

Copyright is owned by the Author of the thesis. Permission is given for a copy to be downloaded by an individual for the purpose of research and private study only. The thesis may not be reproduced elsewhere without the permission of the Author.

**Studies on the Material
Responsible for Activity
Attributed to the
Glucose Tolerance Factor**

A thesis presented in partial fulfilment
of the requirements for
the degree of

Doctor of Philosophy
in Chemistry

at Massey University
New Zealand

Peter Robin SHEPHERD
1989

Massey University Library

Thesis Copyright Form

Title of thesis: Studies ~~of~~ on the Material Responsible
for Activity Attributed to the Glucose
Tolerance Factor

(1) (a) I give permission for my thesis to be made available to readers in the Massey University Library under conditions determined by the Librarian.

~~I do not wish my thesis to be made available to readers without my written consent for _____ months.~~

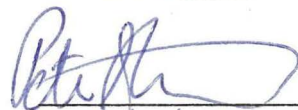
(2) (a) I agree that my thesis, or a copy, may be sent to another institution under conditions determined by the Librarian.

~~(b) I do not wish my thesis, or a copy, to be sent to another institution without my written consent for _____ months.~~

(3) (a) I agree that my thesis may be copied for Library use.

~~(b) I do not wish my thesis to be copied for Library use for _____ months.~~

Signed



Date

23/2/89

The copyright of this thesis belongs to the author. Readers must sign their name in the space below to show that they recognise this. They are asked to add their permanent address.

NAME AND ADDRESS

P. Shepherd
P.O. Box 200
Kaitiaki



DATE

23/2/89

Abstract

An extract that was known to have activity attributed to the glucose tolerance factor (GTF) was isolated from brewers yeast and this was used as the starting point in attempts to isolate GTF.

The initial extract from brewers yeast was shown to be far from pure as it was separated into 15 fractions using high voltage paper electrophoresis. GTF activity was initially monitored using a simple yeast fermentation assay and the more active of these fractions were further purified using reverse phase high performance liquid chromatography. The most active fraction was anionic and contained little chromium or amino acid material although mass spectroscopy revealed the presence of adenine. However the exact nature of the material remained elusive.

Due to doubts about the specificity of the simple yeast assay a modified version of the yeast assay was investigated which measured the ability of the sample to stimulate the metabolism of yeast cells above the level that was accounted for by cell proliferation. This assay were shown to be very reproducible although the most active fraction from the simple yeast assay was not active in this assay.

The low chromium rat epididymal adipocyte assay was investigated as a possible means of verifying the results of the modified yeast assay. The importance of diet as a determinant of whether adipocytes would respond to GTF was investigated using 4 different diets. The adipocytes from rats fed on a torula yeast diet produced the maximum potentiations and it was found that a unique feature of these cells was a reduced ability to convert glucose to fatty acids via the glycolytic pathway. The potentiations seen in these cells were most obvious in the conversion of 1-¹⁴C-glucose to CO₂ and fatty acids and it was concluded that this was due to either a potentiation of glucose transport or of acetyl-CoA carboxylase.

An extract of torula yeast was prepared in a similar method to that used to isolate the initial extract from brewers yeast. This extract showed high levels of activity in the both assay systems which indicated that these assay was not measuring GTF as originally defined. This was further indicated by the finding that no one compound was responsible for the activity in any of the assays investigated.

The chromium contained in the original yeast extract was also spread amongst many fractions and the chromium content of these fractions bore no correlation to the activity in the assay systems indicating that the active fractions were not chromium complexes.

Overall these results show that there is no unique factor responsible for the activity in the simple yeast assay and the low chromium rat adipocyte assay. Further it was concluded that none of the active material represented chromium complexes. As the activity in these assays was thought to be due to the presence of a GTF this firstly strongly argues against GTF being a chromium complex and secondly it questions the existence of GTF at all.

Acknowledgements

I have many people to thank for making this thesis possible and only a very small space to do it in. However I could start by saying;

I wish to sincerely thank my supervisors, Dr Len Blackwell and Dr Paul Buckley for their advice, guidance and above all else for their patience and understanding with regard to my unusual working hours.

I would also like to sincerely thank Professor Clint Elwood (State University of New York, Syracuse) and Emeritus Professor Eric Holdsworth (University of Tasmania) whose advice during their period of sabbatical leave here was an invaluable stimulus at a time when morale was flagging.

Thanks also to Professor Dick Hodge, Dr Graham Midwinter and Mr Dick Poll all of the Department of Chemistry and Biochemistry at Massey University for their assistance with chemical and biochemical techniques

I would like to particularly thank my mother and father who have indirectly been major investors in this project and who, as yet, have not seen any dividends.

I would also especially like to thank my grandfather, Mr Herbert Crownshaw, for instilling in me an interest and confidence in science from early age.

Other names which spring to mind are Anna Woolf, Laurel, Marilyn, Tim Jackson, Dr Kerry Loomes, Dr Neill Haggarty, Dr David Palmer, Mark Gall, Mark Smith, Dr Rose Motion, Mr Fitzherbert, MUSA and there are no doubt many more.

Finally I would like to thank the typist, Mr Peter Shepherd, despite all the mistakes that undoubtedly remain in this thesis.

Table of Contents

	<u>Page</u>
ABSTRACT	ii
ACKNOWLEDGEMENTS	iii
TABLE OF CONTENTS	v
LIST OF FIGURES	x
LIST OF TABLES	xii

Chapter 1 Review of Previous Investigations Related to GTF

1.1	Introduction	1
1.2	Assays for GTF	2
	1.2.1 In vivo Assays	3
	1.2.2 In vitro Assays	4
1.3	Studies of the Structure of GTF	7
	1.3.1 Attempts to Isolate GTF	7
	1.3.2 Attempts to Synthesize GTF	15
	1.3.2.1 Synthetic Chromium Complexes Investigated for GTF Activity	15
	1.3.2.2 Synthetic Chromium Complexes Not Investigated for GTF activity	18
1.4	Studies of the Function of GTF	18
	1.4.1 Does GTF Assist the Binding of Insulin to the Cell ?	18
	1.4.2 Does GTF Potentiate the Transport of Glucose ?	20
	1.4.3 Does GTF Act Intracellularly ?	21
	1.4.4 Discussion	21
1.5	Possible Alternative Explanation for Potentiation of Insulin Action by GTF Fractions	22
	1.5.1 Insulin Potentiators	22
	1.5.2 Insulin Mimickers	23
	1.5.3 Non Specific Effects	24
	1.5.4 Trace Elements	24
	1.5.5 Discussion	26
1.6	In vivo Effects of Chromium and GTF Supplementation	27
1.7	Aims of This Thesis	33

Chapter 2 The Standard Yeast Assay for GTF

	<u>Page</u>	
2.1	Introduction	34
2.2	Methods and Materials	
	2.2.1 Yeast Growth and Harvesting	34
	2.2.2 The Yeast Assay	36
	2.2.3 Calculations	37
	2.2.4 Improvements to Methodology	38
2.3	Results	
	2.3.1 Reproducibility of Results	38
	2.3.2 Effect of Ammonia on the Assay System	38
2.4	Discussion	39

Chapter 3 A New Approach to Separating GTF Fractions from Brewers Yeast

3.1	Introduction	41
3.2	Methods and Materials	
	3.2.1 Isolation of Crude GTF Extracts	42
	3.2.2 Paper Electrophoresis	42
	3.2.3 Elution of Fractions From Paper	43
	3.2.4 HPLC of Electrophoresis Fractions	43
	3.2.5 Amino Acid Analysis of Fractions	44
	3.2.6 Trace Metal Analysis of Fractions	44
	3.2.7 Mass Spectrometry	44
	3.2.8 UV/Visible Absorbance Spectrum	45
	3.2.9 Determination of GTF Activity	45
3.3	Results	
	3.3.1 Crude GTF Extracts	45
	3.3.1.1 Composition and General Characteristics	45
	3.3.1.2 Activity by the Yeast Assay	46
	3.3.2 Electrophoresis Fractions From the Crude GTF Extracts	
	3.3.2.1 Naming of Fractions	47
	3.3.2.2 Elution of Fractions	47
	3.3.2.3 Activity by the Standard Yeast Assay	49
	3.3.2.4 Composition and General Characteristics of Electrophoresis Fractions	49
	3.3.3 HPLC Fractions of Electrophoresis Fractions	52

	<u>Page</u>
3.3.3.1 Isolation and Activity of HPLC Fractions	52
3.3.3.2 Composition and General Characteristics of HPLC Fractions of B-A-3	53
3.4 Discussion	59
3.4.1 Purification of Extracts With GTF Activity From Brewers Yeast	59
3.4.2 Comparison of Extracts With GTF Activity From Brewers Yeast With Similar Extracts from Torula Yeast	66

Chapter 4 A Modified Yeast Assay

4.1 Introduction	69
4.2 Materials and Methods	
4.2.1 Yeast Cell Growth and Harvesting	70
4.2.2 Assay Protocol	70
4.2.3 Yeast Cell Concentration Determination	71
4.2.4 Calculations	71
4.2.5 Preparation of Chromium Complexes	71
4.3 Results	
4.3.1 Characteristics of the Chromium Complex	72
4.3.2 Measurements of Differences in the Rate of Cell Proliferation	72
4.3.3 Investigation of the Parameters of the Modified Assay	72
4.3.4 Assay of Samples by the Modified Yeast Assay	72
4.4 Discussion	76

Chapter 5 Investigations of the Low Chromium Rat Epididymal Adipocyte Assay

5.1 Introduction	83
5.2 Methods and Materials	
5.2.1 Animals and Diet	84
5.2.2 The Adipocyte Assay	
5.2.2.1 Materials	86
5.2.2.2 Preparation of Isolated Adipocytes	87
5.2.2.3 Assay Incubation	88
5.2.2.4 Standard Assay Protocol	89
5.2.2.5 Measuring Metabolic Products	89
5.2.2.6 Determination of the Potentiation of Sub Optimal Insulin Concentrations	91

	<u>Page</u>	
5.3	Results	
5.3.1	Standard Curve for Insulin	93
5.3.2	Investigation of the Potentiation of the Action of Sub Optimal Insulin Concentrations on the Metabolism of Various Substrates to CO ₂ , Total Lipid, Fatty Acids and Glycerol.	94
5.4	Discussion	
5.4.1	Significance of Assay Results	95
5.4.2	Observations About the Metabolism of Adipocytes Isolated from Rats on the Torula Yeast Diet	101
5.4.3	Site of Action of the Material Causing Potentiation of the Adipocyte Assay	106
5.4.4	Implications of Investigations of the Site of Action of GTF Fractions for the Adipocyte Assay System	109
5.4.5	Implications of the Potentiation Activity Shown by the Crude Extract From Torula Yeast	109

Chapter 6 The Effect of Diet on the Response of the Rat Epididymal Assay System to GTF

6.1	Introduction	111
6.2	Methods and Materials	
6.2.1	Animals and Diet	112
6.2.2	Adipocyte Assay	112
6.2.3	Amino Acid Analysis	113
6.2.4	Trace Metal Analysis	113
6.3	Results	
6.3.1	Growth Rate of Rats	113
6.3.2	Adipocyte Assays	113
6.3.3	Amino Acid Analysis	114
6.3.4	Trace Element Analysis of the Diet	114
6.4	Discussion	
6.4.1	Relationship Between Growth Rate and GTF Response	115
6.4.2	Observations About the Metabolism of Adipocytes Isolated From Rats Raised on Different Diets	123
6.4.3	Importance of Different Diets in the Production of Adipocytes in Which the Potentiations Caused by GTF Extracts Can be Observed	124

6.4.4 Dietary Explanations for the Different Responses Seen in Different Tissues	124
6.4.5 Conclusions	127

Page

Chapter 7 Purification of Fractions That Cause Potentiation of the Action of Sub Optimal Amounts of Insulin in the Low Chromium Epididymal Rat Adipocyte Assay System

7.1	Introduction	129
7.2	Methods and Materials	
	7.2.1 Isolation of Crude GTF Extracts	130
	7.2.2 Fractionation of Crude GTF Extracts	130
	7.2.3 Amino Acid, Trace Element and UV/Visible Spectral Analysis of Fractions	130
	7.2.4 Mass Spectrometric Analysis of Fractions	130
	7.2.5 Determinations of GTF Activity	131
7.3	Results	
	7.3.1 Electrophoresis Fractions	131
	7.3.2 HPLC of Fraction B-B-I	131
	7.3.3 Amino Acid Analysis of Purified Fractions	134
	7.3.4 Chromium Analysis of Purified Fractions	138
	7.3.5 UV/Visible Spectra of Purified Fractions	138
	7.3.6 Mass Spectral Analysis of Purified Fractions	138
7.4	Discussion	143

Chapter 8 Overview

8.1	Overview	146
	LIST OF ABBREVIATIONS USED	150
	APPENDIX 1	151
	BIBLIOGRAPHY	153

List of Figures

3.1	Electrophoresis Bands of Crude GTF Extract B	48
3.2	Comparison of Electrophoretic Separation of GTF Fractions	50
3.3	HPLC Profiles of B-A-3	54
3.4	HPLC Profile of B-A-4	55
3.5	HPLC profile of B-B-4	55
3.6	Amino Acid Analysis of Hydrolysed and Unhydrolysed B-A-3-3	56
3.7	Mass Spectra of B-A-3-3	58
3.8	UV/Visible Spectra of B, B-A-3, B-A-3-3	60
3.9	UV/Visible Spectra of ATP and Adenosine	61
3.10	UV/Visible Spectra of NAD and Nicotinic Acid	62
4.1	Cell Proliferation Due to Crude GTF Extract B	73
4.2	Cell Proliferation Caused by Arginine	73
4.3	Cell Proliferation Caused by B-A-3	74
4.4	Cell proliferation Caused by the Chromium Complex	74
4.5	Investigation of Cellular CO ₂ Evolution With Time in the Yeast Assay	75
4.6	Modified Yeast Assay of Crude GTF Extract B	77
4.7	Modified Yeast Assay of B-A-3	78
4.8	Modified Yeast Assay of Arginine	79
4.9	Modified Yeast Assay of the Chromium Complex	78
5.1	Photographs of Rat Housing	85
5.2	Arrangement of Incubation Flask	89
5.3	Calculation of the Potentiation of Sub Optimal Insulin Action by GTF Samples	92
5.4	Standard Curve for the Action of Insulin on the Metabolism of D-[U- ¹⁴ C]-glucose	93
5.5	Effects of Crude GTF Extracts B & T on the Insulin Stimulated Conversion of D-[U- ¹⁴ C]-glucose to ¹⁴ CO ₂ and ¹⁴ C-Fatty Acids	96
5.6	Effects of Crude GTF Extracts B & T on the Insulin Stimulated Metabolism of D-[1- ¹⁴ C]-glucose to ¹⁴ CO ₂ and ¹⁴ C-Fatty Acids	97
5.7	Effects of Crude GTF Extract B on the Insulin Stimulated Conversion of D-[U- ¹⁴ C]-glucose, D-[1- ¹⁴ C]-glucose, D-[6- ¹⁴ C]-glucose and ¹⁴ C-acetate to ¹⁴ CO ₂ , ¹⁴ C-Total Lipid, ¹⁴ C-Fatty Acids and ¹⁴ C-Glycerol in Adipocytes Isolated From Rats on the 30% Torula Yeast Diet	98
5.8	Schematic Representation of Metabolism of Glucose to Fatty Acids in Adipocytes	102
6.1	Comparison of Growth Rates of Rats on Different Diets	114

6.2	Effects of Crude GTF Extract B on the Insulin Stimulated Conversion of D-[U- ¹⁴ C]-glucose, D-[1- ¹⁴ C]-glucose and D-[6- ¹⁴ C]-glucose into ¹⁴ CO ₂ , ¹⁴ C-Total Lipid, ¹⁴ C-Fatty Acids and ¹⁴ C-Glycerol in Adipocytes Isolated From Rats on the Normal Diet	117
6.3	Effects of Crude GTF Extract B on the Insulin Stimulated Conversion of D-[U- ¹⁴ C]-glucose, D-[1- ¹⁴ C]-glucose and D-[6- ¹⁴ C]-glucose into ¹⁴ CO ₂ , ¹⁴ C-Total Lipid, ¹⁴ C-Fatty Acids and ¹⁴ C-Glycerol in Adipocytes Isolated From Rats on the 30% Brewers Yeast Diet	119
6.4	Effects of Crude GTF Extract B on the Insulin Stimulated Conversion of D-[U- ¹⁴ C]-glucose, D-[1- ¹⁴ C]-glucose and D-[6- ¹⁴ C]-glucose into ¹⁴ CO ₂ , ¹⁴ C-Total Lipid, ¹⁴ C-Fatty Acids and ¹⁴ C-Glycerol in Adipocytes Isolated From Rats on the 30% Casein Diet	121
7.1	Effect of the Electrophoresis Fractions of Crude GTF B on the Insulin Stimulated Conversion of D-[U- ¹⁴ C]-glucose to ¹⁴ CO ₂ in Adipocytes of Rats Raised on the Torula Yeast Diet	132
7.2	Effects of the Electrophoresis Fractions of Crude GTF B on the Insulin Stimulated Conversion of D-[1- ¹⁴ C]-glucose to ¹⁴ CO ₂ and ¹⁴ C-Fatty Acids in Adipocytes of Rats Raised on the Torula Yeast Diet	133
7.3	HPLC Profiles of B-B-I and B-B-I-1	135
7.4	Effects of the HPLC Fractions of B-B-I on the Insulin Stimulated Conversion of D-[U- ¹⁴ C]-glucose to ¹⁴ CO ₂ and ¹⁴ C-Fatty Acids in Adipocytes of Rats Raised on the Torula Yeast Diet	136
7.5	Effects of the HPLC Fractions of B-B-I on the Insulin Stimulated Conversion of D-[1- ¹⁴ C]-glucose to ¹⁴ CO ₂ and ¹⁴ C-Fatty Acids in Adipocytes of Rats Raised on the Torula Yeast Diet	137
7.6	Mass Spectra of B-B-I-1	139
7.7	Effects of the Fraction B-B-I-1-2 on the Insulin Stimulated Conversion of D-[U- ¹⁴ C]-glucose to ¹⁴ CO ₂ and ¹⁴ C-Fatty Acids in Adipocytes of Rats Raised on the Torula Yeast Diet	140
7.7	Effects of the Fraction B-B-I-1-2 on the Insulin Stimulated Conversion of D-[1- ¹⁴ C]-glucose to ¹⁴ CO ₂ and ¹⁴ C-Fatty Acids in Adipocytes of Rats Raised on the Torula Yeast Diet	141
7.9	UV/Visible Spectra of B-I and B-B-I-1	142

List of Tables

1.1	Effect of Chromium and GTF Supplementation in Humans	28
1.2	Effect of Chromium and GTF Supplementation on Animals	31
2.1	Composition of Mineral and Essential Vitamin Solutions	35
2.2	Interassay Variation of Assays With Arginine	39
2.3	Effect of Ammonia on the Standard Yeast Assay	39
3.1	Amino Acid Composition of Crude GTF Extracts B & T and of the Yeasts From Which They Were Extracted	45
3.2	Trace Element Composition of Crude GTF Extracts B & T	46
3.3	Activity of Crude GTF Extracts in the Standard Yeast Assay	47
3.4	Comparison of Various Solvents for Eluting Electrophoresis Fractions, Isolated From Crude GTF Extract of Brewers Yeast, From Paper	51
3.5	Activity in the Standard Yeast Assay of the Fractions Separated by Electrophoresis	49
3.6	Amino Acid Analysis of the Most Active Electrophoresis Fractions from Crude GTF Fraction B	52
3.7	Chromium Content of Electrophoresis Fractions	52
3.8	Characteristics of HPLC Fractions of B-A-3	57
3.9	Activity of Various Compounds Implicated in the Makeup of B-A-3-3	57
3.10	Intra Assay Comparison of Active Electrophoresis Fractions	64
4.1	Enhancement of the Rate of Cellular CO ₂ Evolution in the Modified Yeast Assay	76
5.1	Potentiation of the Action of a Sub Optimal Insulin Concentration by Crude GTF Fractions	94
5.2	Potentiation of the Action of a Sub Optimal Insulin Concentration by Crude GTF Fraction B	95
6.1	Potentiation, by Crude GTF Extract B, of the Action of Sub Optimal Insulin Concentrations in Adipocytes of Rats Raised on Different Diets	115
6.2	Amino Acid Analysis of Diets	116
6.3	Trace Element Composition of Diets as Determined by ICP	116
6.4	Estimation of Fibre Content of Rat Diets	125
7.1	Potentiation of the Action of Sub Optimal Concentrations of Insulin by Electrophoresis Fractions of Crude GTF Extract B	132
7.2	Potentiation of the Action of Sub Optimal Concentrations of Insulin by HPLC Fractions of B-B-I	134
7.3	Potentiation of the Action of Sub Optimal Concentrations of Insulin by Fraction B-B-I-2	138