

http://researchspace.auckland.ac.nz

ResearchSpace@Auckland

Copyright Statement

The digital copy of this thesis is protected by the Copyright Act 1994 (New Zealand).

This thesis may be consulted by you, provided you comply with the provisions of the Act and the following conditions of use:

- Any use you make of these documents or images must be for research or private study purposes only, and you may not make them available to any other person.
- Authors control the copyright of their thesis. You will recognise the author's right to be identified as the author of this thesis, and due acknowledgement will be made to the author where appropriate.
- You will obtain the author's permission before publishing any material from their thesis.

To request permissions please use the Feedback form on our webpage. <u>http://researchspace.auckland.ac.nz/feedback</u>

General copyright and disclaimer

In addition to the above conditions, authors give their consent for the digital copy of their work to be used subject to the conditions specified on the Library Thesis Consent Form.

THE METABOLISM OF STEROIDS BY HUMAN

MAMMARY TISSUES

Ronald A. F. Couch

I agree to this thesis being consulted and/or photocopied for the purpose of research or private study provided that due acknowledgment is made where appropriate and that my permission is obtained before any material is published.

> Thesis submitted to the Department of Surgery in partial fulfilment of the requirements for the degree of Doctor of Philosophy of the University of Auckland.

October, 1980

TABLE OF CONTENTS

		Page
	LIST OF TABLES	vi
	LIST OF FIGURES	viii
	ACKNOWLEDGEMENTS	x
	SUMMARY	xii
	ABBREVIATIONS	xv
CHAPTER 1	INTRODUCTION	
1.1	The Physiology of the Human Breast	1
1.2	The Occurrence of Neoplastic Breast Lesions	1
1.3	Historical Developments	3
1.4	Early Predictive Tests	5
1.5	The Source of Plasma Steroids	7
1.6	The Uptake and Binding of Steroids by Mammary Tissues and Receptors	13
1.7	The Use of Radioactive Tracers	17
1.8	Steroid Metabolism by Breast Tissue	18
1.9	Steroid Hydroxylase Reactions and Subsequent Metabolism	22
1.10	The Purpose of This Thesis	25
		Ÿ
CHAPTER 2	MATERIALS AND METHODS	
2.1	Solvents and Reagents	27
2.2	Non-radioactive and Radioactive Steroids	27
2.3	Chromatographic Materials	39
2.4	Miscellaneous	41
2.5	Chromatography Systems and Visualization Techniques	42
2.6	Tissue Specimens	43
2.7	In Vitro Incubations Using Sliced Tissue	44
2.8	Isolation and Quantitation of the Tritiated Water	46

2.9	Extraction of the Steroids	46
2.10	Quantitation of Radioactivity	48
2.11	Recovery Estimations of Carrier Steroids	50
2.12	Microchemical Reactions	52
2.13	Co-crystallization	53
2.14	Estimation of DNA in Tissue Samples	54
2.15	Estimation of Protein in Tissue Samples	54
2.16	Enzyme Assays of the 3β-Sulphatase, 7α-Hydroxylase and 7α-Hydroxysteroid dehydrogenase Enzymes	54
2.16.1	Incubation	55
2.16.2	Extraction	56
2.17	Sub-cellular Localization of Enzymes	57
2.17.1	Fractionation of the Sub-cellular Organelles	57
2.17.2	In Vitro Incubation of the Sub-cellular Fractions	58
	· •	
CHAPTER 3	SYNTHESIS OF STEROIDS	
3.1	Introduction	60
3.2	Experimental	61
3.2.1	The 7-Hydroxy Epimers of Dehydroepiandrosterone (DHA)	61
3.2.2	The 7-Hydroxy Epimers of Cholesterol	62
3.2.3	7a-Hydroxyandrostenedio1	63
3.2.4	7β-Hydroxyandrostenedio1	63
3.2.5	7-Ketoandrostenediol	64
3.2.6	7-Dehydro DHA	65
3.2.7	7α-Hydroxyandrostenedione	65
3.2.8	7α-Hydroxy DHA-3β-Sulphate	66
3.2.9	Pregnenolone-38-Sulphate	67
3.2.10	Androstenedio1-38-Sulphate	67
3.2.11	7α-Hydroxyestradiol	68

iii

Page

	3.2.12	7-Ketoestrone	68
	3.2.13	B-ring Unsaturated Estrogens	68
	3.2.14	[1,2- ³ H] 7 α and 7 β -Hydroxy DHA	69
	3.2.15	$[4-14C]$ 7 α -Hydroxy DHA	71
	3.2.16	[7- ³ H] Androstenedio1-3β-Sulphate	71
	3.2.17	Investigation of Hydrolysis Techniques for 7α-Hydroxy DHA-3β-Sulphate	71
	3.3	Discussion	74
CH	APTER 4	THE IN VITRO SYNTHESIS OF 7α-HYDROXYDEHYDROEPIANDROSTERONE FROM DEHYDROEPIANDROSTERONE SULPHATE BY HUMAN MAMMARY TISSUES	
	4.1	Introduction	78
	4.2	Experimental	80
	4.2.1	Incubations with [³ H] DHA-sulphate	80
	4.2.2	Characterization of the Polar Steroid	83
	4.2.3	Metabolism of [³ H] DHA-sulphate to 7α-Hydroxy DHA and Tritiated Water by Human Mammary Tissues	86
	4.2.4	Investigation of a Sulphate Conjugate Biosynthetic Pathway of Seven Hydroxylation in Mammary Tissue	88
	4.3	Discussion	88
<u>CH</u>	APTER 5	METABOLISM OF DEHYDROEPIANDROSTERONE BY HUMAN MAMMARY TISSUES	
	5.1	Introduction	92
	5.2	Experimental	92
	5.2.1	Metabolism of $[7(n)-{}^{3}H]$ Dehydroepiandrosterone	92
	5.2.2	Metabolism of $[7(n)-{}^{3}H$, $4-{}^{14}C$] Dehydroepiandrosterone	97
	5.2.3	Metabolism of [1,2-3H] Dehydroepiandrosterone	101
	5.3	Discussion	107

Page CHAPTER 6 THE METABOLISM OF 7-HYDROXYDEHYDROEPIANDROSTERONE BY HUMAN MAMMARY TISSUES 6.1 Introduction 121 6.2 Experimental 125 6.2.1 Metabolism of [1,2-3H] 7a-Hydroxy DHA 125 Metabolism of [1,2-³H] 7β-Hydroxy DHA 6.2.2 135 6.3 Discussion 136 CHAPTER THE METABOLISM OF ANDROSTENEDIONE, 7 DIHYDROTESTOSTERONE AND THE SULPHATE

CONJUGATES OF CHOLESTEROL, PREGNENOLONE

AND ESTRONE

7.1 Introduction 141 7.2 Experimental 143 Metabolism of [1,2-³H] Androstenedione 7.2.1 143 Metabolism of [1,2-3H] Cholesterol Sulphate 7.2.2 148 Metabolism of [7-3H] Pregnenolone Sulphate 7.2.3 154 Metabolism of [1,2-3H] Dihydrotestosterone 7.2.4 158 Metabolism of [6,7-³H] Estrone Sulphate 7.2.5 161 7.3 Discussion 167

CHAPTER	8	PROPERTIES OF THE SULPHATASE, 7α -HYDROXYLASE AND 7-HYDROXYSTEROID DEHYDROGENASE ENZYMES	
8.1		Introduction	175
8.2		Experimental .	175
8.2.1		Sulphatase .	175
8.2.2		7α-Hydroxylase	181
8.2.3		7α-Hydroxysteroid Dehydrogenase	185

iv

8.2.4	Sub-cellular Localization of the 3β-Sulphate Sulphatase Enzyme System in Human Mammary Tissue	185
8.2.5	Sub-cellular Localization of the 7α-Hydroxylase Enzyme System in Human Mammary Tissue	185
8.3	Discussion	190
	REFERENCES	205
	APPENDIX	221
	PUBLICATIONS	225

et e

Page

TABLES

CHAPTER 2	· · · · ·	Page
Table 2.1	Steroids	28
Table 2.2	Radioactive Steroids	40
Table 2.3	Classification of the Human Mammary Tissues Obtained from Twenty Five Female Patients	45
Table 2.4	Paper Chromatography of Steroids	49
Table 2.5	Gas Chromatography of Neutral Hydroxylated Steroids as the Trimethylsilyl Ether Derivatives	51
CHAPTER 3		
Table 3.1	Isolation of Tritiated 7α-Hydroxy DHA and 7β-Hydroxy DHA by Column Chromatography	70
Table 3.2	Crystallization of Products D, C and A with Carrier Steroids	70
CHAPTER 4		
Table 4.1	Percentage Conversion of DHA-Sulphate by Malignant and Adjacent Normal Human Mammary Tissues	82
Table 4.2	The Specific Activities of 7α-Hydroxy DHA After Recrystallization	87
Table 4.3	Percentage Conversion of DHA-sulphate to DHA, 7α-Hydroxy DHA and Tritiated Water by Human Mammary Tissues	87
Table 4.4	Percentage Conversion of [7(n)- ³ H] DHA-sulphate to 7α-Hydroxy DHA 3-sulphate, Free Steroids and Tritiated Water, by Human Mammary Tissue	89
CHAPTER 5		1
Table 5.1	The Metabolism of [7(n)- ³ H] Dehydroepiandrosterone to Tritiated Water and Steroid Metabolites by Human Mammary Tumour Tissue	96
Table 5.2	The ³ H/ ¹⁴ C Ratio of 7-Keto Dehydroepiandrosterone and its Acetate Derivative After Recrystallization	98
Table 5.3	The 7-Hydroxylation/Oxidation of [7- ³ H, 4- ¹⁴ C] DHA By Human Mammary Tumour Tissue	100
Table 5.4	Metabolism of [1,2- ³ H] Dehydroepiandrosterone by Human Mammary Tissues	103
Table 5.5	The Specific Activities of Estradiol After Recrystallization	106

vi

CHAPTER 6

CHAPTER 6	2.	Page
Table 6.1	Percentage Conversions of [1,2- ³ H] 7α-Hydroxy DHA to Metabolites by Human Mammary Tissue	127
Table 6.2	Thin Layer Chromatography of Estrogens	132
CHAPTER 7		
Table 7.1	Carrier Steroids Added to the [1,2- ³ H] Androstenedione Incubation Media	144
Table 7.2	Percentage Conversion of [1,2- ³ H] Androstenedione To Metabolites by Benign Hyperplastic Tissue	147
Table 7.3	The Specific Activity of 7-Ketocholesterol on the Paper Chromatogram	152
Table 7.4	The Metabolism of [7(n)- ³ H] Pregnenolone Sulphate to Seven Oxygenated Metabolites and Tritiated Water by Human Mammary Tissue	157
Table 7.5	The Metabolism of Dihydrotestosterone by Human Mammary Tissue	160
Table 7.6	The Metabolism of [6,7- ³ H] Estrone Sulphate By Human Mammary Malignant Tissue	165
CHAPTER 8		
Table 8.1	Activity of the 3β-Sulphate Sulphatase in Sub- cellular Fractions of Human Mammary Tissue	187
Table 8.2	Activity of the 7α-Hydroxylase in Sub-cellular Fractions of Human Mammary Tissue	187
Table 8.3	Apparent Michaelis Constants of Substrates Cleaved by the Steroid Sulphatase Enzyme System	193
Table 8.4	Concentration of Steroids in the Peripheral Circulation, Breast Tumours and Breast Secretions of Women	194

vii

FIGURES

CHAPTER 1	· ·	Page
Figure 1.1	Steroid Metabolism in the Human Adrenal	9
Figure 1.2	Steroid Metabolism in the Human Ovary	11
CHAPTER 3		
Figure 3.1	Degradation of 7α-Hydroxy DHA during a Strong Acid Hydrolysis Procedure	73
Figure 3.2	Steroidal Dimer	76
CHAPTER 5		
Figure 5.1	The Release of the Tritium Label from [7(n)- ³ H] DHA by Seven Hydroxylation	109
Figure 5.2	The Release of the Tritium Label from [7(n)- ³ H] DHA by Seven Oxidation	109
Figure 5.3	Enzymic Pathways for the Production of Androgens and Estrogens from Dehydroepiandrosterone	116
CHAPTER 6		
Figure 6.1	The Metabolism of DHA and DHA-sulphate by Human Mammary Tumour Tissue In Vitro	122
Figure 6.2	The Formation of Cholecalciferol (Vitamin D ₃) from 7-Dehydrocholesterol	124
Figure 6.3	The Postulated Reaction whereby a C ₁₉ Vitamin D3 Analogue could be derived from 7-Dehydro DHA	124
Figure 6.4	The Formation of Hydroxylated Metabolites of Cholecalciferol	124
Figure 6.5	The Postulated C ₁₉ Analogue of 1,25-Dihydroxycholecalciferol	124
Figure 6.6	Pathways Involving the Metabolism of 7α-Hydroxy DHA by Human Mammary Tissue	140
CHAPTER 7		
Figure 7.1	Radioscan of the Phenolic Fraction from the -[1,2- ³ H] Androstenedione Incubation	149
Figure 7.2	Radioscan of the Phenolic Fraction from the [6,7-3H] Estrone Sulphate Incubation	162
Figure 7.3	Metabolic Pathways of Steroid Metabolism in Human Mammary Tissue	174
	N	

С	Н	A	P	Τ	E	R	8

APTER 8		Page
Figure 8.1	Human Mammary Tissue Sulphatase Activity and DNA Content	177
Figure 8.2	DHA-sulphate, Androstenediol Sulphate and Estrone Sulphate: Sulphatase, Lineweaver-Burk Plots	179
Figure 8.3	DHA-sulphate, Pregnenolone Sulphate: Sulphatase, Lineweaver-Burk Plots	180
Figure 8.4	Dehydroepiandrosterone: 7α-Hydroxylase, Lineweaver-Burk Plot	183
Figure 8.5	Androstenediol and Dehydroepiandrosterone: 7α-Hydroxylase, Lineweaver-Burk Plots	184
Figure 8.6	7α-Hydroxydehydroepiandrosterone: 7α-Hydroxysteroid Dehydrogenase, Lineweaver-Burk Plot	186

ix

ACKNOWLEDGEMENTS

I wish to thank my supervisors, Dr. S. J. M. Skinner (Department of Surgery, University of Auckland) and Professor R. C. Cambie (Department of Chemistry, University of Auckland) for their invaluable guidance during this study.

It is with gratitude that I acknowledge the active co-operation of the following members of the Department of Surgery:-

Mr. M. F. Flint, Consultant Surgeon, from whose patients I obtained the specimens of mammary benign hyperplastic and ptotic tissue, and with whose help and that of Dr. A. Poole, the photographic plates were obtained.

Mrs. R. Richards, Mrs. K. Speak and Mrs. M. Lyons for histological staining of tissue specimens.

Dr. G. C. Gillard for invaluable discussion and Miss J. Brindle for assistance with protein assays.

I am most grateful to Surgeons of the Auckland and Greenlane Hospitals from whose patients malignant and fibroadenomatous breast tissue was obtained, to the histological services of these hospitals and to Mrs. P. Trindall and Miss B. Mason for obtaining patient data.

Helpful advice was offered by colleagues in the Biochemistry Department. Nuclear magnetic resonance spectra were produced by Mr. D. J. Calvert (Department of Chemistry). Dr. H. Young (Department of Scientific and Industrial Research, Auckland) produced some of the mass spectra while others were obtained from the Tenovus Institute for Cancer Research, Cardiff.

This thesis was typed by my wife Lynnette whose helpful participation and support throughout this study were a constant stimulus.

Financial support was provided by the Medical Research Council of New Zealand and the Isaacs Medical Research Fellowship.

The author dedicates this thesis to the Late Mr. T. W. Doouss who originally established the Steroid Breast Cancer Research Group from within which this study arose.

SUMMARY

1.

2.

Human mammary tissue was incubated *in vitro* with $[7-^{3}H]$ dehydroepiandrosterone sulphate (DHA-sulphate) and in agreement with other investigators this steroid conjugate was metabolized to DHA and other steroid products. Sulphatase activity was greater in the malignant than the non-malignant tissues and was found to be a function of the tissue cellularity. One of the major products, a "polar steroid" necessitated identification.

The "polar steroid" was identified principally as 7α -hydroxy DHA by chemical modification techniques and co-crystallization of the purified steroid metabolite with carrier 7α -hydroxy DHA. This carrier required synthesis and characterization.

3. Similarly incubation experiments carried out with the substrates $[7-^{3}H]$ DHA and $[7-^{3}H, ^{14}C]$ DHA showed that the tritium label was partially displaced from the C₇ position as a result of 7-oxygenation. The metabolites 7-keto DHA and 7\beta-hydroxy DHA were identified. When the substrate was $[1,2-^{3}H]$ DHA the label remained on the steroid and 7-keto DHA as well as 7α -hydroxy DHA were identified as major products.

4. During the incubations with $[7-^{3}H]$ DHA and $[1,2-^{3}H]$ DHA the C₁₉ steroids androstenediol, androstenedione, androstanedione and testosterone were isolated as metabolites. Evidence was obtained for the minimal metabolism of DHA to DHA-sulphate and to estradiol. No metabolic pathways were found to occur selectively in the malignant or non-malignant tissues.

xii

Other Δ^5 -3 β -hydroxy steroids (cholesterol sulphate and pregnenolone sulphate) were also found to be suitable substrates for the sulphatase and 7 α -hydroxylase enzyme systems. The 7 α -hydroxy and 7-keto metabolites of both substrates were identified. However, the Δ^4 -3-ketosteroid androstenedione and the 5 α -reduced steroid dihydrotestosterone did not undergo 7-oxygenation, but were metabolized to other steroids containing two oxygen functions.

5.

6.

.8.

High specific activity $[1,2-^{3}H]$ [7 α -hydroxy DHA and $[1,2-^{3}H]$ 7 β -hydroxy DHA were synthesized and incubated *in vitro* with human mammary tissues. These substrates were metabolized principally to 7-keto DHA and minor quantities of the respective 3 β ,7,17 β -triols. 7 α -Hydroxy DHA was sulpho-conjugated at the 3 β position. Also there was evidence for an epimerase converting 7 α -hydroxy DHA to 7 β -hydroxy DHA and vice versa. There was no evidence for the metabolism to 7-dehydro DHA, the B-ring unsaturated estrogens or 7-hydroxylated estrogens, and no evidence for a selective pathway of metabolism in malignant or non-malignant tissues.

7. It was concluded that 7-oxygenated derivatives of DHA were not precursors of potent physiologically active steroids. Alternatively, this metabolic pathway may act either as an alternative to the production of androgens and estrogens from DHA or, by the production of 7 α -hydroxyandrostenediol, may act to inhibit the antagonistic effect of androstenediol towards estradiol at the estrogen receptor.

The sulphatase activity previously found to hydrolyse the sulphate conjugates of C_{19} , C_{21} and C_{27} steroids was also found to cleave estrone sulphate. The principal metabolites were estrone and estradiol.

xiii

The apparent Michaelis Constants were determined for the hydrolysis of estrone sulphate, DHA-sulphate, androstenediol sulphate and pregnenolone sulphate by human mammary tissue sulphatase(s). Apparent Michaelis Constants were also obtained for the 7 α -hydroxylation of DHA and androstenediol and for the conversion of 7 α -hydroxy DHA to 7-keto DHA. This data indicates that all these metabolic steps can occur at the physiological concentration of the steroid substrate.

9.

10. The sulphatase and the 7α -hydroxylase enzyme systems were found to be located in the microsomal fraction of the human mammary tissue cellular preparation.

11. Plasma steroids directly influence the steroid micro-environment of body tissues. However, this study has shown that in mammary neoplastic lesions this environment is further determined by enzymic steroid reactions carried out by the neoplastic tissue. These pathways of steroid metabolism involve the production of estrogenic and androgenic steroids, estrogen agonist and antagonist steroids, steroid sulphate conjugates and steroids which are probably non-hormonal.

xiv

ABBREVIATIONS

-

n	as in '[7(n)- ³ H]' denotes that the steroid was nominally labelled with tritium at the C7 carbon atom.
В	as in 'B-ring' denotes the steroid 'hexagon' formed by the C5 to C10 carbon atoms.
CDC13	deuterated chloroform
C ₅ D ₅ N	deuterated pyridine
МеОН	methanol
EtOH	ethanol
NaBH4	sodium borohydride
TCA	trichloroacetic acid
<u>M</u> +.	mass of the molecular ion
m/z	mass of molecular ion fragments
3	extinction coefficient
d.p.m.	disintegrations per minute
f	femto
λ	ultraviolet absorption maxima
ν	infrared absorption maxima
m.p.	melting point

4