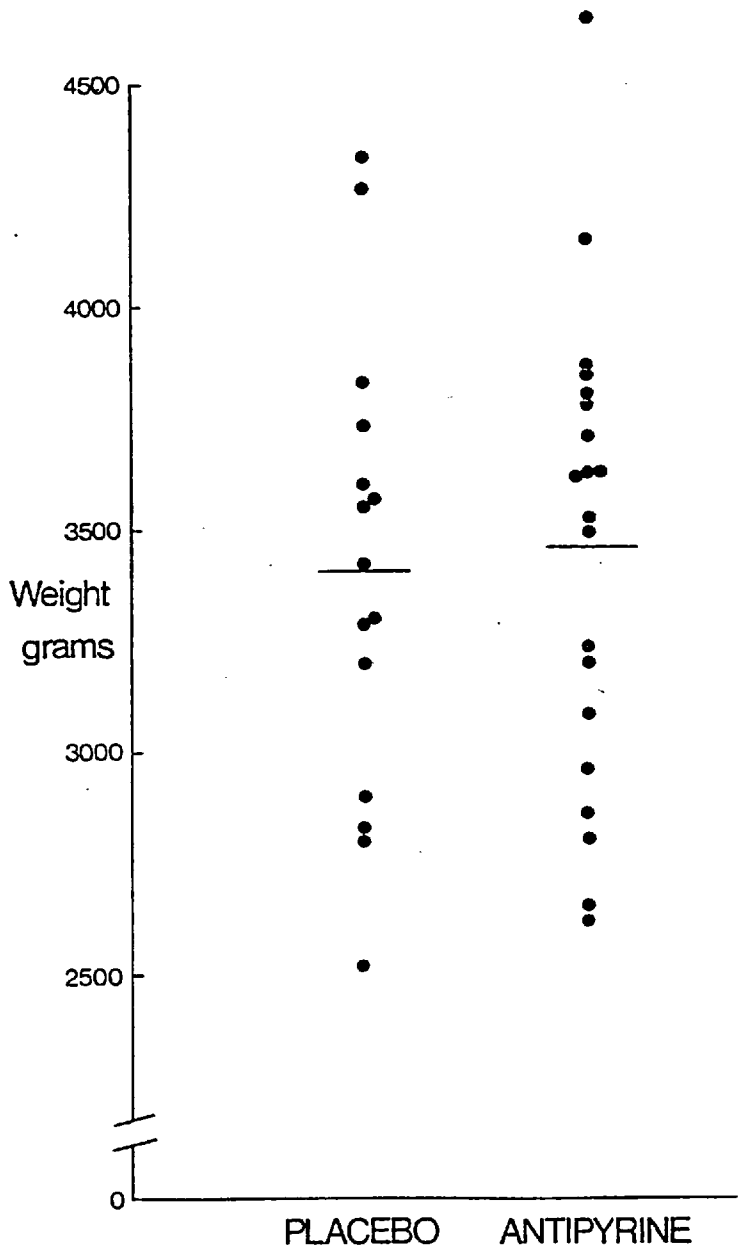


Figure 8:3 Birth weights of infants with means whose mothers received either placebo or antipyrine

Side effects in mothers

Two women complained of 'side effects'. One felt sick each time she took a tablet, she was subsequently found to be taking the placebo tablets. One women stopped after 8 tablets after complaining to the obstetrician at the clinic of black faeces. Her faeces were not black on iron alone and on rectal examination no abnormality was detected. This patient was subsequently telephoned at home after discharge when she admitted that her husband had not wanted her to take the tablets and that she had 'diarrhoea' when taking them.

Side effects in infants

No apparent side effects were observed in the infants of mothers who had taken antipyrine. The mother whose infant had a congenital heart defect had been taking antipyrine. However, the infant was suffering from transposition of the great arteries which could not be a result of a drug treatment which started at the 38th week of gestation.

DISCUSSION

In an ongoing double blind study on the use of antipyrine as prophylaxis for neonatal jaundice, analysis of the data showed that treatment of pregnant women from 38 weeks gestation until delivery resulted in lower fourth day serum bilirubin levels in their newborn infants than in the newborn infants of untreated mothers.

The birth weights, gestational ages and sex ratios of the newborns were similar. A wide variation in serum bilirubin levels was observed even in the newborns who received the antipyrine. This finding is reminiscent of animal work in which a large variability in susceptibility to enzyme induction of the rabbit and rat to phenobarbitone was observed (Pantuck, Conney and Kuntzman, 1968).

Mean serum bilirubin levels were reduced by 56 µmol/l. This is greater than the reduction of 39 µmol/l observed at 4 days in one study, in infants of mothers treated with 30 mg daily phenobarbitone from the 32nd week of pregnancy (Halpin, Jones, Bishop and Lerner, 1972).

However, in another study in which pregnant women were treated with phenobarbitone (30-120 mg daily) for two weeks or longer prior to delivery, mean serum bilirubin levels in the drug treated group on the 4th day were reduced by 58 µmol/l (Maurer, Wolff, Finster, Poppers, Pantuck, Kuntzman and Conney, 1968). Antipyrine therefore appears to be as effective at lowering serum bilirubin levels as is phenobarbitone even in the low dose used.

It can be seen from the literature that effective prophylactic agents seem to produce earlier peak bilirubin levels in those infants of treated mothers than in the infants of control mothers. This has been shown for phenobarbitone (Maurer, Wolff, Finster, Poppers, Pantuck, Kuntzman and Conney, 1968), bucolome (Segni, Polidori and Romagnoli, 1977), uridine diphosphate - glucose (Careddu and Marini, 1968) and

ethanol (Waltman, Bonura, Nigrin and Pipat, 1969).

It is therefore likely that since antipyrine reduces neonatal serum bilirubin levels it also results in an earlier peak bilirubin. If this is the case, then by comparing the bilirubin levels in the two groups the true effect of the drug may be amplified.

Refusal to participate in trial subsequent to agreement

Thirty nine percent of the recruited women who subsequently dropped out had refused to take the tablets following prescription. On investigation it was found that these women had decided not to participate either as an independent decision or because of the husband's refusal. This high drop-out rate is almost certainly due to the pregnant woman's reluctance to take drugs during pregnancy even if they are medically prescribed and particularly if there is any suggestion that the treatment is untried or experimental. These attitudes have been nicely documented by Baric and Macarthar (1977) who investigated the expectations about using medications in a sample of pregnant women. Some 75% thought that a pregnant women should not take any medicines without first consulting a doctor, 19% thought that some special care should be taken and 6% perceived a lack of social expectations concerning medication. Some 67% of the pregnant women said they 'just knew' about the dangers of medication.

Antipyrine toxicity

Antipyrine has been indicted as a possible cause of analgesic induced renal papillary necrosis. The use of antipyrine was popular in Sweden and the use of Hjorton's powders (containing 0.15 g caffeine, 0.5 g antipyrine and 0.5 g phenacetin) was very popular with workers of the Huskvarna factory. A high incidence of renal papillary necrosis has been described in these workers (Grimlund, 1963). It is difficult to know which of the drugs in these powders is the causative agent and it is therefore of interest that Axelsson (1958) has described one case of renal papillary necrosis following the prolonged ingestion of antipyrine and caffeine. There have, however, been many cases of renal papillary necrosis following phenacetin ingestion and since the sale of phenacetin was banned in 1961, the incidence of this disease has progressively fallen, whilst the sale of antipyrine has increased (Michielsen, de Schepper, De Broe, Valkenborgh and Tricot, 1972). It thus seems unlikely that antipyrine is toxic to the kidney.

Antipyrine is not as toxic as many of the pyrazolone compounds, such as amidopyrine or phenylbutazone, whose basic chemical structure is very similar. It does cause an occasional skin rash and it has been indicted as a rare cause of the Stevens-Johnson syndrome (Carroll, Bryan and Robinson, 1968). Agranulocytosis is an occasional complication of therapy with amidopyrine or phenylbutazone and while it has not been reported after antipyrine alone, studies indicate that leucocytoxic antibody directed against amidopyrine will cross react with antipyrine (Magis, Barge and Dausset, 1968). This implies that antipyrine cannot

be regarded as a perfectly safe drug, but its widespread use on the continent of Europe with very few side effects indicates that it is an acceptably safe one.

Kernicterogenic potential of antipyrine

Any drug which is used in the perinatal period may be kernicterogenic by displacing bilirubin from serum albumin (see Chapter 6). One such drug is bucolome a documented enzyme inducer which has been found to precipitate kernicterus in Gunn rats (Sembo, Sato and Yamamura, 1978).

A plasma half-life for antipyrine of 42 hours in a group of 7 normal infants on the first day of life (Murdock, Thorgeirsson, Rossiger and Davies, 1975) indicates that there is probably still appreciable levels of drug present in the neonate at birth. However, the last dose of antipyrine taken by the mother was probably at least 24 hours prior to delivery unlike bucolome which was administered directly to the infants from birth (Segni, Polidori and Romagnoli, 1977). Furthermore, antipyrine itself did not displace bilirubin from human pooled cord serum using Sephadex Gel filtration, but this does not rule out the possibility of the formation of a kernicterogenic metabolite.

Transplacental enzyme induction

Phenobarbitone (Trolle, 1968) and antipyrine (this thesis) given prenatally to women have been found to reduce serum bilirubin levels in the newborn.

a) Animal studies

The ability of drugs given to the mother to induce fetal enzymes has frequently been demonstrated in animal studies. In 1968, Pantuck, Conney and Kuntzman showed that treatment of pregnant rats or rabbits with phenobarbitone during the last week of pregnancy stimulated the activity of hepatic enzymes that metabolise pentobarbital and meperidine in the newborn.

In a study by Halac and Sicignano (1969), the activity of glucuronyl transferase in newborn rats was more than doubled by the administration of phenobarbitone to the mother during pregnancy.

The herbicide contaminant 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) given to rats in a single dose of 2.5 µg/kg on day 17 of gestation has been found to produce considerable induction of aryl hydrocarbon hydroxylase activity in maternal and fetal livers (Berry, Zachariah, Namkung and Jachau, 1976).

b) Human studies

Human studies are few and there appears to be some controversy as to whether induction of fetal liver enzymes in humans can be achieved. Pelkonen, Jouppila and Kärki (1973) were unable to demonstrate statistically different increases in the metabolism of chlorpromazine or p-nitrobenzoic acid by fetal livers obtained from mothers pre-treated with phenobarbitone (100 mg daily) for 7-25 days. The age of the fetus ranged from 9-22 weeks, it is therefore possible that the receptor for phenobarbitone had not yet developed.

However induction of human placental benzpyrene hydroxylase activity by smoking has been demonstrated (Welch, Harrison, Gommi, Poppers, Finster and Conney, 1969) and human fetal livers have been shown to be inducible in vitro (Rüih and Schwartz, 1973; Pelkonen, Korhonen, Jouppila and Kärki, 1975).

Studies of drug half lives in neonatal plasma can also provide information concerning transplacental induction. Murdock, Thorgeirsson, Rossiger and Davies (1975) measured antipyrine plasma half-lives in normal infants and in infants of barbiturate treated mothers. No difference was found in the antipyrine half-lives in the two groups as one might have expected if antipyrine metabolism had been induced.

In a similar study it was shown that amylobarbitone plasma half-lives were similar in infants whether the mothers had received a single dose of amylobarbitone immediately before delivery or multiple inducing doses during the pregnancy. Neither did prior treatment with phenobarbitone have any significant effect on plasma half-lives of amylobarbitone (Draffan, Dollery, Davies, Krauer, Williams, Clare, Trudinger, Darling, Sertel and Hawkins, 1976).

However, reduced plasma half-lives of drugs in infants whose mothers have received inducing agents have been demonstrated by several groups of workers. Rane, Garle, Borga and Sjöqvist (1974) measured the plasma half-lives of infants of epileptic mothers treated with the enzyme

inducer diphenylhydantoin throughout pregnancy. The half-lives were found to be the same as those reported in adults and much shorter than those normally found in neonates. Similar findings were noted with the anticonvulsant carbamazepine, also an inducer, when the drug was used for the management of epilepsy in the mother (Rane, Bertilsson and Palmer, 1975).

In another study, Sereni, Mandelli, Principi, Tognoni, Pardi and Morselli (1973) measured diazepam plasma half-lives in one preterm and two full-term newborn infants whose mothers received both phenobarbitone and diazepam during pregnancy. It was found that the half-lives were considerably faster in these infants than in control newborns of approximately the same chronological and gestational age.

Further studies are needed before the way in which enzyme inducers reduce serum bilirubin levels in the human neonate is elucidated. Moreover, until the total potential of antipyrine for inducing or influencing other developing enzyme systems is known, it should be used with caution.

CHAPTER NINE

CONCLUSION: INFLUENCE OF PERINATAL DRUG USAGE ON JAUNDICE IN THE NEONATE

With the rapid growth of the pharmaceutical industry and the increased interest that obstetricians now have in drug treatments rather than in surgical manipulation, the fetus and the neonate began to be subjected to many potentially toxic substances whose therapeutic benefits were often directed at the mother.

The widespread adoption of oxytocin for induction of labour and bupivacaine for epidural anaesthesia in the 1970's meant that any adverse effects which occurred simultaneously such as the rise in neonatal jaundice reported from some hospitals were inevitably attributed to these drugs.

Studies in this thesis suggest that the incidence has not increased, at least on a world wide basis although localised epidemics may have occurred. However, in view of the incidence of neonatal jaundice at present at Queen Charlotte's Hospital, studies have been carried out to discover which obstetric and other factors influence neonatal jaundice and the manner in which drug treatments may exert their influence has been explored.

Multiple regression analysis data suggest that oxytocin and bupivacaine do result in increased neonatal jaundice rates and elevated mean bilirubin levels but their influence is small and less important than the gestational age of the fetus or race. It is proposed that the effect that these drugs have on neonatal jaundice does not justify their

withdrawal, especially in the light of the advantages that they offer to the mother.

Attempts at elucidating the way in which oxytocin and bupivacaine are icterogenic were unsuccessful in so far as they were not found to inhibit bilirubin conjugation in vitro. It is suggested that oxytocin use may result in the delivery of a chronologically mature but physiologically immature fetus who has failed to initiate parturition.

Pethidine and para-amino benzoic acid were two other perinatal drug treatments which did not result in significant inhibition of bilirubin conjugation in vitro. Nevertheless one can speculate that since the metabolites of many drugs are glucuronated, certain drug treatments may result in enzyme inhibition in vivo. However as long as the newborn is not being directly treated with the icterogenic drug, the concentration of drug in serum at a time when peak bilirubin levels are likely to occur will be low.

Drugs may be kernicterogenic if they displace bilirubin from albumin resulting in passage of free bilirubin into the brain. It is suggested that flucloxacillin and cloxacillin, two highly protein-bound semi-synthetic penicillins commonly used during the neonatal period displace bilirubin from serum albumin. However the concentrations needed to do this are well above therapeutic levels and until clinical evidence

points to kernicterus resulting from their use one cannot recommend their withdrawal.

Sephadex Gel filtration appears to be a reliable method for detecting potentially kernicterogenic drugs in vivo. One can speculate that in the future all new highly protein bound drugs likely to be used in neonates might be tested using this method. If therapeutic serum concentrations of the drug significantly displace bilirubin from human cord serum then they should be proscribed, as is sulphafurazole during the neonatal period.

From work done in this thesis it appears that very few drug treatments are either icterogenic, like novobiocin, or kernicterogenic at therapeutic concentrations, as is sulphafurazole. However, the incidence of neonatal jaundice at Queen Charlotte's Hospital is significant. Preliminary results from a double-blind trial suggest that antipyrine, a known enzyme inducer effectively reduces serum bilirubin levels in neonates whose mothers are treated for 9 days or more prior to delivery. One cannot speculate on the long term consequences to the mother or newborn of treatment with this drug. However, it appears from the literature to be relatively safe with few side-effects, moreover a low dose taken for a short period of time was effective.

Since only a small percentage of neonates have severe enough jaundice to warrant management, the routine treatment of all women with antipyrine

cannot be recommended as yet. However, the mothers of neonates most at risk of neonatal jaundice, such as those with rhesus incompatiblity might be treated. Further studies are required before the minimum dosage and length of treatment needed to significantly lower serum bilirubin levels are established.

APPENDIX

The work described in this thesis was carried out at the Institute of Obstetrics and Gynaecology, Queen Charlotte's Hospital as part of an interdisciplinary research programme founded by the Wellcome Trust with the intention of studying the clinical pharmacology of drug usage in obstetrics. Additional support for consumable materials was provided by Beecham Research Laboratories and Cilag Chemie Foundation for Therapeutic Research.

The laboratory work herein described was carried out by me single handed, protocols being discussed with my supervisor, Dr P J Lewis.

Statistical work involved in the survey of factors which influence neonatal jaundice was carried out jointly with Dr C J Bulpitt and Miss Pam Clifton of the London School of Hygiene and Tropical Medicine, London.

In the antipyrine trial I was aided by Mrs Evelyn Moniram, Research Midwife and the routine biochemistry laboratory, Queen Charlotte's Hospital.

Some of the work described in this thesis has been published and copies of two papers are included.

REFERENCES

Allison, A C (1955): Danger of vitamin K to newborn. Lancet, 1, 669

Anton, A H (1973): Increasing activity of sulfonamides with displacing agents: A review. Ann. N.Y. Acad. Sci., 226, 273

Arias, I M, Gartner, L, Furman, M and Wolfson, S (1963): Studies of the effect of several drugs on hepatic glucuronide formation in newborn rats and humans. Ann. N.Y. Acad. Sci., 111, 274

Arkans, H D and Cassady, G (1978): Estimation of unbound serum bilirubin by the peroxidase assay method: Effect of exchange transfusion on unbound bilirubin and serum bindings. J. Pediat., 92, 1001

Athanassiadis, S, Chopra, D R, Fisher, M A and McKenna, J (1974): An electrophoretic method for detection of unbound bilirubin and reserve bilirubin binding capacity in serum of newborns. J. Lab. clin. Med., 83, 968

Axelsson, A U (1958): Papillitis nectroticans renalis och hemolytisk anemi hos fenaxonmissbrukare. Nord. Med., 59, 903

Ballowitz, L, Hanefeld, F, Jerofke, R, Keller, P, Korn, J and Schweitzer, U (1978): Sephadex-gel filtration (SGF) in infant and adult Gunn rats. Biol. Neonate, 33, 13

Baric, L and Macarthur, C (1977): Health norms in pregnancy Brit. J. prev. soc. Med., 31, 30.

Beazley, J M and Alderman, B (1975): Neonatal hyperbilirubinaemia following the use of oxytocin in labour. Brit. J. Obstet. Gynaec., 82, 265

Beazley, J M and Weekes, A R L (1976): Neonatal hyperbilirubinaemia following the use of prostaglandin E_2 in labour. Brit. J. Obstet. Gynaec., 83, 62

Berry, D L, Zachariah, P K, Namkung, M J and Jachau, M R (1976): Transplacental induction of carcinogen-hydroxylating systems with 2,3,7,8-tetrachlorodibenzo-p-dioxin. Toxicol. appl. Pharmacol., 36, 569

Bevan, B R and Holton, J B (1972): Inhibition of bilirubin conjugation in rat liver slices by free fatty acids with relevance to the problem of breast milk jaundice. Clin. Chim. Acta, 41, 101

Billing, B H, Cole, P G and Lathe, G H (1954): Increased plasma bilirubin in newborn infants in relation to birthweight. Brit. med. J., 2, 1263

Black, M, Billing, B H and Heirwegh, K P M (1970): Determination of bilirubin UDP-glucuronyl transferase activity in needle-biopsy specimens of human liver. Clin. Chim. Acta, 29, 27

Blanc, W A and Johnson, L (1959): Studies on kernicterus: relationship with sulfonamide intoxication. Report on kernicterus in rats with glucuronyl transferase deficiency and review of pathogenesis. J. Neuropath. exp. Neurol., 18, 165

Boggs, T R, jun., Hardy, J B and Frazier, T M (1967): Correlation of neonatal serum total bilirubin concentrations and developmental status at the age of eight months. J. Pediat., 71, 553

Boylan, P (1976): Oxytocin and neonatal jaundice. Brit. med. J., 2, 564

Bratlid, D (1972): The effect of antimicrobial agents on bilirubin binding by human erythrocytes. Scand. J. clin. Lab. Invest., 30, 331

British Births 1970 (1975): Volume 1 (London: Heinemann)

Calder, A A, Moar, V A, Ounsted, M K and Turnbull, A C (1974): Increased bilirubin levels in neonates after induction of labour by intravenous prostaglandin E_2 or oxytocin. Lancet, 2, 1339

Caldwell, J, Moffat, J R, Smith, R L, Lieberman, B A, Cawston, M O and Beard, R W (1976): Pharmacokinetics of bupivacaine administered epidurally during childbirth. Brit. J. clin. Pharmacol., 3, 956P

Caldwell, J, Notarianni, L J and Hawes, E M (1978): Fate of bupivacaine in man. Unpublished data.

Caldwell, J, Notarianni, L J and Smith, R L (1978): Impaired metabolism of pethidine in the human neonate. Brit. J. clin. Pharmacol., 5, 362P

Caldwell, J, Wakile, L A, Notarianni, L J, Smith, R L, Lieberman, B A, Jeffs, J, Coy, Y and Beard, R W (1977): Transplacental passage and neonatal elimination of pethidine given to mothers in childbirth. Brit. J. clin. Pharmacol., 4, 716P

Campbell, N, Harvey, D and Norman, A P (1975): Increased frequency of neonatal jaundice in a maternity hospital. Brit. med. J., 2, 116

Careddu, P and Marini, A (1968): Stimulating bilirubin conjugation. Lancet, 1, 982

Carroll, D M, Bryan, P A and Robinson, R J (1968): Stevens-Johnson syndrome and long-acting sulphonamides. In: Drug induced diseases, 3, 165. Eds. Meyler, L and Peck, H M. Excerpta Medica, Amsterdam

Cashore, W J, Horwich, A, Laterra, J and Oh, W (1977): Effect of postnatal age and clinical status of newborn infants on bilirubin-binding capacity. Biol. Neonate, 32, 304

Catz, C and Yaffe, S J (1962): Pharmacological modification of bilirubin conjugation in the newborn. Amer. J. Dis. Child, 104, 516

Chalmers, I, Campbell, H and Turnbull, A C (1975): Use of oxytocin and incidence of neonatal jaundice. Brit. med. J., 2, 116

Chew, W C and Swann, I L (1977): Influence of simultaneous low amniotomy and oxytocin infusion and other maternal factors on neonatal jaundice: a prospective study. Brit. med. J., 1, 72

Chignell, C F, Vesell, E S, Starkweather, D K and Berlin, C M (1971): The binding of sulfaphenazole to fetal neonatal and adult human plasma albumin. Clin. Pharmac. Ther., 12, 897

Cohen, M D, Raeburn, J A, Devine, J, Kirkwood, J, Elliott, B, Cockburn, F and Forfar, J O (1975): Pharmacology of some oral penicillins in the newborn infant. Arch. Dis. Child., 50, 230

Conney, A H, Davison, C, Gastel, R and Burns, J J (1960): Adaptive increases in drug metabolising enzymes induced by phenobarbital and other drugs. J. Pharmacol. exp. Ther., 130, 1

Coutinho, C B, Lucek, R W, Cheripko, J A and Kuntzman, R (1973): A new approach to the determination of protein-bound bilirubin displacement and its applications. Ann. N.Y. Acad. Sci., 226, 238

Crigler, J F and Gold, N I (1966): Sodium phenobarbital induced decrease in serum bilirubin in an infant with congenital non-haemolytic jaundice and kernicterus. J. Clin. Invest., 45, 998

Culley, P, Powell, J, Waterhouse, J and Wood, B (1970): Sequelae of neonatal jaundice. Brit. med. J., 3, 383

Dahms, B B, Krauss, A N, Gartner, L M, Klain, D B, Soodalter, J and Auld, P A M (1973): Breast feeding and serum bilirubin values during the first 4 days of life. J. Pediat., 83, 1049

Davies, D P, Gomersall, R, Robertson, R, Gray, O P and Turnbull, A C (1973): Neonatal jaundice and maternal oxytocin infusion. Brit. med. J., 3, 476

Davis, D R and Yeary, R A (1975): Effect of sulfadimethoxine on tissue distribution of ^{14}C bilirubin in the newborn and adult hyperbilirubinaemic Gunn rat. Pediat. Res., 9, 846

Davies, D S, Gigon, P L and Gillette, J R (1969): Sex differences in the kinetic constants for the N-demethylation of ethylmorphine by liver microsomes. Biochem. Pharmacol., 17, 1865

Diamond, I and Schmid, R (1966): Experimental bilirubin encephalopathy The mode of entry of bilirubin-^{14}C into the central nervous system. J. clin. Invest., 45, 678

Draffan, G H, Dollery, C T, Davies, D S, Krauer, B, Williams, F M, Clare, R A, Trudinger, B J, Darling, M, Sertel, H and Hawkins, D F (1976): Maternal and neonatal elimination of amobarbital after treatment of the mother with barbiturates during late pregnancy. Clin. Pharmacol. Ther., 19, 271

Eden, O B (1976): Neonatal jaundice in association with operative delivery. Brit. med. J., 1, 461

Eden, O B, Revolta, A D and Adjei, S K (1974): Factors influencing neonatal jaundice. Brit. med. J., 3, 573

Fenwick, J D (1975): Neonatal jaundice as a cause of deafness. J. Laryng., 89, 925

Frenzel, J, Sander, I and Schramm, D (1973): Comparative analysis between albumin-binding capacity and the concentration of unbound bilirubin in hyperbilirubinaemic sera of newborn infants. Z. Kinderheilk, 115, 187

Friedman, L A and Lewis, P J (1977): Maternal drug therapy and neonatal jaundice. In: Therapeutic problems in pregnancy, p. 141 Ed. Lewis, P J. Lancaster, Medical and Technical Publishing.

Friedman, L, Lewis, P and Harvey, D (1977): Oxytocin and neonatal jaundice. Brit. med. J., 2, 1223

Friedman, L, Lewis, P J, Clifton, P and Bulpitt, C J (1978): Factors influencing the incidence of neonatal jaundice. Brit. med. J., 1, 1235

Friedman, E A and Sachtleben, M R (1976): Neonatal jaundice in association with oxytocin stimulation of labour and operative delivery. Brit. med. J., 1, 198

Gate, J M (1978): Syntometrine as a possible cause of neonatal jaundice. Brit. med. J., 1, 49

Ghosh, A (1975): Oxytocin agents and neonatal morbidity. Lancet, 1, 453

Ghosh, A and Hudson, F P (1972): Oxytocin agents and neonatal hyperbilirubinaemia. Lancet, 2, 823

Gluck, L and Kulovich, M V (1973): Lecithin/sphingomyelin ratios in amniotic fluid in normal and abnormal pregnancy. Amer. J. Obstet. Gynec., 115, 539

Griffiths, W C, Diamond, I and Dextraze, P (1975): The albumin binding of unconjugated bilirubin in serum. Clin. Biochem., 8, 254

Grimlund, K (1963): Phenacetin and renal damage at a Swedish factory. Acta med. Scand., 174, Suppl. 405, 1

Grossman, M and Ticknor, W (1965): Serum levels of ampicillin, cephalothin, cloxacillin and nafcillin in the newborn infant. Antimicrobial agents and Chemotherapy, p. 214. Ed. G L Hobby. Am. Soc. Microbiology, Ann Arbor, Michigan

Halac, E and Sicignano, C (1969): Re-evaluation of the influence of sex, age, pregnancy and phenobarbital on the activity of UDP-glucuronyl transferase in rat liver. J. Lab. clin. Med., 73, 677

Halpin, T F, Jones, A R, Bishop, H L and Lerner, S (1972): Prophylaxis of neonatal hyperbilirubinaemia with phenobarbital. Obstet. Gynecol, 40, 85

Hanefeld, F and Ballowitz, L (1976): Flucloxacillin and bilirubin binding. Lancet, 1, 433

Hargreaves, T and Holton, J B (1962): Jaundice of the newborn due to novobiocin. Lancet, 1, 839

Hargreaves, T and Piper, R F (1971): Breast milk jaundice: Effect of inhibitory breast milk and 3α,20β-pregnanediol on glucuronyl transferase. Arch. Dis. Child, 46, 195

Horiguchi, T and Bauer, C (1975): Ethnic differences in neonatal jaundice: Comparison of Japanese and Caucasian newborn infants. Amer. J. Obstet. Gynec., 121, 71

Hsia, D Y-Y, D, Dowben, R M and Riabov, S (1963): Inhibitors of glucuronyl transferase in the newborn. Ann. N.Y. Acad. Sci., 111, 326

Jacobsen, J (1969): Binding of bilirubin to human serum albumin - determination of dissociation constants. FEBS Letters, 5, 112

Jacobsen, J and Wennberg, R P (1974): Determination of unbound bilirubin in the serum of newborns. Clin. Chem., 20, 783

Jeffares, M J (1977): A multifactorial survey of neonatal jaundice. Brit. J. Obstet. Gynaec., 84, 452

Jirsova, V, Jirsa, M, Heringova, A, Koldovsky, O and Weirichova, J (1967): The use and possible diagnostic significance of Sephadex gel filtration of serum from icteric newborn. Biol. Neonat., 11, 204

Jouppila, P, Koivisto, M and Suonio, S (1973): Ethanol in the prevention of neonatal hyperbilirubinaemia. Acta paediat. Scand., 62, 501

Kapitulnik, J, Blondheim, S H and Kaufmann, N A (1972): Sephadex adsorption of bilirubin from neonatal and adult serum. Clin. Chem., 18, 43

Kaufmann, N A, Kapitulnik, J and Blondheim, S H (1969): The adsorption of bilirubin by Sephadex and its relationship to the criteria for exchange transfusion. Pediatrics, 44, 453

Klaasen, C D (1976): Effect of microsomal enzyme inducers on the biliary excretion of an exogenous load of bilirubin in newborn rats. Proc. soc., exp. Biol. Med., 153, 370

Krasner, J (1973): Fluorescent properties of bovine serum albumin - bilirubin complex. Biochem. Med., 7, 135

Krasner, J, Giacoia, G P and Yaffe, S J (1973): Drug-protein binding in the newborn infant. Ann. N.Y. Acad. Sci., 226, 101

Kreek, M J and Sleisenger, M H (1968): Reduction of serum unconjugated bilirubin with phenobarbitone in adult congenital non-haemolytic unconjugated hyperbilirubinaemia. Lancet, 2, 73

Kunin, C M (1967): Clinical significance of protein binding of the penicillins. Ann. N.Y. Acad. Sci., 145, 282

Lathe, G H and Ruthven, C R J (1958): Factors affecting the rate of coupling of bilirubin and conjugated bilirubin in the van den Bergh reaction. J. clin. Path., 11, 155

Lee, K, Gartner, L M and Zarafu, I (1975): Fluorescent dye method for determination of the bilirubin-binding capacity of serum albumin. J. Pediat., 86, 280

Loria, C J, Echeverria, P and Smith, A L (1976): Effect of antiobiotic formulations in serum protein: bilirubin interaction of newborn infants. J. Pediat., 89, 479

Lucey, J F, Hibbard, E, Behrman, R E, Esquivel de Gallardo, F O and Windle, W F (1964): Kernicterus in asphyxiated newborn rhesus monkeys. Exp. Neurol., 9, 43

Magis, C C, Barge, A and Dausset, J (1968): Serological study of an allergic agranulocytosis due to nor-amidopyrine. Clin. exp. Immunol., 3, 989

Maurer, H M, Wolff, J A, Finster, M, Poppers, P J, Pantuck, E, Kuntzman, R and Conney, A H (1968): Reduction in concentration of total serum-bilirubin in offspring of women treated with phenobarbitone during pregnancy. Lancet, 2, 122

Menken, M, Barrett, P V D, Swarm, R L and Berlin, N I (1966): Kernicterus: Development of an experimental model using bilirubin ^{14}C. Arch. Neurol., 15, 68

Meuwissen, J A T P and Heirwegh, K P M (1970): Transfer of adsorbed bilirubin to specific binding proteins. Biochem. J., 200, 19P

Michielsen, P, De Schepper, P J, De Broe, M, Valkenborgh, G and Tricot, J P (1972): Renal diseases due to drugs In: Drugs induced diseases, 4, p. 261. Eds. Meyler, L and Peck, H M. Excerpta Medica, Amsterdam

Mulder, G J (1974): On non-specific inhibition of rat liver microsomal UDP glucuronyl transferase by some drugs. Biochem. Pharmacol., 23, 1283

Nakamura, H and Lee, Y (1977): Microdetermination of unbound bilirubin in icteric newborn sera: An enzymatic method employing peroxidase and glucose oxidase. Clin. chim. Acta, 79, 411

Nathenson, G, Cohen, M I and McNamara, H (1975): The effect of Na benzoate on serum bilirubin of the Gunn rat. J. Pediat., 86, 799

Nymand, G (1974): Maternal smoking and neonatal hyperbilirubinaemia Lancet, 2, 173

Odell, G B (1959a): Studies in kernicterus. I. The protein binding of bilirubin. J. clin. Invest., 38, 823

Odell, G B (1959b): The dissociation of bilirubin from albumin and its clinical implications. J. Pediat., 55, 268

Odell, G B (1970): The distribution and toxicity of bilirubin. Pediatrics, 46, 16

Odell, G B (1973): Influence of binding on the toxicity of bilirubin. Ann. N.Y. Acad. Sci., 226, 225

Odell, G B, Cohen, S N and Kelly, P C (1969): Studies on kernicterus II. The determination of the saturation of serum albumin with bilirubin. J. Pediat., 74, 214

Ohnhaus, E G, Thorgeirsson, S S, Davies, D S and Breckenridge, A (1971): Changes in liver blood flow during enzyme induction. Biochem. Pharmacol., 20, 2561

Orme, M L'E., Davies, L and Breckenridge, A (1974): Increased glucuronidation of bilirubin in man and rat by administration of antipyrine (phenazone). Clin. Sci. molec. Med., 46, 511

Ounsted, M K, Boyd, P A, Hendrick, A M, Mutch, L M M, Simons, C D and Good, F J (1978): Induction of labour by different methods in primiparous women. II. Neuro-behavioural status of the infants. Early human Develop., 2/3, 241

Pantuck, E, Conney, A H and Kuntzman, R (1968): Effect of phenobarbital on the metabolism of pentobarbital and meperidine in fetal rabbits and rats. Biochem. Pharmacol., 17, 1441

Pelkonen, O, Jouppila, P and Kärki, N T (1973): Attempts to induce drug metabolism in human fetal liver and placenta by the administration of phenobaribital to mothers. Arch. int. Pharmacodyn. Ther., 202, 288

Pelkonen, O, Korhonen, P, Jouppila, P and Kärki, N T (1975): Induction of aryl hydrocarbon hydroxylase in human fetal liver cell and fibroblast hydroxylase in human fetal liver cell and fibroblast cultures by polycyclic hydrocarbons. Life Sci., 16, 1403

Petite, J P, Etienne, J P and Housset, E (1968): Liaisons albumin-bilirubin non conjugee. Etude experimentale chez le rat Gunn. Biologie et Gastro-Enterologie, 2, 137

Polacek, K (1966): Risk of kernicterus in newborn infants with a high level of conjugated bilirubin. Acta paediat. Scand., 55, 401

Porter, E G and Waters, W J (1966): A rapid micromethod for measuring the reserve albumin binding capacity in serum from newborn infants with hyperbilirubinaemia. J. Lab. clin. Med., 67, 660

Räihä, N C R and Schwartz, A L (1973): Enzyme induction in human fetal liver in organ culture. Enzyme, 15, 330

Ramos, A, Silverberg, M and Stern, L (1966): Pregnanediols and neonatal hyperbilirubinaemia. Amer. J. Dis. Child., 111, 353

Rane, A, Bertilsson, L and Palmer, L (1975): Disposition of placentally transferred carbamazepine (Tegretol) in the newborn. Eur. J. clin. Pharm., 8, 283

Rane, A, Garle, M, Borga, O and Sjöqvist, F (1974): Plasma disappearance of transplacentally transferred diphenylhydantoin in the newborn studied by mass fragmentography. Clin. Pharmacol. Ther., 15, 39

Reyes, H, Levi, A J, Gatmaitan, Z and Arias, I M (1969): Organic anion-binding protein in rat liver: Drug induction and its physiologic consequence. Proc. nat. Acad. Sci. (Wash), 64, 168

Rozdilsky, B (1966): Kittens as experimental model for study of kernicterus. Amer. J. Dis. Child, 111, 161

Satge, P, Colin, J, Voyer, M, Crumiere and Charlas, J (1974): Place des dosages de la bilirubine non liée et de l'albumine serique Arch. Franc. Péd., 31, 201

Scatchard, G (1949): The attractions of proteins for small molecules and ions. Ann. N.Y. Acad. Sci., 51, 660

Scheidt, P C, Mellits, E D, Hardy, J B, Drage, J S and Boggs, T R (1977): Toxicity to bilirubin in nenonates: Infant development during first year in relation to maximal neonatal serum bilirubin concentration. J. Pediat., 91, 292

Schenker, S, McCandless, D W and Zollman, P E (1966): Studies of cellular toxicity of unconjugated bilirubin in kernicteric brain J. clin. Invest, 45, 1213

Schiff, D, Chan, G and Stern, L (1971): Fixed drug combinations and the displacement of bilirubin from albumin. Pediatrics, 48, 139

Schiff, D, Chan, G and Stern, L (1972): Sephadex G-25 quantitative estimation of free bilirubin potential in jaundiced newborn infants sera: A guide to the prevention of kernicterus. J. Lab. clin. Med., 80, 455

Schmid, R, Diamond, I, Hammaker, L and Gundersen, C B (1965): Interaction of bilirubin with albumin. Nature, 206, 1041

Schmorl, G (1903): Verhandlunger der Deutschen Pathologischen Gesellschaft, 6, 109

Schulz, J, Van Creveld, S (1958): Stuart Prower factor in newborn infants. Etud. néo-natal, 7, 133

Segni, G, Polidori, G and Romagnoli, C (1977): Bucolome in prevention of hyperbilirubinaemia in preterm infants. Arch. Dis. Child, 52, 549

Semba, R, Sato, H and Yamamura, H (1977): Induction of kernicterus by bucolome in rats. Lancet, 2, 1134

Semba, R, Sato, H and Yamamura, H (1978): Danger of bucolome in infants with hyperbilirubinaemia. Arch. Dis. Child., 53, 503

Sereni, F, Mandelli, M, Principi, N, Tognoni, G, Pardi, G and Morselli, P L (1973): Induction of drug metabolizing enzyme activities in the human fetus and in the newborn infant. Enzyme, 15, 318

Silverman, W A, Anderson, D H, Blanc, W A and Crozier, D N (1956): A difference in mortality rate and incidence of kernicterus among premature infants allotted to two prophylactic antibacterial regimens. Pediatrics, 18, 614

Sims, D G and Neligan, G A (1975): Factors affecting the increasing incidence of severe non-haemolytic neonatal jaundice. Brit. J. Obstet. Gynaecol., 82, 863

Singhi, S and Singh, M (1977): Oxytocin induction and neonatal hyperbilirubinaemia. Brit. med. J., 2, 1028

Smith, M N and Wilson, R G (1978): Oxytocin and neonatal jaundice. Brit. med. J., 1, 50

Sudlow, G (1978): Specific binding sites on albumin. 7th Int. Con. Pharmacol. Abstracts, 2, 892

Sutherland, J M and Keller, W H (1961): Novobiocin and neonatal hyperbilirubinaemia. Amer. J. Dis. Child., 101, 447

Tan, J S, Trott, A, Phair, J P and Watanakunakorn, C (1972): A method for measurements of antibiotics in human interstitial fluid J. Infect. Dis., 126, 492

Thomas, C R (1976): Routine phenobarbital for prevention of neonatal hyperbilirubinaemia. Obstet. Gynec., 47, 304

Thompson, R P H, Eddleston, A L W F and Williams, R (1969): Low plasma-bilirubin in epileptics on phenobarbitone. Lancet, 1, 21

Trolle, D (1968a): Decrease of total serum bilirubin concentration in newborn infants after phenobarbitone treatment. Lancet, 2, 705

Van Roy, F P and Heirwegh, K P M (1968): Determination of bilirubin glucuronide and assay of glucuronyltransferase with bilirubin as acceptor. Biochem. J., 107, 507

Wallace, S (1976): Factors affecting drug-protein binding in the plasma of newborn infants. Brit. J. clin. Pharmac., 3, 510

Wallace, S (1977): Altered plasma albumin in the newborn infant. Brit. J. clin. Pharmac., 4, 82

Waltman, R, Bonura, F, Nigrin, G and Pipat, C (1969): Ethanol in prevention of hyperbilirubinaemia in the newborn. Lancet, 2, 1265

Waters, W J, Dunham, R and Bowen, W R (1958): Inhibition of bilirubin conjugation in vitro. Proc. Soc. exp. Biol. Med.; 99, 175

Welch, R M, Harrison, Y E, Gommi, B W, Poppers, P J, Finster, M and Conney, A H (1969): Stmulatory effect of cigarette smoking on the hydroxylation of 3,4-benzpyrene and the N-demethylation of 3-methyl-4-monomethylaminoazobenzene by enzymes in human placenta. Clin. Pharmacol. Ther., 10, 101

Wilson, J T (1969): Phenobarbital in the neonatal period. Pediatrics, 43, 324

Wysowski, D K, Flynt, J W, Goldfield, M, Altman, R and Davis, A T (1978): Epidemic neonatal hyperbilirubinaemia and use of a phenolic disinfectant detergent. Pediatrics, 61, 165

Yaffe, S J, Levy, G, Matsuzawa, T and Baliah, T (1966): Enhancement of glucuronide conjugating capacity in a hyperbilirubinaemic infant due to apparent enzyme induction by phenobarbital. New Eng. J. Med., 275, 1461

Yeary, R A and Davis, D R (1974): Protein binding of bilirubin: Comparison of in vitro and in vivo measurements of bilirubin displacement by drugs. Toxicol. appl. Pharmacol., 28, 269

Yeung, C Y and Field, C E (1969): Phenobarbitone therapy in neonatal hyperbilirubinaemia. Lancet, 2, 135

Zamet, P, Nakamura, H, Perez-Robles, S, Larroche, J C and Minkowski, A (1975): The use of critical levels of birth weight and 'free bilirubin' as an approach for prevention of kernicterus. Biol. Neonate, 26, 274

Oxytocin and neonatal jaundice

SIR,—Several UK centres have reported a recent increase in neonatal jaundice.[1 2] Oxytocin or some as yet unidentified obstetric practice has been held responsible.[3 4] However, there is some doubt that a genuine increase in the incidence of neonatal jaundice has occurred since serial figures[4 5] are few and the incidence of jaundice increases with the proportion of neonates whose plasma bilirubin is measured. We have sent questionnaires to paediatricians in Europe (E) and North America (A) asking their recent experience of jaundice rates.

Sixty-six members of the European Society for Paediatric Research were circularised; 52 replied. A random 1 in 5 sample of the American Pediatric Society, total 135, were also circularised; 100 replied. Thirty-nine of the Europeans and 51 of the North Americans were in active practice in neonatology. The majority, 66% (E) and 60% (A), thought there had been no increase in neonatal jaundice since 1970. Only 23% (E) and 24% (A) thought jaundice had increased. The remainder were uncertain. The respondents' estimate of the present percentage of neonates having a peak plasma bilirubin concentration of 206 μmol/l (12 mg/100 ml) or greater in the first 7 days after birth varied between 2 and 30%. The averages of all the replies, 14·2% (E) and 12·6% (A), were similar to the published incidence in unselected series.[5] We asked also about the use of phenobarbitone in late pregnancy for the prophylaxis of neonatal jaundice. Only 13% (E) and 8% (A) paediatricians used this occasionally; none used it routinely.

If oxytocin were icterogenic then we would have expected most paediatricians in North America and Europe to have noticed some increase in neonatal jaundice. The fact that they have not casts further doubt on this association.

LOUISE FRIEDMAN
PETER LEWIS
DAVID HARVEY

Institute of Obstetrics and Gynaecology, London

[1] Campbell, N, *et al*, *British Medical Journal*, 1975, 2, 548.
[2] Sims, D G, and Neligan, G A, *British Journal of Obstetrics and Gynaecology*, 1975, 82, 863.
[3] Beazley, J M, and Alderman, B, *British Journal of Obstetrics and Gynaecology*, 1975, 82, 265.
[4] Chew, W C, and Swann, I L, *British Medical Journal*, 1977, 1, 72.
[5] Lewis, P J, *Therapeutic Problems in Pregnancy*, p 141. Lancaster, Medical and Technical Publishing, 1977.

BRITISH MEDICAL JOURNAL 13 MAY 1978 1235

PAPERS AND ORIGINALS

Factors influencing the incidence of neonatal jaundice

LOUISE FRIEDMAN, P J LEWIS, PAMELA CLIFTON, C J BULPITT

British Medical Journal, 1978, 1, 1235-1237

Summary and conclusions

A retrospective study of 12 461 single births confirmed an association between maternal oxytocin infusion and neonatal jaundice. The effect of oxytocin on jaundice was independent of gestational age at birth, sex, race, epidural anaesthesia, method of delivery, and birth weight, each of which was significantly associated with neonatal jaundice.

The effect of oxytocin was, however, small, producing a calculated mean increase in peak plasma bilirubin concentration of 8·6 μmol/l (0·5 mg/100 ml); this excess was independent of sex and less than the effect of the baby being born one week earlier.

Introduction

Although mild jaundice of the newborn is accepted as normal, severe or prolonged jaundice is a potential cause of mental or motor impairment.[1] Several workers have suggested recently that the incidence of considerable neonatal jaundice has increased.[2,3] Changes in obstetric practice have been blamed for this increase, particularly the increased use of oxytocin for inducing labour.[4,5] But this increased incidence of neonatal jaundice is largely based on clinical impression, and the role of oxytocin in producing the increase is difficult to confirm since oxytocin use is associated with other icterogenic factors such as preterm delivery, instrumental delivery, and epidural anaesthesia. We therefore analysed neonatal jaundice rates in one obstetric hospital over five years. The rates were examined for the factors associated with neonatal jaundice, and we measured the relative contribution of the icterogenic factors identified.

Institute of Obstetrics and Gynaecology, Queen Charlotte's Hospital, London W6 0XG

LOUISE FRIEDMAN, BSC, research assistant

P J LEWIS, MD, MRCP, senior lecturer in clinical pharmacology

London School of Hygiene and Tropical Medicine, London WC1E 7HT

PAMELA CLIFTON, RN, nurse research assistant

C J BULPITT, MD, MRCP, senior lecturer in epidemiology

Methods

The study population was drawn from the 15 932 live births at Queen Charlotte's Hospital in 1971-5. Cases of multiple birth, rhesus incompatibility, ABO incompatibility, and G6PD deficiency were excluded, as were those in which the infant did not remain in hospital for seven days or more after delivery (in whom jaundice may have developed after discharge). The study group was thus composed of 12 461 infants without haematological predisposition to neonatal jaundice who were observed in hospital for the first week after birth. The date of delivery, sex, race, birth weight, gestational age, and method of delivery were recorded as were presence or absence of amniotomy, oxytocin infusion (whether for induction or acceleration of labour), epidural anaesthesia, and general anaesthesia. When plasma bilirubin had been measured the peak plasma bilirubin concentration in the first seven days after delivery was recorded.

The proportion of neonates with significant jaundice (a peak plasma bilirubin concentration of 205 μmol/l (12 mg/100 ml) or greater) was calculated for each variable. Statistical differences between groups were calculated using the χ^2 test. Each factor showing a relationship with jaundice was also studied using a linear discriminant function (0 = no jaundice, 1 = bilirubin > 205 μmol/l (12 mg/100 ml)) to determine whether the factor had an influence dependent on or independent of other factors. A multiple regression analysis was carried out to determine the numerical influence of each independent variable on the peak neonatal bilirubin concentration, assigning an arbitrary peak plasma bilirubin value of 77 μmol/l (4·5 mg/100 ml) to those infants who had not had their bilirubin concentrations measured because they had had no clinical sign of jaundice.

Results and comments

SERIAL FIGURES

The proportions of neonates with peak plasma bilirubin concentrations of 205 μmol/l (12 mg/100 ml) or 291 μmol/l (17 mg/100 ml) or greater over the five years are shown in fig 1. The incidence of jaundice was lowest in 1971 and remained steady thereafter.

INDIVIDUAL FACTORS

The seven factors that were associated with a significantly different incidence of jaundice are shown in table I. Four other factors

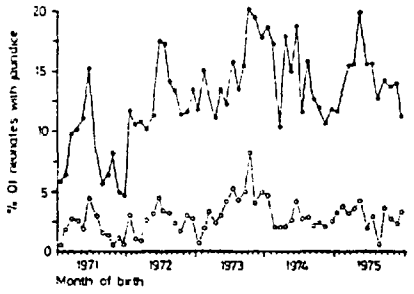


FIG 1—Percentage of neonates with plasma bilirubin concentrations of >205 μmol/l (12 mg/100 ml) (●) and >291 μmol/l (17 mg/100 ml) (○) month by month over five years.

TABLE I—*Proportion of neonates with jaundice (peak bilirubin >205 μmol/l (12 mg/100 ml)) according to various factors*

Factor	Neonates with factor		Neonates without factor		P value (χ^2 test)
	Total No	% with jaundice	Total No	% with jaundice	
Asiatic race*	220	29·1	12 241	12·6	<0·001
Breech delivery	265	22·6	12 196	12·6	<0·001
Ventouse extraction	502	21·3	11 959	12·5	<0·001
Oxytocin	2 820	15·3	9 641	12·1	<0·001
Epidural anaesthesia	5 915	15·0	6 546	10·8	<0·001
Male sex	6 140	14·9	6 296	10·6	<0·001
Negro race	911	7·0	11 550	13·3	<0·001

*Asians of Chinese or Japanese origin excluding Indian race.
Conversion: SI to traditional units—Bilirubin: 1 μmol/l ≈ 0·058 mg/100 ml.

investigated—caesarean section, forceps delivery, general anaesthesia, and amniotomy—were not associated with significantly different rates of jaundice. Linear discriminant function analysis, where the numerical factors birth weight in grams and gestational age in weeks were also included, gave slightly different results. The analysis confirmed an independent influence of six of the factors previously noted—Asiatic race, ventouse extraction, oxytocin, male sex, epidural anaesthesia, and Negro race—but did not definitely confirm breech delivery ($P>0·09$) and introduced general anaesthesia as an independent factor ($P<0·005$). Gestational age in weeks was the most important of all factors considered. Birth weight was also significantly related to neonatal jaundice as a factor independent of gestational age. The proportion of variation in the incidence of jaundice explicable in terms of the nine factors totalled 7·6%.

A second multiple regression analysis in which the bilirubin concentration of 77 μmol/l (4·5 mg/100 ml) was assigned to non-jaundiced infants gave nearly identical results. The partial regression coefficients of this equation are shown in table II; these values can be equated to the mean increase in peak bilirubin concentration in μmol/l attributable to each of the independent variables or a unit increase in that variable.

SMALL-FOR-DATES INFANTS

An interesting relationship between birth weight, gestational age, and neonatal jaundice was noticed. When mean peak bilirubin concentration was plotted against gestational age the expected negative correlation was seen. But when the relationships for infants of different birth weights were considered separately (fig 2) an unexpected pattern was seen for infants of gestational age less than 36 weeks. The lighter infants had a lower mean bilirubin concentration than those of heavier weight. This is contrary to what might have been expected and may represent another example of accelerated physiological maturity in small-for-dates infants.

TABLE II—*Influence of various factors on peak neonatal plasma bilirubin concentrations (μmol/l) calculated by multiple regression analysis*

Factor	Partial regression coefficient	Significance (F value)
Asiatic race	+20·5	25·96 (P<0·0001)
Ventouse extraction	+15·4	32·01 (P<0·0001)
Negro race	−13·0	38·48 (P<0·001)
Oxytocin	+8·6	39·72 (P<0·001)
Female sex	−8·6	60·43 (P<0·001)
Epidural anaesthesia	+6·8	35·66 (P<0·001)
General anaesthesia	−6·2	10·87 (P<0·01)
Gestational age (weeks)	−9·4	603·79 (P<0·001)
Birth weight (kg)	−3·4	8·00 (P<0·01)

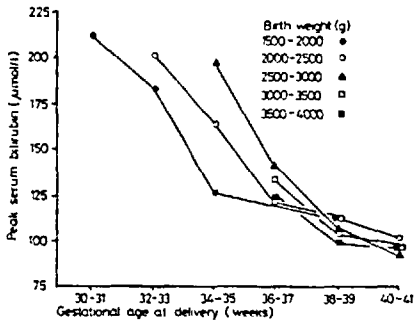


FIG 2—Mean peak neonatal serum bilirubin values according to gestational age for groups of infants with different birth weights. Points have been omitted where fewer than 20 infants fitted the category.

Discussion

The serial figures for the incidence of neonatal jaundice show a possible increase in 1971 and a stable rate thereafter. Among several reports quoting a recent increase in neonatal jaundice only two include serial incidence rates. Sims and Neligan[4] quote an increase from 2·2% to 5·5% in the proportion of infants with bilirubin concentrations of 15 mg/100 ml [257 μmol/l] or greater between 1972 and 1973. Campbell *et al*[5] quoted a progressive increase in yearly jaundice rates between 1969 and 1973. They also used data from Queen Charlotte's Hospital but their method of sampling was not as rigorous as ours since they included all infants born in the hospital except those with rhesus disease. If neonatal jaundice had genuinely increased in recent years as a result of some obstetric innovation then the present incidence (about 12·9% of neonates have a peak bilirubin value of 205 μmol/l (12 mg/100 ml) or greater) should be greater than figures quoted in earlier reports. These surveys are few but Trolle[6] measured plasma bilirubin concentrations in a sample of 1469 untreated infants in 1968. Altogether 15·8% had serum bilirubin concentrations of 205 μmol/l or more.

A recent postal survey of neonatal paediatricians in Europe and the USA confirmed that most had seen no evidence of an increased incidence of neonatal jaundice since either 1970 or 1965.[7] Jaundice rates may fluctuate according to the interest taken in the condition and hence the measurements made of bilirubin concentrations. The availability of phototherapy and the continuing controversy over the role of oxytocin in jaundice may have led paediatricians to suspect significant jaundice more often.

We have analysed certain factors suggested to have been associated with this "increase" in neonatal jaundice, notably oxytocin and epidural anaesthesia.[2 3] The technique of multiple regression analysis enables these factors to be examined independently of their frequent association with one another and with other known icterogenic factors such as prematurity and instrumental delivery. Our results show that both oxytocin and epidural anaesthesia have a small effect, which probably explains

why some authors have found these factors to be associated with jaundice[2 5 6] while others have not.[7 10]

The mechanism of the icterogenic effect of these drug treatments is obscure. Neither bupivacaine, the local anaesthetic used in epidural blocks, nor oxytocin has any direct effect on glucuronidation of bilirubin in vitro.[11] The hypo-osmotic effect of oxytocin has been implicated,[12] as has the increased placental transfusion of fetal blood resulting from oxytocin.[13] So far as oxytocin induction is concerned this relationship may be due to an association between the use of induction in chronologically mature but physiologically immature infants who fail to initiate parturition.

The finding that premature, small-for-dates infants were protected from jaundice by their low weight suggests that hepatic glucuronyl transferase is prematurely developed in small-for-dates infants. This development recalls the accelerated surfactant production by the lung in small-for-dates infants, which has been attributed to intrauterine stress.[14] A teleological explanation would be that early maturation of liver and lung enzymes may prepare small-for-dates infants for premature delivery.

Neonatal jaundice is undoubtedly a time-consuming clinical problem. Severe physiological jaundice is associated with mild brain damage,[15 16] although classical kernicterus is rare. Our results indicate that the problem is unlikely to be resolved by changes in obstetric practice, for even elimination of epidural anaesthesia and oxytocin use would have little impact. These considerations may suggest to paediatricians that they should look again at prophylactic prenatal drug treatment for neonatal jaundice. Though phenobarbitone is effective,[9] its adverse effects may be greater than the morbidity and medical costs of a significant rate of neonatal jaundice; other enzyme-inducing agents might be found with fewer side effects.

We are grateful to Miss E C Curtis, Queen Charlotte's Hospital, and the computing advisory service, London School of Hygiene and Tropical Medicine. This study was supported by the Wellcome Trust.

References

1 Scheidt, P C, *et al*, *Journal of Pediatrics*, 1977, 91, 292.
2 Sims, D G, and Neligan, C A, *British Journal of Obstetrics and Gynaecology*, 1975, 82, 863.
3 Campbell, N, Harvey, D, and Norman, A P, *British Medical Journal*, 1975, 2, 548.
4 Davies, D P, *et al*, *British Medical Journal*, 1973, 3, 476.
5 Beazley, J M, and Alderman, B, *British Journal of Obstetrics and Gynaecology*, 1975, 82, 265.
6 Trolle, D, *Lancet*, 1968, 2, 705.
7 Friedman, L, Lewis, P, and Harvey, D, *British Medical Journal*, 1977, 2, 1223.
8 Chew, W C, and Swann, I L, *British Medical Journal*, 1977, 1, 72.
9 Friedman, E A, and Sachtleben, M R, *British Medical Journal*, 1976, 1, 199.
10 Boylan, P, *British Medical Journal*, 1976, 2, 564.
11 Lewis, P J, *Therapeutic Problems in Pregnancy*, p 141. Lancaster, Medical and Technical Publishing, 1977.
12 Singhi, S, and Singh, M, *British Medical Journal*, 1977, 2, 1028.
13 Gate, J M, *British Medical Journal*, 1978, 1, 49.
14 Gluck, L, and Kulovich, M V, *American Journal of Obstetrics and Gynecology*, 1973, 115, 539.
15 Boggs, T R, Jun, Hardy, J B, and Frazier, T M, *Journal of Pediatrics*, 1967, 71, 553.
16 Penwick, J D, *Journal of Laryngology and Otology*, 1975, 89, 925.

(*Accepted 27 February 1978*)

High prevalence of hyperuricaemia and gout in an urbanised Micronesian population

P Z ZIMMET, S WHITEHOUSE, L JACKSON, K THOMA

British Medical Journal, 1978, 1, 1237-1239

Summary and conclusions

The prevalence of hyperuricaemia and gout was investigated in the Micronesian inhabitants of the highly urbanised central Pacific island of Nauru. Sixty-four per cent of men and 60% of women aged 20 years and over had hyperuricaemia—the highest prevalence rates yet reported for a population. The hyperuricaemia in men was accompanied by a high prevalence of clinical gout (6·9%).

While the hyperuricaemia is probably genetic, the high prevalence of gout may be related to the environmental change from the traditional island style of living to one of almost complete Westernisation.

Department of Metabolic Medicine and Epidemiology, Southern Memorial Hospital, Melbourne 3162, Australia

P Z ZIMMET, PHD, FRACP, director
S WHITEHOUSE, computer fellow
L JACKSON, MB, BS, research associate
K THOMA, DMS, director of medical services, Nauru

Introduction

As the Third World nations change from subsistence to cash economies, drastic changes have occurred in the social, cultural, and health aspects of their societies. With the shedding of the traditional lifestyle for the seemingly more glamorous Western style of living and eating, there has been a dramatic rise in the prevalence of chronic degenerative diseases such as diabetes mellitus, hypertension, heart disease, and gout—conditions previously unknown or rare in these populations.[1-4] Nowhere have these changes been more apparent than in the developing countries of the Pacific.

Nevertheless, while diabetes, hypertension, and heart disease are rare in populations maintaining traditional lifestyles and appear only in their urbanised counterparts,[4] hyperuricaemia has been reported in Pacific islanders both affected and unaffected by Western influences.[1] Thus, while the clinical expression of hyperuricaemia may be influenced by the change to a modern lifestyle, the actual metabolic abnormality is present in both environments.

In one particular Micronesian population the results of Western influence have been disastrous. In a recent survey of the population of the urbanised central Pacific island of Nauru we found that 34·4% of the people aged 15 years and over had diabetes.[3] This figure is comparable with that of the American Pima Indians—a population with the highest prevalence of diabetes ever reported.[4]