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**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

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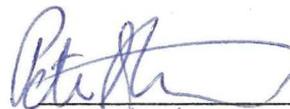
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## Abstract

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-<sup>14</sup>C-glucose to CO<sub>2</sub> 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.

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# 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 <sub>2</sub> , 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 <sub>2</sub> 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- <sup>14</sup> C]-glucose	93
5.5	Effects of Crude GTF Extracts B & T on the Insulin Stimulated Conversion of D-[U- <sup>14</sup> C]-glucose to <sup>14</sup> CO <sub>2</sub> and <sup>14</sup> C-Fatty Acids	96
5.6	Effects of Crude GTF Extracts B & T on the Insulin Stimulated Metabolism of D-[1- <sup>14</sup> C]-glucose to <sup>14</sup> CO <sub>2</sub> and <sup>14</sup> C-Fatty Acids	97
5.7	Effects of Crude GTF Extract B on the Insulin Stimulated Conversion of D-[U- <sup>14</sup> C]-glucose, D-[1- <sup>14</sup> C]-glucose, D-[6- <sup>14</sup> C]-glucose and <sup>14</sup> C-acetate to <sup>14</sup> CO <sub>2</sub> , <sup>14</sup> C-Total Lipid, <sup>14</sup> C-Fatty Acids and <sup>14</sup> 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- <sup>14</sup> C]-glucose, D-[1- <sup>14</sup> C]-glucose and D-[6- <sup>14</sup> C]-glucose into <sup>14</sup> CO <sub>2</sub> , <sup>14</sup> C-Total Lipid, <sup>14</sup> C-Fatty Acids and <sup>14</sup> 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- <sup>14</sup> C]-glucose, D-[1- <sup>14</sup> C]-glucose and D-[6- <sup>14</sup> C]-glucose into <sup>14</sup> CO <sub>2</sub> , <sup>14</sup> C-Total Lipid, <sup>14</sup> C-Fatty Acids and <sup>14</sup> 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- <sup>14</sup> C]-glucose, D-[1- <sup>14</sup> C]-glucose and D-[6- <sup>14</sup> C]-glucose into <sup>14</sup> CO <sub>2</sub> , <sup>14</sup> C-Total Lipid, <sup>14</sup> C-Fatty Acids and <sup>14</sup> 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- <sup>14</sup> C]-glucose to <sup>14</sup> CO <sub>2</sub> 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- <sup>14</sup> C]-glucose to <sup>14</sup> CO <sub>2</sub> and <sup>14</sup> 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- <sup>14</sup> C]-glucose to <sup>14</sup> CO <sub>2</sub> and <sup>14</sup> 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- <sup>14</sup> C]-glucose to <sup>14</sup> CO <sub>2</sub> and <sup>14</sup> 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- <sup>14</sup> C]-glucose to <sup>14</sup> CO <sub>2</sub> and <sup>14</sup> 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- <sup>14</sup> C]-glucose to <sup>14</sup> CO <sub>2</sub> and <sup>14</sup> 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 <sub>2</sub> 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

# CHAPTER 1

## REVIEW OF PREVIOUS INVESTIGATIONS RELATED TO GTF

### 1.1 Introduction

The enigma of the putative Glucose Tolerance Factor (GTF) can be traced back to the observations of Glaser and Halpern (1929) who noticed that yeast extracts potentiated the action of insulin in lowering blood sugar but that the yeast press juice by itself had no effect. Subsequently Mertz and Schwarz (1955) reported that extracts of Brewers yeast were able to reverse the impairment of glucose tolerance seen in rats fed a certain torula yeast based diet; they called this fraction Factor 3. Further work by the same workers resulted in the separation of Factor 3 into a selenium containing component and a component restoring glucose tolerance. On the basis of these results the existence of a separate Glucose Tolerance Factor was postulated (Schwarz and Mertz 1957). GTF was defined as being a factor, obtained from brewers yeast or defatted pork kidney powder, responsible for the maintenance of normal glucose tolerance in rats.

Later work (Schwarz and Mertz 1959) showed that the GTF activity of the yeast fractions was not destroyed by wet ashing with  $\text{HNO}_3/\text{H}_2\text{SO}_4$  which is in apparent conflict with the observation in the same paper that GTF fractions readily lost activity on storage at 4 °C. However these authors took this observation to indicate that a trace element was the active ingredient and 43 simple inorganic complexes were therefore screened for GTF activity. Only chromium (III) compounds showed significant activity as judged by the ability to restore normal glucose removal rates in rats where an impairment in this parameter had been induced (Schwarz & Mertz 1959). Since this time GTF has been inextricably linked to chromium and GTF has been assumed to be the biologically active form of chromium. It has also led to chromium being considered an essential trace element in human metabolism.

GTF lacks a succinct definition. A list of five criteria which a compound or fraction must fulfil to be called GTF is outlined by Mertz *et al.* (1974). Specifically these are:

1. Potentiation of the action of insulin *in vitro*
2. Ability to restore an induced impairment of glucose tolerance
3. Facilitation of an increase in intestinal absorption of chromium

4. Facilitation of an increase in the incorporation of chromium into specific pools
5. Facilitation of the transport of chromium across the placenta.

In this definition it is assumed that GTF is a biologically active form of chromium and as such displays the biological and metabolic properties attributed to simple chromium compounds, but to a greater degree. Since the structure of GTF is unknown it can only be recognised on the basis of its supposed effects and in practice the first criterion is the one most commonly used as a measure of GTF activity. Until the nature of the substance or substances causing these effects is identified a more specific definition is impossible and thus different researchers may well be working with different materials all of which are collectively referred to as GTF. This practice will be continued in this thesis where any fraction which shows activity in a defined assay for GTF will be referred to as a GTF containing fraction.

There have been many reviews of the field (Mertz 1969, Guthrie 1982, Mertz 1983, Wallach 1985, Barret *et al.* 1985, Anderson 1986) but most have concentrated on the association between chromium and GTF rather than reviewing the causes of the observed activity that is attributed to GTF. These reviews show that chromium is believed to have some undiscovered, but essential role, in human metabolism and that GTF is thought of as the predominant form of biologically active chromium.

However there is a growing realisation that GTF activity is not necessarily associated with chromium and in recent years a number of workers (Haylock *et al.* 1983a & b, Donaldson *et al.* 1985, Davies *et al.* 1985 a & b, Hwang *et al.* 1987) have reported a separation of chromium from fractions that still give what has traditionally been accepted as GTF activity. This has forced a re-evaluation of previous data and there is now a growing opinion that a biologically active form of chromium (BAC) and the material traditionally identified as GTF may be different compounds and that GTF material may not be a chromium complex (Offenbacher *et al.* 1988, Anderson 1988). Irrespective of whether these conclusions are correct there is a need for further investigation of the factors causing the metabolic effects attributed to GTF.

## **1.2 Assays for GTF**

As discussed above the definition of GTF activity is critical to investigations into its structure and function. The original definition that it is a fraction able to restore impaired glucose tolerance in rats fed a diet inducing dietary liver necrosis (Mertz 1969) is an operationally unwieldy definition of the function of GTF. This definition has been further

extended to imply that GTF is a chromium containing compound (Mertz 1969), without ever firmly establishing the link between chromium and the original definition of GTF. Neither the specific site of action nor the specific function of the putative GTF molecule have ever been established so all assays can ultimately only be tested against the original definition of GTF. It is therefore of paramount importance in GTF research to use an assay that specifically measures GTF activity as originally defined.

### **1.2.1 In Vivo Assays**

Originally Mertz and co-workers (Schwarz and Mertz 1957 and 1959) used an assay system whereby rats which had developed an impairment of their glucose tolerance were given an oral dose of a test fraction 18 hours before a glucose tolerance test was carried out. The rate of removal of excess blood glucose was calculated and the GTF activity in a sample was inferred from its ability to return the impaired glucose removal rates to those of control animals. However several problems have been encountered by workers attempting to use this assay to identify and purify GTF fractions, these being; (1) problems in making statistically significant measurements on small populations of whole animals (Sheriff 1983, Mertz 1969) (2) conclusions drawn from the data can depend on the method used to interpret the blood glucose data (Wooliscroft *et al.* 1977) (3) even the mild stress animals are subjected to during intravenous glucose tolerance tests was sufficient to produce glucose disappearance rates of a similar order of magnitude to those claimed to be due to chromium deficiency (Shah *et al.* 1977) and (4) the serious restriction that this assay system is unable to cope with large numbers of samples. The discovery of these limitations subsequent to the postulation of the existence of a GTF based on results from this assay system must call into question the original conclusion that a unique GTF exists (Schwarz and Mertz 1957 and 1959).

An alternative method for assaying GTF activity *in vivo* is the method of Tuman *et al.* (1977) who measured the ability of fractions to lower the non fasting blood glucose levels in normal and genetically diabetic mice. Animals were injected intraperitoneally and the effect of the sample in lowering non fasting plasma glucose levels was calculated and compared with controls. This system is also rather complicated and not suited to analysing large numbers of fractions and the mere fact that a substance lowers blood sugar cannot be taken as evidence of GTF activity since glucose homeostasis can be influenced at many points.

Nath and Sidhu (1979) investigated fractions for GTF activity using a modification of the rat diaphragm insulin bioassay. Rats were injected intraperitoneally with combinations of insulin, GTF fractions and saline and the effects of glucose incorporation into glycogen in

the liver and diaphragm were measured. No attempt was made to induce chromium deficiency in the rats prior to the assay as had been the case in previous assay systems (Mertz 1969) nor was there any attempt to use rats with impaired glucose metabolism of any other sort. Hence the relevance of the results from these assays to identifying GTF is doubtful.

An *in vivo* assay in humans has been investigated by Vinson *et al.* (1985). This assay claims to measure the effect of biologically active chromium (BAC), by monitoring the decrease in fasting serum glucose following oral ingestion of various samples. However this assay is more a measure of the hypoglycemic activity of a sample than of the ability to restore an impairment of glucose tolerance as all subjects were healthy adults.

More recently an *in vivo* assay system has been reported in humans which has been used to look at the effects of chromium supplementation and could possibly be used in a similar manner to assay GTF fractions (Elias *et al.* 1984). This system uses the artificial  $\beta$  cell insulin delivery system and insulin sensitivity is measured by the dose of insulin required to maintain a euglycemic state at various glucose levels. The rationale of this assay is that GTF would be able to improve insulin sensitivity in a patient that had a resistance to insulin.

### **1.2.2 In Vitro Assays**

The need for an *in vitro* assay suitable for screening large numbers of samples led Mertz and co workers to develop an assay for GTF activity based on the epididymal rat fat pad assay for insulin (Rodbell 1964, Glieman 1965). It was observed that chromium (III) salts potentiated the action of suboptimal doses of insulin on the uptake and conversion of glucose to fat (Mertz *et al.* 1961) and CO<sub>2</sub> (Mertz *et al.* 1965a) by rat epididymal adipose tissue *in vitro*. The effects were only reported where the rats had been raised on a special torula yeast diet, found to be low in chromium, and where the rats had developed an impairment in glucose tolerance. A similar experiment, carried out under conditions allowing strict control of trace element contamination, including chromium, resulted in more pronounced results (Mertz *et al.* 1965b). Since GTF was believed to be a chromium complex this system appeared to be ideal as a bioassay.

Further investigations seemed to confirm this as the epididymal adipose tissue of rats, fed on the torula yeast diet that had been concurrently supplemented with chromium, had a greater rate of basal glucose uptake than the tissue of rats not supplemented with chromium (Mertz *et al.* 1961 and 1965b) and in the chromium supplemented rats impaired glucose clearance rates were returned to near normal levels (Mertz *et al.* 1965a and

1965b). This apparently confirmed that chromium had an important role in the glucose metabolism of rats and that the adipose tissue was a sensitive site for measuring the response to the effects of chromium. As chromium was implicitly linked to GTF it was assumed that the same would apply for GTF and later it was found that brewers yeast extracts attributed with GTF activity, by the repairing of impaired glucose clearance rates assay, gave a response similar to that of chromium in adipose tissue of rats on the torula yeast diet (Roginski *et al.* 1970, Mertz & Roginski 1971).

These results form the basis of what has become the standard assay for GTF activity. In this assay GTF activity is assessed on the basis of the ability of a fraction to potentiate the conversion of  $^{14}\text{C}$ -glucose to  $^{14}\text{CO}_2$  by sub optimal doses of insulin, in the epididymal fat pads of rats kept on a high sucrose/torula yeast diet (Mertz 1969, Mertz and Roginski 1971). This assay initially judged GTF activity on the ability of a fraction to potentiate the stimulation caused by a sub optimal insulin concentration of 25  $\mu\text{U}/\text{ml}$  insulin (Mertz 1969 p217) where potentiation describes the ability of a sample to increase the effectiveness of sub optimal insulin concentrations. Therefore, if the effects of insulin are increased by addition of a certain fraction, the potentiation is defined as the component of that increase that is over and above any effect the fraction has on basal cell metabolism in the absence of insulin (i.e minus any insulin like effects). It is interesting to note that the data presented in that report indicates that yeast extracts potentiate not only the sub optimal but also the optimal insulin concentrations whereas chromium (III) only potentiated the action of the sub optimal concentrations of insulin. The rats used for these experiments were kept in a special chromium free environment (Polansky & Anderson 1979) as the levels of chromium thought to be required to provide sufficiency were minute (Mertz 1969). A later development of this assay (Anderson *et al.* 1979) used adipocytes isolated from the epididymal fat pad by a collagenase digestion. This later development has allowed for more consistent results and also a vast increase in the number of samples that can be analysed in an assay. It is important to realise that in this assay GTF activity is defined as the potentiation of the effects of sub optimal doses of insulin which is therefore clearly separated from substances which mimic the action of insulin. Several investigators have used this assay system without maintaining the rats on the special low chromium torula yeast diet and have claimed positive results (Kienle *et al.* 1979, Tokuda *et al.* 1987) however the relevance of these results is questionable due to previous results which showed the necessity of the high sucrose/torula yeast diet for GTF activity to be observed.

The relationship between the ability of a sample to show GTF activity in the adipocyte assay and its ability to restore impaired glucose tolerance in the animals on the torula yeast

diet has never been definitively established. The choice of adipose tissue is obviously based on the ease with which this tissue can be extracted and manipulated and its ready response to insulin. However this tissue doesn't seem to be the most logical choice for use in assaying for an agent affecting glucose homeostasis as adipose tissue is not a quantitatively important site of glucose disposal or insulin resistance *in vivo* (Kahn 1985). Further the symptoms of glucose intolerance in the rats on the torula yeast are more pronounced in the female rats (Schroeder 1966).

Another widely used assay for GTF was developed based on the rationale that as brewers yeast fractions exhibit high levels of GTF activity then it could be expected that the brewers yeast would have some metabolic role for GTF. It was noticed by Burkeholder and Mertz (1967) that a fraction extracted from brewers yeast and which gave activity in the rat fat pad assay, did indeed stimulate the production of CO<sub>2</sub> by fermenting brewers yeast. Mirsky *et al.* (1980) later used this observation to develop an assay for GTF based on yeast fermentation rates. Three strains of yeast were used (*Saccharomyces carlsbergensis*, *Saccharomyces ellipsoideus* and *Saccharomyces cerevisiae*) which had been grown in a chromium free medium. The activity of samples was determined by the ability to increase the rate of CO<sub>2</sub> production as compared to a blank. The parameters of this system were further investigated by Haylock *et al.* (1982). Later work on the same assay system by Holdsworth and Appleby (1984) showed that samples displaying activity in the yeast assay did not necessarily show activity in the rat adipocyte assay but that fractions showing activity in the adipocyte assay always gave activity in the yeast assay. This implied that the yeast assay could be used as a technique for rapidly and cheaply screening large numbers of samples for GTF activity which could then be analysed in the adipocyte assay.

A number of other methods have been used to estimate GTF activity. These include a microbial assay using *Flavobacterium Rhenanum* (Gutierrez *et al.* 1974). This bacterium was grown on a medium containing vitamin free casein hydrolysate, glucose and vitamins and the growth of samples inoculated with GTF fractions was compared with blanks by monitoring turbidity readings. The activity of the sample was estimated by a plot of culture growth versus sample concentration. A good correlation was found between results obtained by this method and results obtained by the rat fat pad assay indicating that both assays may have been measuring the same parameter. However this assay does not appear to have been developed further.

An assay in which normal animals, as opposed to those with impaired glucose tolerance, were used was that described by Kienle *et al.* (1979). Here epididymal rat adipocytes

and subcutaneous human adipocytes were used and GTF activity was estimated by the ability of a sample to potentiate the actions of insulin on glucose oxidation, lipolysis and glucose incorporation into lipid fractions.

In summary several *in vitro* assay systems for measuring GTF activity have been investigated however no in depth studies have been made into the methods by which the material deemed to possess GTF activity exerts its effect and so the relationship of this material to the putative GTF is unknown. There is no firm evidence that the material showing activity in the *in vitro* assay systems is the same material that gave the response originally defined as being due to GTF in the *in vivo* studies carried out by Schwarz and Mertz (1959) and so caution must be exercised when interpreting the results of these assays.

## **1.3 Studies of the Structure of GTF**

Since its existence was first postulated many attempts have been made to isolate a pure sample of GTF. Despite these attempts it remains a major deficiency in the case for the existence of a GTF that no single identifiable compound has been conclusively shown to be solely responsible for the effects attributed to GTF.

Investigations into the composition and structure of GTF have fallen into two distinct categories. Some workers have attempted to isolate GTF in a pure form from biological starting materials while others have taken the composition indicated by analysis of these GTF extracts and attempted to create GTF synthetically. A large amount of information has been obtained using both of these approaches.

### **1.3.1 Attempts to Isolate GTF**

Most attempts to purify GTF have used brewers yeast (Toepfer *et al.* 1977, Mirsky *et al.* 1980, Haylock *et al.* 1983, Davies *et al.* 1985a) as a starting material as this has been acknowledged as being a rich source of the factor (Mertz 1975). However the occurrence of GTF activity in other substances including black pepper corns, molasses, wheat bran and sage (Haylock 1981), defatted pork kidney powder (Mertz *et al.* 1959), liver extracts (Mertz *et al.* 1962), beer and its components (Anderson *et al.* 1983 b) and a range of 25 different foods (Toepfer *et al.* 1974) has also been investigated. GTF activity was found in some extracts from these substances but levels were not as high as those seen in yeast extracts.

The first attempts to isolate fractions with GTF activity were undertaken by Schwarz and Mertz (1957 and 1959). Brewers yeast and de-fatted pork kidney powder were subjected to various combinations of extraction procedures which resulted in a fraction which improved the impairment of glucose tolerance induced in rats fed the torula yeast/ sucrose diet. The exact extraction procedures were not fully elucidated in the publications; merely that they involved concentration in a salt cake, extraction with phenol and iso-butanol, adsorption onto charcoal and the use of ion exchange resins. It would appear that this procedure did not result in a high degree of purification. The material isolated was water soluble, cationic and apparently lost no activity when wet ashed in  $\text{HNO}_3:\text{H}_2\text{SO}_4$  which was originally taken to indicate the involvement of a trace element in GTF. On the basis of unspecified colorimetric methods it was suggested that chromium was also present in the sample although later work (Cornelis and Wallaey 1985) has highlighted the difficulties which existed in analysis for low levels of chromium in biological material in that era and casts some doubt over the conclusion. However since that time GTF has been considered to be a chromium complex.

An 8 step purification scheme beginning with brewers yeast was then described by Burkeholder and Mertz (1967) and resulted in a fraction which stimulated both the conversion of glucose to  $\text{CO}_2$  in yeast cells and also potentiated the insulin stimulated conversion of glucose to  $\text{CO}_2$  in epididymal tissue from rats raised on the torula yeast/sucrose diet. However these fractions were not tested against the original definition of GTF activity, namely that they could repair dietarily induced impaired glucose metabolism.

Further work by this group (Mertz 1969 p216) describes the fractionation by gel filtration, of the alcohol extracts of yeast that had been grown on a  $^{51}\text{Cr}$  labeled medium. The  $^{51}\text{Cr}$  containing material was closely associated with a biological activity peak, as measured by the ability to potentiate the insulin stimulated conversion of glucose to  $\text{CO}_2$  in the adipose tissue of rats grown on a torula yeast ration. At a concentration of  $100\ \mu\text{g}$  of solid material/ml this material maximally potentiated the action of a sub optimal insulin concentration of  $25\ \mu\text{U}/\text{ml}$ . The presence of  $1\ \mu\text{g}/\text{ml}$  chromium in the fraction was taken to indicate the presence of a chromium complex however such a level is hardly likely to indicate the presence of a low molecular weight chromium complex as chromium would constitute between 1 and 10% by weight of such a complex and so with the isolation of even a semi pure complex a chromium concentration several orders of magnitude higher would be expected. The chromium containing material was not the only peak of biologically active material isolated. Another non-chromium containing peak showing

GTF activity was also consistently seen although the nature of the material in this peak was not investigated.

The first really detailed description of attempts to isolate material with GTF activity was described by Votava *et al.* (1973). Their procedure involved the growing of yeast in a medium containing  $^{51}\text{Cr}$ . The harvested yeast was then subjected to butanol/water extraction and the material obtained from this was then chromatographed on a gel column. This resulted in a single peak of  $^{51}\text{Cr}$  containing material which had a molecular weight of 400-600 . This material was then chromatographed on a Dowex 50W cation exchange column and a Dowex 1 anion exchange column which yielded a single peak of anionic  $^{51}\text{Cr}$  containing material. Further work showed that this material also contained at least 6 amino acids (Asp,Ala,Glu,Pro,Ser and Cys). It was noted that the binding of the  $^{51}\text{Cr}$  to the other material was reversible. Unfortunately no biological assays were conducted to assess the GTF activity of these fractions hence the relationship of this material to GTF cannot be judged.

Mertz and co-workers later published a refined version of their methods used to isolate fractions with GTF activity from brewers yeast (Mertz *et al.* 1974 and Toepfer *et al.* 1977). Their procedure involved the extraction of brewers yeast with hot ethanol and then the adsorption of material onto charcoal. The eluate from the charcoal was then somewhat surprisingly subjected to reflux in 5 N HCl for 18 hours and then applied to a Dowex 50 W X8 cation exchange column. This column was then eluted with 0.1 N HCl resulting in a cationic fraction eluted as a broad peak. The material absorbed strongly at 262 nm, showed GTF activity in the rat fat pad assay and was found to contain three amino acids namely cysteine, glycine and glutamic acid. Atomic absorption spectroscopy revealed the presence of chromium and a combination of mass spectrometry, UV and IR spectroscopy was claimed to reveal the presence of nicotinic acid. On the basis of these studies GTF was proposed as a complex consisting of a central chromium (III) atom with 2 axial nicotinic acids and 4 planar amino acids ligated to it; however confirmation of this model has not been achieved in the decade since it was first postulated and it seems unlikely on chemical grounds (Gerdorn and Goff 1982). This isolation procedure suffers from several major deficiencies the most obvious of which is the 18 hour reflux step. This seems a rather harsh step to subject biological material to in an attempt to purify it, particularly if the presence of peptides is suspected. It is worth noting though that even this harsh step doesn't destroy the biological activity although it would undoubtedly affect the structure and composition of any complex similar to that postulated for GTF which may have been present originally. A second criticism is that in light of later work showing the complexity of the components present in the yeast extracts (Haylock 1983a

& b) it is clear that this extraction procedure would produce at best only an impure GTF fraction and so conclusions as to the structure of GTF based on this work seem premature.

Kumpulainen *et al.* (1978) developed a scheme similar to that of Votava *et al.* (1973) using yeast grown in a medium containing radioactively labeled chromium. The yeast was extracted with ether and the resultant material then chromatographed on Sephadex G-25 gel and Dowex 50W X8 cation exchange resin followed by Sephadex QAE-A-25 anion exchange and then finally, separation by thin layer chromatography. It was found that the chromium containing material separated from the ninhydrin reactive material indicating a separation of chromium from the peptide material. A major shortcoming of this work was again that no attempt was made to assay the fractions for GTF activity and the only estimation of biological activity was a measurement of the binding of the fractions to insulin. None of the fractions found was able to bind to insulin. It was stated that no material resembling the GTF fraction described by Mertz *et al.* (1974) was found and it is significant to later observations that the chromium was separated from the peptide material. Interestingly they found that chromium added to the yeast growth medium was able to spontaneously form complexes with elements of that growth medium. This binding ability of chromium is discussed later.

The first attempt to follow both chromium content and biological activity concurrently in an extraction procedure was that of Mirsky *et al.* (1980). They used a four step purification scheme starting with powdered yeast extract and following activity by the yeast fermentation assay and chromium content by atomic absorption spectroscopy. The yeast was extracted with a butanol:H<sub>2</sub>O mixture and the aqueous phase was separated and dialysed using a membrane with a MW 3500 cutoff which removed larger molecular weight materials. The dialysate was then chromatographed on a DEAE-cellulose anion exchange column to remove the anionic material which was assumed not to be active. It was found that the active cationic material could be eluted with water. This was then chromatographed on a Dowex 50W X8 cation exchange column and a number of fractions were collected. Only the fraction eluted by 0.25 N NH<sub>4</sub>OH showed biological activity in the yeast assay. The material in this fraction absorbed strongly at 262 nm, was stable to acid (2 N HCl), stable to base (2 N NH<sub>4</sub>OH), stable to heat (100 °C for 30 minutes) and also contained a large proportion of the chromium which was originally contained in the yeast powder. These properties were similar to the properties of the GTF extract reported by Toepfer *et al.* (1977) and hence the fraction was identified as GTF. The fact that the exact nature of the material was unable to be determined means that it can

only be regarded as a crude preparation containing GTF activity as indicated by the standard yeast assay for GTF.

Haylock *et al.* (1983a) reported a detailed purification procedure using mainly ion-exchange chromatography. An aqueous extract of whole yeast or of powdered yeast extract was chromatographed on a sequence of columns using Dowex 50W X12 cationic resin, Dowex 50W X8 anionic resin, Sephadex G-10 Biogel and Biogel P-2 with the chromium content of the fractions being monitored. A total of 11 chromium containing fractions were isolated of which 3 were anionic, 4 were cationic and 4 were amphoteric. When tested in the yeast assay all the cationic and one of the anionic fractions were active. It is important to note that the two most active fractions (labeled P-3 and P-4) contained only 3.3% of the total chromium extracted from the yeast. The reason that this isolation procedure seems to have been able to separate so many fractions is due to the choice of elution buffer systems. Here a gradient of  $\text{Na}_2\text{HPO}_4/\text{Na}_3\text{PO}_4$  was used as opposed to previous schemes which had used a straight  $\text{NH}_4\text{OH}$  elution and had resulted only in a single broad band of chromium containing material. This purification procedure clearly showed that previously obtained GTF fractions were not pure which casts serious doubt on the structure for GTF postulated by Mertz *et al.* (1974). Another important point which arose from this work was the observation that elution of a compound from a column using  $\text{NH}_4\text{OH}$  was able to confer activity on previously inactive fractions (Cooper *et al.* 1985). This observation may also have relevance to the observations of Mirsky *et al.* (1980) since their active fractions were also eluted with  $\text{NH}_4\text{OH}$ .

Further work by Haylock *et al.* (1983b) confirmed the observations of Kumplainen *et al.* (1978) that a number of the chromium containing fractions were actually artifactual complexes formed by the reaction of chromium with components of the medium in which the yeast was grown. This is not surprising as chromium is a good complexing agent. Of the biologically active fractions isolated from the yeast only 2 cationic fractions (named P-3 and P-4) were not attributable to such complexing reactions. It was concluded therefore that only these fractions could represent GTF material synthesized by the yeast. Even these fractions were however still far from pure and hence were regarded as crude GTF preparations. However it was subsequently shown that P-4 was 90% tyramine although tyramine by itself was not active in either the yeast assay (Haylock *et al.* 1983b) or the adipocyte assay (E.S.Holdsworth, personal communication). When fractions P-3 and P-4 were further purified on Dowex 50W X2 cationic exchange resin and Sephadex G-15 gel it was found that the biological activity separated from the chromium containing fractions. This result directly challenges the 25 year old assumption that chromium is an

implicit part of the structure of GTF isolated from yeast; an assumption which had strongly influenced the direction of research in this field in those years.

These results have recently been supported by several other groups (Davies *et al.* 1985a & b, Held *et al.* 1984, Hwang *et al.* 1984, Barseghian 1987) all of whom describe equally assiduous isolation procedures to obtain GTF containing fractions from yeast. In the procedure of Holdsworth and co-workers (Davies *et al.* 1985a & b) whole yeast cells were harvested and extracted with 0.2 N NH<sub>4</sub>OH/14% n-butanol. This extract was chromatographed on a series of anionic and cationic ion exchange columns followed by gel chromatography and paper chromatography. The procedure yielded a cationic /amphoteric fraction which on further purification gave three fractions active in a yeast assay for GTF activity (Davies *et al.* 1985a). Of these one was also active in the adipocyte assay, this last fraction constituting most of the GTF activity. The material from this fraction was white, water soluble, stable to heating and most significantly contained no chromium. This material has since been shown to contain mostly ornithine (Davies *et al.* 1985b) although ornithine is only mildly active in the low chromium epididymal adipocyte assay and thus only partially explains the observed activity of the fraction. The anionic fraction on further purification also yielded material which was active in both assay systems which also contained negligible chromium and this is thought to contain  $\epsilon$ -N-glutaryllysine.

Work by Held *et al.* (1984) also describes the isolation of GTF-containing fractions from whole yeast and the subsequent separation of biological activity from chromium. Their separation procedure involved extraction of the yeast with 0.1 M NH<sub>4</sub>OH, dialysis, sequential chromatography on Dowex 1 X8 and Sephadex G-15 gel and finally ion pairing HPLC. The biological activity was determined by the low chromium epididymal adipocyte assay. The purified active material was found to have a symmetric absorbance peak at 254 nm but to contain no chromium.

A purification scheme for GTF has been patented by Barseghian (1987). In this procedure a commercially obtained yeast extract was separated on a Sephadex G-75 column and the activity of the 6 fractions collected was determined by an adipocyte assay system. The active fractions were subjected to acid-ethanol extraction, the supernatant of which had increased activity in the yeast assay. This material was then further separated using Biogel P-6 and P-2 columns. The most active fraction obtained by this process also showed a hypoglycemic effect in mice. This material was separated still further using C-18 reverse phase HPLC resulting in a cationic fraction which had a marked insulin like effect and a hypoglycemic effect in normal mice. It did not contain chromium

but was readily able to bind to it. The material absorbed strongly at 301.5 nm, 258.5 nm and 139.0 nm when dissolved in 0.1 N HCl and mass spectrometry indicated a molecular mass of 174. This data combined with NMR data confirmed that the material was a quinoline derivative. While this purification scheme is certainly assiduous the methods used to assay the fractions may mean that it has not really identified GTF but merely an insulin mimicker that results in a hypoglycemic effect. There are several reasons for this assertion: (1) No attempt was made to induce an impairment in glucose tolerance in the rats used for the assay (2) The adipocyte assay measured insulin like activity as opposed to potentiation of insulin activity (3) No attempt was made to induce an impairment of glucose tolerance in the mice used for the *in vivo* studies.

An attempt to isolate fractions containing GTF activity by isolating chromium containing fractions from brewers yeast has been reported by Hwang *et al.* (1987). Yeast was grown on a  $^{51}\text{Cr}$  containing medium and chromium complexes were extracted using dialysis, ion exchange and gel filtration. None of the chromium containing fractions displayed GTF activity as measured by the potentiation of the action of insulin in rat epididymal adipocytes. Some complexes did however display insulin like activity but further investigation showed that identical chromium complexes were obtained when chromium was added to a yeast extract indicating that the formation of the complexes observed was not a function of the living cells but a spontaneous chemical reaction. This reinforces the conclusions of Kumplainen *et al.* (1978) and Haylock *et al.* (1983b) that most of the chromium complexes isolated from brewers yeast are artifactual.

Further information on the fate of chromium in yeast cells is contained in the report of Gonzalez-Vergara *et al.* (1982) that a fraction was isolated from yeast containing all the chromium but not containing nicotinic acid, glutamic acid, glycine or cysteine although it did appear to contain tryptophan. The report does not comment on the biological activity of the final extract but it does show that GTF probably does not exist in the form previously thought (Mertz *et al.* 1974).

Concurrently with the above work on yeast extracts Japanese workers have been investigating the occurrence of chromium complexes in other systems including urine (Wu & Wada 1981a), dog liver (Wu & Wada 1981b, Wada *et al.* 1983), rabbit liver (Yamamoto *et al.* 1987a) and bovine colostrum (Yamamoto *et al.* 1987b & 1988). An anionic chromium containing fraction isolated from the liver of dog (Wada *et al.* 1983) was found to contain glycine, glutamic acid and cysteine and to have a molecular mass of approx 1500. Apart from the anionic property of the complex this is consistent with the postulated composition of GTF (Mertz *et al.* 1974) however the GTF activity of this

complex was not tested. More recently Yamamoto *et al.* (1987a) have looked at chromium compounds isolated from the livers of rabbits which had been administered doses of chromium in the form of  $K_2Cr_2O_7$ . A significant proportion of the chromium was found in the form of an anionic, low molecular mass chromium compound again containing cysteine, glutamic acid and glycine as well as aspartic acid, which they called Low Molecular Weight Chromium Complex (LMCr). This was found to significantly stimulate incorporation of  $^{14}C$ -glucose into  $CO_2$  and also of  $^3H$ -glucose into lipids in the rat epididymal adipocyte assay, where presumably the rats had been maintained on a low chromium diet. It was postulated that the LMCr played an essential role in both the glucose metabolism and the detoxification of invaded chromium in the body. However the fact that none of the body compartments that LMCr was isolated from are important sites of insulin action indicates that its major role is detoxification rather than potentiation of insulin action. In light of the observations of Kumplainen *et al.* (1978) and Haylock *et al.* (1983b) the GTF activity ascribed to LMCr may be an artifact resulting from incomplete purification of the fractions.

Further work on bovine colostrum by the same workers (Yamamoto *et al.* 1987b) described a detailed separation procedure which yielded 2 cationic and 3 anionic chromium containing fractions. One of these, an anionic fraction of molecular mass 1500, exhibited GTF activity in the epididymal adipocyte assay (Anderson *et al.* 1978). This fraction was further purified (Yamamoto *et al.* 1988) and the final product also displayed GTF activity. It also had a molecular mass of 1500 and was found to contain chromium and the amino acids cysteine, glycine, glutamic acid and aspartic acid in a ratio of 1:2:4:5. An ultraviolet absorption maxima at 260 nm which could have been due to the presence of nicotinic acid was also reported. This purified fraction was labeled Milk Low Molecular Weight chromium Complex (M-LMCr) and its properties were similar to those attributed to GTF (Mertz *et al.* 1974).

The fact that the identity of a substance responsible for GTF activity has never been elucidated after 25 years of research in the field is surprising if such a factor exists. Only in recent years have serious attempts been made to isolate pure fractions of GTF-containing material but despite this there is still little more known about the nature of GTF, except that it now appears that chromium is not necessarily a component of the GTF found in brewers yeast.

In summary it appears that material ascribed with GTF activity is of low molecular weight and water soluble. Both anionic and cationic fractions with GTF activity have been isolated. It appears to be present in a number of organisms although some variations in

its form are evident. It also appears to contain amino acids and is stable to heat, acid and base. As procedures for further purification of yeast extracts have developed it now appears more than likely that chromium is not an implicit component of those fractions containing the most GTF activity. A major failing of many of the above separation procedures therefore is that they follow primarily the chromium content of the fractions rather than the biological activity. This is a reflection of the fact that while chromium analysis is relatively cheap and easy, no cheap and easy assay of GTF activity has yet been developed. If GTF does exist it is vital that the biological activity of all fractions isolated during purification be ascertained so that a true picture of the relationship between the GTF activity and chromium can be found. It is also important that a coherent picture of the diverse range of compounds exhibiting GTF activity can be built up and compared in an effort to determine whether a single identifiable Glucose Tolerance Factor actually exists.

### **1.3.2 Attempts to Synthesize GTF**

Early work on GTF concentrated on the *in vitro* and *in vivo* effects of simple chromium compounds (Mertz *et al.* 1961 & 1965b) due to the activity exhibited by chromium(III) salts. Early attempts to isolate GTF consistently revealed chromium, nicotinic acid and the amino acids glycine, cysteine and glutamic acid as components of semi pure GTF fractions (Mertz *et al.* 1974) and these compounds were present in proportions suggesting the possibility of a complex of the structure discussed earlier in this introduction. As complete purification has remained elusive attempts have been made to synthesize complexes using the compounds implicated in the GTF structure. This work aims to either (a) produce synthetic versions of GTF or a molecule with analagous properties so that the properties of GTF can be investigated in the absence of a homogenous sample of purified GTF or (b) investigate the general chemical properties of complexes in this category so as to be able to predict the likely stability and behaviour of such complexes *in vivo*. Much is known about the chemistry of chromium complexes with biologically significant substances and this information and how it might relate to GTF has been reviewed by Barrett *et al.* (1985) and Larkworthy *et al.* (1987).

#### **1.3.2.1 Synthetic Chromium Complexes Investigated For GTF Activity**

Because of the implicit association of chromium with GTF (Mertz 1969) much of the work has concentrated on chromium complexes. The first attempts at synthesizing such complexes were undertaken by Mertz and co-workers (Mertz *et al.* 1974, Roginski *et al.* 1974, Toepfer *et al.* 1977). Appropriate proportions of chromium (III), nicotinic acid, glutamic acid, cysteine and glycine were refluxed in 6 N H<sub>2</sub>SO<sub>4</sub> for 18 hours and then

partially purified by ion exchange chromatography. The product obtained from this procedure was held to be a synthetic form of GTF as it supposedly had properties very similar to a GTF fraction isolated from brewers yeast (Toepfer *et al.* 1977). The complex was prepared and partially purified using the same reflux and ion exchange procedures that the yeast extract was subjected to. It is therefore not surprising for two solutions of roughly similar initial composition, and which are subjected to the same conditions, that similar products accrue. The synthetic preparation had GTF activity in the low chromium rat epididymal adipocyte assay system (Toepfer *et al.* 1977, Steele *et al.* 1977) with the synthetic preparation actually showing more activity than the comparable GTF preparation from yeast. The same synthetic GTF has been shown to lower plasma glucose and triglycerides in normal and diabetic mice (Tuman *et al.* 1978) although in this case it was not as active as a yeast extract. The effects of the complex were also investigated in swine adipose tissue *in vitro* (Steele *et al.* 1977) and while no effect was noted on glucose oxidation or lipogenesis it was found to enhance the hypoglycemic action of insulin *in vivo*.

Attempts have been made to further separate the components of the synthetic complex of Toepfer *et al.* (1977) which is undoubtedly a complex mixture. Kienle *et al.* (1979) prepared a solution in exactly the same manner as Toepfer *et al.* (1977) and attempted further purification. They found two distinct components in the crude complex solution but were unable to separate them. The crude complex was found to have no effect on the binding of insulin to subcutaneous human adipocytes and it did not potentiate insulins antilipolytic effect. The complex did possess some intrinsic antilipolytic effect in these cells although it is possible that this was a result of free nicotinic acid in the complex solution. There was however some small potentiation of insulins action in a rat epididymal adipocyte assay although the rats had not been maintained on a torula yeast/sucrose diet. Further attempts at separating the components of the crude complex solution were made by Cooper *et al.* (1985) using a Dowex 50W X12 cation exchange column. Six chromium containing fractions were isolated all of which contained amino acids and all but one of which contained nicotinic acid. However whereas the crude complex had had activity in a yeast assay system none of the six chromium complexes isolated had such activity. This raised the possibilities that; the active complex remained bound to the column, it was altered while on the column or that some thing other than a chromium complex was responsible for the observed activity. Anderson *et al.* (1978 & 1979) realised that the ratios of amino acids in the GTF preparations of Toepfer *et al.* (1977) corresponded to those of glutathione and an attempt was made to prepare a chromium-nicotinic acid-glutathione complex using a method similar to Toepfer *et al.* (1977) and a chromium-nicotinic acid-glycine complex was also prepared. It was found

that both solutions gave significant GTF activity in the rat epididymal adipocyte assay however no attempt was made to characterise the complexes and thus nothing can be deduced about the possible structure of GTF. This and other work on chromium-glutathione complexes is reviewed by Larkworthy *et al.* (1987).

Another important finding from this work (Anderson *et al.* 1978 & 1979) was that little if any GTF activity was induced by the individual components of the reaction mixture (glutathione, glycine, CrCl<sub>3</sub>, chromium acetate or nicotinic acid), so it was concluded that the activity observed was indeed dependent on the formation of a complex. This is supported by the observations of Haylock *et al.* (1983a & b) and Cooper *et al.* (1984b) who used the yeast assay and found no effect with low concentrations of chromium (III), cysteine, glycine and nicotinic acid and only a small effect with low levels of glutamic acid.

A similar chromium-nicotinic acid-glutathione complex was investigated by Nath and Sidhu (1979) using an assay measuring the ability of the complex to potentiate the effect of insulin in the liver and the diaphragm of rats fed a normal lab chow diet. It was found that the complex greatly increased the insulin stimulated incorporation of glucose into glycogen in the diaphragm but not in the liver where glycogen synthesis is not responsive to insulin. As no attempt had been made to induce a GTF deficient state in the rats and as no other work on GTF has been done using this assay system it is difficult to ascertain the relevance of these observations.

Cooper *et al.* (1984a) prepared and characterised a range of chromium-nicotinic acid complexes and found that biological activity as measured by the yeast assay was only present where the nicotinic acid was O-coordinated to the chromium and not in those with N-coordination. Further work (Cooper *et al.* 1984b) investigated a range of chromium-nicotinic acid-amino acid and chromium-amino acid complexes. Three complexes were found to be active in the yeast assay, these being a chromium-nicotinic acid-glycine complex of unspecified formula, a Cr(Gln)<sub>2</sub>(H<sub>2</sub>O)<sub>2</sub><sup>+</sup> complex and a Cr(Gly)<sub>2</sub>(H<sub>2</sub>O)<sub>4</sub><sup>3+</sup> complex. The overall conclusion of their work was that the structural factor necessary for biological activity was two free trans configured nitrogens and it was also noted that 1,4 diguanadino butane, which fulfils these criteria, is a well known hypoglycemic agent. If this is the case it may be that the action of chromium complexes is reliant on a fortuitous conformation of trans nitrogens and hence GTF may not necessarily be a chromium complex. It is interesting to note that Cooper (1982) has also reported a chromium hexa ammonia complex with activity in the yeast GTF assay.

### **1.3.2.2 Synthetic Chromium Complexes Not Investigated For GTF Activity**

As well as the above work relating chromium complexes to GTF there have been a number of studies of chromium complexes of similar composition where the relationship to GTF was not investigated. These should be noted as they contain information on the synthesis and chemical properties of these complexes and are thus of relevance to discussions on the plausibility of the structural hypothesis put forward by Mertz *et al.* (1974).

Syntheses in this category which have been reported are chromium (III) complexes with glycine and alanine (Gillard *et al.* 1974, Wallace *et al.* 1982), nicotinic acid (Chang *et al.* 1983, Gonzalez-Vergara *et al.* 1982, Purser *et al.* 1987, Gerdorn *et al.* 1982, Nelson *et al.* 1981), methyl nicotinate (McArdle *et al.* 1982), methionine and cysteine (Maslowska *et al.* 1981, McAuliffe *et al.* 1966, de Meester *et al.* 1977), iminodiacetic acid, aspartic acid, glutamic acid and cysteine (Venkatachalapathi *et al.* 1982), glucose (Brown, D.H. *et al.* 1976), aspartic acid (Grouhhi-Witte *et al.* 1976), and glutathione (Barret *et al.* 1985)

## **1.4 Studies of the Function of GTF**

Despite the fact that the existence of a Glucose Tolerance Factor was first postulated 25 years ago very little is known about the mechanism or mechanisms by which the effects attributed to the factor are brought about. Any proposals on such mechanisms must take into account the original definition of GTF (Section 1.1).

One thing that does seem certain is that fractions with GTF activity have a direct effect on peripheral tissues (Anderson *et al.* 1978, Roginski *et al.* 1970). The nature of this action at the cellular level remains unknown although several possibilities have been investigated.

### **1.4.1 Does GTF Assist the Binding of Insulin to the Cell ?**

The most commonly expounded theory of GTF action is that it acts extracellularly to aid the binding of insulin to the cell membrane (Mertz 1969). Since it was believed that GTF was a chromium(III) complex it was postulated that chromium (III) provided by GTF may act to form a ternary complex with two sulphide groups on the insulin and two on the cell surface. This suggestion was supported by a polarographic study (Christian *et al.* 1963) in which it was claimed that when chromium was added to insulin polarographic

waves were seen which indicated the reduction of the sulphide groups, thus presumably supporting the formation of the hypothetical ternary complex. They were however using very high concentrations of chromium and insulin to achieve this effect.

If GTF acts as a mediator for insulin binding it would be expected that GTF would bind to insulin *in vitro*. This experiment has been attempted several times with widely differing results. The binding of insulin to adipose tissue was reportedly slightly increased by incubation with inorganic chromium (Mertz 1969). Results of circular dichroism and gel chromatography studies of the interactions of zinc (II) and chromium (III) with insulin indicate that chromium (III) has a dissociative effect on insulin aggregates (Belusko 1977). Other work by the same investigators showed that addition of chromium (III) to insulin solutions changed the UV spectrum of the solutions as compared with a blank (Mc Donald and Belusko 1976) and this was held to indicate that insulin bound chromium under physiological conditions. The effect of biological extracts has also been investigated with a stable GTF extract-insulin complex being reported by Mertz and Thurman (1968) and it was reported by Evans *et al.* (1973) that a crude extract of yeast with GTF activity was able to bind to insulin. Similarly Anderson *et al.* (1978b) suggested that a synthetic complex, claimed to have GTF activity, was also able to bind firmly to insulin.

These results are however contradicted by those of Kumpulainen *et al.* (1978) who grew yeast on a  $^{51}\text{Cr}$  labeled medium but none of the chromium containing fractions isolated from this yeast was able to bind to insulin. It was also shown by Davies *et al.* (1985a) that a fraction with GTF activity that had been isolated from yeast neither aided nor hindered the binding of insulin to human placental membrane and Kienle *et al.* (1979) found that a synthetic chromium complex with apparent GTF activity did not aid the binding of insulin to either human adipocytes or epididymal adipocytes from rats.

Thus it seems that there is no conclusive evidence that GTF or chromium(III) complexes aid in the binding of insulin to the cell surface. Also in the period corresponding to the above studies great advances have been made in our knowledge of the method of action of insulin (Simpson and Cushman 1986, Kahn 1985) and no co-factor requirement for insulin binding to the cell, as postulated by GTF theorists, has yet been demonstrated. From the chemical point of view the non labile nature of chromium (III) complexes would appear to mitigate against its involvement in the formation of a ternary insulin-chromium-receptor complex as the rates of exchange between chromium and its ligands are too slow for any such reactions to be of physiological importance. The burden of proof therefore

lies with those who maintain that GTF is a chromium complex and who propose a ternary insulin-chromium-receptor complex.

### **1.4.2 Does GTF Potentiate the Transport of Glucose ?**

There has also been investigation into the effect of GTF on glucose transport. The results of Mirsky *et al.* (1981) indicated that GTF had a marked stimulatory effect on glucose transport in yeast cells, although Holdsworth *et al.* (1984) did not notice any such effects. In the adipocyte assay system Anderson *et al.* (1979) found no stimulation of glucose transport by a GTF fraction while the same fraction gave noticeable stimulation of the metabolism of glucose to CO<sub>2</sub>.

It has recently been postulated that GTF has a direct stimulatory effect on glucose transport by significantly lowering the  $K_s$  for glucose transport (Tokuda *et al.* 1987). This work has shown that a crude GTF extract from brewers yeast had a marked insulin like effect on the transport of 3-O-methylglucose into the isolated epididymal adipocytes of rats fed a normal diet. Basal 3-O-methylglucose transport was stimulated 2.2 fold by the yeast extract in the absence of insulin but no such effect was noted in the presence of insulin. It was concluded that the effect was due to a novel insulin like effect in which the increase in glucose transport was due to a lowering of the  $K_s$  for glucose whereas all previously observed insulin mimickers had exerted their effect by increasing  $V_{max}$  with little effect on  $K_s$ . This insulin mimicking role for GTF seems to directly contradict the previously held tenet that GTF is mainly an insulin potentiator (Mertz 1969). Further work was being done at the time of writing to deduce whether the same effects could be elicited using purified GTF extracts and until this is done the true relationship between these observations and the mechanism of GTF cannot be ascertained.

An even more novel theory has been put forward by Brown, D.H *et al.* (1986). They were able to form chromium-sugar complexes and a vast extrapolation of this achievement envisaged that the nicotinic acid of the originally postulated GTF structure (Mertz *et al.* 1974) swapped with glucose molecules to facilitate either transport or metabolism of the glucose. No evidence has been presented for this theory and it seems unlikely that the small amounts of chromium present in living organisms could facilitate the huge amounts of glucose transport and metabolism taking place in the organisms although it could perhaps explain the small attenuations in glucose metabolism caused by GTF.

Thus there is no conclusive proof for or against GTF having a role in the stimulation of glucose transport and this is an area requiring further investigation.

### **1.4.3 Does GTF Act Intracellularly ?**

It had been observed that some enzyme systems were stimulated by chromium (III) and these were reviewed by Mertz (1969). Because of the then implicit association of chromium with GTF this led to speculation of a possible intracellular role for GTF. The possible link between GTF and the enzyme systems mentioned in that review however appear never to have been followed up.

Later work by Holdsworth and Appleby (1984) did however show that a preparation containing GTF activity was able to stimulate pyruvate carboxylase and pyruvate decarboxylase in yeast, suggesting a possible intracellular role. It has been reported that insulin has a direct stimulatory effect on pyruvate dehydrogenase in a plasma membrane and mitochondrial fraction of rat adipocytes (Seals and Jarett 1980). This indicates the possibility of a direct intracellular effect by insulin on pyruvate dehydrogenase and it may be that GTF potentiates this function.

However it has been noted by Mirsky *et al.* (1980) that a GTF preparation had no effect on a cell free yeast extract and that in fact intact cells were required for activity to be noticed in the yeast assay system. This indicates that GTF is not directly stimulating any of the intra cellular enzyme systems in yeast but favours an effect on glucose transport.

### **1.4.4 Discussion**

There is little unequivocal evidence for either an intracellular or extracellular site of GTF action. In light of recent advances in knowledge of the structure and function of insulin receptors and the subsequent stimulation of production of glucose transporters (Kahn 1985, Simpson and Cushman 1986) it would appear that theories of the mechanism of GTF action need to be re evaluated. Obvious experiments would be to test the effects of combinations of GTF and insulin on the translocation of glucose transporters in the cell. Insulin is known to have a wide range of physiological effects (Kahn 1985) but the effect of GTF on the action of insulin *in vitro* has mainly concentrated on the stimulation of glucose oxidation in epididymal rat adipocytes and detailed studies should be instigated into the potentiation of other noted effects of insulin.

## **1.5 Possible Alternative Explanations for Potentiation of Insulin Action by GTF Fractions in Isolated Adipocytes**

As most GTF preparations to date have been relatively crude extracts, the possibility must be raised that the effects attributed to GTF in adipocyte assays are in fact due to other components of the extract, or indeed to the individual molecules postulated to be components of the GTF moiety. It would thus be logical to investigate the GTF like activity of all components of GTF extracts, however the exact composition of the various crude GTF extracts has proved very difficult to ascertain. The more easily quantified components such as amino acids and trace elements have generally been determined but do not account for all of the material present. Nicotinic acid has also been implicated as a component of GTF (Mertz 1974) and adenine has been seen in fractions active in the yeast assay (Chapter 3 this thesis).

By previous definition, anything causing a GTF like effect would act only to potentiate the effects of sub-optimal amounts of insulin, as opposed to giving an insulin like effect. In practice however the delineation between a potentiation and an insulin like effect has sometimes become blurred. The GTF potentiation effect is only very small in absolute terms and so the superimposition of the effects of various components of an impure fraction on the various processes of insulin stimulated glucose metabolism can lead to erroneous conclusions being drawn from the results. It is thus important to be aware of the substances possibly present in the GTF extracts which could have insulin potentiating or insulin mimicking properties and also of physical parameters of the assays which could lead to errors.

### **1.5.1 Insulin Potentiators**

The effects of **nicotinic acid**, an identified component of GTF extracts, on glucose metabolism of the rat adipocyte system *in vitro* are well known (Simpson and Cushman 1986). Significantly, nicotinic acid has been shown to lower blood glucose levels in normal rats (Mirsky *et al.* 1957), at higher doses to aggravate hyperglycemic states in humans (Gaut *et al.* 1971), to potentiate the action of insulin on glucose transport (Taylor and Halperin 1979) and to potentiate the action of insulin on the rate of CO<sub>2</sub> evolution in a GTF assay (Roginski *et al.* 1974). The latter work concluded that the effects of chromium-nicotinic acid complexes was far greater. Nicotinic acid supplementation was found to have no effect in lowering blood glucose levels in healthy humans but was able to exert such an effect by what appeared to be a synergistic mechanism with chromium

(Urberg and Zemel 1987). However neither nicotinic acid nor a combination of nicotinic acid and chromium was able to potentiate the action of insulin *in vitro* in the low chromium rat epididymal adipocyte assay (Anderson 1979) and nicotinic acid was found to have only a small effect on that assay by Holdsworth and co-workers (Davies *et al.* 1985a). Further it was noted that the small effect nicotinic acid did elicit was found in both normal rats and those fed on the torula yeast diet whereas the effect of GTF was only found in tissue from rats on the torula yeast diet (Davies *et al.* 1985a).

**Adenosine** is a known hyperglycemic agent (Suarez *et al.* 1987) and also has a marked effect on the metabolism of rat adipocytes (Simpson and Cushman 1986). It has been shown to markedly potentiate the actions of sub optimal amounts of insulin on glucose transport and oxidation in isolated adipocytes (Taylor and Halperin 1979, Schwabe *et al.* 1974) and these effects are also seen with adenosine analogues (Souness *et al.* 1981). It has been found that the effects of adenosine can be removed by the addition of adenosine deaminase to the incubation media (Simpson 1986). It has also been noted that the loss of adenosine effect on addition of adenosine deaminase can be countered by the addition of a stable adenosine analogue such as N-6 phenyl isopropyladenosine (Rennie 1984).

**Biotin**, a well known component of yeast extracts, may also have an effect on the blood glucose levels of both insulin dependent and non-insulin dependent diabetics (Reddi *et al.* 1988). **Isoprenaline** is another compound which has been shown to potentiate the actions of sub-optimal amounts of insulin (Joost *et al.* 1985) and of course the **sulfonylurea compounds** have been widely used as hypoglycemic agents. They are found to potentiate the action of insulin (Maloff *et al.* 1984) possibly by potentiating the recruitment of the glucose carrier (Jacobs 1985a). An example of this is the ability of glyburide, which is one of the most potent sulfonylurea compounds, to potentiate glucose transport at a level of 7 nM insulin by 30-40% while having no effect on basal transport (Jacobs *et al.* 1985b).

### **1.5.2 Insulin Mimickers**

The oxidising agents **Vitamin K<sub>5</sub>** and **H<sub>2</sub>O<sub>2</sub>** have been shown to mimic the actions of insulin in isolated adipocytes (Simpson and Cushman 1986).

Lockwood *et al.* (1971 and 1974) found that the polyamines **Spermine** and **Spermidine** gave significant insulin like effects in isolated epididymal rat adipocytes and they concluded that this activity was due to the spatial separation of the amino groups. This may have some relevance to the similar observations of Cooper *et al.* (1983b) who postulated that GTF activity requires a similar spatial separation of amino groups.

The effects of amino acids are particularly important as they are significant components of most GTF extracts. **L-tryptophan** and its metabolites have been shown to have an insulin like effect in rat diaphragm (Silverstein *et al.* 1967) and whole rat (Mirsky *et al.* 1957) as have **L-lysine** and **hydroxy-L-proline** (Borrebaek *et al.* 1963). Other amino acids to show hypoglycemic effects include; **glutamic acid** and **arginine** (Anderson and Herman 1967), **leucine** (Fajans *et al.* 1964) and **tryptophan** (Zakim *et al.* 1968).

A case with parallels to the attempts to isolate GTF fractions from yeast is that of *Panax ginseng*. Several components of this plant have been found to possess insulin like activity as measured by their ability to inhibit hormone stimulated lipolysis in adipocytes (Ng *et al.* 1985). These active components include; **adenosine**, an **unidentified carboxylic acid**, a **peptide of MW 1400** which contains glutamic acid, glycine and aspartic acid in a ratio of 3:3:2 (Note: This composition is similar to that of GTF extracts except for the absence of cysteine) and five **glycans**. The investigations of this plant highlights the range of possible compounds present in extracts.

Other examples of extracts from natural sources which possess hypoglycemic activity include oyster extracts (Shibata *et al.* 1986), guar gum (Track *et al.* 1984, Ebeling *et al.* 1988), *poterium spinosum*, which is a member of the rose family, (Kanter *et al.* 1984) and extracts from a number of other plants (Muller *et al.* 1988). These extracts are generally effective after simple purification steps similar to those used for separating crude GTF preparations from brewers yeast.

### **1.5.3 Non Specific Effects**

Another factor which could affect results of GTF assays is variance of the physical parameters of the assays. Physical parameters which have been shown to effect the adipocyte assay system include; **buffer systems** used (Anderson *et al.* 1979), **pH** (Simpson and Cushman 1986, Toyoda *et al.* 1986, Anderson *et al.* 1979), **incubation shaking speed** (Whitesell *et al.* 1986) and **hyperosmolarity** (Toyoda *et al.* 1986).

### **1.5.4 Trace Elements**

As well as chromium a number of other elements have been implicated as having a possible effect on glucose metabolism but there is little published data on the occurrence of these elements in GTF preparations. Some of these elements have similar properties to those claimed for chromium:

**Manganese** Manganese deficient rats have been shown to have impairment of glucose tolerance (Keen *et al.* 1985) and a case has been reported where manganese

administration induced hypoglycemia in a diabetic patient (Rubenstein *et al.* 1962). Administration of manganese (II) in the diet has been shown to lower the blood sugar of white mice by up to 30%, although this effect was less than that caused by similar amounts of chromium (III) (Vakhrusheva 1960). However manganese (II) was unable to induce a lipolytic effect in rats, whereas chromium did (Vakhrusheva 1964). Further, manganese (II) has been reported to demonstrate insulin like effects, stimulating transport of glucose into isolated rat adipocytes *in vitro* (Yamamoto *et al.* 1984, Saggerson *et al.* 1975). In the report which originally identified chromium as the inorganic component of GTF, manganese exerted a similar, but smaller, effect to that of chromium (Mertz 1961).

**Cadmium** Small amounts of cadmium (II) stimulated lipogenesis but not glucose transport in isolated adipocytes (Yamamoto *et al.* 1984).

**Nickel** Nickel (II) was found to have insulin like activity as it decreased adrenalin stimulated lipolysis and stimulated incorporation of glucose into lipids in isolated rat adipocytes (Saggerson *et al.* 1976), although Yamamoto *et al.* (1984) reported that Nickel (II) had no insulin like effect on 3-O-methylglucose transport in adipocytes.

**Copper** Saggerson *et al.* (1976) have reported that copper (II) exhibits some marked insulin like effects. Copper significantly decreased the lipolytic effects of both glucagon and adrenalin and significantly increased the incorporation of glucose into fatty acids and glycerol. An impairment in glucose tolerance due to copper deficiency has been claimed to have been seen in humans (Klevay *et al.* 1986) and in rats (Fields *et al.* 1983b, Hassel *et al.* 1983). The copper deficient rats developed a similar impairment of glucose tolerance to that claimed to be seen in rats fed a torula yeast diet that was apparently low in chromium (Schwarz *et al.* 1955, Mertz *et al.* 1959). It was found that copper (II) exerted a significant hypoglycemic effect in rabbits which was obviated by administration of cysteine while a similar hypoglycemic effect by chromium was unaffected by cysteine administration (Berenshtein 1965). An interesting result of the work of Fields *et al.* (1983b) was that depletion of bodily copper levels was far more pronounced in rats fed a copper deficient sucrose based diet than in rats fed a starch based diet even though the copper levels of both diets were the same. This may also have interesting implications for the high sucrose diet used in the chromium deficient sucrose-torula yeast based diet used in the epididymal rat adipocyte GTF assay. Copper was also found to potentiate the hypoglycemic action of insulin in diabetic rats *in vivo* (Fields 1983 a).

**Vanadium** Vanadium in the form of vanadate has a well known insulin like effect in a number of tissues but the mechanism of this action is not understood. Tolman *et al.* (1979) demonstrated insulin like activity in a number of tissues of rat including the ability to stimulate glucose oxidation and transport in isolated epididymal adipocytes, to stimulate glycogen synthesis in the liver and diaphragm and also to inhibit hepatic gluconeogenesis. Clark *et al.* (1985) have shown that vanadate also mimics insulin's

stimulation of glucose oxidation in the skeletal muscles of rats. Vanadate has also been shown to lower plasma cholesterol levels (Curran *et al.* 1959) and to return blood glucose levels of diabetic rats to the levels of a non-diabetic control group (Heyliger *et al.* 1985).

**Zinc** Animals fed a zinc deficient diet have been shown to develop a severe impairment of glucose tolerance (Prasad 1979). While zinc affects a wide range of metabolic processes it also has an *in vitro* effect on the rat adipocyte system. Zinc has been found to potentiate insulin stimulated glucose uptake in the adipocytes of fasted normal rats *in vitro* (Quaterman 1972). In these experiments chromium was also noted to have an insulin potentiating effect although it was small compared to zinc. Further it has been found that zinc (II) has a direct stimulatory effect on the uptake of 3-O-methyl glucose into rat adipocytes (Yamamoto *et al.* 1984).

### **1.5.5 Discussion**

The reports summarised in this section indicate that a number of extracts from natural sources exert a hypoglycemic effect similar to that caused by extracts of brewers yeast. Furthermore it can be seen that a number of compounds probably present in GTF preparations cause similar effects. The situation in adipocyte assays is similar with a range of substances which are clearly unrelated to GTF either potentiating insulin action or causing an insulin like effect which under certain circumstances could be confused with a potentiation effect. This possible confusion is particularly important when it is considered that the majority of GTF preparations are impure and hence some contaminants mentioned above could give results falsely indicating GTF activity and this could lead to false indications of potentiation.

While it is true that the effects observed above were not investigated in the tissues of rats fed the torula yeast diet, the results do cast grave doubt over the specificity of the adipocyte assay as a measure of GTF activity. By implication the existence of a discrete glucose tolerance factor is called into question when fractions under test are impure.

## **1.6 In Vivo Effects of Chromium and GTF Supplementation**

If chromium has an essential role in carbohydrate metabolism it would be expected that defects of carbohydrate metabolism attributed to a deficiency of chromium would respond to either supplementation with chromium or GTF preparations over a period of time. The ability to show conclusively that chromium or GTF preparations are able to consistently

repair such defects would be a major step in establishing chromium as an essential trace element and confirming a role for GTF in glucose metabolism.

A number of studies have been undertaken over the years into the effect of various chromium and GTF supplementation regimes on the metabolism of humans (see Table 1.1) and animals (see Table 1.2). The implications of these studies have been discussed by several authors (Rabinowitz 1983, Wallach 1985, Anderson 1986) but in general the results of the studies have been equivocal.

The most convincing evidence presented by these studies are the results of chromium supplementation in a small number of documented cases of chromium deficiency in long term total parenteral nutrition (Jeejeebhoy 1977, Freund 1979, Brown, R.O 1986). In these cases patients had developed symptoms including impaired glucose tolerance, peripheral neuropathy, brain disorders and abnormalities of nitrogen metabolism, and inorganic chromium supplementation was found to dramatically reverse these symptoms. However countering these observations is a report (Nutrition Reviews 1988) where chromium supplementation in a similar situation had little effect. It has also been reported that children on a long term total parenteral nutrition regimen which resulted in very low body levels of chromium did not develop any of the symptoms mentioned above (Dahlstrom *et al.* 1986).

An interesting observation with possible relevance to GTF mechanism is that reported by (Urberg *et al.* 1987) where neither chromium nor nicotinic acid alone significantly lowered blood glucose but when the two were combined a synergistic effect was noticed. This has relevance to GTF as many GTF extracts contain chromium and nicotinic acid.

The results of 32 chromium and GTF supplementation studies in humans and 21 in animals are summarised in Tables 1.1 and 1.2. Despite the large number of studies no significant conclusions can be drawn from this information. Most of the studies concluded that there was no significant effect of supplementation on the parameters measured while others claimed there were effects of supplementation although the significance of some of these is marginal. The lack of conclusive results is not surprising bearing in mind the difficulties and possible sources of error in running studies

Table 1.1 Effect of Chromium and GTF Supplementation on Humans

Reference	Patient Status	Daily Supplement	Study Length	Trial Type	Blood Glucose	Serum Insulin	Serum Chol	HDL Chol	Total TG
Glinsmann <i>et al</i> (1966)	6 diabetics	150 µg -1mg CrCl <sub>3</sub>	15-120 days		Some improvement in OGTT of 3 patients				
Carter <i>et al</i> (1968)	34 infants with kwashiorkor	250 µg CrCl <sub>3</sub>	Single Dose		NC				
Hopkins <i>et al</i> (1968)	Malnourished Infants	250 µg CrCl <sub>3</sub>	Single Dose		Improved Glucose Tolerance				
Levine <i>et al</i> (1968)	10 elderly adults, abnormal OGTT	150 µg CrCl <sub>3</sub>	4 months		NC	NC			
Sherman <i>et al</i> (1968)	4 Normal,10 Diabetic Adults	50 µg CrCl <sub>3</sub> or Placebo	4 months		DBC	NC			
Gurson <i>et al</i> (1973)	28 malnourished infants	250 µg CrCl <sub>3</sub>	Single Dose		Imp				
Freiberg <i>et al</i> (1975)	a) 38 Adults , Diabetic	"Yeastamin"	2-15 months		- 12%	+ 9%			
	b) 15 Adults , Doubtful	1-2 Teaspoons			- 15%	NC			
	c) 17 Adults , Normal	per day			NC	NC			
Jeejeebhoy (1977)	Adult on TPN , Hyperglycemic	250 µg CrCl <sub>3</sub>	2 weeks		Imp+				
Wise (1978)	9 Adults,abnormal fasting glucose	1 mg CrCl <sub>3</sub>	6 days		++				
Liu & Morris(1978)	a) 15 Adults, Normal OGTT	5 g BY	3 months		NC	- 23%	- 12%		NC
	b) 12 Adults, Abnormal OGTT	5 g BY	3 months		NC	- 35%	- 12%		NC
Doisy <i>et al</i> (1976)	16 Normal Adults	10 g BY	1 month		NC	-20%	-16%		
Nath <i>et al</i> (1979)	12 diabetic adults	500 µg Cr(III)	2 months		-21%	-22%	-9 %		NC
Freund <i>et al</i> (1979)	Adult on TPN , Hyperglycemic	150 µg CrCl <sub>3</sub>			Imp+				

BY=Brewers Yeast,TPN= Total Parenteral Nutrition,OGTT=Oral Glucose Tolerance Test,Imp=Some Improvement in Glucose Tolerance Found , Chol=Cholesterol,DBC=Double Blind Crossover Imp+=Large improvement in Glucose Tolerance found,+ =Small increase, += large increase, - =small decrease, NC =No Significant Change, HDL =High Density Lipoprotein, DB=Double Blind

Table 1.1 cont.

Reference	Patient Status	Daily Supplement	Study Length	Trial Type	Blood Glucose	Serum Insulin	Serum Chol	HDL Chol	Total TG
Offenbacher <i>et al</i> (1980)	a) 12 elderly adults, var OGTT	9 g BY	2 months		- 13%	NC	- 12%		NC
	b) 12 elderly adults, var OGTT	9 g Torula	2 months			NC	NC	NC	
Riales & Albrink (1981)	12 Healthy Adults	200 µg CrCl <sub>3</sub>	3 months		NC	NC	NC	+ 12%	NC
Polansky <i>et al</i> (1981)	a) 58 Adults, Normal OGTT	200 µg CrCl <sub>3</sub>	3 months		NC	NC			
	b) 18 Adults, Abnormal OGTT	200 µg CrCl <sub>3</sub>	3 months		- 15%	NC			
Polansky <i>et al</i> (1982)	a) 30 Adults, Normal OGTT	{200 µg CrCl <sub>3</sub> and }	3 months		NC	NC	NC		NC
	b) 11 Adults, Abnormal OGTT	{10 g BY}	3 months		- 15 %	NC	NC		NC
Elwood <i>et al</i> (1982)	a) 11 Adults, Normal Lipids	20 g BY	2 months				- 10%	+ 9%	NC
	b) 16 Adults, Hyperlipemia	20 g BY	2 months				- 9%	+ 12%	NC
	c) 19 Adults, Normal Lipids	10 g BY	2 months				- 5%	+ 8%	NC
Grant & McMullen (1982)	37 type 2 Diabetics	1.6 g BY	6 weeks	DBC	NC		NC	+ 36%	NC
Rabinowitz <i>et al</i> (1983)	43 Diabetic Adults	Placebo	4 months	DBC	NC	NC	NC	NC	NC
		or 150 µg CrCl <sub>3</sub>	on each		NC	NC	NC	NC	NC
		or 8.35 g Yeastamin			NC	NC	NC	NC	NC
		or 8.35 g BY			NC	NC	NC	NC	NC
Mossop (1983)	26 Diabetic Adults	2 mg CrCl <sub>3</sub>	3 months		- 50%		NC	+ 30%	
Anderson (1983 a)	a) 20 adults judged hyperglycemic	Placebo	3 months	DBC	-15%	NC	NC	NC	NC
	b) 35 adults judged normal	or 200 µg CrCl <sub>3</sub>			NC	NC	NC	NC	NC
	c) 21 adults judged hypoglycemic				+14%	NC	NC	NC	NC

BY=Brewers Yeast, TPN= Total Parenteral Nutrition, OGTT=Oral Glucose Tolerance Test, Imp=Some Improvement in Glucose Tolerance, Chol=Cholesterol, DBC=Double Blind Crossover  
 Imp+=Large improvement in Glucose Tolerance, + =Small increase, ++= large increase, - =small decrease, NC =No Significant Change, HDL =High Density Lipoprotein, DB=Double Blind

Table 1.1 cont.

Reference	Patient Status	Daily Supplement	Study Length	Trial Type	Blood Glucose	Serum Insulin	Serum Chol	HDL Chol	Total TG
Uсутupa <i>et al</i> (1983)	10 diabetics	200 µg CrCl <sub>3</sub>	6 weeks	DBC	NC	-	NC	NC	NC
Vinson <i>et al</i> (1984)	23 Adults of various status	100 µg High Cr BY	6 months		Cholesterol/HDL ratio significantly lowered for Hyperglycaemics but not for Diabetic or normal subjects				
Wang <i>et al</i> (1984)	High Cholesterol Adults	Placebo or 50 µg CrCl <sub>3</sub> or 9 g BY	3 months				NC - NC	NC NC	
Elias <i>et al</i> (1984)	6 Adult Diabetics	52 g BY	2 weeks		Increased insulin sensitivity and glucose tolerance				
Martinez <i>et al</i> (1985)	85 elderly women, vari status	200 µg CrCl <sub>3</sub>	10 weeks	DB	NC	NC			
Bourn <i>et al</i> (1985)	32 healthy elderly women	Placebo or 200 µg CrCl <sub>3</sub>	10 weeks	DBC	-	NC	NC	+	NC
Hunt <i>et al</i> (1985)	39 Diabetics & 39 normal Adults	BY to give 68 µg of Cr/day	3 months	DBC	NC	NC	NC	NC	NC
Offenbacher <i>et al</i> (1985)	23 Healthy Elderly Adults	Placebo or 200 µg CrCl <sub>3</sub> or 500 mg BY	10 weeks	Blind	NC	NC	NC		NC
Brown <i>et al</i> (1986)	Adult on TPN, Hperglycemic	200 µg CrCl <sub>3</sub>	2 weeks		Imp+				
Anderson <i>et al</i> (1987)	8 Hypoglycemic Females	200 µg CrCl <sub>3</sub>	3 months	DBC	Improved Hypoglycemic Symptoms				
Urberg <i>et al</i> (1987)	16 Healthy Adults	200 µg CrCl <sub>3</sub> 100 mg NA CrCl <sub>3</sub> + NA	1 month		NC	NC			
					NC	NC			
					-14%	NC			

BY=Brewers Yeast, TPN= Total Parenteral Nutrition, OGTT=Oral Glucose Tolerance Test, Imp=Some Improvement in Glucose Tolerance Found, Chol=Cholesterol, DBC=Double Blind Crossover  
Imp+=Large improvement in Glucose Tolerance found, + =Small increase, ++= large increase, - =small decrease, NC =No Significant Change, HDL =High Density Lipoprotein, DB= Double Blind

Table 1.2 Effect of Chromium and GTF Supplementation on Animals

Reference	Species	Diet	Supplement	Effects
Mertz <i>et al</i> (1961)	Rat	Purina Chow, Low Cr	10 ppm CrCl <sub>3</sub>	Increased glucose removal rates.
Schwarz <i>et al</i> (1961)	Rat	Low Cr	Various Cr Complexes	had various effects on glucose clearance rates.
Mertz <i>et al</i> (1965a)	Rat	Torula Yeast Low Cr	Single IV dose CrCl <sub>3</sub>	Increased glucose removal rates.
Mertz <i>et al</i> (1965b)	Rat	Low Cr	5ppm Cr in H <sub>2</sub> O	Generally improved glucose tolerance.
Davidson <i>et al</i> (1968)	Squirrel Monkey	Stock Diet	10ppm Cr in H <sub>2</sub> O	Improved glucose tolerance after 22 days supplementation.
Staub (1969)	Rat	Stock Diet	5ppm Cr in H <sub>2</sub> O	Lowered plasma cholesterol.
Roginski & Mertz (1969)	Rat	Soy Protein Low Cr	2ppm Cr in H <sub>2</sub> O	a) Raised tissue glycogen levels b) No change in fasting glucose levels c) Potentiated hypo glycemc action of insulin.
Tuman <i>et al</i> (1975)	Diabetic Mice	Stock Diet	Single IP dose 5.0 mg CrCl <sub>3</sub>	No effects on glucose levels.
Mickail <i>et al</i> (1976)	Rat	Low Protein High Protein	50µg CrCl <sub>3</sub> 50µg CrCl <sub>3</sub>	Raised K <sub>g</sub> glucose 70% in Cr Suppl vs un-suppl Cr suppl had no effect
Preston <i>et al</i> (1976)	Guinea Pig	Torula Yeast Low Cr	0.5 ppm Cr in diet 50 ppm Cr in diet	No Effect on Glucose Tolerance or Serum Cholesterol No Effect on Glucose Tolerance or Serum Cholesterol
Wooliscroft <i>et al</i> (1977)	Rat	Torula Yeast Low Cr	5 ppm Cr in H <sub>2</sub> O	Effect on glucose tolerance depended on method of calculation used
Tuman & Doisy (1977)	Diabetic Mouse	Stock Diet	Single dose IP GTF	a) 14% drop in plasma glucose and synergistic effect on action of insulin. b) 16% drop in plasma TG and synergistic effect on the action of insulin. c) 21% drop in plasma Cholesterol over controls

IP =Intraperitonially, IV = Intravenously, TG = Triglyceride , NA = Nicotinic Acid

Table 1.2 cont

Reference	Species	Diet	Supplement	Effects
Tuman <i>et al</i> (1978)	Diabetic Mouse	Stock Diet	<u>Single dose IP</u>	
			a) Yeast GTF extract	29% drop in plasma glucose, 56% drop in plasma TG.
			b) Synthetic GTF	18% drop in plasma glucose, 26% drop in plasma TG.
	Normal Mouse	Stock Diet	<u>Single dose IP</u>	
a) Yeast GTF extract			36% drop in plasma glucose.	
		b) Synthetic GTF	18-20% drop in plasma glucose.	
O'Flaherty <i>et al</i> (1978)	Rat	Low Cr Diet	5 ppm Cr in H <sub>2</sub> O	22% drop in plasma glucose
	Rat	Stock Diet	for 8 weeks	22% drop in plasma glucose
Ghafghazzi <i>et al</i> (1979)	Rat	Stock Diet	Single dose IP Cr	Rise in blood glucose levels.
			Cr Suppl for 23 days	No change in plasma glucose.
Rosebrough <i>et al</i> (1981)	Turkey Poult	Stock Diet	20 ppm Cr in diet	No change in blood glucose
			20ppm Cr + NA	No change in blood glucose
Steele <i>et al</i> (1980)	Turkey Poult	Stock Diet	20 ppm Cr in diet	No change in blood glucose but 60% increase in lipogenesis
Donaldson (1985)	Rats	Low Cr, high sucrose and casein as protein	5 ppm Cr in diet until	a) No significant difference in glucose levels & high cholesterol diet age 18 months
				b) No significant change in total plasma cholesterol levels
				c) No significant change in plasma TG levels.
Schrauzer <i>et al</i> (1986)	Mouse	Stock Diet	Brewers Yeast	Prevented drop in food energy utilisation
Ranhotra <i>et al</i> (1986)	Rat	Low Cr Diet	Bakers Yeast	Lowered blood glucose, Cholesterol and Triglycerides.
			CrCl <sub>3</sub>	No effect on blood glucose, Cholesterol or Triglycerides.
Yi-Ching <i>et al</i> (1986)	Obese Mouse	Low Cr, Casein Diet	1.8 ppm Cr	Some lowering of plasma glucose and cholesterol. Reported a significant lowering of hepatic lipid

IP =Intraperitonially, IV = Intravenously, TG = Triglyceride , NA = Nicotinic Acid

with so many variables especially when the effects observed are often relatively small and the number of subjects studied was generally too low for statistically significant results. It has been suggested (Wooliscroft 1977) that conclusions reached may largely depend on the method of interpreting the data. It would appear therefore that if conclusive results are to be obtained in this area that properly designed, large scale studies, such as that carried out by Anderson *et al.* (1983a), need to be undertaken. However, the data summarised in Table 1.1 and 1.2 does not show the effects expected if chromium is an essential trace element and once again the burden of proof rests with those who claim that it is.

## **1.7 Aims of this Thesis**

In view of the confusion which reigns in this field and the recent work (Haylock *et al.* 1983a & b) which shows that purified GTF extracts from brewers yeast do not contain physiologically significant levels of chromium it was considered worthwhile to attempt to purify and characterise the material responsible for the effects attributed to GTF. It was decided to begin with a crude extract of yeast that was known to have GTF activity and for this purpose the relatively impure extract described by Toepfer *et al.* (1977) was used. It was aimed to more fully investigate the nature of this impure extract to more clearly resolve whether the most pure GTF fraction from this was a chromium complex. Since it is probable that chromium is not an integral component of GTF, the degree of purification obtained in the fractions of interest was judged by the fractions ability to show activity in *in vitro* assays for GTF rather than by measuring chromium levels. The standard yeast assay for GTF and the low chromium rat epididymal adipocyte assay were used to determine GTF activity.

Concurrently it was decided to make preliminary investigation into occurrence of GTF activity in fractions from torula yeast since torula yeast has long been assumed to be low in GTF and this is the protein source in diets inducing the GTF deficient state. Comparisons between the fractions of the extracts of brewers yeast and the corresponding fractions from torula yeast may allow conclusions to be drawn as to which fractions are showing true GTF activity.

While the low chromium rat epididymal adipocyte assay system is the most widely accepted assay for GTF still little is known about the mechanism by which GTF exerts its potentiation of insulin action in the adipocytes. It was an aim of this thesis to investigate this and to use this information to find the most specific way of measuring GTF activity.

## **CHAPTER 2**

# **THE STANDARD YEAST ASSAY FOR GTF**

### **2.1 Introduction**

To be able to follow the GTF activity in a purification scheme it is first necessary to have an assay system which gives a measure of GTF activity in the large number of fractions obtained during such a fractionation process. Of the assay systems investigated to date the standard yeast assay system (Mirsky *et al.* 1980, Haylock *et al.* 1982) seemed best suited to screening large numbers of samples (Section 1.2.2). GTF activity in the yeast assay is measured by the ability of a fraction to enhance the rate at which yeast, that has been grown in a chromium deficient medium, is able to convert glucose to CO<sub>2</sub>. While the assay does not measure GTF activity alone, it has been found to be a useful screening procedure for identifying fractions worth further analysis (Davies *et al.* 1985a). It was shown by Davies *et al.* (1985a) that, while not all fractions showing activity in the yeast assay show GTF activity in the more definitive low chromium rat epididymal adipocyte assay, all fractions active in the rat assay did show activity in the yeast assay. Thus the yeast assay is a rapid, cheap and easy assay for identifying fractions with potential GTF activity.

The work in this chapter investigates the reproducibility of the yeast assay system and an assay procedure is described which allows more consistent results and better comparison of results to be obtained from different assays. Investigations of the interferences caused by ammonia are also described.

### **2.2 Methods and Materials**

#### **2.2.1 Yeast Growth and Harvesting**

The standard yeast assay used here is essentially as described by Haylock *et al.* (1982). The assay yeast was grown in 3 consecutive chromium free media to allow removal of residual chromium from the yeast which was then grown on a final defined medium for use in the assay. To accomplish this a yeast growth medium containing 0.66% yeast nitrogen base without amino acids (Difco) and 2% glucose monohydrate (Glaxo) was prepared in doubly distilled deionised water. Three 100 cm<sup>3</sup> aliquots were sterilised by boiling under 15 lb/in<sup>2</sup> pressure for 10 minutes.

The three media flasks were allowed to cool. One of the flasks was taken for inoculation and the other 2 were stored at 4 °C for later use. The first flask was inoculated with an agar grown single cell colony of the assay yeast, *Saccharomyces ellipsoideus*, using an inoculating loop and employing aseptic transfer techniques. The inoculated flask was then incubated at 30 °C for 24 hours. Aseptic transferral techniques were then used to transfer a 0.1 ml aliquot of the resulting yeast suspension to the second flask of the prepared growth medium. This was then also incubated for 24 hours at 30 °C. Using a 0.1 cm<sup>3</sup> aliquot from this growth the same process was repeated for the third flask of the prepared media.

To prepare the final growth medium 2 g glucose monohydrate (Glaxo) was dissolved in 90 cm<sup>3</sup> doubly distilled water and to this was added 10 cm<sup>3</sup> of a stock solution containing essential elements (see Table 2.1). This was sterilized by boiling for 10 minutes under 15 lb/in<sup>2</sup> pressure. To the cooled solution was added 0.1 cm<sup>3</sup> of a stock solution of essential vitamins (See Table 2.1) and this then constituted the final defined growth medium. This medium was inoculated with a 0.1 cm<sup>3</sup> aliquot from the third growth flask using aseptic techniques and then incubated at 30 °C for 18 hours. This lesser incubation time was employed so as to obtain a yeast suspension in its logarithmic growth phase and thus with a minimum of dead cells. Minimisation of dead cells was important as a set number of cells per assay flask were used.

Table 2.1 Composition of Mineral and Essential Vitamin Solutions

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Essential elements:	CaCl <sub>2</sub> .6H <sub>2</sub> O (0.14 g l <sup>-1</sup> ), (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> (5.0 g l <sup>-1</sup> ), MgSO <sub>4</sub> .7H <sub>2</sub> O (0.5 g l <sup>-1</sup> ), NaCl (0.1 g l <sup>-1</sup> ), KH <sub>2</sub> PO <sub>4</sub> (0.875 g l <sup>-1</sup> ) and K <sub>2</sub> HPO <sub>4</sub> (0.125 g l <sup>-1</sup> )
Essential Vitamins:	thiamine hydrochloride (1.25 mg l <sup>-1</sup> ), vitamin B <sub>12</sub> (1.25 mg l <sup>-1</sup> ), calcium pantothenate (1.25 mg l <sup>-1</sup> ), nicotinic acid (1.25 mg l <sup>-1</sup> ), pyridoxal phosphate (0.25 mg l <sup>-1</sup> ), para-amino benzoic acid (0.25 mg l <sup>-1</sup> ), inositol (0.25 mg l <sup>-1</sup> ), folic acid (0.25 mg l <sup>-1</sup> ) and biotin (0.20 mg l <sup>-1</sup> )

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Source Haylock *et al.* 1982

Cells were harvested by centrifuging the yeast suspension at 4000 rpm for three minutes and the supernatant was removed. The cell pellet was resuspended in doubly distilled de-ionized water, re-centrifuged and the supernatant discarded. This was repeated a total of

three times so that all the yeast growth medium was removed. The resulting yeast pellet was suspended in 50 cm<sup>3</sup> of phosphate buffer (pH 5.75, 0.62 M) and stored at 4 °C.

To determine the concentration of cells in this final solution the absorbance of the solution was determined at 540 nm. The concentration was determined by comparison with a pre-determined standard curve (Haylock 1981). The solution was then diluted using phosphate buffer to give a final concentration of 1.5 x 10<sup>8</sup> yeast cells per cm<sup>3</sup>. This solution was used directly in the assay.

### **2.2.2 The Yeast Assay**

The yeast assay measures the rate at which a set number of chromium deficient yeast cells produce CO<sub>2</sub> anaerobically at 30 °C. GTF activity is determined by the amount by which a sample was able to enhance CO<sub>2</sub> evolution from the assay as compared with the effect of a buffer blank. The CO<sub>2</sub> production is measured using Warburg manometric techniques.

To the base of each manometer flask was added 2 cm<sup>3</sup> of the yeast suspension (pH 5.75, 1.5 x 10<sup>8</sup> cells per cm<sup>3</sup>). To the side arm of the flask was added 0.4 cm<sup>3</sup> of a 2% glucose monohydrate (Glaxo) solution and 0.1 cm<sup>3</sup> of the sample solution which was dissolved in doubly distilled de-ionized water. The flasks used as controls had 0.1 cm<sup>3</sup> of the phosphate buffer in place of the sample and the thermobar flasks, which were used to adjust for atmospheric fluctuations, contained 2.5 cm<sup>3</sup> of water pipetted directly into the bottom of the flask. All additions were made using a sterile pipette.

The filled flasks were then prepared for incubation by first inserting the stopcock in the side arm. This was achieved by greasing the stopcock with anhydrous lanolin and gently grinding it into the joint so as to achieve an airtight seal. The stopcock was then adjusted so that the air vent hole was in a position where gas could pass from the side arm through the stopcock. Occasionally in these manipulations lanolin became lodged in the stopcock air vent hole and this could be removed by sucking gently through the stopcock. The ground glass joint on the manometer was then greased, also using anhydrous lanolin, and the flask was attached to the manometer. The joint was made airtight by gently grinding the lanolin into the joint and the flask was attached to the manometer using a rubber band.

Oxygen was purged from the system by flushing with oxygen free dry nitrogen for three minutes and the stopcock and the manometer tap were then closed in that order. If no leaks were present in the system the manometer fluid flowed slightly from the right hand bore to the left hand bore due to the elevated pressure in the flask. The flasks were then immersed in a Warburg bath set at 30 °C. The sudden increase in temperature resulted in

a pressure build up resulting in a rise in manometer levels. This pressure was released by quickly opening the manometer tap. All the manometer levels were adjusted so that the right hand bore read approximately 15 and the left hand bore read approximately 5. The flasks were equilibrated for 15 minutes.

Before starting the assay the agitator was started and run for 5 minutes. A reading was then taken by adjusting the right hand bore to read 15 and the reading on the left hand bore was recorded. This step was repeated every 5 minutes until readings were constant, taking into account atmospheric changes registered by the Thermobars. If readings continued to rise a leak was indicated and this was corrected.

To start the assay the flask was tipped by inverting the manometer so that the contents of the sidearm were mixed with the yeast suspension. While this mixing was occurring it was important to block the manometer overflow so the manometer fluid did not escape. The manometer was placed back in the water bath and an initial reading for this time ( $t_0$ ) was taken. Readings were subsequently taken every 20 minutes up to a total elapsed time of 300 minutes.

### 2.2.3 Calculations

To determine the rate of  $\text{CO}_2$  evolution the individual readings were first converted to micromoles of  $\text{CO}_2$  evolved using a standard formula (Manometric Techniques 3rd edition) :

$$\text{micromoles CO}_2 = \frac{H \times \{ [(V_f + V_m) - V_s] \times (273/300) + (V_s \times S_{\text{CO}_2}) \}}{22.4}$$

Where  $H$  = corrected manometer height = { (reading at time  $t$  - reading at time 0) - (Thermobar reading at time  $t$ ) - (Thermobar reading at time 0) }

$V_f$  = volume of the Warburg flask ( $\text{cm}^3$ )

$V_m$  = volume of the manometer ( $\text{cm}^3$ )

$V_s$  = volume of test solution ( $2.5 \text{ cm}^3$ )

$S_{\text{CO}_2}$  = solubility of  $\text{CO}_2$  at  $30^\circ\text{C}$  ( $0.665 \text{ cm}^3$ )

This was routinely calculated using a standard computer program and then a graph of micromoles of  $\text{CO}_2$  produced versus time was then drawn for each flask for the last 140 minute period of each assay. The activity of the sample was determined by the percentage by which a sample was able to enhance  $\text{CO}_2$  production over the rate of the control. This was calculated as follows:

$$\% \text{ Enhancement} = \frac{\mu\text{moles CO}_2 / \text{min (sample)} - \mu\text{moles / minute (control)}}{\mu\text{moles CO}_2 / \text{min (control)}} \times \frac{100}{1}$$

### **2.2.4 Improvement in Methodology**

For the present work two Thermobar manometers were employed per assay instead of one. This was to try and get a more precise correction for the effect of atmospheric pressure changes during the assay. It was also decided to run at least two control flasks per assay and to use the average of these in the calculations. This was done to minimise the effect of variations in the blank rate on the results and to allow better inter assay comparisons. It was also decided to use arginine as the internal standard as it gave high levels of activity in the yeast assay and its behaviour in the assay system was known (Jackson, 1985).

## **2.3 Results**

### **2.3.1 Reproducibility of Results**

An investigation was made of the reproducibility of results by assaying identical samples on different days. For this purpose arginine was chosen since it is stable, regularly showed a considerable degree of GTF like activity in the yeast assay (Jackson, 1985) and it was not thought to be a component of GTF. The concentration of the arginine standard was important as arginine displayed a saturation effect (Jackson, 1985) and it was important to choose a concentration on the plateau of this curve. A final concentration of  $0.2 \text{ mg/cm}^3$  in the assay solution was chosen since arginine is maximally active at this concentration (Jackson, 1985). A series of assays were run to test the variation in results in assays run on different days and the results are shown in Table 2.2 .

### **2.3.2 Effect of Ammonia on the Assay System**

Ammonia (BDH analar) was assayed at a range of concentrations and the results are shown in Table 2.3. From these results it can be seen that ammonia has a concentration dependant effect on the rate of  $\text{CO}_2$  evolution from cells. Included as a comparison is the result of a determination of the activity of a crude GTF extract to give an idea of the level of activity involved.

Table 2.2 Interassay Variation of Assays With Arginine (Conc 0.2 mg/cm<sup>3</sup>)

Assay No	Assay Results	Average
10	480 , 434 , 397 , 422 , 510	450 ± 60%
11	672 , 715 , 672 , 620 , 766	689 ± 70%
12	438 , 451 , 433 , 544 , 425	458 ± 20%
13	350 , 350 , 385 , 382 , 348	363 ± 15%
14	272 , 342 , 429 , 320 , 287	330 ± 60%
15	377 , 377 , 383 , 390	382 ± 10%
16	440 , 440 , 445 , 451	444 ± 5%

NB all results expressed as percentage enhancements.

## 2.4 Discussion

The results of assays with arginine standards demonstrate that while intra assay comparisons are generally reasonable, problems arise when comparing results from different assays. Previous workers using the same strain of yeast (Haylock *et al.* 1981, Cooper *et al.* 1984a) do not mention such problems. It is likely that they did not encounter reproducibility problems as they were not continually re assaying the same sample. This inter assay variations highlights the need for the use of an internal standard, such as arginine, in each assay for the purposes of valid comparisons between different samples. In this way it is easier to identify the fractions containing the most GTF activity.

Table 2.3 Effect of Ammonia on the Yeast Assay

Conc of NH <sub>3</sub> in assay	Percentage Enhancement
1 x 10 <sup>-3</sup> M	700%
5 x 10 <sup>-4</sup> M	725%
3 x 10 <sup>-4</sup> M	600%
1 x 10 <sup>-4</sup> M	400%
5 x 10 <sup>-5</sup> M	310%

In all subsequent assays, 2 flasks containing arginine at a concentration of  $0.2 \text{ mg/ cm}^3$  were run to act as internal standards. It would be possible to normalise results obtained in different assays using the results from arginine standards. However errors were estimated at 20% for both the estimation of arginine activity and the activity of the sample, hence normalised results would not necessarily be any more precise. It was concluded therefore that as the yeast assay was being used as a screening process an absolute value for GTF activity was not as important as the ability to roughly compare and rank the GTF activities of different samples. The use of arginine standards to gain improved comparability between assays increased the number of flasks used for various controls from 2 to 6 thus decreasing the number of samples that could be tested in each assay. This comprises one of the main benefits of using this assay, the ability to assay a large number of samples.

This work also reveals that ammonia has a direct effect on the yeast assay. The change of pH due to the ammonia is undoubtedly at least partially responsible but it also appears that ammonia is exerting a direct effect on the assay system, probably by acting as a nitrogen source for the starved yeast cells. Previous workers (Haylock *et al.* 1983a) claimed that exposure of a sample to ammonia increased its activity in the yeast assay. Other work (Cooper *et al.* 1985) showed that fractions eluted from an ion exchange column with ammonia had higher activity. It was not concluded whether this activity was due to a direct effect on the assay caused by any ammonia that may have remained after freeze drying or whether the ammonia induced structural changes in the samples which increased their activity. The results shown here tend to favour the former postulate and highlight the dangers of using ammonia in the separation procedures for GTF.

The standardised yeast assay using arginine as an internal standard is referred to in later work as the standard yeast assay where it was used as a screening assay to monitor the biological activity of fractions isolated from brewers yeast.

## CHAPTER 3

# A NEW APPROACH TO SEPARATING GTF FRACTIONS FROM BREWERS YEAST

### 3.1 Introduction

Before any meaningful investigations of the properties of GTF can be made it is imperative that a pure sample of the factor is isolated. Despite the development of complex purification schemes, the identification of a single factor responsible for GTF activity has remained elusive (Section 1.3.1). Many of the previous isolation procedures concentrated on monitoring chromium content as a simple indication of likely GTF activity in a fraction. However, since chromium is not necessarily associated with GTF activity of fractions obtained from brewers yeast it is important to repeat the purification work of Haylock *et al.* (1983a & b) and Davies *et al.* (1985a) using the yeast bioassay to monitor the GTF activity of the fractions. However, for the purposes of the current separation work it was decided to begin with the initial extraction procedure described by Toepfer *et al.* (1977) as this method derives directly from those used to obtain the material on which the initial observations underpinning the case for GTF were made (Schwarz and Mertz 1955 & 1957). It was also decided to investigate in parallel the separation of fractions from both brewers yeast (*Saccharomyces cerevisiae*) and torula yeast (*Candida utilis*) using the same method. Torula yeast is assumed to be deficient in GTF as it is used as the sole protein source in the diets which are used to create a GTF deficient state in animals. Surprisingly however there has never been an investigation of torula yeast fractions for GTF activity. If the torula yeast is found to contain no GTF then a comparison of the fractions obtained from the torula yeast and the brewers yeast would be helpful in identifying brewers yeast fractions which were giving spurious GTF activity.

The work in this chapter describes a fractionation procedure for the yeast with the target being to follow the fractions showing the most activity in the standard yeast assay.

## **3.2. Methods and Materials**

### **3.2.1 Isolation of Crude GTF Extract**

The isolation procedure began with various starting materials. These were live brewers yeast cells (Lion Breweries, Palmerston North, New Zealand), dried powdered brewers yeast (Dominion Breweries, Mangatainoka, New Zealand) and dried torula yeast granules (Sigma cat YCU). The whole yeast cells were harvested and washed as for the assay yeast (Section 2.2.1) before the initial extraction while the other yeasts were used directly in the extraction.

One kilogram of yeast was suspended in 4 litres of 50% ethanol and this was then heated just to boiling and allowed to cool overnight. The yeast was filtered off and the ethanol was removed from the eluate using a rotary evaporator. The remaining aqueous solution was adjusted to pH 3.5 with HCl and treated with 250 g of activated charcoal. After one hour the charcoal was filtered off and the cake was washed with doubly distilled deionised water and 95% ethanol and the filtrates were discarded. The crude GTF was then eluted from the charcoal using 2 litres of diethyl ether: concentrated  $\text{NH}_4\text{OH}$  (1:1) for three hours after which the charcoal was again filtered off and the diethyl ether and the  $\text{NH}_4\text{OH}$  were removed from the filtrate using a rotary evaporator. The separation up to the stage was based on the procedure of Toepfer *et al.* (1977) and the material obtained is referred to in further work as the crude GTF extracts.

### **3.2.2 Paper Electrophoresis**

To further purify the crude GTF extract it was decided to use preparative high voltage paper electrophoresis. It has been claimed that GTF is a cationic compound (Mertz 1969, Toepfer 1977) and so separation on the basis of charge is important. It was decided to run the electrophoresis in pH 6.5 buffer to achieve separation of the cationic, amphoteric and anionic components of the crude GTF extract. This technique also had the advantage of separating the crude GTF into a reasonably large number of fractions and it had the capacity to process relatively large amounts of extract so that sufficient material would be available for further purification.

In this procedure up to 200 mg of the crude GTF extract was dissolved in doubly distilled deionized water and loaded in a band along an origin line which evenly divided a sheet of Whatman 3 MM chromatography paper in two. A margin of 50 mm was left on the line on each edge of this band. In this space on each side were loaded two individual standard chromatography marker spots. One of these contained the amino acids asp,

glu, thr, ser, ile, ala, his and gly and the other contained tyr, phe, met, leu, val, arg and lys. Following this, spots of a fluorescent marker containing dansyl-arg and dansyl-OH were placed at 50 mm intervals over the entire length of the line so that the movement of the migrating electrophoretically separated bands could be monitored. The paper was then wetted with the pH 6.5 buffer which was composed of 10% pyridine and 0.4% acetic acid v/v. The buffer was applied by pipette in such a way that the liquid migrated by capillary action through the paper and converged from both sides of the sample band so as to focus the sample material onto the origin as it became wetted. The wetted electrophoretogram was then lowered into the electrophoresis tank so that each end was in contact with the reservoirs of pH 6.5 buffer and that the centre section of it was in an inert petroleum spirits medium. A potential of 3 kV was then applied across the electrophoretogram, with the current varying between 200 and 300 mA, for 35 minutes and it was then removed from the tank and allowed to dry. The location of the separated bands was revealed by cutting a strip from each side of the paper which included both markers and an approximately 25 mm wide strip of the separated sample material. As GTF was thought to contain peptides or amino acids it was decided to reveal the separated material on the margin strips using ninhydrin.

### **3.2.3 Elution of Fractions From Paper**

Material was eluted from the paper by gravity fed capillary action and the material collected was freeze dried and weighed. As elution of ion exchange columns with  $\text{NH}_4\text{OH}$  had shown the ability to activate previously inactive fractions (Haylock 1983a) and had also shown a direct effect upon the yeast assay (section 2) it was decided to investigate alternative elutants. Other elutants investigated were  $\text{H}_2\text{O}$  and 1% acetic acid.

### **3.2.4 HPLC of Electrophoresis Fractions**

Electrophoresis fractions showing high levels of activity in the yeast assay were further purified by reverse phase high performance liquid chromatography (HPLC). The system used consisted of two Waters 6000a pump units controlled by a Waters model 660 solvent programme. The sample was injected using a UK6 injection system and the column used was a Waters 8MBC18-10  $\mu\text{m}$  (RCM).

Varying amounts of sample were dissolved in water and applied to the column and they were eluted using a linear gradient (1-100%) starting with 0.1 M ammonium bicarbonate and changing to 0.1 M ammonium bicarbonate : isopropanol : acetonitrile (1:1:1) with a flow rate of 1  $\text{cm}^3/\text{min}$  for 40-60 minutes. The eluted material was detected by monitoring absorbance at 220 nm and material was pooled according to the peaks shown by this monitoring.

### **3.2.5 Amino Acid Analysis of Fractions**

Samples were analysed on a Beckman 119 BL Amino Acid Analyser which gave an analogue output from which the quantities of amino acids present were calculated by comparison of peak areas with those of a standard. Samples were prepared by weighing an amount into a hydrolysis tube and adding 50  $\mu$ l of glass distilled 5 M HCl. The tube was sealed under vacuum and hydrolysed at 100 °C for 18 hours. The tube was then cooled and opened and the contents dried under vacuum. The contents were taken up in a pH 3.25 sodium citrate loading buffer and applied to the column of the analyser.

### **3.2.6 Trace Metal Analysis of Fractions**

#### **Inductively Coupled Argon Plasma Emission Spectrometry**

For samples where more than about 1 gram of material was available for analysis the preferred method of analysis was by radio frequency inductively coupled argon plasma emission spectrometry (ICP). This method was preferred as it enabled 23 elements to be determined simultaneously in the sample. The analysis was carried out on an ARL 36000 at the DSIR, Palmerston North, New Zealand. Samples were prepared by wet ashing a weighed sample in 20 ml of 1:1 conc H<sub>2</sub>SO<sub>4</sub>: conc HNO<sub>3</sub> (both BDH analar). Samples were digested at 100 °C until they were nearly dry and the residue was dry ashed at 600 °C for 18 hours in a muffle furnace. The trace elements were then taken up in 10 ml of 2 M HCl (BDH analar) by soaking overnight and this solution was used for analysis.

#### **Graphite Furnace Atomic Absorption Spectrometry (GFAA)**

For purified samples where there were smaller amounts of material available for analysis graphite furnace atomic absorption spectrometry for chromium was attempted. Initially a GBC 902 atomic absorption spectrometer was used. This instrument used a pyrolytically coated graphite furnace which was much smaller than those used in instruments produced by other manufacturers. Chromium was measured at the 357.9 nm wavelength using a process very similar to that described by Guthrie *et al.* (1978). However this method proved impossible to use operationally in this instrument as massive chromium contamination was found in all the batches of the special furnaces used. Graphite furnace analysis was thereafter carried out by the the Chemistry Division of the DSIR, Wellington, New Zealand.

### **3.2.7 Mass Spectrometry**

Investigations were carried out on a AEI MS902 mass spectrometer. Both a Fast Atom Bombardment (FAB) source and a 70 eV Electron Impact source were used in the investigations. The sample was taken up in glycerol.

### 3.2.8 UV/Visible Absorbance Spectra

Spectra were determined and analysed using a Hewlett Packard 8452A Diode Array Spectrophotometer. Samples were dissolved in distilled water and measurements were made using a 0.5 ml quartz cuvette with a 1 cm path length.

### 3.2.9 Determination of GTF Activity

GTF activity was determined using the standard yeast GTF assay as described in Section 2. As it was impossible to determine the molarity of isolated fractions all fractions were assayed at a concentration of 0.2 mg/cm<sup>3</sup> as previous work (Jackson 1985) had shown that most compounds were at or near their peak activity at this weight/volume concentration.

## 3.3 Results

### 3.3.1 Crude GTF Extracts

The crude GTF extracts obtained by the procedure described in Section 3.2 were either a tan or a greenish brown coloured hygroscopic solid, depending on whether live or dead yeast cells were used. The crude GTF extract obtained from brewers yeast was labeled B and the crude extract obtained from torula yeast was labeled T. For both yeasts typically 2 grams of crude extract were obtained from 1 kg of starting material.

#### 3.3.1.1 Composition and General Characteristics

##### Amino Acid Composition

The amino acid composition of the crude GTF extracts B & T are shown in Table 3.1. It can be seen from this data that the composition of B shows similarities to that reported by Toepfer *et al.* (1977) for a similar extract. The ratio of Glu:Gly:Asp:Ala:Arg was approximately 3:2:2:1:1 and there were only trace levels of other amino acids present in this sample. Extract T contained a wider range of amino acid constituents with the major amino acids present being Gly:Phe:Ala:Val:Glu:Thr:Ile:Leu in a ratio of 7:6:4:3:2:1:1:1.

Table 3.1 Amino Acid Composition of Crude GTF Extracts B & T and of the Yeasts From Which They Were Extracted

Substance	Amino Acids Present (n moles / mg solid)															
	Asp	Thr	Ser	Glu	Pro	Gly	Ala	Val	Met	Ile	Leu	Tyr	Phe	Lys	His	Arg
Brewers Yeast	310	150	160	360	120	225	270	185	40	135	205	70	90	200	50	155
B	180	-	-	460	-	295	275	-	-	-	-	-	-	-	-	150
Torula Yeast	270	155	135	285	115	260	285	210	40	115	265	80	125	215	55	125
T	40	60	40	70	45	290	170	115	-	65	85	-	250	-	-	-

## Trace Metal Composition

The trace element composition of the crude GTF extracts B and T, as determined by ICP, are shown in Table 3.2. The levels of selenium were below the detection limit for the instrument and so are not quoted. The levels for nickel were close to the detection limit and cannot be quoted accurately but are included to give some indication of nickel content.

Table 3.2 Trace Element Composition of Crude GTF Extracts B & T  
(All results expressed as  $\mu\text{g/g}$  of solid)

Element	B	T
Aluminium	363.0	641.0
Chromium	44.0	16.2
Copper	3.5	3.5
Iron	24.0	26.1
Manganese	0.4	0.6
Nickel	>0.1	>0.1
Tin	14.0	7.1
Zinc	0.6	0.8

## UV/Visible Absorbance Spectrum

The UV visible spectrum of B is shown in Figure 3.8. There is little detail in the spectrum although peaks are revealed at 194 nm and 260 nm. The 260 nm peak in particular is consistent with a similar peak seen in the spectrum of the crude GTF extract obtained by Toepfer *et al.* (1977) where that peak was held to show the involvement of nicotinic acid in the GTF structure.

### 3.3.1.2 Activity by the Yeast Assay

Table 3.3 shows that a freshly prepared sample of extract B had an extremely high level of activity in the yeast assay, although this activity diminished over a period of several months. Further it shows that there was variation in the activity of different batches of the crude GTF preparation, despite the fact that exactly the same starting material and methods were used. Hydrolysing crude GTF extract B in 5 M HCl in vacuo for 18 hours drastically reduced its activity as did ashing it in a muffle furnace at 500 °C for 18 hours (Table 3.3).

The extract from the torula yeast, T, also displayed a large amount of activity and this was of a similar magnitude to that of B.

Table 3.3 Activity of Crude GTF Extracts in the Yeast Assay

Extract	Concentration in assay (mg/cm <sup>3</sup> )	% Enhancement by Sample	% Enhancement by Arginine Standard
B (First prep)	0.4	910	390
B (First prep)	0.2	900	390
B (First prep)	0.2	350	140
B (Second Prep)	0.2	910	140
B (Ashed)	Ash from 16 mg of B	0	140
B (Hydrolysed)	0.2	80	110
T	0.2	365	140

### 3.3.2 Electrophoresis Fractions From the Crude GTF Extracts

#### 3.3.2.1 Naming of Fractions

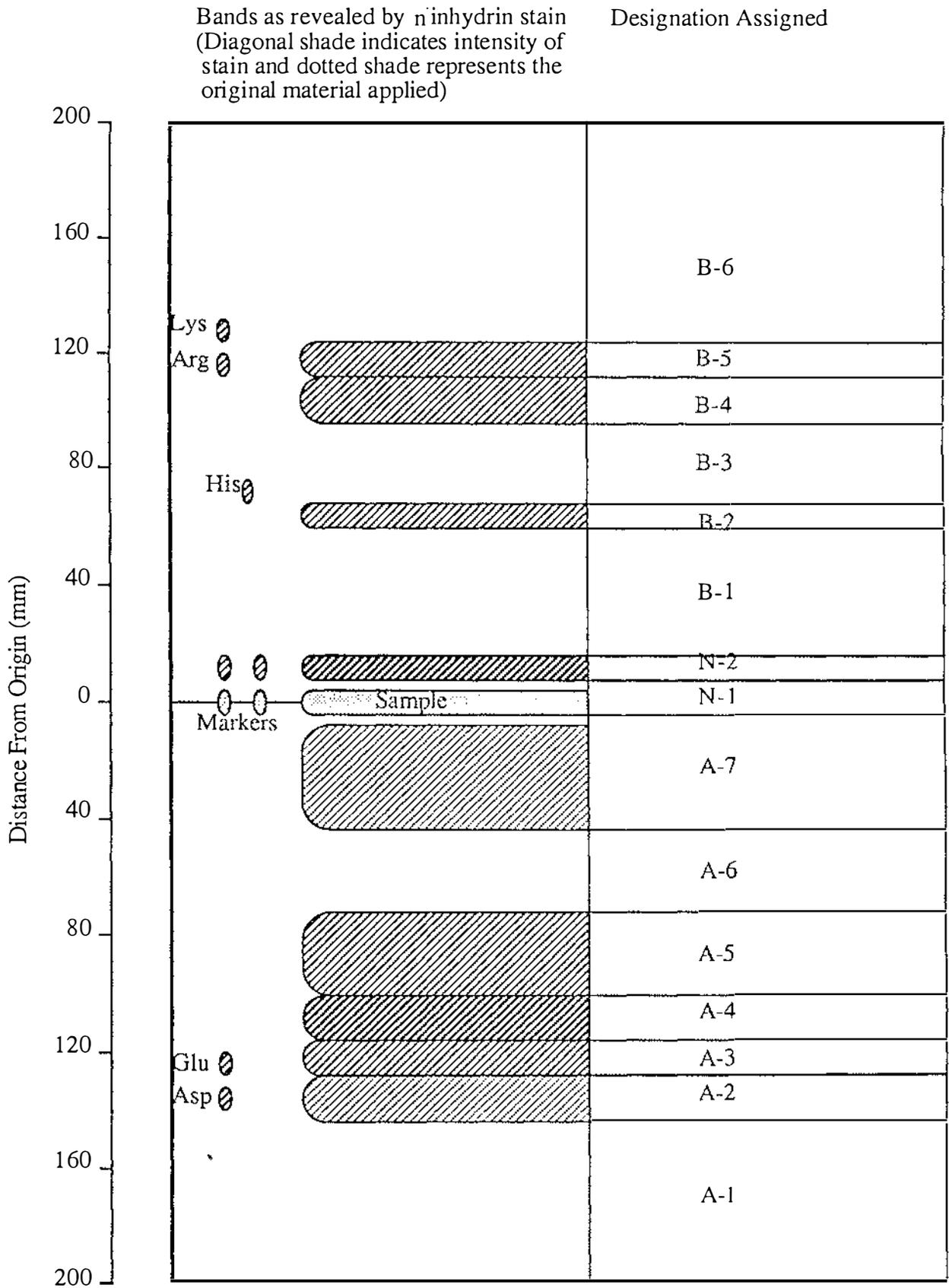
When the crude brewers yeast GTF extracts were subjected to electrophoresis, as described in section 3.2.2, 15 identifiable bands were located on the strips cut from the edge of the electrophoresed material. The seven bands that migrated toward the anode and which were hence anionic (acidic) were labeled A-1 to A-7 as shown in the Figure 3.1. The two fractions which barely moved from the origin were assigned as containing neutral material and were labeled N-1 and N-2 and the 6 bands that were found to migrate toward the cathode and hence contained cationic (basic) material were labeled B-1 to B-6.

When subjected to electrophoresis the crude GTF extract from torula yeast (T) showed many similarities to that from brewers yeast (B) but it was not exactly the same (Figure 3.2). However to make comparisons between the separations of the two extracts easier the bands on the separated torula yeast extract were allocated by comparison with the position of the bands seen in the separation of brewers yeast extract. The position of these bands being located using the markers and the ninhydrin reactive bands in the electrophoresed material.

#### 3.3.2.2 Elution of Fractions

The elutant of choice was NH<sub>4</sub>OH but as previous work (Chapter 2) had shown that this interfered dramatically with the results of yeast assays it was decided to investigate the effect of several other elutants. An electrophoresis of 200 mg of crude extract B was run and the dried paper sheet was divided up laterally into the fractions described previously. Then each of these pieces of paper was divided longitudinally into three equal portions. Each of these was eluted by one of three elutants, these being 0.1 M NH<sub>4</sub>OH,

Figure 3.1 Electrophoresis Bands of Crude GTF Extract B



1 % acetic acid and doubly distilled deionised water. The eluted material was then freeze dried and weighed. The results of using these three elutants on fractions separated from 66 mg crude GTF extract B are shown in Table 3.4.

The yield of solid material eluted from the bands did not vary greatly between the 2 crude GTF fractions and the overall percentage recovery of material applied was very similar. On the basis of these results it was decided to use water as the elutant for all fractions as water was shown to be as effective an elutant as anything else and it eliminated the possibility of the elutant interfering in the final result.

### 3.3.2.3 Activity By the Standard Yeast Assay

The activity of the various isolated bands is summarised in Table 3.5. It is seen that the most active fractions are contained in the acidic fractions. In general about 60% of the isolated material by weight was from the acidic bands , 10 % from the neutral bands and 30 % from the basic bands.

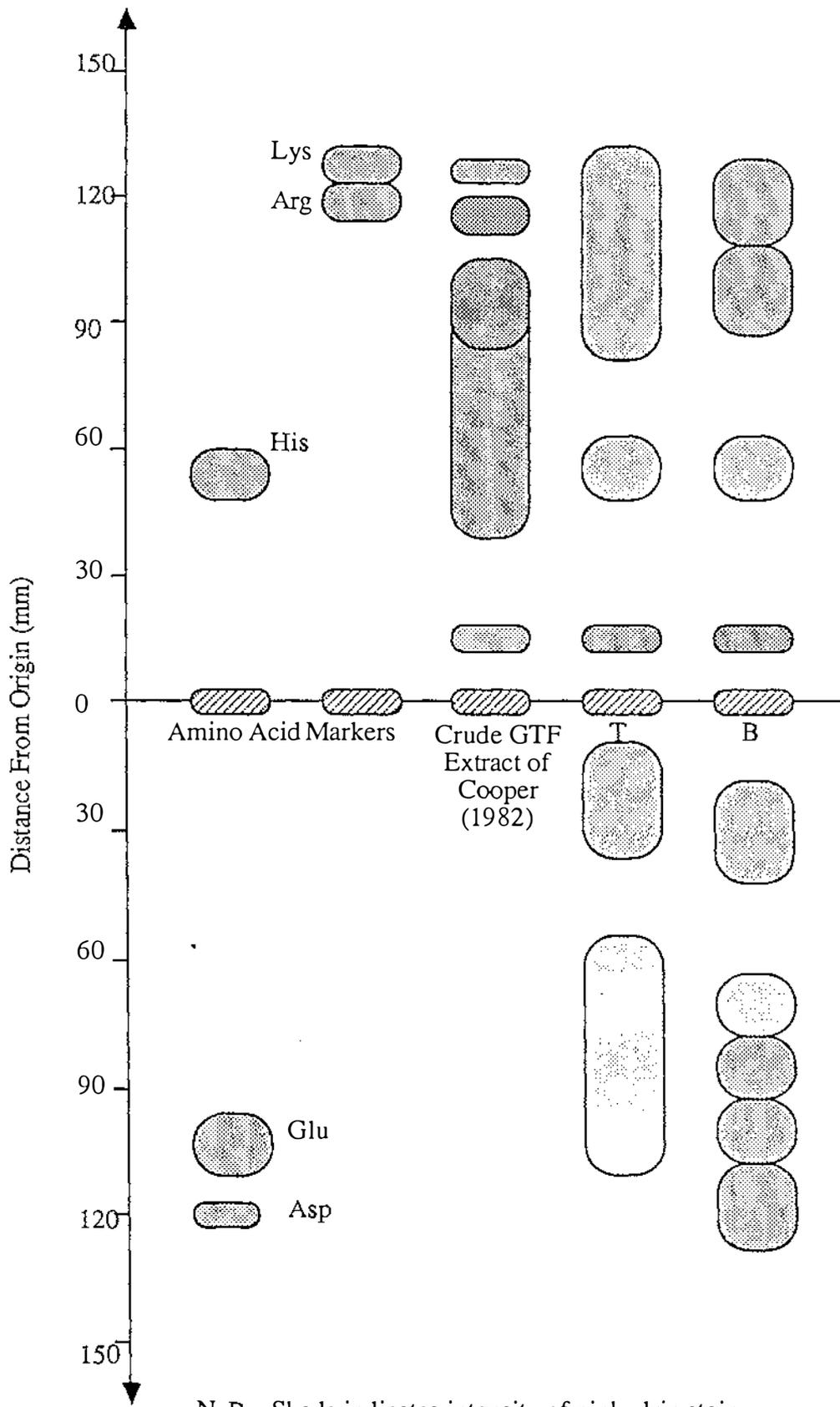
Table 3.5 Activity in the Yeast Assay of the Fractions Separated by Electrophoresis

Fraction	From Extract B	Arginine Standard	From Extract T	Arginine Standard
B-A-1	190	450	590	340
B-A-2	220	450	610	340
B-A-3	400	450	690	340
B-A-4	320	450	790	340
B-A-5	190	450	790	340
B-A-6	180	450	500	340
B-A-7	200	450	495	340
B-N-1	160	450	125	270
B-N-2	80	125	660	270
B-B-1	20	125	430	270
B-B-2	55	125	220	270
B-B-3	20	125	170	270
B-B-4	90	125	105	270
B-B-5	40	125	70	270
B-B-6	20	125	45	270

(all results are expressed as percentage enhancement and all fractions were assayed at a concentration in the assay of 0.2 mg/cm<sup>3</sup>)

### 3.3.2.4 Characteristics of Electrophoresis Fractions Amino Acid Analysis

The fractions showing activity were analysed for amino acid composition and the results are shown in Table 3.6. It can be seen from these results that the anionic fractions contained substantially more amino acid material than did the cationic fractions, particularly Glu, Gly and Cys which are thought by some workers (Toepfer *et al.* 1977) to be associated with GTF.



N.B. Shade indicates intensity of ninhydrin stain

Table 3.4 Comparison of Various Solvents for Eluting Electrophoresis Fractions, Isolated From a Crude GTF Extract of Brewers Yeast, From Paper

Fraction	Elution Solvent					
	0.1 M NH <sub>4</sub> OH		Water		1% Acetic Acid	
	Yield (mg)	Activity (%)	Yield (mg)	Activity (%)	Yield (mg)	Activity (%)
A - 1	4.0	390	3.6	270	4.4	360
A - 2	1.1	400	1.1	410	1.0	400
A - 3	2.4	460	2.2	370	2.7	365
A - 4	3.1	410	2.6	330	3.8	410
A - 5	2.9	430	2.4	410	2.8	460
A - 6	2.3	550	2.1	310	2.6	550
A - 7	2.4	590	1.3	350	2.0	540
N - 1	0.9	1030	1.1	370	1.9	535
N - 2	8.6	600	13.0	585	7.3	895
B - 1	1.5	870	2.0	500	1.6	330
B - 2	0.7	550	0.4	495	1.0	410
B - 3	1.9	610	0.5	330	1.5	360
B - 4	0.8	410	0.6	335	0.8	460
B - 5	1.0	550	0.2	250	1.0	580
B - 6	0.8	220	0.5	5	1.1	170
Total Recovered (mg)	34.4		33.6		35.5	
Percentage Recovered	52.2		50.9		53.8	

Notes :

1. All fractions were assayed at a concentration of 0.2 mg/ml where possible and activity is expressed as percentage enhancement determined by the standard yeast assay.
2. Activity values shown were determined in 4 separate assay runs but as the correlation between the Arginine internal standards in these assays was good (Between 160% and 190%) the results from the different assays are directly comparable.
3. A total of 66 mg of Crude GTF was used in each of the electrophoresis runs.

Table 3.6 Amino Acid Analysis of the Most Active Cationic and Anionic Electrophoresis Fractions from Crude GTF Fraction B

Fraction	Amino Acids ( $\mu$ moles / mg solid)						
	Asp	Arg	Cys	Glu	Gly	Lys	Ser
B-A-2	0.70	-	0.64	1.80	1.06	-	-
B-A-3	0.09	-	-	0.12	0.36	-	-
B-A-4	0.10	-	1.26	1.50	1.99	-	-
B-B-4	0.01	0.21	-	0.02	0.02	0.02	0.01
B-B-5	0.01	0.05	-	0.01	0.02	0.05	0.01
B-B-6	0.11	-	0.12	0.34	0.44	-	-

### Chromium Content of Electrophoresis Fractions

The chromium concentration in the electrophoresis fractions as determined by graphite furnace atomic absorption spectrometry is shown in Table 3.7. No fraction of B was found to have a higher chromium concentration than B itself and chromium concentration did not correlate with the activity in the yeast assay (Table 3.5).

Table 3.7 Chromium Content of Electrophoresis Fractions of Crude GTF B

Fraction	A-1	A-2	A-3	A-4	A-5	A-6	A-7	N-1	N-2	B-1	B-2	B-3	B-4	B-5	B-6
Cr content ( $\mu$ g/g)	17.4	32.8	17.0	18.4	13.0	12.0	10.7	6.8	8.5	1.4	4.8	2.2	2.3	2.8	2.5

### UV/Visible Absorbance Spectrum of B-A-3

The UV/Visible absorbance spectrum of B-A-3, the most active electrophoresis fraction, is shown in Figure 3.8. It can be seen that the absorbance spectrum of B-A-3 is very similar to that of B with peaks at 194 nm and 260 nm. It is possible that B-A-3 contains all the UV absorbing material seen in the crude extract B.

### 3.3.3 HPLC of Electrophoresis Fractions

The most active fractions from the electrophoresis were still not pure and were therefore subjected to HPLC for further purification. Most effort was devoted to separation of fraction B-A-3 which had shown by far the highest levels of activity in the yeast assay (Table 3.5).

#### 3.3.3.1 Isolation and Activity of HPLC Fractions

Investigative HPLC runs were done with low levels of several fractions showing activity in the yeast assay. The profile of fraction B-A-3 is shown in Figure 3.3a and profiles of B-A-4 (Figure 3.4) and B-B-4 (Figure 3.5) are also shown. As can be seen from these elution profiles the fraction B-A-3 was a fairly complex mixture of components but HPLC achieved good separation of these. The other fractions were less complex mixtures and

not surprisingly the fraction B-A-4 showed many similarities with that of B-A-3. A series of 4 preparative chromatographic runs, each with 5 mg of B-A-3, was carried out and the elution profile is shown in Figure 3.3b. The eluted material was collected in 10 fractions as indicated in Figure 3.3b and these fractions were pooled, freeze dried and then assayed. The characteristics of these fractions are shown in Table 3.8. From this data it can be seen that fraction B-A-3-3 was by far the most active fraction and this result is supported by a trend towards this in the activity of the fractions on either side of this fraction. The fraction B-A-3-3 corresponds with a large and clean peak of material which absorbed at 220 nm and it was this fraction which was selected for further analysis.

### **3.3.3.2 Characteristics of HPLC Fractions of B-A-3**

#### **Amino Acid Analysis**

Amino acid analysis was undertaken of all the fractions identified in the HPLC runs and the results are shown in Table 3.8. It can be seen from these results that the most active fractions contained very little amino acid material. The most active fraction, B-A-3-3, contained 5.5% glycine and 2% glutamic acid by weight. B-A-3-3 did however contain a very large peak of ammonia. To determine whether the amino acids that were present in B-A-3-3 were free or bound as a complex or a peptide a sample was applied to the amino acid analyser unhydrolysed. The results are shown in Figure 3.6. From this it can be seen that the amino acids do indeed appear to be bound. Despite the fact that the low levels of amino acid were unlikely to be responsible for the observed activity the effects of these were investigated. As most of the amino acid material present was glycine it was decided to assay a polyglycine peptide to see whether such a compound showed more activity than the inactive glycine (see Table 3.9).

#### **Mass Spectroscopy of Fraction B-A-3-3**

As fraction B-A-3-3 was so active and little light was shed on its composition by amino acid analysis it was decided to use mass spectrometric methods of identification. Since GTF material is thought to involve a peptide complex and to have a molecular mass of between 400 and 1000 (Mertz *et al.* 1974) the sample was first analysed using the Fast Atom Bombardment source for the mass spectrometer. However this method revealed no peaks at all indicating that the components of B-A-3-3 were probably smaller than previously thought. Using an Electron Impact source some molecular peaks were obtained but not until the temperature of the source had reached in excess of 350 °C. The spectrum is shown in Figure 3.7a and it contains a major peak at 135.0545 and when the overlying spectrum of the glycerol solvent is removed and the spectrum is compared with library spectra it was found to very closely match the spectrum of adenine (Figure 3.7b).

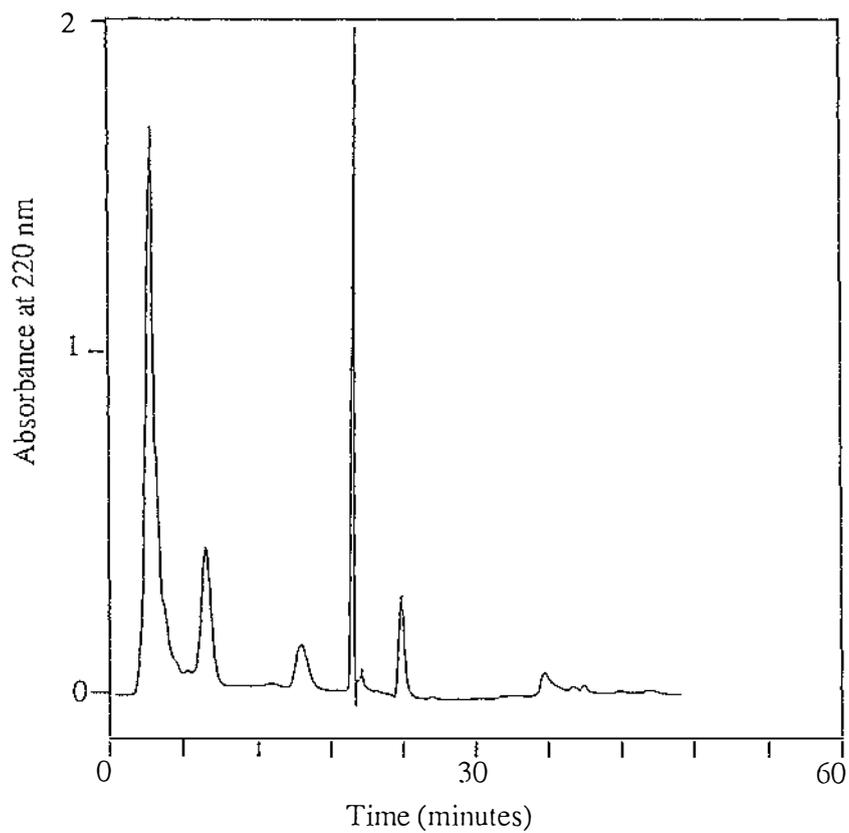
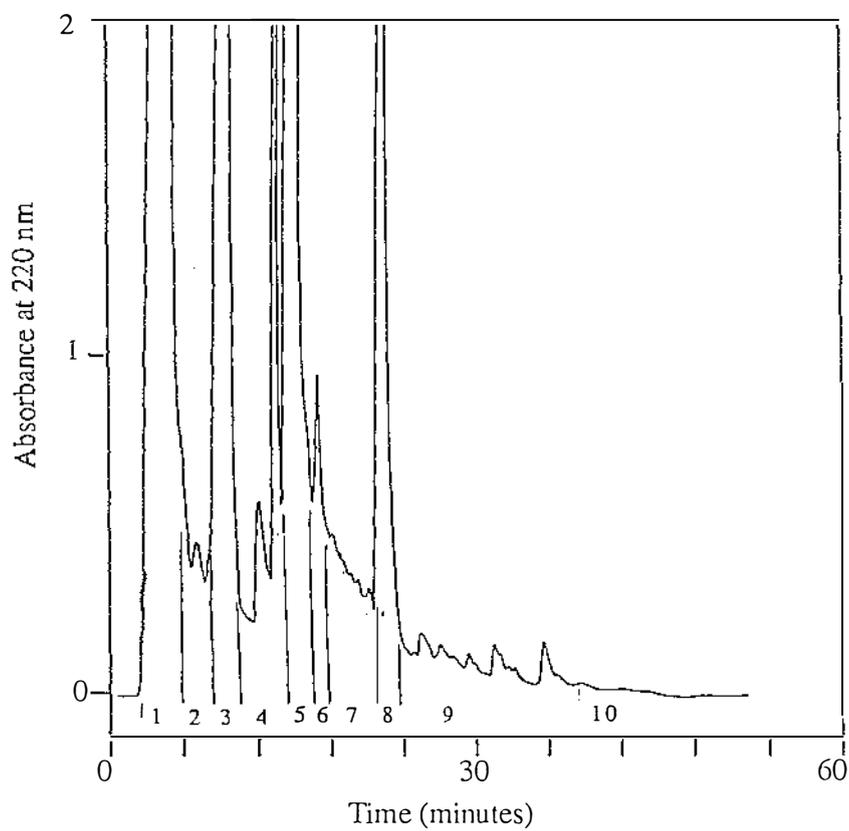
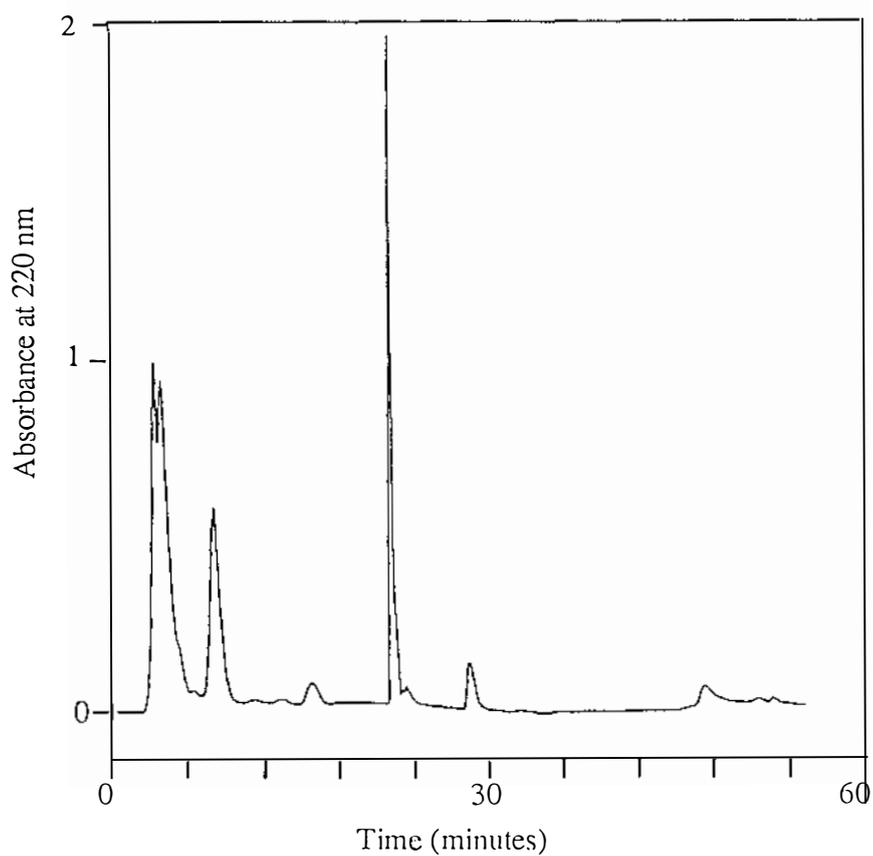
Figure 3.3a HPLC Profile of Investigative Run of B-A-3Figure 3.3b HPLC Profile of Preparative Run of B-A-3

Figure 3.4 HPLC Profile of Investigative Run of B-A-4



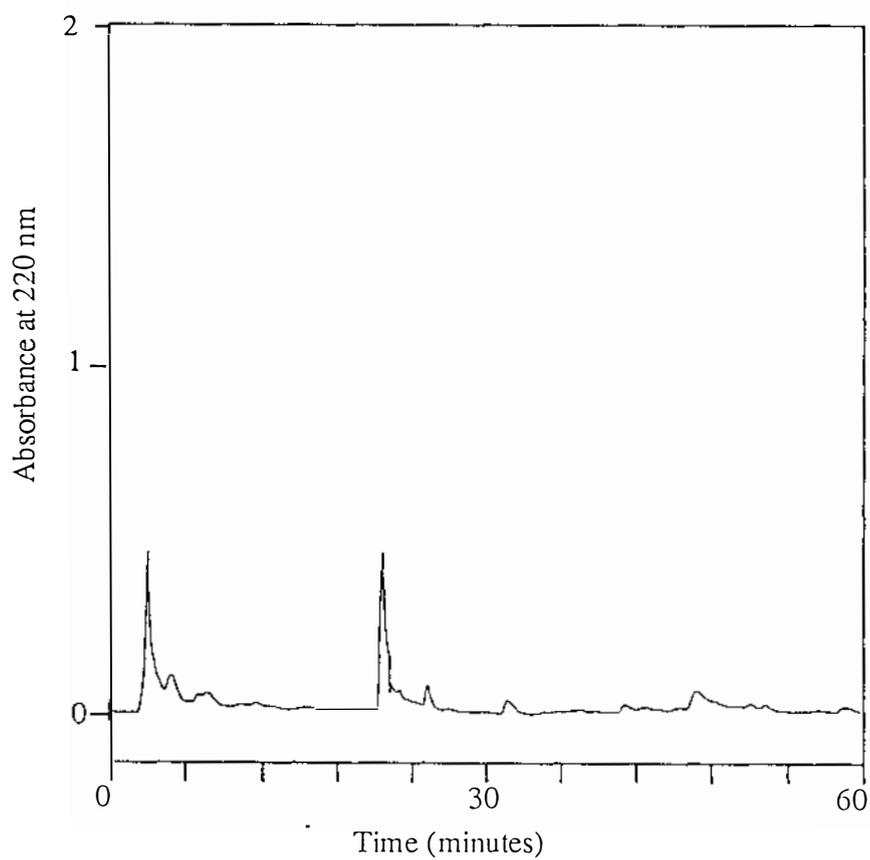
Sample: B-A-4

Time of run: 60 min

Amount Loaded: 400 $\mu$ g

Volume Loaded: 400 $\mu$ l

Figure 3.5 HPLC Profile of Investigative Run of B-B-4



Sample: B-B-4

Time of run: 60 min

Amount Loaded: 400 $\mu$ g

Volume Loaded: 400 $\mu$ l

Figure 3.6a Amino Acid Analysis of Unhydrolysd B-A-3-3

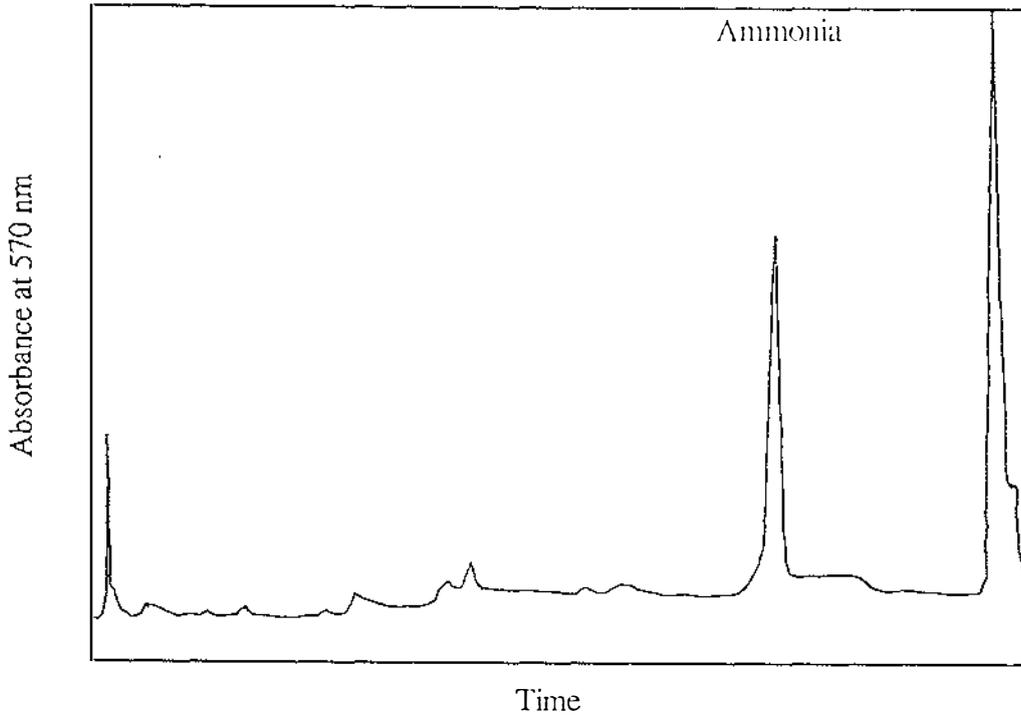


Figure 3.6b Amino Acid Analysis of Hydrolysd B-A-3-3

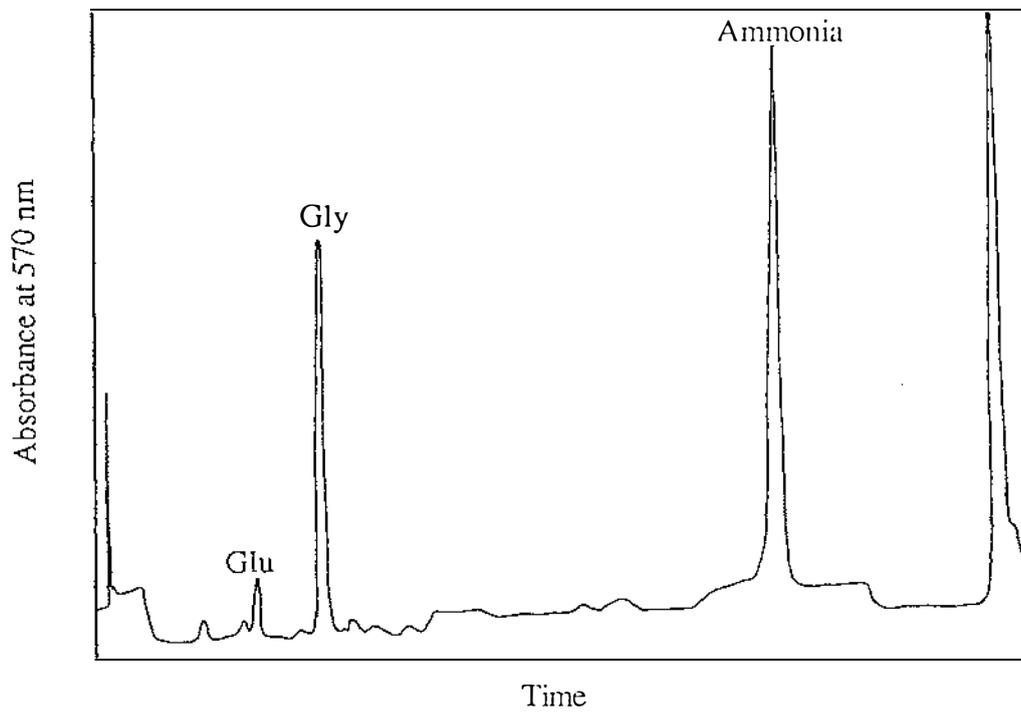


Table 3.8 Characteristics of HPLC Fractions of B-A-3

Fraction	Amount (mg)	Conc (mg/ml)	Activity	Amino Acid Analysis					
				n moles of A.A per 100µg				Area of peaks per 100µg	
				Asp	Ser	Glu	Gly	Unknown Amino Acid	NH3
B-A-3-1	6.1	0.2	280	32	45	158	73	100	2995
B-A-3-2	0.6	0.2	330	11	-	37	34	175	510
B-A-3-3	1.8	0.2	740	-	-	15	70	-	2000
B-A-3-4	0.3	0.2	380	7	-	26	30	38	450
B-A-3-5	2.2	0.2	295	16	-	58	61	-	1295
B-A-3-6	0.1	0.04	120						
B-A-3-7	0.1	0.04	50						
B-A-3-8	0.1	0.04	50						
B-A-3-9	1.5	0.2	200	-	-	-	-	-	-
B-A-3-10	0.1	0.04	185	5	9	10	9	-	-

Table 3.9 Activity of Various Compounds Implicated in the Makeup of B-A-3-3

Substance (all 0.2 mg/cm <sup>3</sup> )	% Enhancement	
	by sample	by Arginine Standard
Glycine	58	130
Glycyl-glycyl-glycine	20	130
Adenine	60	280
Adenosine	65	280

Figure 3.7a Mass Spectrum of B-A-3-3

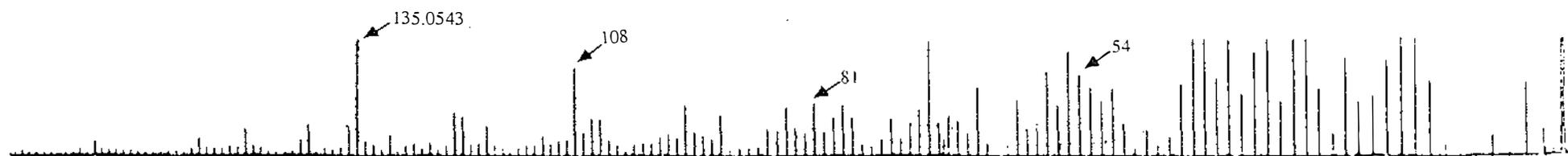


Figure 3.7b Library Mass Spectrum of Adenine

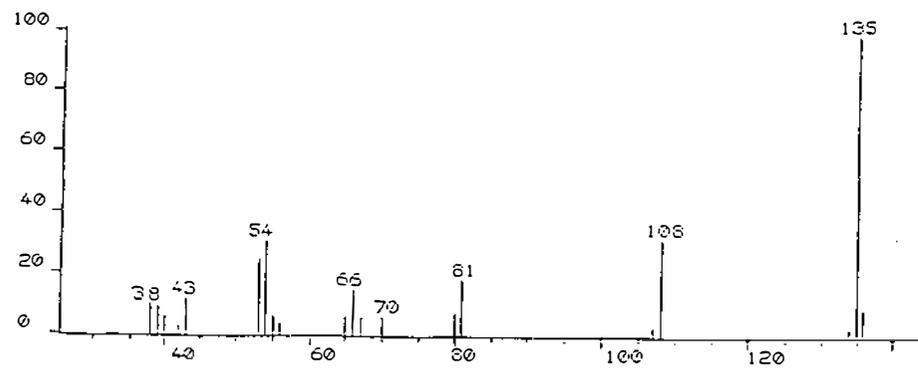
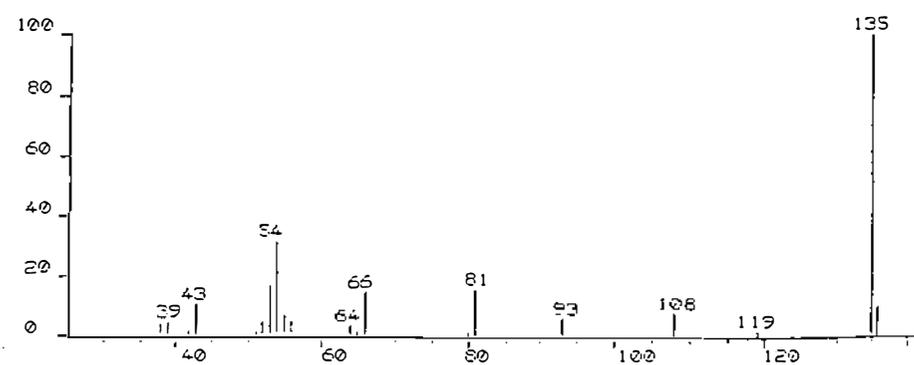


Figure 3.7c Library Mass Spectrum of 1H-Pyrazolo(4,3)Pyrimidin-7-Amine



### UV/Visible Absorbance Spectrum of B-A-3-3

The UV/visible absorbance spectrum of B-A-3-3 is shown in Figure 3.8. While the spectra of B and B-A-3 were fairly similar it can be seen that the spectrum of B-A-3-3 differs in that the 194 nm peak is of greatly reduced magnitude revealing instead a peak at 206 nm. This had previously contributed to the shoulder on the 194 nm peak seen on the spectra of B and B-A-3. The 260 nm peak seen in the spectra of B and B-A-3 remains. These spectra can be compared to those of the adenine based compounds, adenosine triphosphate (ATP) and adenosine (Figure 3.9), and to those of nicotinamide adenine dinucleotide (NAD<sup>+</sup>) and nicotinic acid (Figure 3.10).

### Chromium Concentration of B-A-3-3

The chromium concentration of B-A-3-3 was determined by graphite furnace atomic absorption spectroscopy as described in section 3.2.6. The concentration of chromium in B-A-3-3 was found to be 35.7 µg/g.

## 3.4 Discussion

### 3.4.1 Purification of Extracts With GTF Activity From Brewers Yeast

The crude GTF extracts were obtained from yeast using the the initial steps of the methods of Toepfer *et al.* (1977). These have been termed crude GTF extracts as further work has shown that are far from pure, contrary to the views of the originators of the scheme. However the extract does provide a good starting point for purification of GTF as it is known to have GTF activity in the most definitive *in vitro* assay, the low chromium rat epididymal adipocyte assay (Anderson *et al.* 1978). Different batches of the crude extract from brewers yeast (labeled B) showed consistently high levels of activity in the standard yeast assay system used in this work (see Table 3.3). This is consistent with the observations of other workers who also produced crude GTF extracts from brewers yeast which showed high levels of activity in the standard yeast assay (Haylock *et al.* 1983a , Holdsworth and Appleby 1984).

The crude GTF extract from the brewers yeast (B) showed a similar amino acid profile (Table 3.1) to that seen for the extracts of Toepfer *et al.* (1977), which were obtained using the same methods. The UV/visible absorbance spectrum of B (Figure 3.8) was also similar to that of Toepfer's extract, with B having peaks at 194 nm and 260 nm while Toepfer *et al.* described a 262 nm peak for their extract. B was also consistently very active in the yeast assay (Table 3.3) while Toepfer *et al.*'s fraction had been found to show consistent activity in the rat adipocyte assay. However ashing or hydrolysis of B

Fig 3.8 UV/Vis Spectra of Extracts

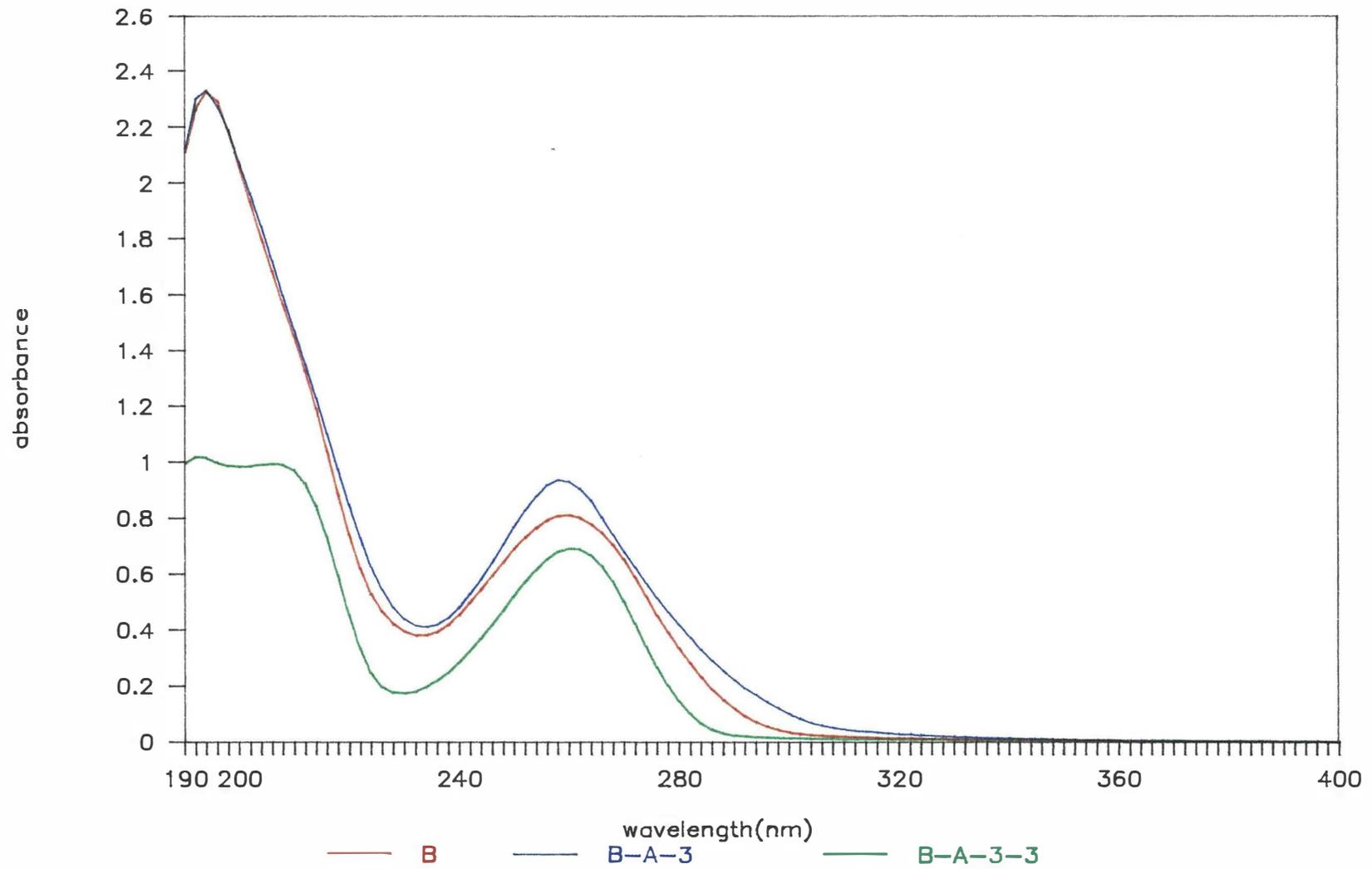


Fig 3.9a UV/Vis Spectrum of Adenosine

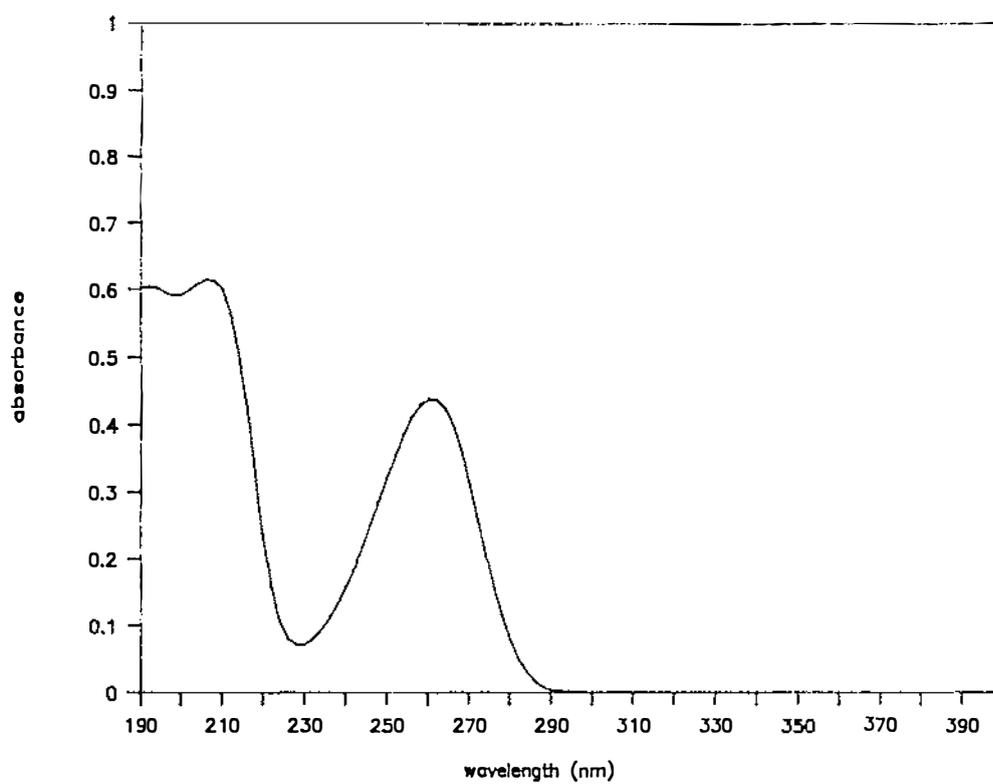


Fig 3.9b UV/Vis Spectrum of ATP

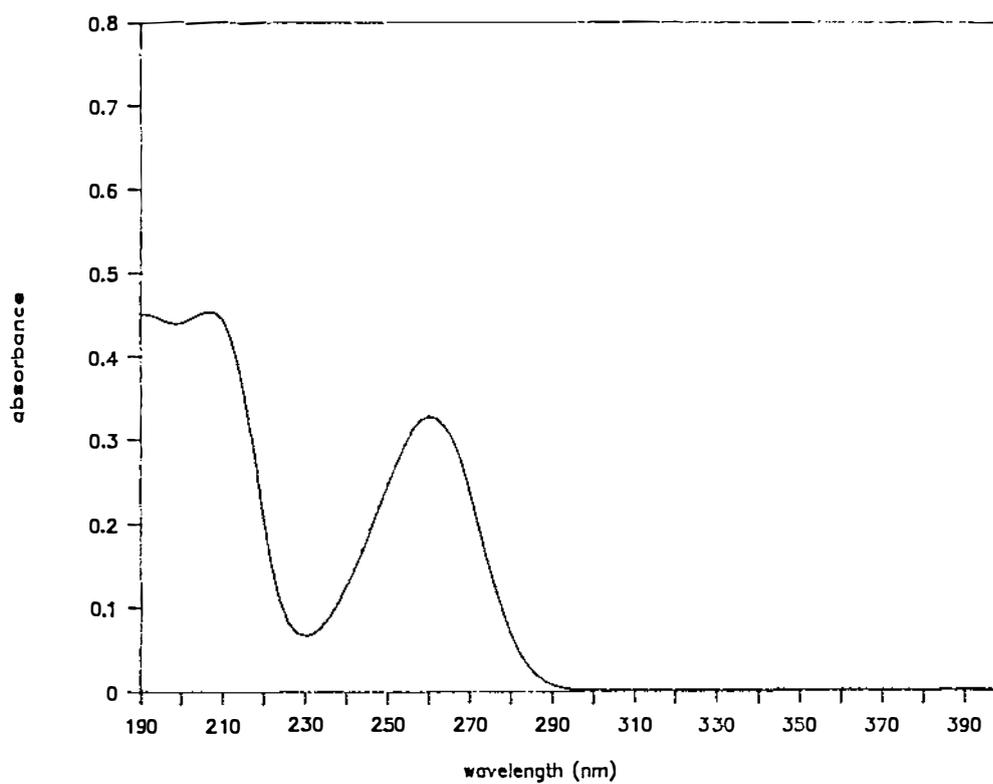


Fig 3.10a UV/Vis Spectrum of NAD

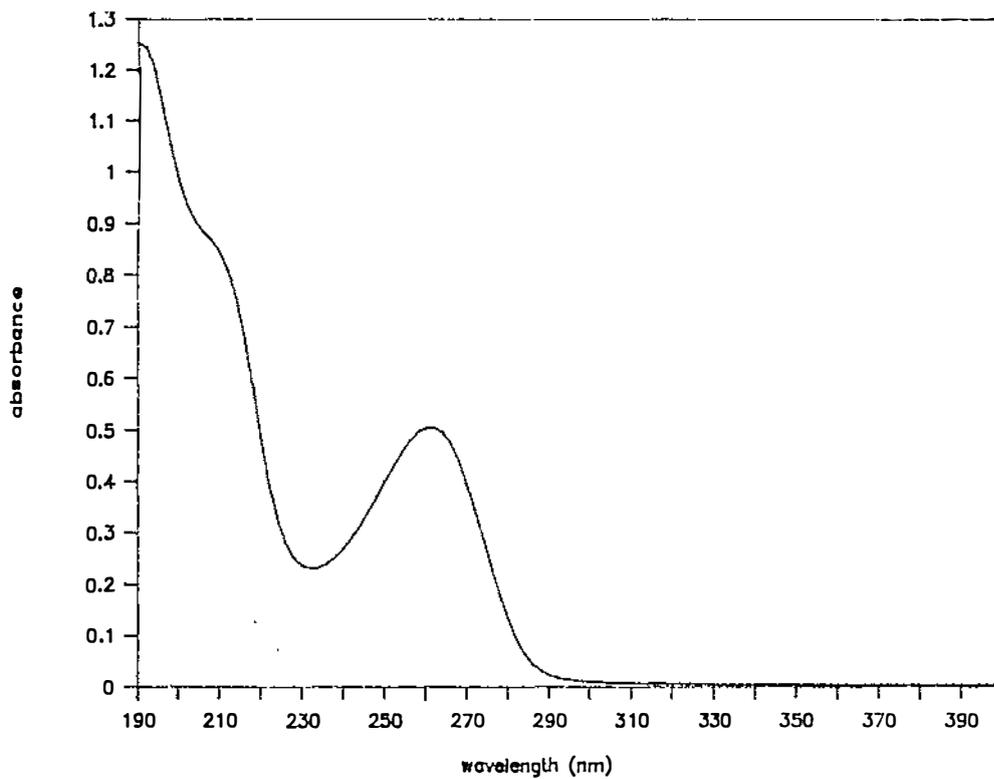
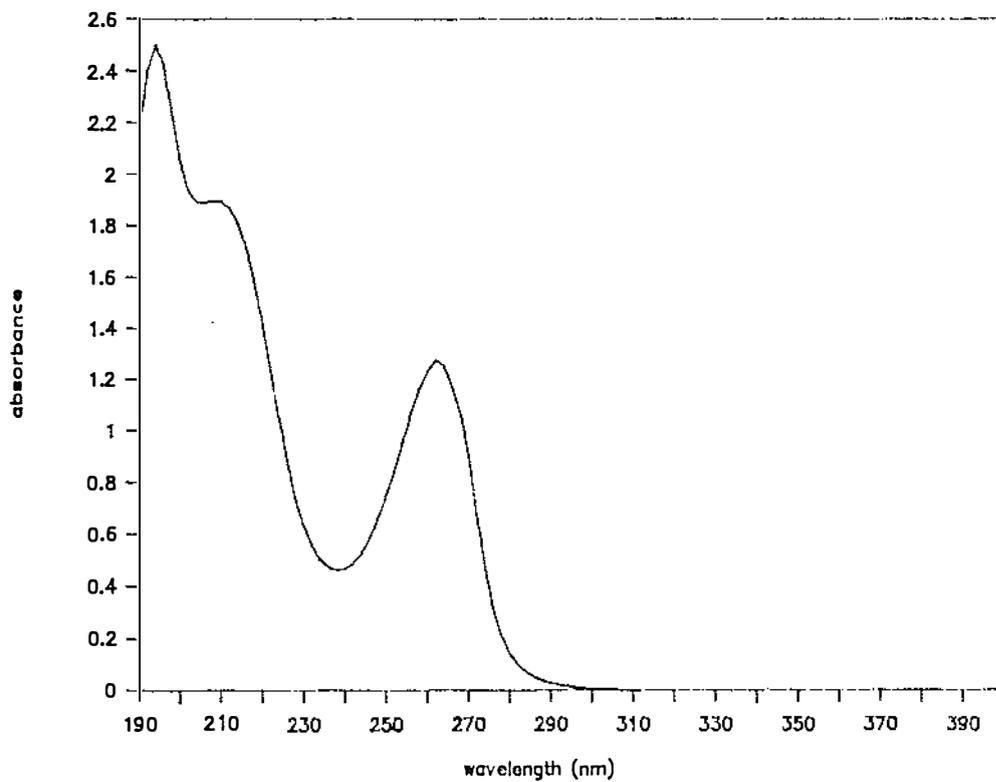


Fig 3.10b Nicotinic Acid Spectrum



largely destroyed its activity in the yeast assay (Table 3.3) whereas other workers have claimed that this does not affect GTF activity (Mertz 1969). For this crude GTF extract the results of chromium analysis (Table 3.2) are also consistent with the results of Toepfer *et al.* in that the extraction procedures have acted to concentrate the chromium content of the yeast.

High voltage paper electrophoresis of the crude GTF extract B at pH 6.5 enabled ninhydrin sensitive components to be separated on the basis of charge. This is important as previous workers have suggested that GTF is a cationic substance (Mertz *et al.* 1974, Toepfer *et al.* 1977, Mirsky *et al.* 1980, Haylock *et al.* 1983a). In this separation 6 cationic fractions, 2 neutral fractions and 7 anionic fractions were revealed as shown in Figure 3.1.

Investigations into which elutant was best to elute fractions from the paper are summarised on Table 3.4. It can be seen that there was consistently higher activity in the fractions eluted with  $\text{NH}_4\text{OH}$ . While this may be due to the fact that this solvent is eluting more active substances from the paper this is unlikely as similar amounts of material were eluted by all elutants. Also the extra activity in the ammonia elutions was observed in most samples and not just in isolated cases. Combined with earlier observations; i) of the effect of ammonia elutions on activity of samples in the yeast assay (Cooper *et al.* 1985), ii) that pretreatment of GTF samples with ammonia gave higher activity (Haylock *et al.* 1983a) and iii) the direct effects of  $\text{NH}_4\text{OH}$  on this assay system (Chapter 2), it seems that this elutant should be avoided. Hence  $\text{H}_2\text{O}$  was chosen as the elutant as it was least likely to give spurious results and yet still gave good yields of material.

Because variability of the yeast assay prevented direct quantitative comparison of the results from different assays the six most active fractions from B were run in a single assay to determine exactly which of the fractions of B was most active, these results are shown in Table 3.10. It can be seen that there was consistently more activity in the anionic fractions, with fraction B-A-3 being the most active of all. This is at odds with the stated properties of GTF as previously it has been assumed that GTF material was cationic (Toepfer *et al.* 1977) although other workers have previously isolated active anionic fractions (Davies *et al.* 1985b). While none of these fractions exhibited as much activity as the crude extract B the rationale of the purification scheme was to follow the fractions with the most activity in an effort to isolate GTF. In light of this decision, fraction B-A-3 was chosen for further investigation despite the fact that it was anionic. The UV/visible absorbance spectrum of B-A-3 (Figure 3.8) showed many similarities to

that of B although the amino acid composition had changed markedly (Table 3.6) indicating that some separation, and hence purification, had been achieved.

Table 3.10 Intra-assay Comparison of Active Electrophoresis Fractions From B

Substance	Concentration(mg/cm <sup>3</sup> )	% Enhancement
B-A-2	0.2	40
B-A-3	0.2	230
B-A-4	0.2	120
B-B-2	0.2	50
B-B-4	0.2	95
B-B-5	0.2	15
Arginine	0.2	150
Crude B	0.2	570

Fractionation of B-A-3 using HPLC resulted in 10 sub fractions labeled B-A-3-1 to B-A-3-10 (Figure 3.3b). However despite having achieved significant purification, even the most active sub fractions still did not have as much activity as the crude yeast GTF extract B (Table 3.7). If indeed GTF does exist in the yeast then it would be expected that separation of the yeast into its components would increase the specific activity of the components carrying the GTF. In fact what we do see is more a dispersion of activity as measured by the yeast assay.

This dispersion could be due to a number of factors;

- (a) it is possible that an inhibitor restricting full expression of the activity of the GTF co-purifies with the fraction containing GTF
- (b) it may be that while the standard yeast assay is measuring GTF activity it is also being stimulated by other substances thus giving false indications of GTF activity. It has been demonstrated in section 2.3 that both arginine and ammonia gave indications of activity in the yeast assay although it is very unlikely that they represent GTF material. These observations are consistent with work by Davies *et al.* (1985a) which showed that fractions that were active in the low chromium rat epididymal adipocyte assay were always active in the yeast assay although the reverse was not always true. This can be taken to indicate the presence in the extracts of factors causing spurious activity in the yeast assay. If this is the case then the less pure fractions will have a background activity in the yeast assay which could be obscuring the fraction actually containing GTF.
- (c) It may be in fact that the effects attributed to GTF are in fact really due to synergistic or cumulative interactions of a number of diverse components of the yeast extracts and

that separation is merely dispersing these. There are parallels in other systems for this, such as in the case of the hypoglycemic effects present in *Panax ginseng* (Ng *et al.* 1985). In that example 8 different hypoglycemic agents were isolated from the plant including adenosine, a low molecular weight peptide, a carboxylic acid and 5 different glycans.

It cannot be denied however that at the highest level of purification obtained in the procedures described here fraction B-A-3-3 did possess considerable activity in the yeast assay (Table 3.8). This fraction appears relatively pure as it corresponds to a relatively clean peak on the HPLC trace (Figure 3.3a). When analysed it was found to contain very little amino acid, about 5.5% w/w glycine and about 2 % w/w glutamic acid. The activity does not seem due to the glycine nor to any peptide of glycine (Table 3.9). While earlier work by Cooper *et al.* (1984b) claimed that glutamic acid had no activity in the standard yeast assay at a concentration of 0.1 mg/cm<sup>3</sup>, a study on the effects of this amino acid on the same assay system (Jackson 1985) showed that glutamic acid did show significant activity in the yeast assay. However this activity was not observed at concentrations as low as 0.004 mg/cm<sup>3</sup>, which is the concentration of glutamic acid in an assay of B-A-3-3 at a total concentration of 0.2 mg/cm<sup>3</sup>. Therefore the glutamic acid content of B-A-3-3 is unlikely to be the cause of the observed activity. Further when a sample of B-A-3-3 was run through the amino acid analyser unhydrolysed no significant peaks were observed (Figure 3.6) so it is assumed that the small amount of amino acids are in fact part of some other structure.

The component of B-A-3-3 that was able to be identified was adenine. The adenine was probably associated with another unidentified compound as adenine was not revealed in the mass spectrum until the source temperature was over 350 °C. Adenine alone would normally volatilise at a much lower temperature indicating that in this sample it is released as a pyrolysis product of a non volatile substance. The adenine could be released as a breakdown product of adenosine which would be expected to be present in yeast extracts in reasonably large amounts. The UV/visible absorbance spectrum of B-A-3-3 shows peaks at 260 nm, 206 nm and 194 nm (Figure 3.8). While the 260 nm peak is consistent with a range of compounds containing the adenine or nicotinamide moieties, the nature of the peaks at 206 nm and 194 nm is more consistent with it containing adenine compounds (see Figures 3.9 and 3.10), further confirming the mass spectroscopy data. However the similar spectra obtained for various adenine containing compounds makes it difficult to conclude from these spectra what form the adenine moiety is in. It is unlikely that either adenine or adenosine were directly responsible for the activity of B-A-3-3 as neither alone showed significant activity in the yeast assay (see table 3.9).

On the basis of the results presented here the postulate that the material present in B represents a chromium complex (Toepfer *et al.* 1977) cannot be supported. While B does contain 44  $\mu\text{g/g}$  chromium this is an impure fraction and so further purification is required before the chromium can be said to be associated with any particular complex. However, it is very interesting to note that none of the electrophoresis fractions of B show any spectacular accumulations of chromium (Table 3.7) as would be expected if the postulated chromium complex form of GTF was being purified and in fact the crude GTF extract B had a higher chromium concentration than any of the electrophoresis fractions. For example while B-A-3-3, most active purified fraction obtained, contained a significant chromium concentration (35.7  $\mu\text{g/g}$ , section 3.3.3.2) this was less than that found in the crude extract B (44.0  $\mu\text{g/g}$ , Table 3.2). Because of the much smaller total weight of the B-A-3-3 material, this amount represented only a small fraction of the total original chromium found in B. It would be expected that if the substance responsible for GTF activity that was being purified was a chromium complex then there would have been a concomitant increase in chromium concentration with the purification of the fraction. Further, the final chromium concentration of B-A-3-3 is not sufficient to support the hypothesis that this reasonably pure fraction is a chromium complex. For such a low molecular weight chromium complex chromium would be expected to comprise 1-10% of the total weight of the complex and so a chromium concentration several orders of magnitude higher would be expected.

The most active fraction on the basis of the yeast assay is neither a chromium complex nor a peptide and is anionic at pH 6.5. Although complete identification was not made, B-A-3-3 contained an adenine derivative and comparison of this finding with those of other non chromium containing active fractions from brewers yeast (tyramine (Haylock *et al.* 1983b), ornithine and  $\epsilon$ -glutaryl-lysine (Davies *et al.* (1985a & b) and now adenine-X) reveals no obvious trend in the nature of the substances comprising these fractions. Since none of these compounds completely accounts for the activity observed in the assay system it is difficult to discount the possibility that a small amount of some as yet unrecognised material is responsible for the activity in each case.

### **3.4.2 Comparison of Extracts With GTF Activity From Brewers Yeast with Similar Extracts from Torula Yeast**

A crude fraction was isolated from torula yeast using the same methods used to obtain B and this is the first reported attempt to investigate torula yeast for fractions showing GTF activity. The results show that the initial crude GTF extracts from the torula yeast (T)

exhibited a very high degree of activity in the yeast assay (Table 3.3) and was at least as active as the brewers yeast fraction, the activity of which is thought to be due to the presence of GTF activity. This is curious as torula yeast is the sole protein source in the low chromium diet used to induce the impaired glucose tolerance state in the animals which are used in GTF assays (Anderson *et al* 1978). Feeding brewers yeast to these animals is claimed to repair this state (Ranhotra 1986) and for this reason it has been assumed that the torula yeast is deficient in GTF. However the results presented here dispute this assumption and either torula yeast does contain GTF, which would seem unlikely on the basis of the dietary evidence, or the activity seen in the brewers yeast extract which is assumed to be due to GTF is in fact spurious.

A comparison of the composition of the two extracts shows many similarities between B & T. Trace metal analysis (Table 3.2) reveals that T has lower concentrations of chromium and tin and a higher concentration of aluminium than B while concentrations of iron, manganese, nickel, zinc and copper are similar. Amino acid analysis of extract T (Table 3.1) showed some similarities to the amino acid composition of B although T was lower in the acidic amino acids aspartic acid and glutamic acid.

The electrophoresis pattern of T is very similar to B (Figure 3.2) although there is less anionic material which is consistent with the lack of acidic amino acids mentioned above. Table 3.5 shows the activity of the electrophoresis fractions and it can be seen that for both crude extracts there is a spread in the activity with no one fraction having a spectacular level of activity. The most active fractions were in the acidic material in both cases and there was at least as much activity in the fractions from T as there was in those from B. If only B contained true GTF activity it would be expected that at least one fraction of B would show significantly higher levels of activity than the corresponding fraction from T, however this is not seen. The similarities in activity and composition between the fractions from brewers yeast and torula yeast cast serious doubts on the conclusion that such extracts from brewers yeast represent GTF.

The conclusions drawn on the basis of the yeast assay are confused by the observations of Jackson (1985) made in a parallel investigation of the non chromium containing fraction from the brewers yeast extract of Haylock *et al.* (1983a). This fraction was active in the yeast assay and had been through several purification steps. However, work by Jackson (1985) showed that this fraction consisted mainly of a large number of small peptides, many of which were active in the yeast assay, and control assays showed that many individual amino acids also showed significant activity. The activity of the individual amino acids was found to closely match the order of utilisation as nitrogen

sources which raises the possibility that the activity seen in the yeast assay is at least in part due to an increase in cell numbers. In view of this possibility , a reassessment of the yeast assay is necessary and this forms the basis of the next chapter.

# CHAPTER 4

## A MODIFIED YEAST ASSAY

### 4.1 Introduction

The yeast assay for GTF assumes that the observed stimulation of CO<sub>2</sub> evolution, as compared to a blank, in yeast grown and harvested in a chromium free medium is entirely due to the presence of GTF (Haylock 1982). Since it was assumed at that time that chromium was an integral component of GTF it was believed that growing the yeast in a chromium free medium would make the yeast GTF deficient and hence the system would then react to the introduction of GTF in a sample with an enhanced stimulation of CO<sub>2</sub> evolution.

As discussed in Chapter 3 a large number of fractions exhibited activity in the yeast assay and with the possibility that CO<sub>2</sub> evolved was due to cell proliferation rather than GTF activity it was difficult to differentiate which if any of these were due to GTF activity. This highlighted the need for an assay that would more specifically identify GTF activity. The most obvious solution is to use the more definitive low chromium rat epididymal adipocyte assay but this is a very difficult assay to operate and the number of fractions that can be assayed is restricted. If the specificity problems could be overcome the yeast assay would be preferable as it offers the cheapest and easiest assay method for GTF and is most suitable for assaying large numbers of samples. However for the assay to be useful a method of determining the difference between true GTF activity and that of the possibility that CO<sub>2</sub> evolution is due to cell proliferation is required.

If it is assumed that GTF potentiates the metabolism of the yeast cells (Burkeholder and Mertz 1967) and that CO<sub>2</sub> evolved is not merely due to increases in cell numbers, then it is possible to separate a true GTF response from the effects due to cell proliferation during the course of the assay. While there may always be some cell proliferation, the GTF component of the activity could be recognised as the potentiation of the evolution of CO<sub>2</sub> from the yeast cells over and above that due to yeast cell proliferation.

When assaying a substance for GTF activity all assay flasks, including the reference flask, begin with the same number of yeast cells, and it is assumed that over the course of the assay the number of cells in each flask remains constant or will change at a constant rate. If this is so then of course any increase in CO<sub>2</sub> evolution is due to stimulation of the

yeast metabolism and not due to an increase in the number of metabolising cells. To determine whether this was the case it was decided to follow the proliferation of cells over the period of the yeast assay. If there are differences in the rate of cell proliferation induced by different substrates, it would be necessary to normalise the CO<sub>2</sub> production in terms of the cell numbers in order to assess whether or not there had in fact been any stimulation of the cellular metabolism.

An estimation of the stimulation of cellular metabolism can be gained by measuring the CO<sub>2</sub> evolution and comparing it to the number of cells in a flask at a given time during the assay. If observed CO<sub>2</sub> evolution is in fact only due to cell proliferation a graph of the rate of CO<sub>2</sub> evolution per cell will have the same slope for both blanks and sample. If there is an upward divergence in the slope of the line representing the sample then this indicates a potentiation of the cell metabolism. Such a procedure is obviously not suited to assaying a large number of samples and so is not suitable as a routine assay procedure. It does however offer a possible method for determining whether any activity detected in the yeast assay is in fact due to stimulation of the yeast cell metabolism which is a minimum prerequisite for this activity to be attributed to GTF.

## **4.2 Materials and Methods**

### **4.2.1 Yeast Cell Growth and Harvesting**

*Saccharomyces ellipsoides* was grown in a chromium free medium and harvested as described in Section 2.2.1. The harvested yeast was then diluted in distilled reverse osmosis water to give a final concentration of  $1.5 \times 10^8$  cells per cm<sup>3</sup>.

### **4.2.2 Modified Assay Protocol**

The flasks were prepared as in section 2.2.2 except that instead of investigating a range of samples and a blank only one sample per assay was investigated. A paired series of flasks were prepared. One series contained a series of identical blanks and the other contained an identical series which contained a sample. As well as these flasks two flasks containing arginine as an internal standard were also run. All flasks were incubated at 30 °C as previously and readings of all manometers were taken at 20 minute intervals as described previously but at set time intervals after commencing the incubation, one each of the series of blanks and samples was taken from the water bath after a manometer reading and the flasks were immediately immersed in ice to stop the metabolism of the yeast cells. The contents of the flask were transferred to a glass vial for storage at 4 °C so that cell concentration could be determined at a later time.

### 4.2.3 Yeast Cell Concentration Determination

The concentration of yeast cells in the various flasks was determined by counting the number of yeast cells in a given volume of solution. Cell solutions were diluted appropriately and this was mixed with a few drops of 0.1% methylene blue which stained the dead cells. Cells were counted manually in a improved Neubauer (0.100 mm<sup>2</sup> x 0.0025 mm<sup>2</sup>) counting chamber using a Zeiss optical microscope at 40 x magnification . A count was made of both dead cells and live cells.

### 4.2.4 Calculations for the Modified Yeast Assay

In the modified yeast assay the assay was run as described above. The manometer readings for both series of flasks were converted into  $\mu$ moles of CO<sub>2</sub> evolved using the formula in section 2.2.3. The amount of CO<sub>2</sub> that had been evolved according to the reading taken immediately prior to the removal of the corresponding flask from the assay was divided by the number of cells that were calculated to be in that flask at that time. This gave a value for CO<sub>2</sub> evolved per cell and when this data was then plotted against the time at which the flask was removed from the water bath , a plot of CO<sub>2</sub> evolved per cell versus time resulted. The line of best fit was calculated and plotted using the linear regression function of the Cricket Graph package on the Apple MacIntosh computer.

The activity of a sample was measured by the percentage by which the sample was able to enhance the cellular rate of CO<sub>2</sub> evolution as compared with that of the blank. The equation for this was:

% Enhancement of the rate of cellular CO<sub>2</sub> evolution =

$$\frac{\text{CO}_2 \text{ Evolved / cell /min(sample)} - \text{CO}_2 \text{ evolution/cell/min (blank)}}{\text{CO}_2 \text{ evolution/cell(blank)}} \times \frac{100}{1}$$

### 4.2.5 Preparation of the Chromium Complex

A chromium complex was prepared as described by Cooper (1982) . To 10.6 g of chromium sulphate (BDH) was added 8.0 g of nicotinic acid (Sigma) and this was refluxed for 3 hours. Then at 1/2 hourly intervals were added 4.8 g glycine (US Biochemicals), 6.0 g glutamic acid (BDH) and 5.0 g cysteine (BDH). Following this the pH of the solution was adjusted to pH 4.0 with NaOH and this solution was then refluxed overnight. On cooling a blue green sediment formed and this was filtered off leaving a deep red solution which was left to stand. Purple material precipitated and was collected.

## **4.3 Results**

### **4.3.1 Characteristics of the Chromium Complex**

The chromium complex material was purple and was highly water soluble. When assayed in the yeast assay the complex gave an enhancement of 230% at a concentration of 0.2 mg/ cm<sup>3</sup> in an assay where the arginine standard returned an enhancement of 460%. Amino acid analysis of the unhydrolysed complex revealed that there was negligible free amino acid in the complex to explain the activity observed. The amino acid analysis of the complex revealed that it contained glycine and glutamic acid in a molar ratio of 1:1 indicating a Cr:Gly:Glu complex with the possible addition of nicotinic acid.

### **4.3.2 Measurement of Differences in the Rate of Cell Proliferation**

The results of the investigation of the proliferation of cells in the yeast assay system caused by crude GTF B, fraction B-A-3, arginine and the chromium complex are shown in Figures 4.1 to 4.4.

### **4.3.3 Investigation of Parameters of the Modified Yeast Assay for GTF**

Most of the parameters of the assay have previously been established (Haylock 1981). It was thought important however to investigate the period over which the plots of cellular CO<sub>2</sub> evolution versus time remained linear so that an appropriate period for this assay could be determined. The results of an initial investigative run was run over a period of 500 minutes duration are shown in Figure 4.5. From these results it was decided the assay period would be 0-360 minutes as this represented the straightest period of the graph.

### **4.3.4 Assay of Samples by the Modified Yeast Assay**

Substances assayed by this method included the crude yeast GTF extract B, the semi purified extract B-A-3, arginine and the chromium complex with glycine, glutamic acid,

Figure 4.1 Cell Proliferation Due to Crude GTF B

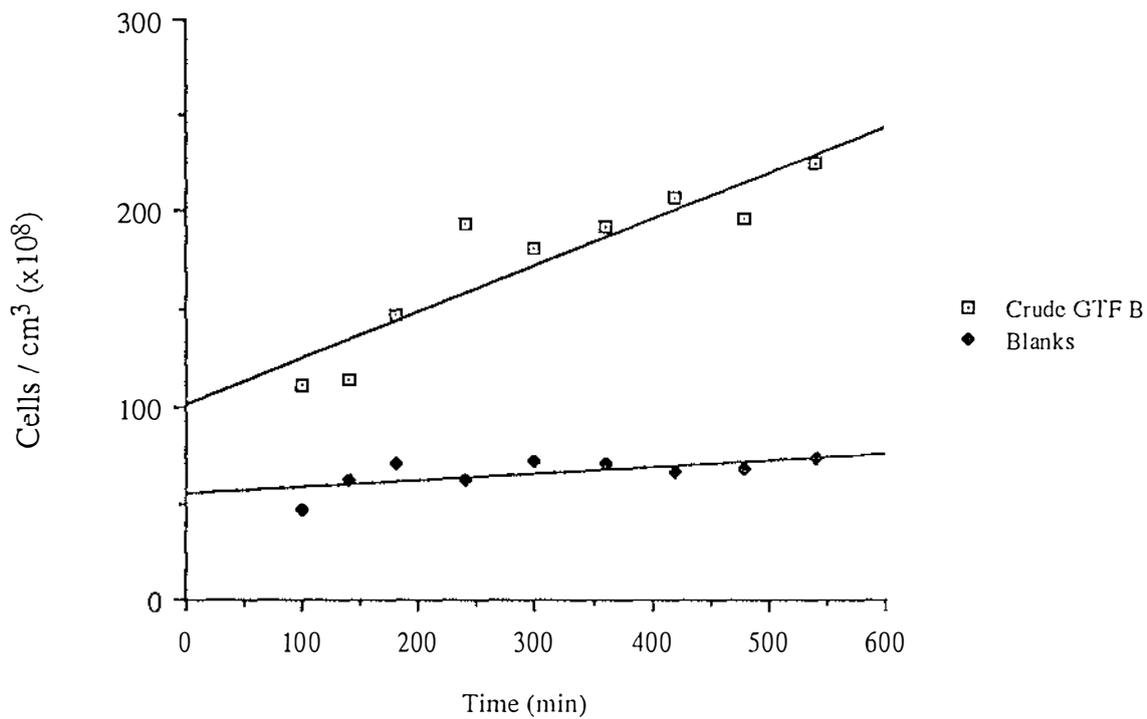


Figure 4.2 Cell Proliferation Caused By Arginine

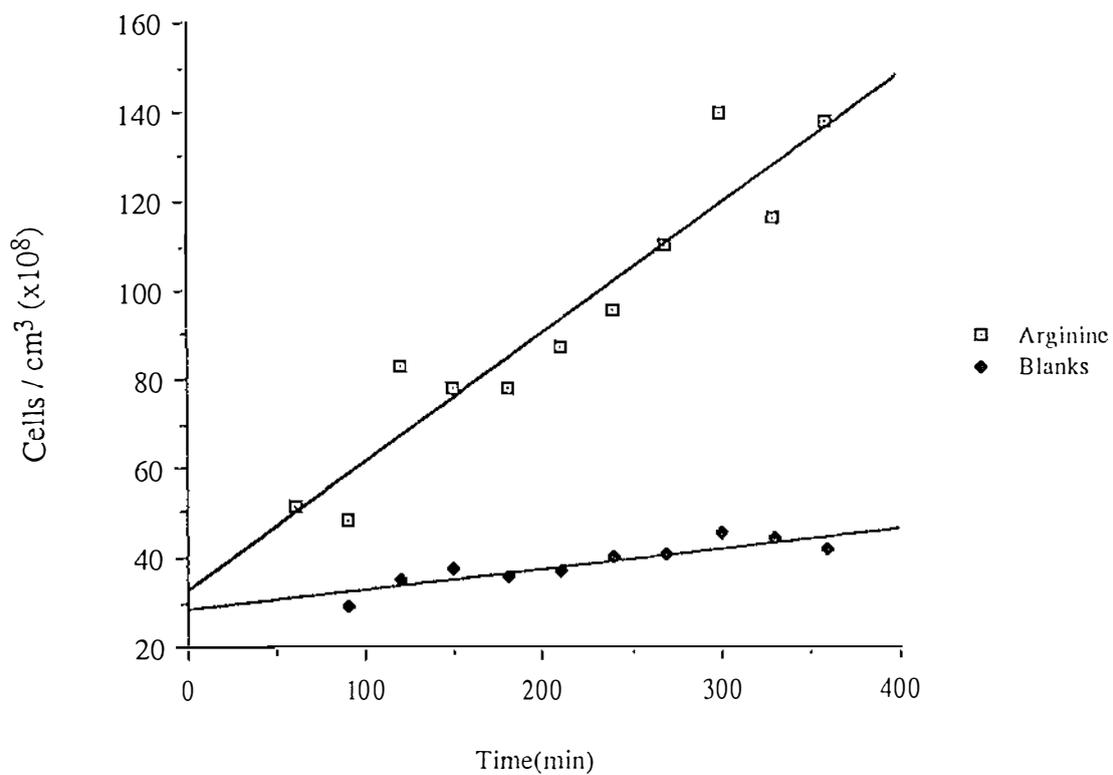


Figure 4.3 Cell Proliferation Due to B-A-3

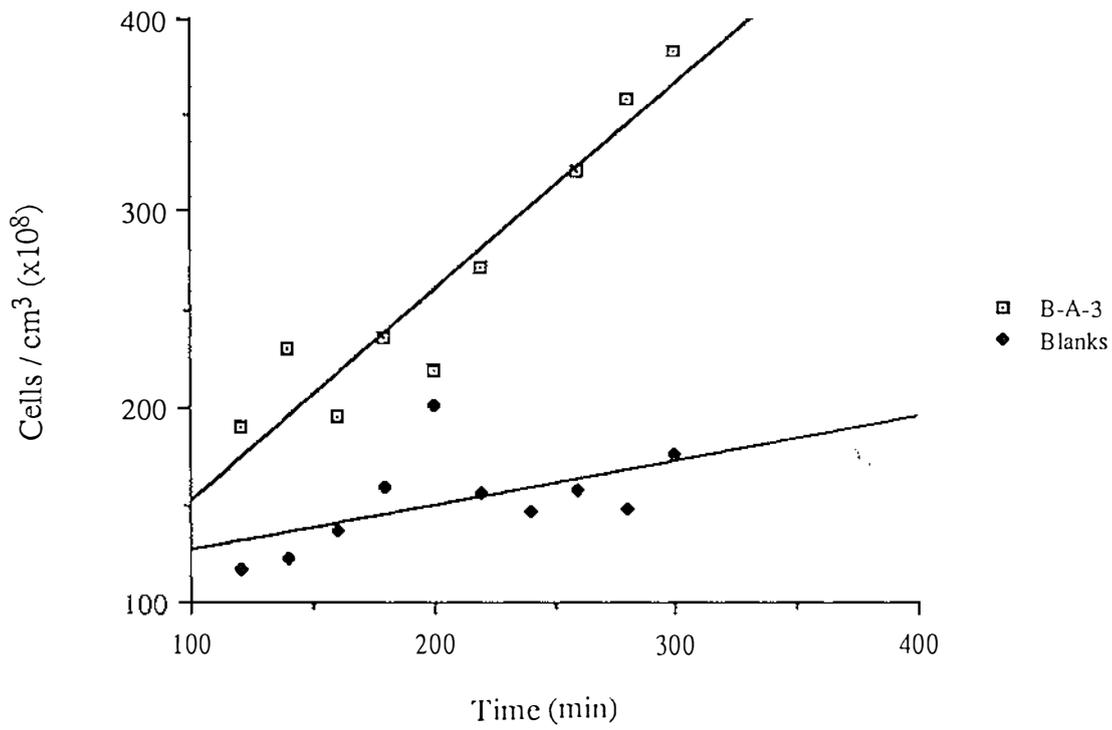


Fig 4.4 Cell Proliferation Caused By Cr-NA-Glu-Gly-Cys Complex

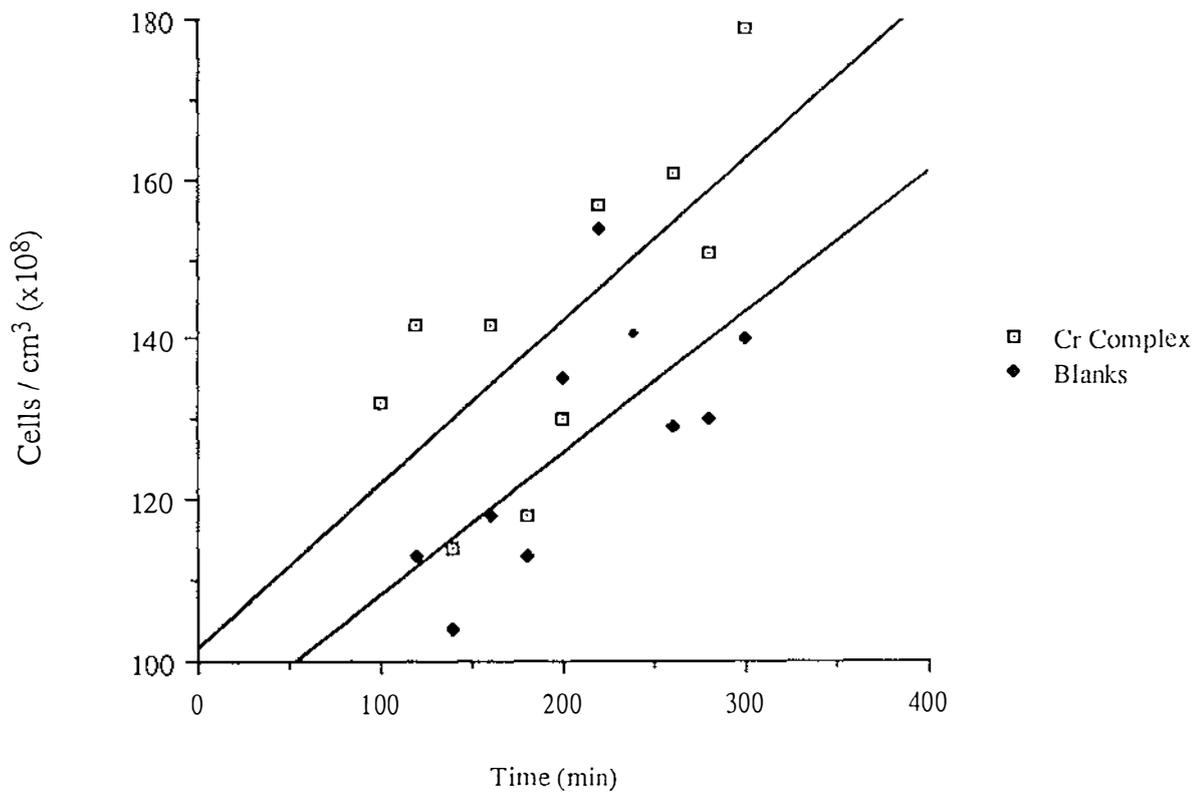
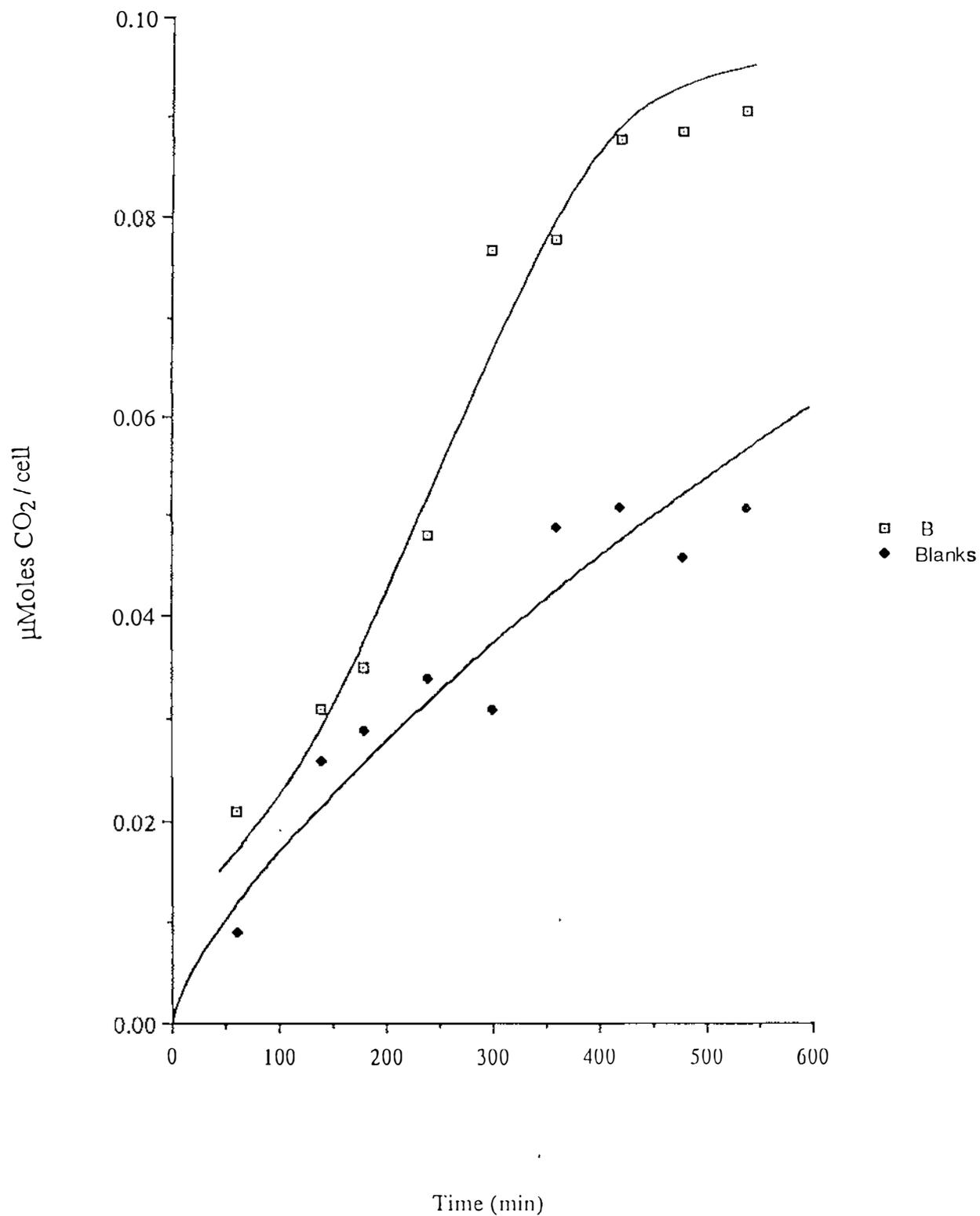


Figure 4.5 Investigation of Cellular CO<sub>2</sub> Evolution With Time in the Yeast Assay

cysteine and nicotinic acid. All were assayed at a concentration of 0.2 mg/0.1 cm<sup>3</sup>. The results and formula of the regression line are shown in Figures 4.6 - 4.9. The calculated enhancements of the rate of cellular CO<sub>2</sub> production are shown in table 4.1.

Table 4.1 Enhancement of the Rate of Cellular CO<sub>2</sub> Evolution  
in the Modified Yeast Assay

Substance	Concentration(mg/cm <sup>3</sup> )	Enhancement
Crude GTF B i)	0.2	91%
ii)	0.2	100%
Arginine i)	0.2	7%
ii)	0.2	- 2%
B-A-3	0.2	- 13%
Cr Complex	0.2	2%

## 4.4 Discussion

The effect on cell proliferation of substances that had previously shown significant activity in the yeast assay was investigated and the results are shown in Figures 4.1-4.4. It can be clearly seen that the crude GTF extract B, fraction B-A-3 and arginine all caused a marked increase in the rate of cell proliferation. This doesn't appear to be the case for the synthetic chromium complex although the variability of the data in Figure 4.4 does not allow firm conclusions to be drawn in that case. These results show the need for modifications to be made to the yeast assay to take account of the fact that a large proportion of the apparent potentiation of CO<sub>2</sub> evolution may be due to cell proliferation. Surprisingly this possibility has never been investigated and no report of an increase in yeast cell numbers during the assay period has yet appeared.

Such a marked cell proliferation would be consistent with the starved state of the yeast used in the assay. The yeast is stored suspended in phosphate buffer and on introduction to the assay it is mixed only with glucose and the sample and so the introduction of fermentable substrates, particularly nitrogen sources, in the sample to be tested is likely to lead to cell growth and proliferation leading to an increased evolution of CO<sub>2</sub>. This is definitely the case for arginine and is probably the case in the impure GTF extracts of yeast.

Figure 4.6a Modified Yeast Assay of Crude GTF B

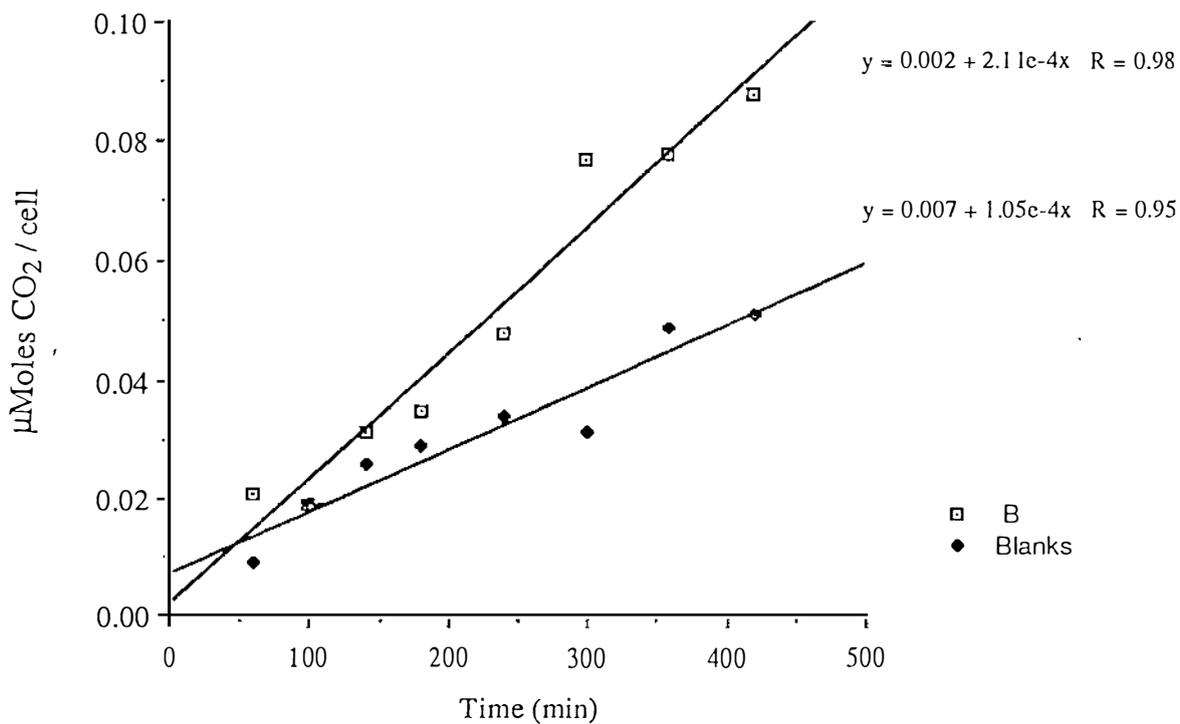


Figure 4.6b Modified Yeast Assay of Crude GTF B

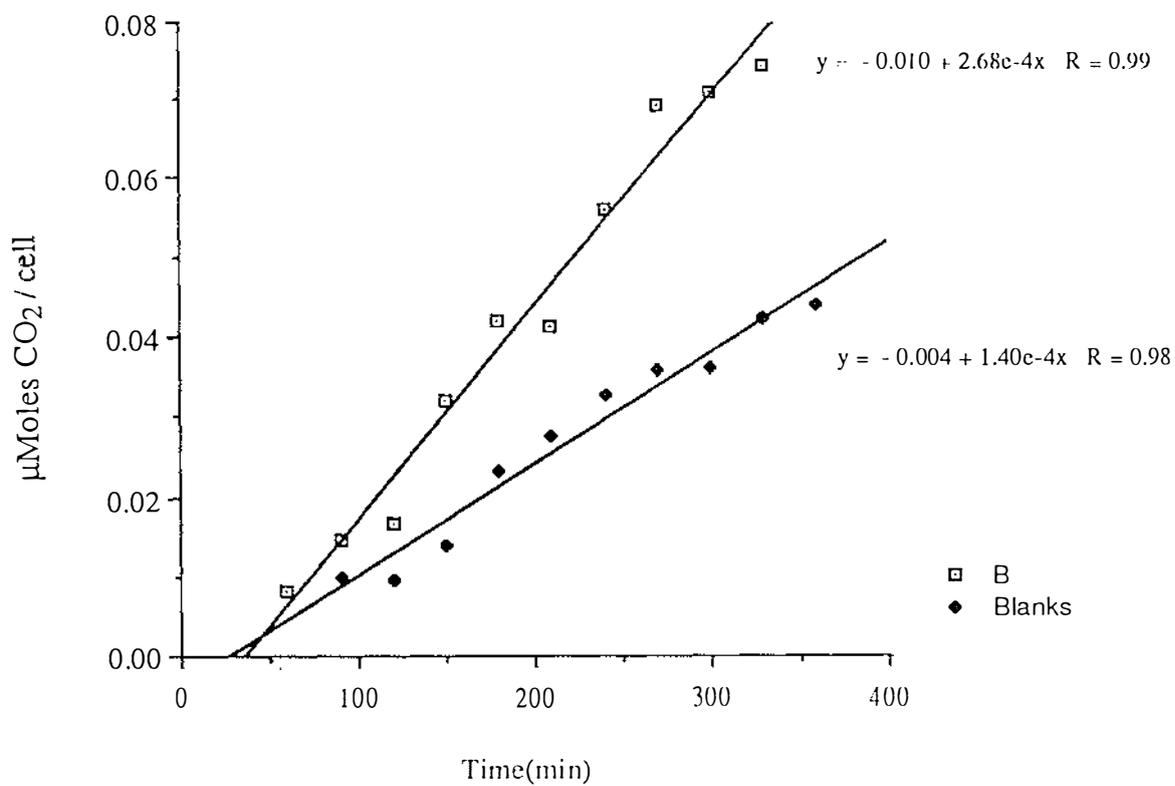


Figure 4.7 Modified Assay of B-A-3

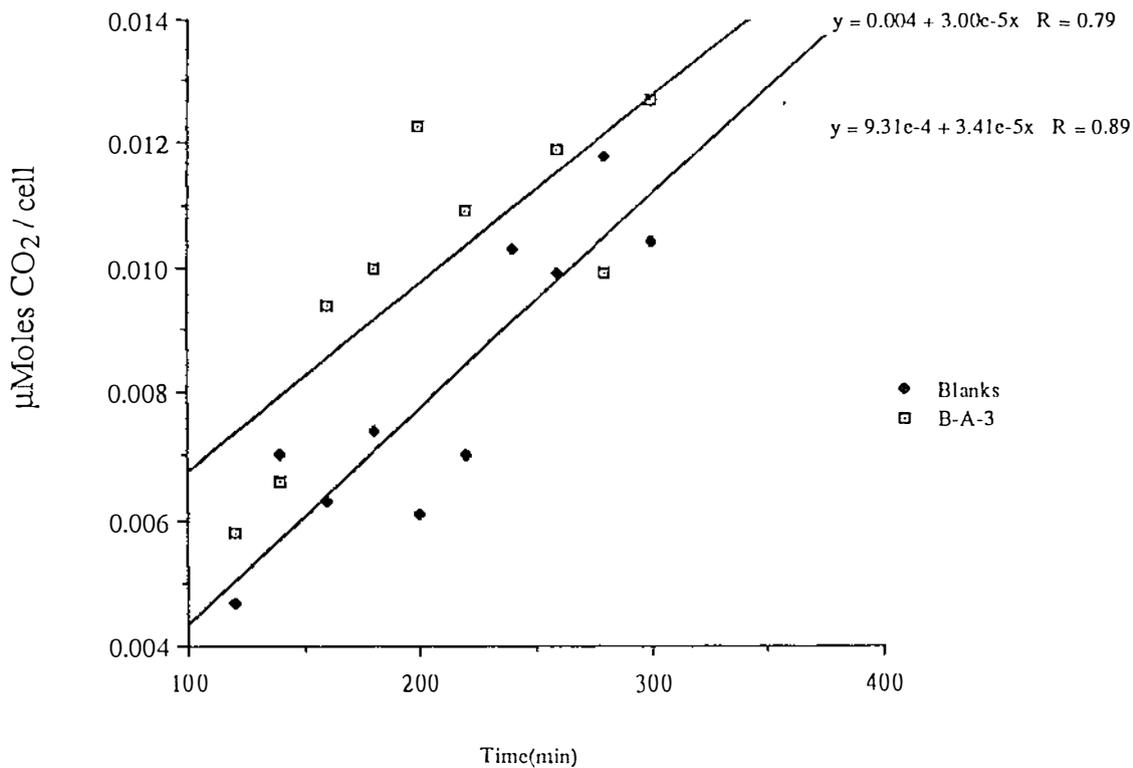


Figure 4.9 Modified Assay of Cr Complex

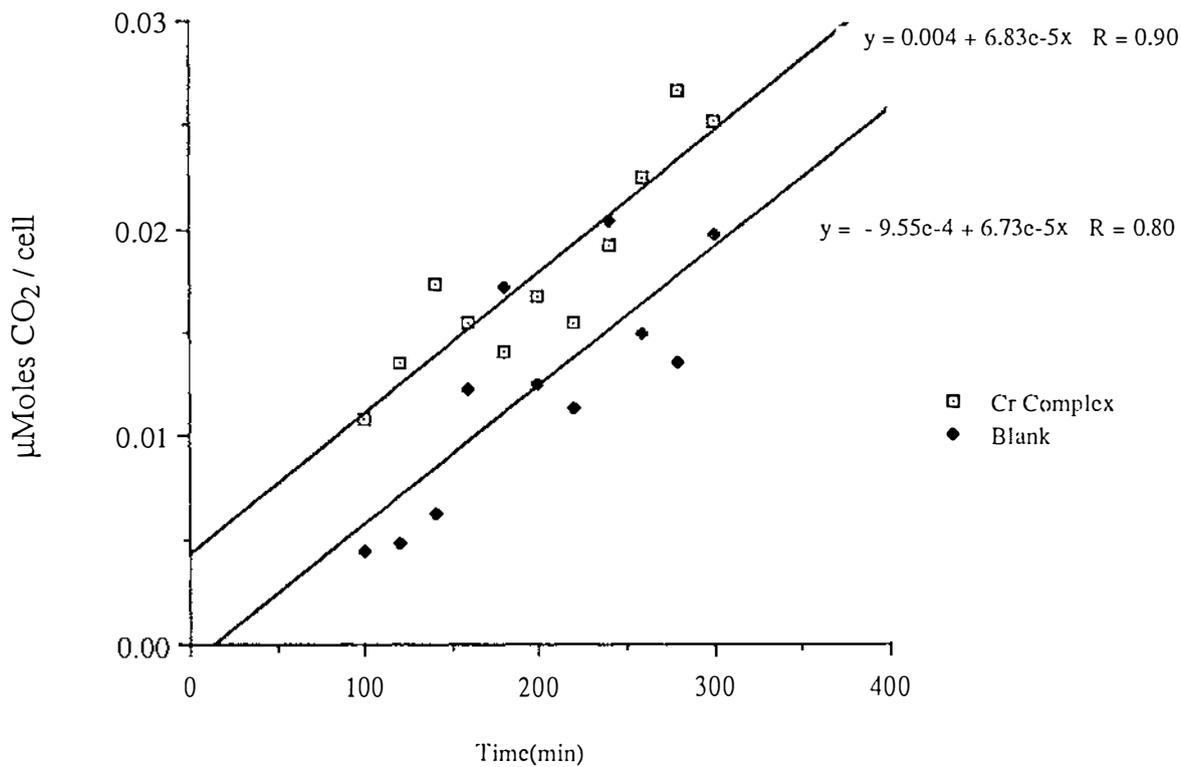


Figure 4.8a Modified Assay of Arginine

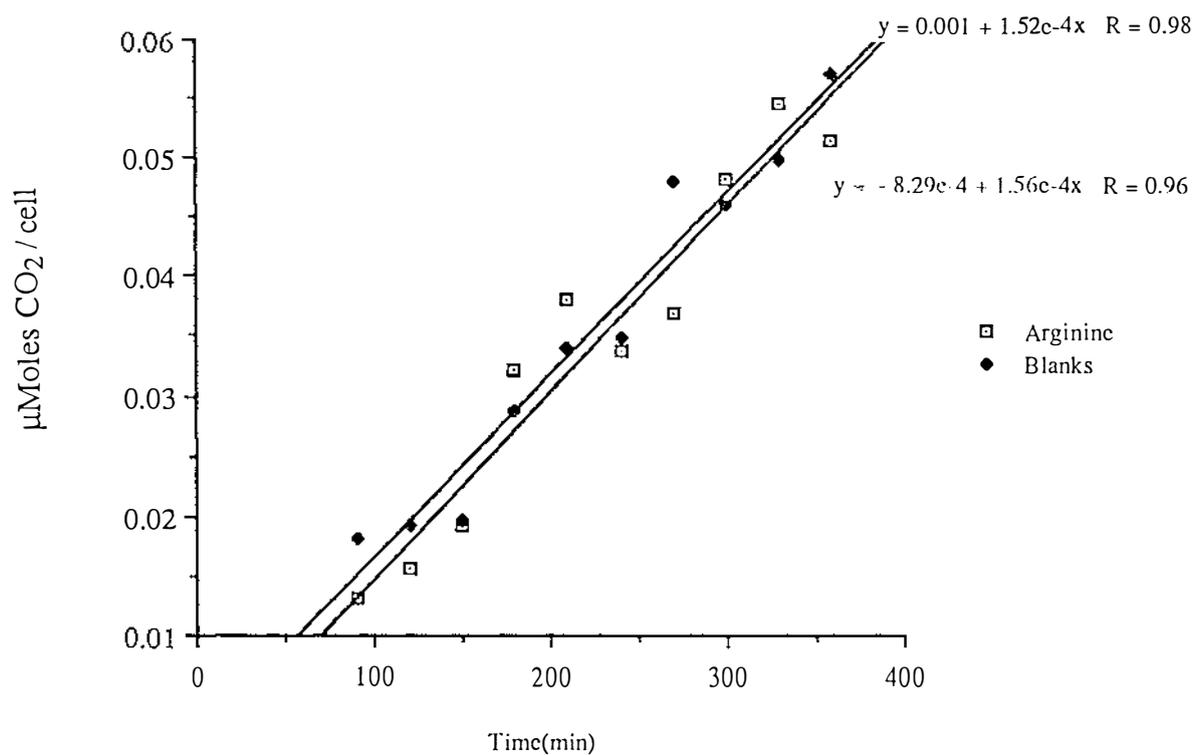
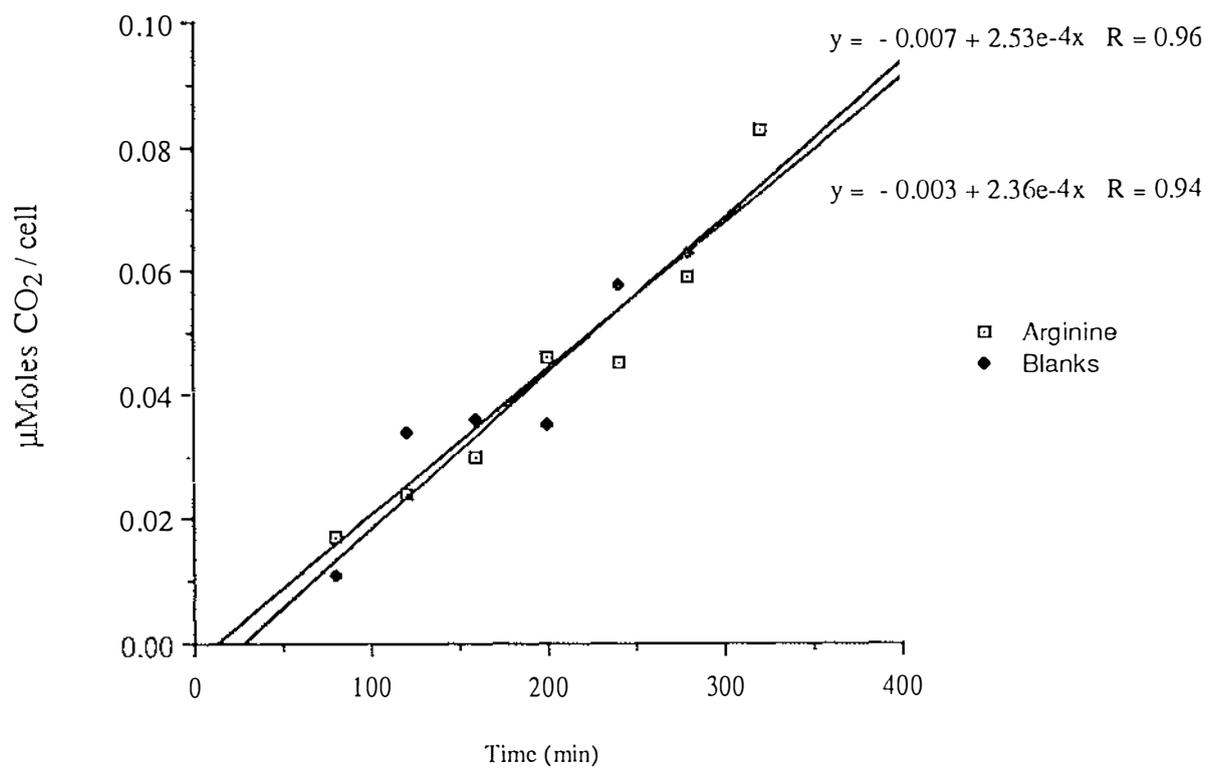


Figure 4.8 b Modified Assay of Arginine



Initial investigations with the modified assay procedure (Figure 4.5) showed that in the case of the crude GTF extract B there was a stimulation of CO<sub>2</sub> production which appeared to be greater than that due to cell proliferation alone. If the near linear segment of the cellular CO<sub>2</sub> evolution curve, between 60 and 400 minutes, was used then an enhancement could be calculated as in the normal yeast assay. In this case of course enhancement in the rate of CO<sub>2</sub> evolution per cell is being calculated. This calculation forms the basis of a new assay for GTF activity which is referred to as the modified yeast assay. If GTF in fact potentiates yeast cell metabolism as claimed by Burkeholder and Mertz (1967), then an enhancement in the cellular CO<sub>2</sub> evolution rate would be expected.

The crude GTF extract B previously exhibited extremely high levels of activity in the standard yeast assay (Chapter 2) and a similar preparation by Toepfer *et al* (1977) had been found to have GTF activity in the adipocyte assay. When GTF extract B was assayed twice in the modified assay at a concentration of 0.2 mg/ml the cellular rate of CO<sub>2</sub> evolution was enhanced 91% in one assay 100% in the second (Figure 4.6a & b). The results were reproducible and clearly suggest that some component in the extract is having a significant potentiating effect on yeast metabolism. However B was still a very crude preparation and this activity may therefore have been due to a combination of a number of components of the extract.

The most active electrophoresis fraction, as determined by the standard yeast assay was the acidic fraction B-A-3. However this displayed no activity at all in the modified assay (Figure 4.7). Thus the activity observed in the standard yeast assay is most likely an artifact which is best explained in terms of material contained in the fraction acting as a source of nutrients to enable cell proliferation. Unfortunately it was not possible to evaluate the HPLC subfractions of B-A-3 in the modified yeast assay due to the relatively large amount of sample required for this assay.

Arginine was also assayed in the modified assay as it was one of the amino acids which had shown high levels of activity in the yeast assay; activity which it was presumed was not related to the activity induced by GTF as GTF fractions were not found to be rich in arginine. The results were very reproducible and clearly show that arginine does not enhance the cellular rate of CO<sub>2</sub> evolution. This confirms the suggestion by Jackson (1985) that arginine was increasing CO<sub>2</sub> evolution by aiding cell proliferation in acting as a nitrogen source for the starved yeast cells. These results are consistent with data which identifies arginine as one of the best amino acid growth sources for the yeast *Saccharomyces* (Strathern *et al* 1982).

The chromium complex assayed was selected for testing on the basis that it had previously shown very high levels of activity in the standard yeast assay (Cooper 1982). Its activity in the standard yeast assay was confirmed here but the results from the modified assay show that the complex does not enhance the the rate of CO<sub>2</sub> evolution per cell (Figure 4.9). Thus the complex is not stimulating the metabolism as would be expected for a true GTF analogue and hence the unpurified chromium complex also must be acting as a source of fermentable substrates. The fact that the crude GTF extract B showed activity in this assay whereas the synthetic chromium complex did not is further evidence against an essential trace element role for chromium.

Overall the modified yeast assay shows promise as a procedure for eliminating spurious fractions from those initially showing GTF activity in the standard yeast assay. The excellent reproducibility between the modified yeast assays done on different days suggests that the inter assay variations observed in the standard yeast assay are probably due to variations in cell proliferation between assays. When the variance in cell numbers between assays is taken into account the results become more consistent. Using the modified yeast assay described here it seems possible to distinguish two separate contributions to the activity observed in the normal yeast assay. One due merely to cell proliferation, such as shown by arginine, and another due to a stimulation of the yeast cells CO<sub>2</sub> production, such as shown by the crude GTF extract B.

It seems likely that a substance containing GTF should show an enhancement in both the standard and the modified yeast assays. By this "yardstick" the crude GTF extract B does show GTF activity but B-A-3 does not. Fraction B-A-3 had been consistently the most active electrophoresis fraction of B in the standard yeast assay and as such it has been further investigated as a candidate for GTF (Chapter 3), however the fact that it did not show activity in the modified yeast assay indicates that this fraction is probably not GTF and has merely been acting as fermentable substrate for yeast.

The observation by Holdsworth and Appleby (1984) that while all fractions that were active in the low chromium rat epididymal adipocyte assay were active in the standard yeast assay, that the reverse was not necessarily true is consistent with these results. The yeast assay system used by Mirsky *et al.* (1980 and 1981) also assumed that the observed activity was not due to the interference of fermentable substrates in the sample. However this assumption is hard to sustain when the system is being used to assay semi purified extracts of yeast for GTF activity. In light of the present results use of the standard yeast assay as a definitive measure of GTF activity, as was done by Haylock *et al.* (1982), is invalidated. As a result the conclusions drawn about the GTF activity of the fractions

obtained from brewers yeast in Chapter 3 must be reassessed. This highlights the difficulty of working in an area where an unknown substance has to be recognised on the basis of its effects determined by an assay system. The importance of having an assay system which identifies material that fulfils the original criteria for GTF (section 1.1) is clearly emphasised.

If the modified yeast assay is indeed able to measure GTF activity then the yeast assay system would still offer a relatively simple method of first screening (using the standard assay) and confirming GTF activity (using the modified assay on active samples) without having to resort to the difficult adipocyte assay system. However the results obtained with the modified yeast assay must now be validated against the more definitive low chromium epididymal rat adipocyte assay (section 5).

# CHAPTER 5

## INVESTIGATIONS OF THE LOW CHROMIUM RAT EPIDIDYMAL ADIPOCYTE ASSAY

### 5.1 Introduction

The modified yeast assay described in Chapter 4 offers a possible means of eliminating artifactual indications of GTF in the yeast assay system that result from proliferation of cell numbers. However the results from that assay must be confirmed against those of a more definitive assay system before they can be taken as a valid measurement of GTF activity. The most widely accepted assay for GTF has been the low chromium rat epididymal adipocyte assay (Anderson *et al.* 1978a & 1979) and it was therefore decided that this system must be established to check the GTF activity of the fractions isolated in Chapter 3 and to be able to make comparisons with the results obtained in Chapter 4. With this information it might then be possible to draw firm conclusions about the nature of the material responsible for causing the effects being attributed to GTF.

The methods used in the current study were based on the procedures of Anderson *et al.* (1978a) with modifications as described by Gliemann (1985) and Dr Kevin Gain, Otago University, Dunedin, New Zealand (Personal communication). In this assay a sample is judged to have GTF activity if it is able to potentiate the action of sub optimal insulin levels on the adipocytes of rats raised on a special torula yeast diet that is low in chromium (Anderson *et al.* 1978a). Generally this has been monitored by measuring the rate of conversion of D-[U- $^{14}\text{C}$ ]-glucose or D-[1- $^{14}\text{C}$ ]-glucose into  $^{14}\text{CO}_2$ . However it has also been observed that GTF has various effects on the insulin stimulated conversion of label from D-[1- $^{14}\text{C}$ ]-glucose, D-[6- $^{14}\text{C}$ ]-glucose and  $^3\text{HIOH}$  into  $^{14}\text{CO}_2$  and  $^3\text{H}$ - and  $^{14}\text{C}$ -fatty acid (Sherriff 1983).

It is vital that readers of the literature have an understanding of the way the term potentiation is used in this field of research. Potentiation of insulin action refers to the ability of a fraction to increase the effectiveness of small amounts of insulin. To determine this in the adipocyte assay system, measurements are made of the individual effects of both insulin and the fraction under test and also of the effect of the two combined. Potentiation is defined as the component of the increase in insulins effect caused by addition of the fraction, over and above any effect that the fraction had on its own. Such

a potentiation would imply that the fraction had a distinct modulating effect on insulins action, as has been postulated for GTF. Thus potentiation is clearly different from the insulin like effects seen with many compounds (section 1.5.1) as an insulin like effect would be seen regardless of the presence of insulin whereas a potentiation of insulins effect is an increase in metabolism seen only in the presence of insulin. Potentiation is also distinct from stimulation as stimulation merely indicates that a substance is able to increase the production of the product being monitored whereas potentiation describes a specific effect on insulins action that is only seen in the presence of both insulin and the sample. For example insulin like effects are a stimulation of metabolism but are not a potentiation. It is important for the reader to realise the significance of these calculated potentiations. The calculated potentiations are not a quantitative measure of overall changes in the metabolism but rather they measure how much the sample has magnified or shrunk the metabolic effects of the sub optimal amounts of insulin. A positive potentiation indicates that a sample increases the effectiveness of insulins action in that particular facet of metabolism being measured while while a negative potentiation indicates the reverse.

This chapter describes the establishment of an adipocyte assay system for GTF. The potentiation of insulin action by GTF fractions is investigated by using the labeled substrates D-[U-<sup>14</sup>C]-glucose, D-[1-<sup>14</sup>C]-glucose, D-[6-<sup>14</sup>C]-glucose and <sup>14</sup>C-acetate and monitoring the conversion of label into metabolic products. The intention was to determine the optimum conditions for measurement of GTF activity, as defined in this assay system, and to make inferences about the mechanism of this potentiation of the metabolism.

## **5.2 Methods and Materials**

### **5.2.1 Animals and Diet**

Male Sprague Dawley rats (Massey University Small Animal Production Unit) were raised from weaning in special low chromium cages based on the design of Polansky and Anderson (1979). In our work the rats were kept in metal free 200 mm x 300 mm glass cages with elevated plastic light diffusor grids as flooring, so as to prevent copography. Water and food containers were also made of glass and consisted of an open cylindrical cup of 70 mm diameter and approx 60 mm in height to which was glued a 90 mm x 90 mm square glass base to prevent rats over turning them (see Figure 5.1). Cages and feeding apparatus were cleaned regularly. The cages were covered by a piece of egg crate panelling which slid into place and was secured by a plastic clothes peg. The cages were



Figure 5.1a Detail of Cage Construction



Figure 5.1b Rat Housing in Use

housed in a room separate from the laboratory although there was no special air filtration system in place.

The rats were kept in pairs and were fed ad libitum the "TD 83155 Torula Yeast Diet for Chromium Studies" (Teklad, Madison, Wisconsin) and water used was deionised and then doubly distilled. The diet was supplied in the powdered form to avoid any chromium contamination during the pelleting process. Diet was stored at 4 °C and kept for up to 6 months under these conditions. However occasionally after several months signs of vitamin E deficiency would appear in the rats and this was remedied by the addition of 200 U/kg vitamin E (BDH) to the diet. Rats raised on this diet gained weight at a much slower rate than rats raised in the same conditions but fed a normal diet (see Chapter 6).

Rats grown under these conditions can only be described as marginally chromium deficient as more severe chromium deficiency is only developed using strictly controlled environmental conditions as described by Schroeder *et al.* (1965 & 1966). However this state is sufficient for producing adipose tissue that exhibits the characteristics required for the eliciting of a response to GTF in the assay (Anderson *et al.* 1978a).

## 5.2.2 The Adipocyte Assay

### 5.2.2.1 Materials

**Buffers:** Stock solutions of 300 mM Hepes and Krebs Ringer Phosphate Buffers were prepared as described below and stored for up to 3 weeks at 4 °C.

Buffer	Component	Supplier	Concentration (moles/litre)
HEPES	HEPES	(Gibco)	0.300

#### Krebs Ringer Phosphate

NaCl	(Dominion Salt Co)	1.200
KCl	(BDH)	0.048
KH <sub>2</sub> PO <sub>4</sub>	(Ajax Chemicals, Sydney)	0.012
MgSO <sub>4</sub> .7H <sub>2</sub> O	(Riedel de Hahn)	0.012
CaCl <sub>2</sub> .6H <sub>2</sub> O	(Riedel de Hahn)	0.013

**Glucose :** A stock solution of 0.38 mM glucose monohydrate (Glaxo) was made up in doubly distilled deionised water and stored frozen.

**Collagenase :** Great variations were found in the ability of different collagenases to produce viable adipocyte preparations. A suitable batch of collagenase was obtained from Cooper Biomedical (CLS 4196, 154 U/mg). A stock solution of a concentration of 2 mg/ml was prepared and frozen for later use.

**Insulin :** A stock solution was prepared by first dissolving 6.02 mg of bovine insulin (Sigma I 5500, 25 insulin units /mg) in a small volume of 0.001M HCl and making up to a volume of 100 ml in a silanised volumetric flask, using 2% albumin solution without glucose (see below). A portion of this solution was taken and diluted 1/100 using 2% albumin solution giving a stock solution of  $1.05 \times 10^{-8}$  M (15 mU/ml) insulin. This stock insulin solution was stored frozen in 1 ml plastic snap top vials for up to 6 weeks and when required it was thawed and diluted 1/10 in 2% buffer.

**Albumin :** The Bovine Serum Albumin used (Sigma A 7906) was a preparation which had been subject to extensive charcoal treatment, to remove fatty acids, and dialysis, to remove small molecular weight inhibitors. This grade was consistently found to be suitable for direct use in the adipocyte assay, unlike many other commercial albumin preparations which required further purification before use (Gliemann 1985).

**Isotope :** D-[U- $^{14}$ C]-glucose (CFB.96, 280 mCi/mmol), D-[1- $^{14}$ C]-glucose (CFA.349, 55.6 mCi/mmol), D-[6- $^{14}$ C]-glucose (CFA.351, 58.5 mCi/mmol) and  $^{14}$ C acetate (CFA.13, 56 mCi/mmol) were all obtained from Amersham .

**Assay Buffers :** Three different buffers for use in the assay were made from stock solutions. To make 100 ml of buffer, 10 ml of each of the HEPES and KRP-salt solutions were pipetted into a beaker and then either 2 g or 4 g of albumin was added, to make 2% by weight or 4% by weight albumin buffers. Each was then made up to 100 ml with doubly distilled deionised water and the pH was adjusted to 7.4 by dropwise addition of 2 M NaOH. This gave a final concentration of 30 mM HEPES and 1.2 mM phosphate in all the buffers. Generally 50 ml of a 4% by weight albumin buffer and 150 ml of a 2% by weight albumin buffer were prepared. To a 75 ml portion of the 2% albumin buffer was added 150  $\mu$ l of the stock glucose solution to give a glucose concentration of 0.5 mM.

**Scintillation Fluid :** Scintillation solvent used was toluene: Triton X-100 (2:1, v:v). The fluors used were 2,5 Diphenyloxanazole (PPO) at a concentration of 4.0 g/l and 1,4-Di-[2-(5-phenyloxazolyl)]-benzene (POPOP) at a concentration of 0.1 g/l. Both fluors were obtained from Park Scientific Ltd.

#### **5.2.2.2 Preparation of Isolated Adipocytes**

Between 9 am and 10 am on the day of the assay, two fed rats weighing 180 - 210 g were taken and killed by spinal dislocation. The distal portion of the epididymal adipose fat pads was quickly removed, taking care to avoid including blood vessels, and weighed.

The fat tissue was then well minced with sharp scissors and transferred to a 25 ml plastic conical flask. To this flask was added stock collagenase so as to give a final concentration of 1 mg collagenase per mg of tissue and this was then diluted with 2.5 ml of the 4% albumin buffer per 1 mg of tissue. This solution was incubated at 37 °C at 150 rpm in a Heto shaking water bath. Incubation was for up to 40 minutes depending on the length of time taken for the tissue to digest.

Once digested the isolated adipocytes were strained, into a graduated plastic centrifuge tube, through a double syringe bore apparatus which had muslin cloth at the end of the first bore and washed nylon stocking mesh at the end of the second. The filtered adipocytes were gently centrifuged at 300 rpm for a brief time and the liquid below the adipocytes was removed by means of a syringe with a length of thin plastic tubing attached. The adipocytes were then resuspended in the 4% albumin buffer and the centrifuging and liquid removal steps were repeated. This washing step was repeated 3 times in all to ensure the removal of all collagenase. In the last wash the volume of the adipocytes was noted and the washed adipocytes were suspended in a volume of the 2% albumin, 50 mM glucose buffer in a plastic conical flask. The cells were diluted so as to give an approximate concentration of 10-15 mg of adipocytes /ml. Generally this required a dilution of about 25 ml of buffer for every 1 ml of adipocytes. This adipocyte suspension for use in the incubation was allowed to equilibrate at 37 °C for 10 minutes before being used in the assay. Four 1 ml aliquots of the adipocyte suspension and four 1 ml aliquots of buffer were pipetted into vials and dried at 110 °C and in this way the weight of cells was determined.

### **5.2.2.3 Assay Incubation**

Incubation was carried out in a series of silicon coated glass 10 ml conical flasks which were re silanized after each assay using Sigmacote (Sigma SL2). Using two water baths up to 48 assay flasks could be incubated simultaneously. All determinations were carried out in triplicate so only 16 different determinations could be carried out simultaneously.

Flasks were prepared by first adding the sample to the bottom of the flask. To this was added the appropriate amount of insulin, taking care to pipette the insulin in plastic tipped pipettes. Sufficient 2% albumin buffer (without glucose) was added to make a total combined volume of 475  $\mu$ l. To this was added 25  $\mu$ l of isotope which gave a specific activity of 0.2  $\mu$ Ci/flask. In the case of D-[U-<sup>14</sup>C]-glucose each flask contained 0.7 nmoles of labeled substrate, in the case of D-[1-<sup>14</sup>C]-glucose and D-[6-<sup>14</sup>C]-glucose and <sup>14</sup>C-acetate each flask contained 3.6 nmoles. This compared with 750 nmoles of cold glucose present from the 0.5 mM glucose 2 % albumin buffer thus giving a tracer quantity

of isotope in each flask. To this was added 1000  $\mu$ l of the adipocyte suspension. The adipocytes were added using a plastic tipped pipette, in which the hole in the tip had been widened to prevent cell disruption, and care was taken to ensure an even suspension was maintained while pipetting.

Once the incubation mixture was complete, each flask consecutively was gassed with 95%  $O_2$  / 5%  $CO_2$  to enable maximum oxidation and then quickly sealed. The seal consisted of a rubber stopper (Kontes K882310) which supported a suspended plastic centre well (Kontes K882320) in which a wick of blotting paper was rolled (see Figure 5.2). The flask was then incubated in a Heto shaking water bath at 37  $^{\circ}C$  and 100 rpm for 90 minutes. Immediately prior to termination of the incubation 0.2 ml of 5 M NaOH was injected into the centre well through the rubber seal. The incubation was terminated after 90 minutes by the injection of 0.3 ml of 2 M  $H_2SO_4$  into the adipocyte suspension.

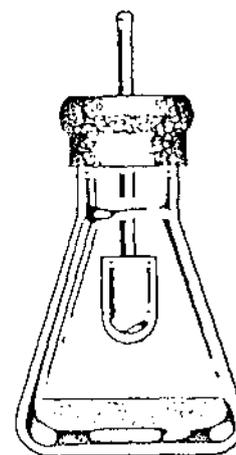


Figure 5.2 Arrangement of the Incubation Flask

#### **5.2.2.4 Standard Assay Protocol**

**Standards:** In all assays a determination was made of a blank, a sub optimal insulin concentration and an optimal insulin concentration. The determination of an optimal insulin concentration was carried out to determine the viability of the assay. If the adipocyte preparation was functioning properly then the optimal insulin concentration should stimulate the oxidation of glucose to  $CO_2$  approximately ten fold over the basal rate (Gliemann 1985).

**Samples:** To determine the ability of a sample to potentiate the action of insulin a determination was first made of the amount of label converted to labeled product in the presence of the sample alone and another determination was made of the amount of label converted to labeled products in the presence of both the sample and a sub optimal amount of insulin.

#### **5.2.2.5 Measuring Metabolic Products**

**Carbon Dioxide:** The acidified flasks were left in the shaking water bath for 45 minutes to allow the complete release of  $CO_2$  and its uptake by the NaOH wick. The flasks were then removed from the bath and the seals removed. The wick was extracted from the centre well with tweezers and placed in a scintillation vial to which was added 8 ml of scintillation fluid. This was then counted on a Beckman LS8000 liquid scintillation counter. It was found that this method was able to collect all the  $CO_2$  evolved from the solution and it was therefore concluded that the counts present in the wick

represented all the  $^{14}\text{CO}_2$  present in the incubation medium at the time the incubation was terminated.

**Total Lipids:** The incorporation of  $^{14}\text{C}$  label into total lipid, glycerol and fatty acids was determined in the acidified adipocyte suspension. The methods used were based on those described by Elwood and Van Bruggen (1961). The acidified contents of the flask were transferred quantitatively to a screw cap culture tube using 3 x 2 ml washes of the Doles extraction solvent (Isopropanol : Heptane :  $\text{H}_2\text{SO}_4$ , 40 : 10 : 1). The lid was screwed on and the contents shaken. The culture tube was then left to stand for at least 30 minutes and 3 ml of heptane was added. This was again shaken then centrifuged to separate the layers. Most of the heptane layer was removed by Pasteur pipette to another culture tube. To ensure quantitative transfer 1 ml of heptane was added to the original solution and this was shaken, centrifuged and the heptane layer transferred to the second culture tube as well. To remove any residual  $^{14}\text{C}$ -glucose the heptane solution was back washed with 0.5 ml of 2 % glucose in 0.05 N  $\text{H}_2\text{SO}_4$ . The glucose solution was removed from heptane with a pasteur pipette. These steps resulted in the isolation of the total lipid fraction which could either be saponified to isolate glycerol and fatty acids or which could be taken up in 10  $\text{cm}^3$  of scintillation fluid and the amount of label incorporated into total lipid determined by counting in a Beckman LS 8000 liquid scintillation counter.

**Fatty Acids and Glycerol:** The volume of the total lipid fraction was reduced almost to dryness by bubbling dry nitrogen through the tube and saponified by heating for 90 minutes at 70 °C with 2 ml of 11 % ethanolic KOH. As it has been found that there were insignificant amounts of  $^{14}\text{C}$  label incorporated into cholesterol and other steroids in this assay system (Jungas 1968 & Elwood, Personal communication) it was decided that it was unnecessary to extract non saponifiable lipids at this point. The saponified material was acidified with 0.5 ml of 6 M  $\text{H}_2\text{SO}_4$  to protonate the fatty acids. To the tube was added an equal volume of hexane. After shaking the hexane layer was removed with a pasteur pipette and transferred to another tube. This step was repeated twice more to ensure quantitative transfer of the fatty acids.

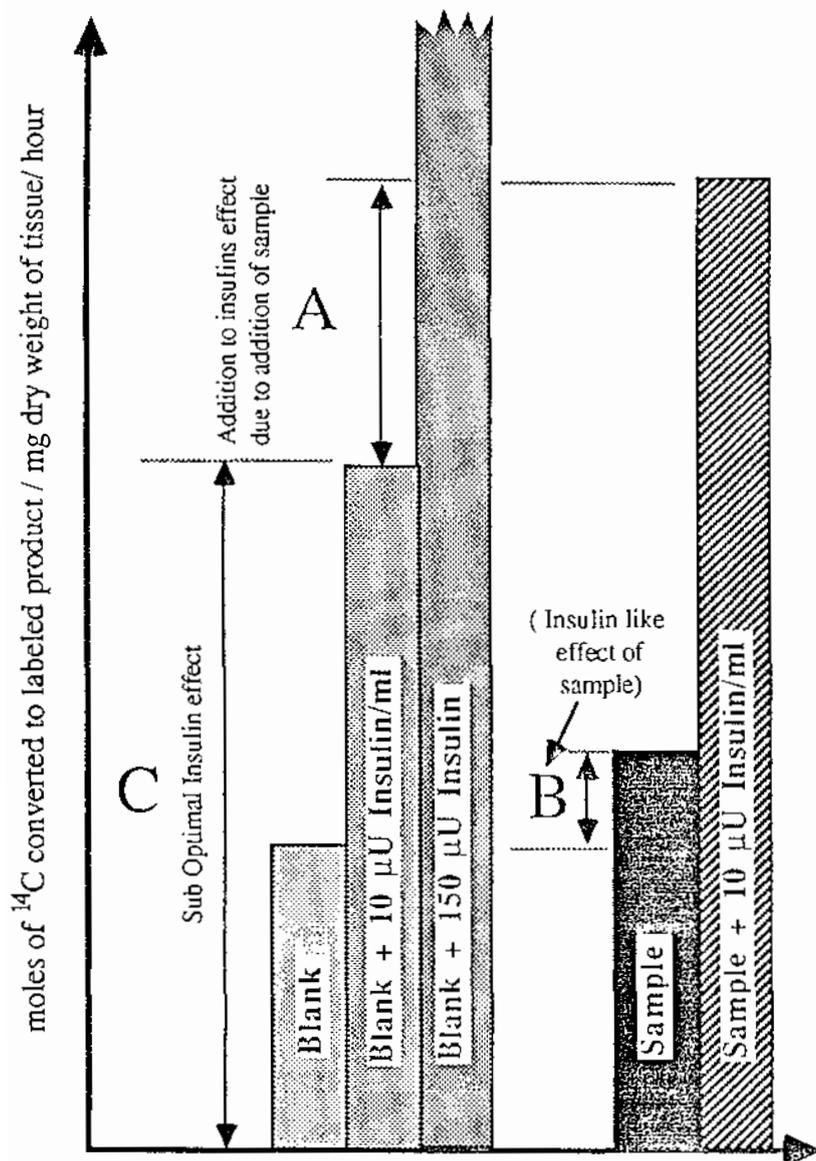
The incorporation of label into glycerol was determined by transfer of the aqueous phase to a scintillation vial and the addition of 12 ml of scintillant. Counts were determined on a Beckman LS8000 liquid scintillation counter.

The hexane extract was backwashed with 0.5 ml of 2 % glycerol in 0.02 N  $\text{H}_2\text{SO}_4$ . The washed hexane layer was transferred to a scintillation vial and 10 ml of scintillation fluid was added to the vial and the label incorporated in the fatty acids was determined on the Beckman LS8000 liquid scintillation counter.

### **5.2.2.6 Determination of the Potentiation of Sub optimal Insulin Concentrations**

Significant interassay variation has been found in the response of different preparations of adipocytes to insulin and GTF (Sherriff 1983). This highlights the need for a system of calculating the potentiation of insulin action that minimises this type of inherent systematic variability. The method previously described for calculating potentiation of the action of sub optimal insulin concentrations (Anderson *et al.* 1978a) takes no account of the insulin like component that a sample may exhibit, which is a particularly important factor when determining the potentiation by crude extracts. To overcome this a new method of calculation was developed. All data were converted from counts per minute to disintegrations per minute (dpm) using a standard curve of II number versus efficiency. From this the number of moles of  $^{14}\text{C}$  converted into labeled product was calculated and this was normalised with respect to the dry weight of cells to eliminate variability between different assays due to differences in cell numbers. As is conventionally done in this field the data was plotted as moles of  $^{14}\text{C}$  converted into labeled product/ g dry weight of tissue/ hour. If it is assumed that there was no isotope effect then this accumulation of the label in particular metabolic pools measured at a fixed time would parallel the corresponding metabolism of unlabeled substrate. Data was plotted on a bar graph and which gave an indication of the ability of a sample to potentiate the action of sub optimal quantities of insulin. However a calculation was required to definitely ascertain whether or not a potentiation in fact existed and to give a quantitative value for such a potentiation which would allow accurate inter assay comparisons. For the purposes of this work such a quantitative calculation of the potentiation of the action of sub optimal insulin was devised and its basis is shown in Figure 5.3. An important feature of the assay protocol used here is that all the determinations required to calculate the potentiation are made in the same assay, thus minimising errors caused by inter assay variations. All results shown are the average of at least 3 determinations and the errors indicate the variance of these results from this average.

Figure 5.3 Calculation of the Potentiation of Sub Optimal Insulin Action by GTF Samples



$$\% \text{ Potentiation of the action of sub optimal insulin concentrations} = \frac{\left( \text{Addition to insulins effect due to addition of sample} \right) - \left( \text{Insulin like effect of sample} \right)}{\text{Sub Optimal Insulin effect}} \times \frac{100}{1}$$

$$= \frac{A - B}{C} \times \frac{100}{1}$$

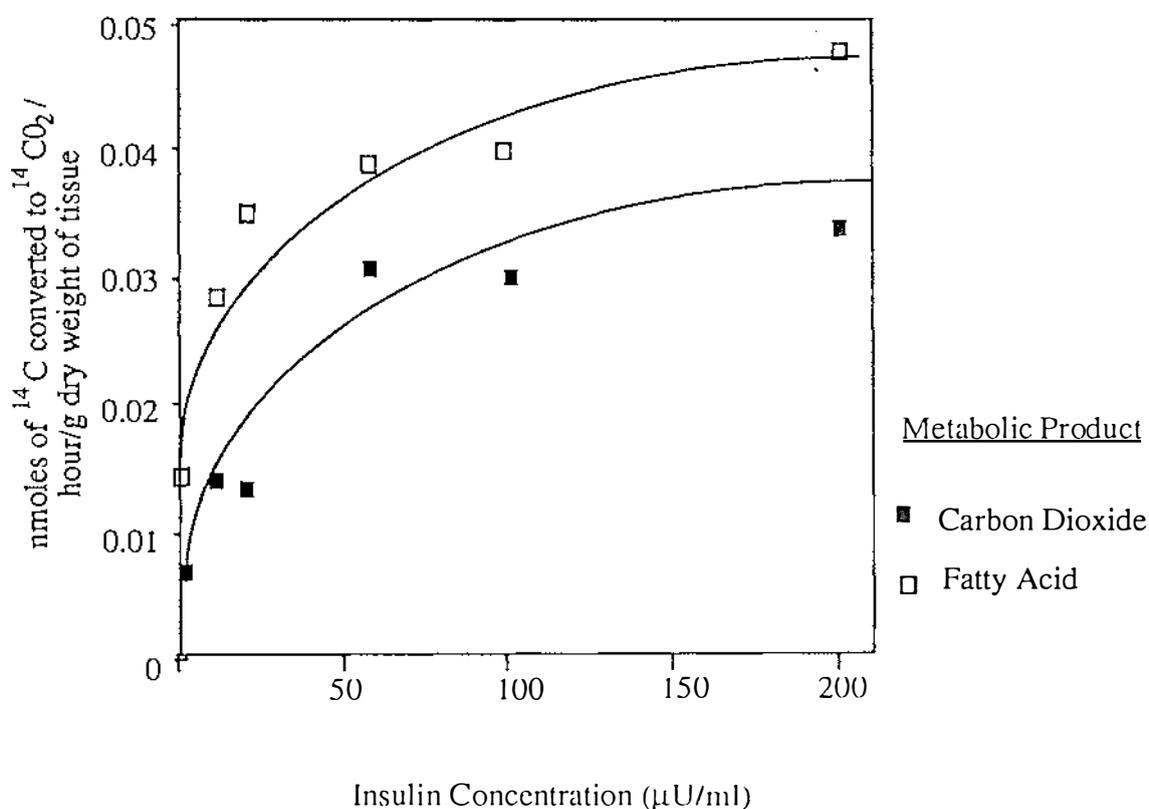
## 5.3 Results

### 5.3.1 Standard Curve for Insulin

Initially it is important to ascertain that the methods used to isolate the adipocytes are producing adipocytes which are viable in the assay system. This is confirmed by the results shown in Figure 5.5a where it can be seen that the maximally insulin stimulated rate of conversion of label from D-[U- $^{14}$ C]-glucose to  $^{14}$ CO $_2$  (dark shaded bar) was 10 times higher than that of the basal rate (lightest shaded bar). This is the ratio that is expected in a viable preparation of adipocytes (Gliemann 1985).

Before establishing a routine assay for the potentiation of the action of sub optimal insulin concentrations in the adipocyte system, it is important to establish a standard curve to determine the response of insulin in the assay system. To achieve this a standard curve was run for concentrations of insulin ranging from 10  $\mu$ U/ml to 200  $\mu$ U/ml (Figure 5.4) to measure insulins ability to stimulate the rate of metabolism of D-[U- $^{14}$ C]-glucose to CO $_2$  and fatty acids. From this curve it was determined that for the purposes of this assay system that suitable optimal and sub optimal insulin concentrations would be 100  $\mu$ U/ml and 10  $\mu$ U/ml respectively.

Figure 5.4 Standard Curve for the Action of Insulin on the Conversion of D-[U- $^{14}$ C]-glucose to  $^{14}$ C-Fatty Acids and  $^{14}$ CO $_2$



### 5.3.2 Investigation of the Potentiation of the Action of Sub Optimal Insulin Concentrations on the Metabolism of Various Substrates to CO<sub>2</sub>, Total Lipid, Fatty Acids and Glycerol

Initial investigations of the insulin potentiating ability of crude GTF extracts B & T were carried out monitoring the rate of production of <sup>14</sup>C labeled CO<sub>2</sub> and fatty acids from D-[U-<sup>14</sup>C]-glucose and D-[1-<sup>14</sup>C]-glucose. The concentration of B & T used was 66 µg/ml (100 µg per assay) as other workers had found this to be a suitable concentration (Elwood, personal communication). The results of these preliminary investigations are shown in Figures 5.5 and 5.6. Visual examination of the graph indicates that the crude GTF extracts increased the amount of <sup>14</sup>C label appearing in CO<sub>2</sub> and fatty acids as a result of the presence of sub optimal concentrations of insulin, thus indicating the possibility of a potentiation of insulins action. However visual estimations of potentiation can be misleading and it is only when the data is calculated as a percentage potentiation using the method described in figure 5.3 (Table 5.1) that it can be seen that both extracts do generally show significant potentiation of the action of insulin.

Table 5.1 Potentiation of the Action of a Sub Optimal Insulin Concentration by Crude GTF Fractions

GTF Extract	Substrate	Potentiation of Conversion of <sup>14</sup> C into:	
		<sup>14</sup> CO <sub>2</sub>	<sup>14</sup> C-Fatty Acid
B	D-[U- <sup>14</sup> C]-glucose	85 ± 30%	155 ± 45%
B	D-[1- <sup>14</sup> C]-glucose	60 ± 25%	100 ± 30%
T	D-[U- <sup>14</sup> C]-glucose	10 ± 10%	85 ± 25%
T	D-[1- <sup>14</sup> C]-glucose	45 ± 10%	110 ± 40%

In an attempt to locate the site of the potentiation by the crude GTF extract B, potentiation of the insulin stimulated conversion of label from D-[U-<sup>14</sup>C]-, D-[1-<sup>14</sup>C]-, D-[6-<sup>14</sup>C]-glucose and [<sup>14</sup>C]-acetate into CO<sub>2</sub>, the total lipid fraction, fatty acids and the glycerol moiety was investigated. The results are shown in Figure 5.7 and the percentage potentiation of the action of a sub optimal insulin concentration is shown in Table 5.2. The figures shown here are the average of results from at least two separate experiments with three flasks of the sample being run in each assay and errors shown indicate the variance of results from this average.

Table 5.2 Potentiation of the Action of a Sub Optimal Insulin Concentration by Crude GTF Fraction B (at a concentration of 66  $\mu\text{g/ml}$ )

Substrate	Product			
	CO <sub>2</sub>	Total Lipid	Fatty Acid	Glycerol
D-[U- <sup>14</sup> C]-glucose	30 $\pm$ 15%	25 $\pm$ 10%	40 $\pm$ 30%	20 $\pm$ 30%
D-[1- <sup>14</sup> C]-glucose	30 $\pm$ 3%	0 $\pm$ 15%	40 $\pm$ 10%	-15 $\pm$ 10%
D-[6- <sup>14</sup> C]-glucose	0 $\pm$ 5%	5 $\pm$ 5%	-50 $\pm$ 15%	0 $\pm$ 10%
[ <sup>14</sup> C]-acetate	-35 $\pm$ 5%	-50 $\pm$ 10%	65 $\pm$ 10%	-80 $\pm$ 20%

## 5.4 Discussion

### 5.4.1 Significance of Assay Results

The definition of potentiation described in section 5.1 has been quantified using the calculations described in Figure 5.3. This is the first reported use of such quantitative calculations of potentiation of insulin's actions by yeast extracts. Use of this calculation is particularly important when looking for potentiation effects in crude extracts of yeast, as has been the case in most GTF research to date, as it is highly likely that a crude extract would contain material causing an insulin like effect which would be difficult to separate from a true potentiation effect on visual analysis of the data plotted as bar graphs. However this calculation is able to separate these two contributory factors.

Preliminary investigations were carried out into the ability of the crude GTF fractions isolated in this work to potentiate the insulin stimulated conversion of <sup>14</sup>C from glucose into <sup>14</sup>CO<sub>2</sub> and <sup>14</sup>C-fatty acids. In this work two isotopically labeled forms of glucose were used, D-[1-<sup>14</sup>C]-glucose and D-[U-<sup>14</sup>C]-glucose, as these were the two forms used in previous adipocyte assays for GTF (Roginski *et al.* 1970, Anderson *et al.* 1978a). The results of the assays are shown in Figures 5.5 and 5.6 and the potentiations of sub optimal insulin calculated from these results are shown Table 5.1. An example of how the bar graphs relate to calculated potentiation is seen from analysis of the results shown in Figure 5.5a. This shows that the rate of conversion of <sup>14</sup>C-glucose to <sup>14</sup>CO<sub>2</sub> by the sub optimal level of insulin (middle shaded bar, Figure 5.5a) is greatly increased by the addition of extract B (diagonally shaded bar, Figure 5.5a). The insulin like effect of B is the difference between the blank (lightest shaded bar, Figure 5.5a) and the addition of B to a blank (back bar, Figure 5.5a) and this is subtracted from the increase of insulin action caused by B described above to give the true potentiation (see Figure 5.3), in this case 85%. This positive potentiation indicates that the sample is making insulin more effective

### Notes on Figures 5.5a and 5.5b

Adipocytes were isolated from rats (180-220 g) which had been maintained on the Torula yeast diet. Adipocytes were incubated for 90 minutes in pH 7.4, 2% Albumin:30 mM HEPES buffer that contained 0.5 mM glucose and 0.2  $\mu$ Ci of labeled substrate. Incubation temperature was 37°C and the flasks were shaken at 100 rpm. Total incubation volume was 1.5 ml. Results are the mean of triplicate estimations. Bars shown on the graph represent a blank (▣), 10  $\mu$ U/ml Insulin (▤), 100  $\mu$ U/ml Insulin (▥), the sample only (▦) and the sample in the presence of 10  $\mu$ U/ml Insulin (▧).

Figure 5.5a Effect of Crude GTF Extracts B & T on the Insulin Stimulated Conversion of D-[U-<sup>14</sup>C]-glucose to <sup>14</sup>CO<sub>2</sub>

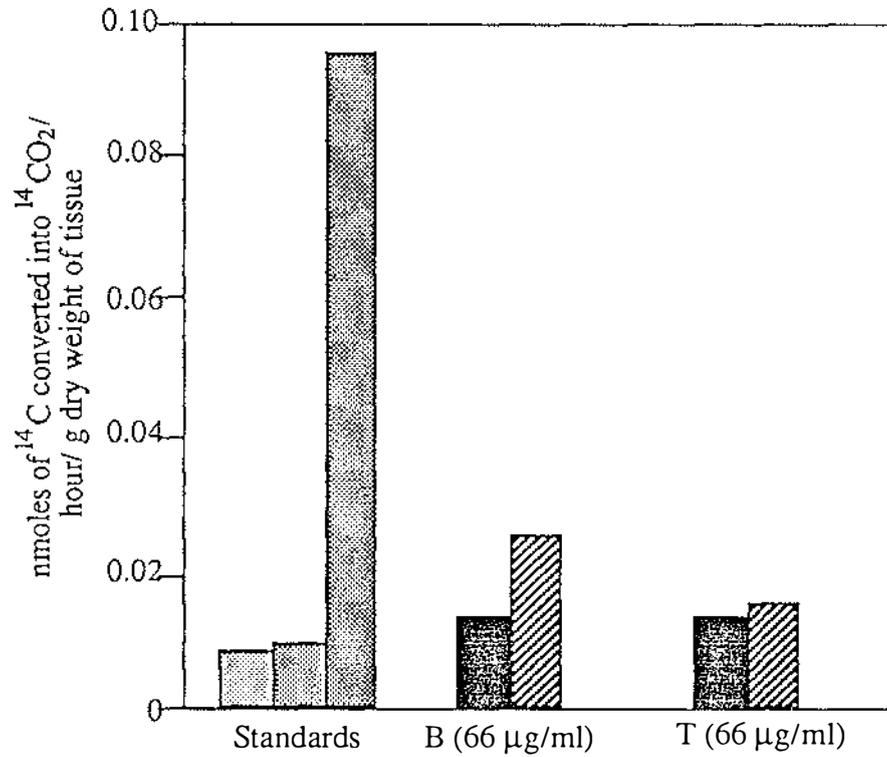
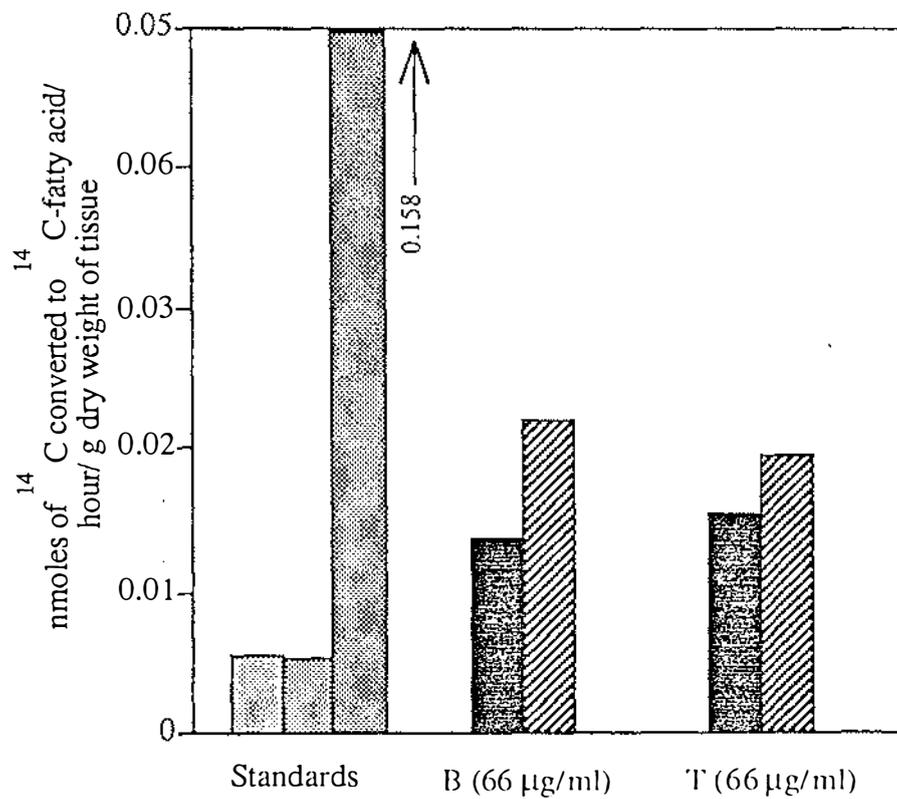


Figure 5.5b Effect of Crude GTF Fractions B & T on the Insulin Stimulated Conversion of D-[U-<sup>14</sup>C]-glucose to <sup>14</sup>C-Fatty Acids



### Notes on Figures 5.6a and 5.6b

Adipocytes were isolated from rats (180-220 g) which had been maintained on the Torula yeast diet. Adipocytes were incubated for 90 minutes in pH 7.4, 2% Albumin:30 mM HEPES buffer that contained 0.5 mM glucose and 0.2  $\mu$ Ci of labeled substrate. Incubation temperature was 37°C and the flasks were shaken at 100 rpm. Total incubation volume was 1.5 ml. Results are the mean of triplicate estimations. Bars shown on the graph represent a blank (▣), 10  $\mu$ U/ml Insulin (▤), 100  $\mu$ U/ml Insulin (▥), the sample only (▦) and the sample in the presence of 10  $\mu$ U/ml Insulin (▧).

Figure 5.6a Effect of Crude GTF Extracts B & T on the Insulin Stimulated Conversion of D-[1-<sup>14</sup>C]-glucose to <sup>14</sup>CO<sub>2</sub>

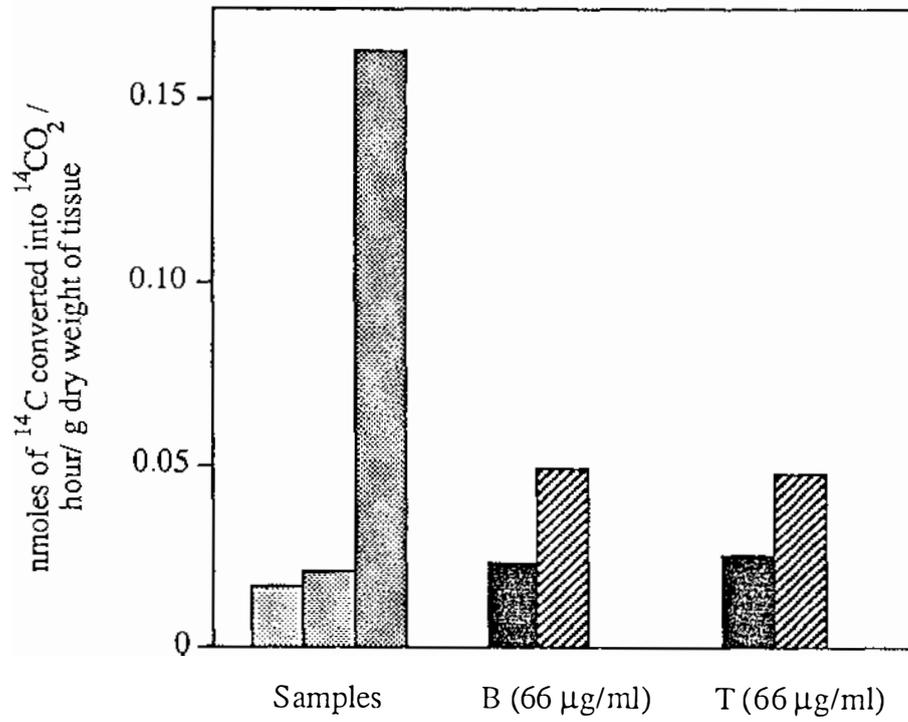
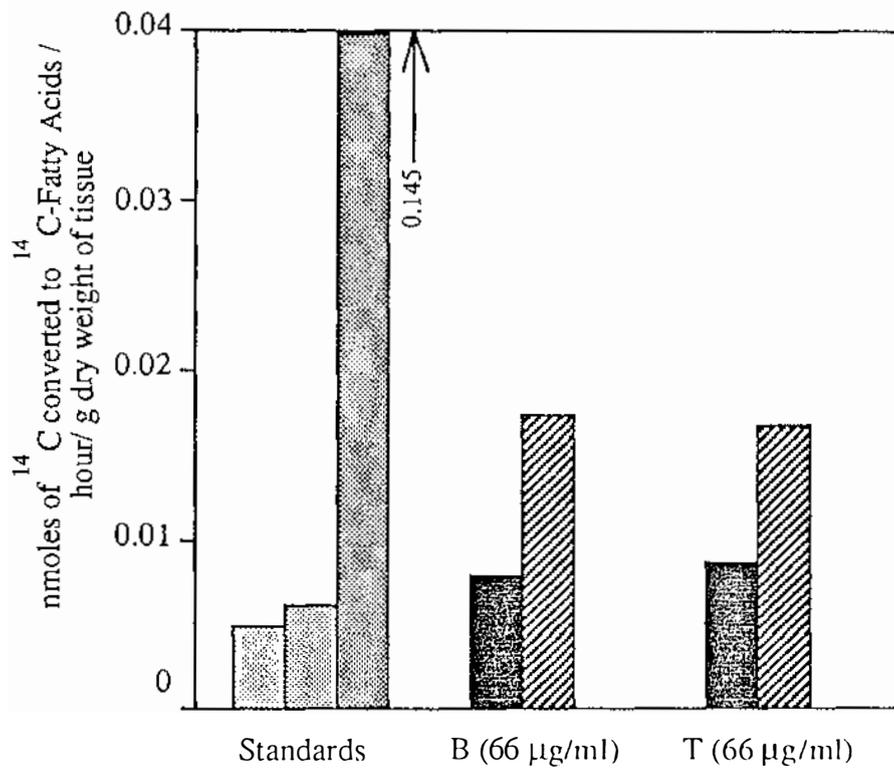


Figure 5.6b Effect of Crude GTF Extracts B & T on Insulin Stimulated Conversion of D-[1-<sup>14</sup>C]-glucose to <sup>14</sup>C-Fatty Acids



### Notes on Figures 5.7a and 5.7b

Adipocytes were isolated from rats (180-220 g) which had been maintained on the Torula yeast diet. Adipocytes were incubated for 90 minutes in pH 7.4, 2% Albumin:30 mM HEPES buffer that contained 0.5 mM glucose and 0.2  $\mu$ Ci of labeled substrate. Incubation temperature was 37°C and the flasks were shaken at 100 rpm. Total incubation volume was 1.5 ml. Results are the mean of triplicate estimations. Bars shown on the graph represent a blank (▣), 10  $\mu$ U/ml Insulin (▤), 100  $\mu$ U/ml Insulin (▥), the sample only (▦) and the sample in the presence of 10  $\mu$ U/ml Insulin (▧).

Figure 5.7a Effect 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 $_2$

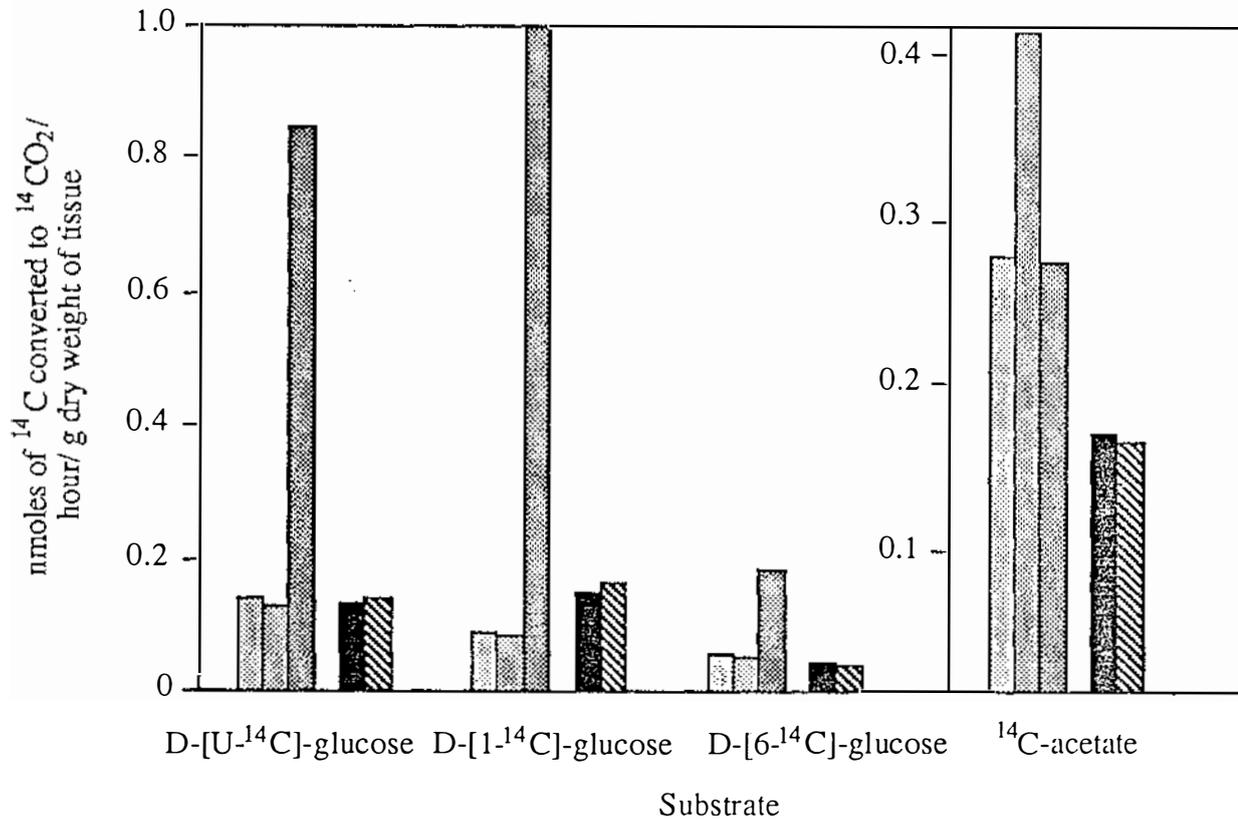
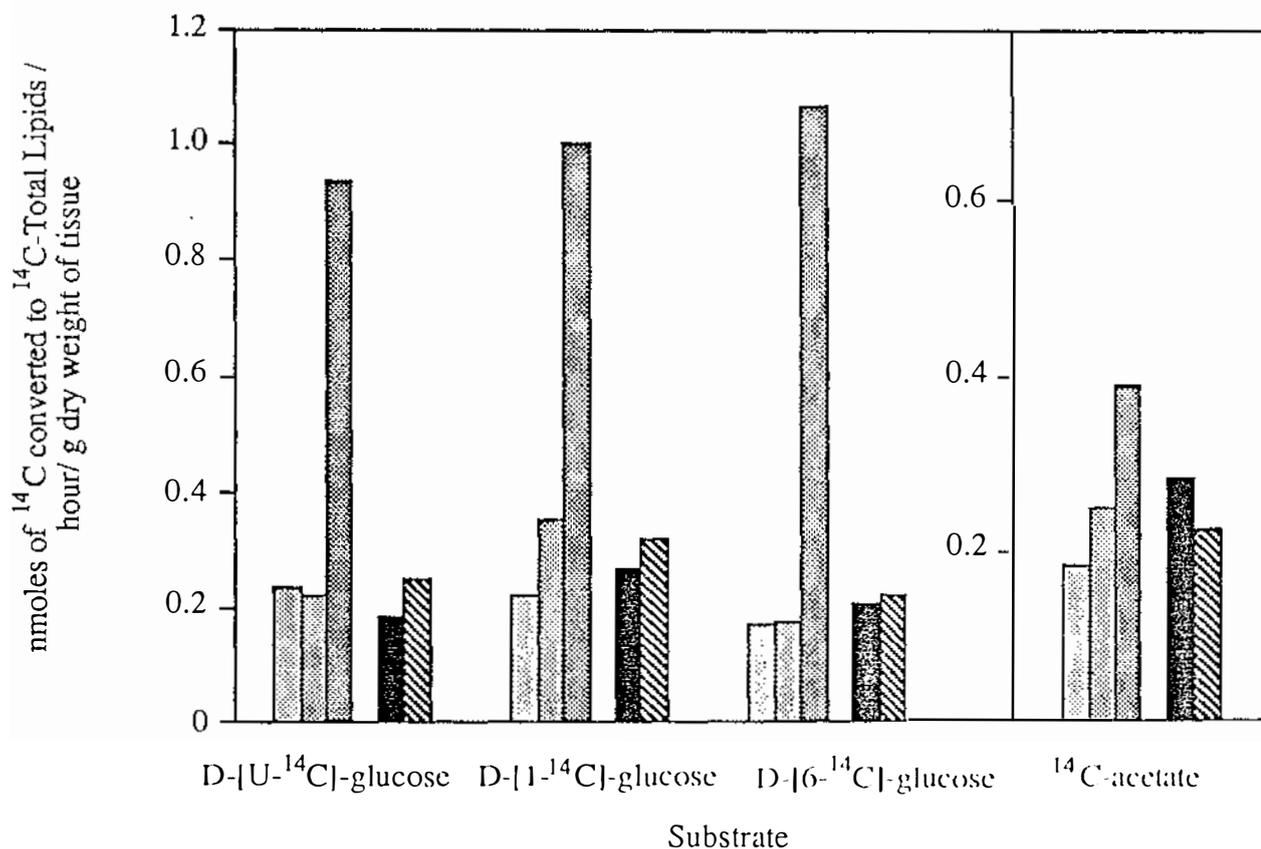


Figure 5.7b Effects of Crude GTF Extract B on the Insulin Stimulated Conversion of D-[U- $^{14}$ C]-glucose, D-[1- $^{14}$ C]-glucose, D-[6- $^{14}$ C]-glucose and  $^{14}$ C-acetate to  $^{14}$ C-Total Lipid



### Notes on Figures 5.7c and 5.7d

Adipocytes were isolated from rats (180-220 g) which had been maintained on the Torula yeast diet. Adipocytes were incubated for 90 minutes in pH 7.4, 2% Albumin:30 mM HEPES buffer that contained 0.5 mM glucose and 0.2  $\mu$ Ci of labeled substrate. Incubation temperature was 37°C and the flasks were shaken at 100 rpm. Total incubation volume was 1.5 ml. Results are the mean of triplicate estimations. Bars shown on the graph represent a blank (▣), 10  $\mu$ U/ml Insulin (▤), 100  $\mu$ U/ml Insulin (▥), the sample only (▦) and the sample in the presence of 10  $\mu$ U/ml Insulin (▧).

Figure 5.7c Effect of Crude GTF Extract B on the Insulin Stimulated Conversion of D-[U-<sup>14</sup>C]-glucose, D-[1-<sup>14</sup>C]-glucose, D-[6-<sup>14</sup>C]-glucose and <sup>14</sup>C-acetate to <sup>14</sup>C-Fatty Acids

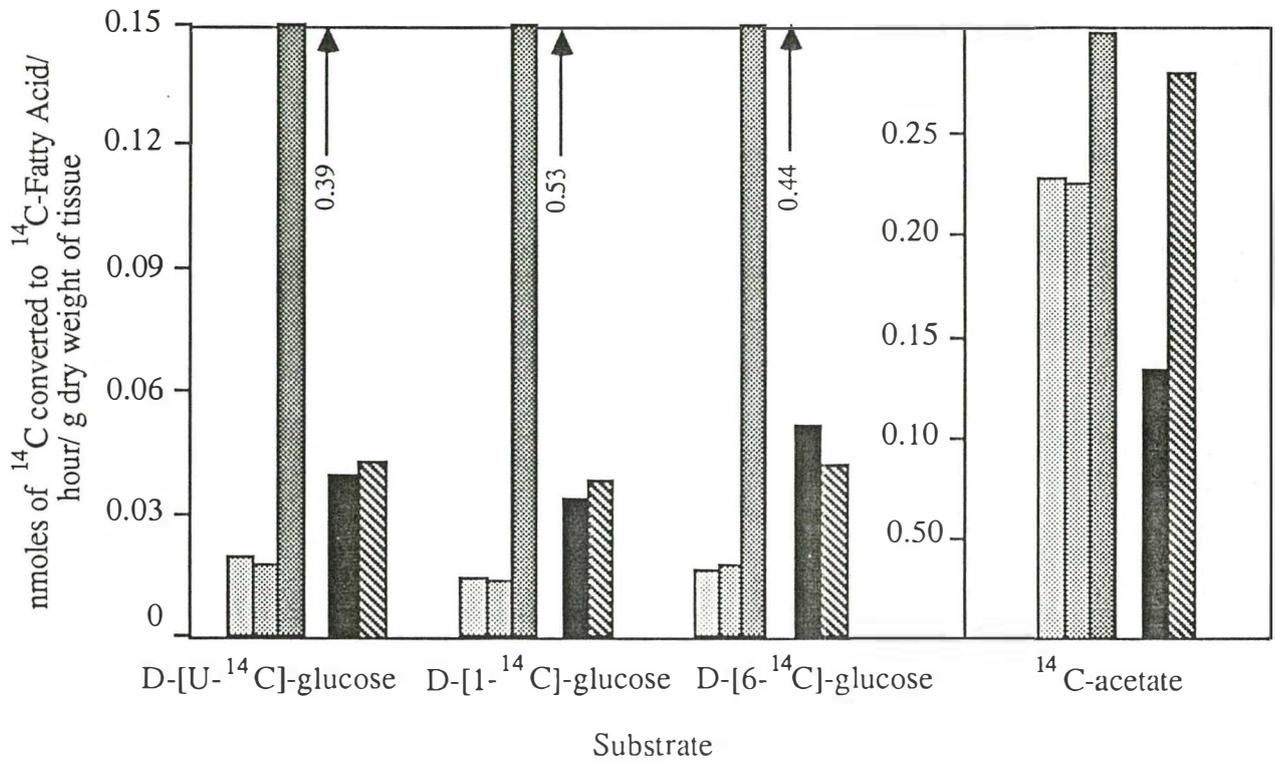
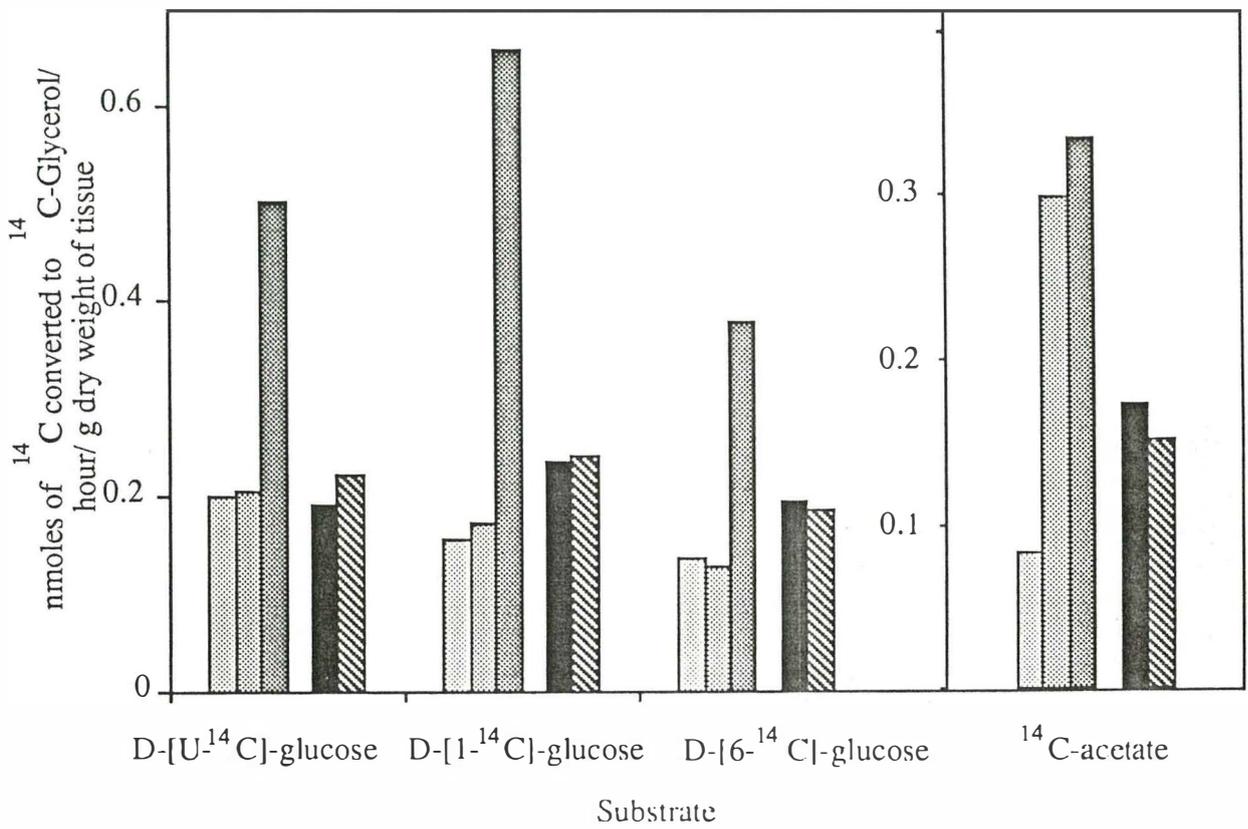


Figure 5.7d Effect of Crude GTF Extract B on the Insulin Stimulated Conversion of D-[U-<sup>14</sup>C]-glucose, D-[1-<sup>14</sup>C]-glucose, D-[6-<sup>14</sup>C]-glucose and <sup>14</sup>C-acetate to <sup>14</sup>C-Glycerol



in the conversion of  $^{14}\text{C}$  from D-[U- $^{14}\text{C}$ ]-glucose into  $^{14}\text{CO}_2$ . while a negative potentiation would have indicated that the sample was making insulin less effective in that role.

However it is important to realise that, while the extracts were giving real and significant potentiation of insulin's action, the absolute magnitude of this amplification of metabolism from which this potentiation was calculated was minimal when compared with the response elicited by maximal insulin concentrations (e.g. compare the diagonally shaded bar and the dark shaded bar in Figure 5.5a). It seems unlikely that such small amplifications of insulin's action could have any great physiological significance. While it is possible that using other conditions such as; more pure GTF fractions, or using lower glucose concentrations in the assay (Gliemann 1985), or using rats showing the greater deficiency symptoms observed by Schroeder (1964 & 1965) would lead to greater amplifications of the metabolism, this seems unlikely. If the material causing the potentiations mentioned above is GTF as originally defined, then it might be expected that much larger modulations of insulin action would be seen. This indicates that, while the material isolated in the current study potentiates the action of sub optimal amounts of insulin in the adipocytes of rats raised on a torula yeast diet, the properties of this material are not totally consistent with those originally postulated for GTF (Mertz 1969).

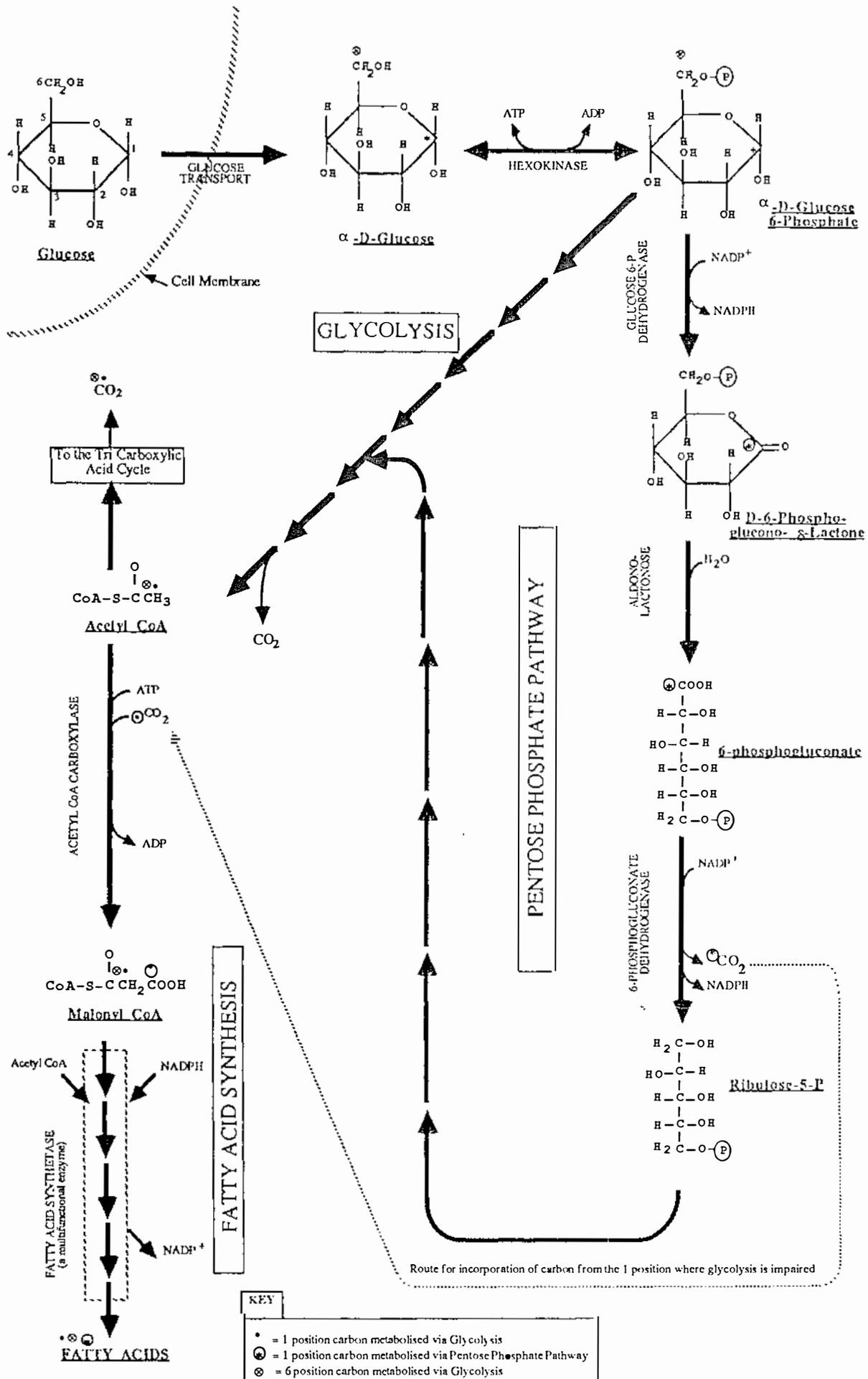
It is possible that substances, present in the crude yeast extracts, presently thought to have only insulinomimetic properties are responsible for the low levels of amplification of insulin action and hence the potentiations that are seen. Most insulinomimetic agents tested to date have been shown to cause an increase in  $V_{\text{max}}$ , indicating a mechanism similar to that of insulin (Simpson and Cushman 1986). These substances are normally thought to merely have an additive effect on the insulin stimulated metabolism of glucose. However if the formula used in this thesis to calculate potentiation of insulin action, was applied to the data of Taylor *et al* (1979), then at high insulin concentrations there is actually a potentiation of insulin stimulated glucose transport by adenosine and nicotinic acid. This potentiation was in the vicinity of 80% for an adenosine concentration of 10  $\mu\text{M}$  (2.6  $\mu\text{g/ml}$ ) and about 70% in the case of nicotinic acid at a concentration of 200  $\mu\text{M}$ , although for both substances the effect was only noted at a maximally stimulating insulin concentration of more than 40 ng/ml. This shows that in normal rat adipocytes some insulinomimetic agents are able to potentiate the action of optimal insulin concentrations. If these agents were causing the GTF effect they don't explain the observation that GTF only potentiates the action of sub optimal insulin concentrations and only then in tissue from rats fed a special diet. However it does show that it is possible that substances previously thought to only possess an insulinomimetic properties may

also be able to potentiate the action of sub optimal quantities of insulin and further investigations are required to see if any of these could be responsible for the properties of the crude GTF yeast extracts. A mechanism to explain how the insulinomimetic substances in yeast extracts act differently to other insulinomimetic agents has been postulated by Tokuda *et al.* (1987). This suggests that while most insulinomimetic substances act to increase  $V_{\max}$  for glucose transport, the insulinomimetic substances present in yeast extracts increase glucose transport by lowering the  $K_s$  for glucose. This would indicate that the yeast extract is acting to assist the binding of glucose to extant glucose transporters and, while such a direct stimulation of glucose transport by the yeast extract is not compatible with the defined mode of action of GTF as a potentiator of insulin stimulated glucose metabolism, it does show that components of GTF extracts do have a different mechanism for exerting metabolic effects. Overall the evidence does support the possibility that the potentiation effects of the yeast extracts, which are attributed to GTF, could be attributable to the effects of known insulinomimetic agents present in the yeast extracts.

#### **5.4.2 Observations About the Metabolism of Adipocytes Isolated From Rats on the Torula Yeast Diet**

Before looking for the sites of potentiation it is important to look at the metabolism of the adipocytes of rats on the torula yeast diet. As it is claimed that this diet is essential for GTF potentiations to be observed, there must be some unique feature of the metabolism of these cells which lends itself to the revelation of these potentiations. To investigate this, adipocyte assays were carried out using different isotopically labeled substrates and the the rate of conversion of  $^{14}\text{C}$  from these into  $^{14}\text{CO}_2$ ,  $^{14}\text{C}$ -fatty acids,  $^{14}\text{C}$ -total lipids and  $^{14}\text{C}$ -glycerol was determined. Universally labeled glucose (D-[U- $^{14}\text{C}$ ]-glucose) was used to study the overall potentiation of glucose metabolism as label from this appeared in all metabolic products of glucose. Glucose specifically labeled at either the 1 or 6 carbon was used to determine the relative flux of glucose through the glycolytic and pentose phosphate pathways. The initial rate at which label from glucose is converted to  $^{14}\text{CO}_2$  by the combined action of glycolysis and the tricarboxylic acid cycle is the same for both types of labeled glucose. However the  $^{14}\text{CO}_2$  produced initially from the action of the pentose phosphate pathway comes exclusively from D-[1- $^{14}\text{C}$ ]-glucose (Lehninger 1975). A comparison between the rates of conversion of  $^{14}\text{C}$  from these two glucose substrates into  $^{14}\text{CO}_2$  therefore gives an indication of the relative flux of glucose through these two pathways. The relevant routes by which  $^{14}\text{C}$  from the different types of labeled glucose are incorporated into various products is shown in Figure 5.8. As well as

Figure 5.8 Schematic Representation of Metabolism of Glucose to Fatty Acids in Adipocytes



isotopically labeled glucose,  $^{14}\text{C}$ -acetate was used to more specifically reveal if there were any effects on fatty acid synthesis.

Analysis of the raw data from these assays (Figure 5.7) shows some important information about the metabolism of the adipocytes. It is apparent that, while maximal insulin stimulates the metabolism of D-[U- $^{14}\text{C}$ ]-glucose and D-[1- $^{14}\text{C}$ ]-glucose to  $\text{CO}_2$  to a similar extent in absolute terms, insulin stimulation of the metabolism of D-[6- $^{14}\text{C}$ ]-glucose to  $\text{CO}_2$  is only about 1/5 of those levels (see dark shaded bars on Figure 5.7a). The disproportionate metabolism of D-[1- $^{14}\text{C}$ ]-glucose to  $\text{CO}_2$  is an indication of the relatively high level of the pentose phosphate pathway activity in adipocytes, which results in preferential release of  $^{14}\text{CO}_2$  from the 1 labeled carbon from hexose sugars (Kather *et al.* 1972a). The importance of the pentose phosphate pathway in adipose tissue is as the source of about 60% of the NADPH reducing equivalents used in the fatty acid synthesis pathways (Figure 5.8) which is very active in this tissue (Kather *et al.* 1972a). The remainder of the NADPH is presumed to come from the decarboxylation of malate catalysed by the malic enzyme (Conover 1987). Since D-[6- $^{14}\text{C}$ ]-glucose is mainly converted to  $^{14}\text{CO}_2$  by the combined action of the glycolytic pathway and the tri carboxylic acid cycle, the low rate of conversion of  $^{14}\text{C}$  into  $^{14}\text{CO}_2$  from D-[6- $^{14}\text{C}$ ]-glucose (Figure 5.7a) indicates the low level of activity of the tricarboxylic acid pathway in the adipocyte. Further it can be seen that  $^{14}\text{C}$  from both D-[1- $^{14}\text{C}$ ]-glucose and D-[6- $^{14}\text{C}$ ]-glucose were converted into  $^{14}\text{C}$ -total lipid,  $^{14}\text{C}$ -fatty acids and  $^{14}\text{C}$ -glycerol at similar rates under conditions of maximal insulin stimulation (see dark shaded bars in Figure 5.7b-d). These results closely match those of Sherriff (1983) who also used rats on the same torula yeast based diet. However the adipocytes from torula yeast rats differ from the metabolism of adipocytes from normal rats in one major area, that being the rate of conversion of label into fatty acids. In normal rats the rate of conversion of  $^{14}\text{C}$  from D-[6- $^{14}\text{C}$ ]-glucose into  $^{14}\text{C}$ -fatty acids is about twice the rate from D-[1- $^{14}\text{C}$ ]-glucose under basal conditions and this trend continues with insulin stimulation (Milstein 1956, Winegrad *et al.* 1958, Kather *et al.* 1972a & b, Taylor *et al.* 1979, Fried *et al.* 1981). A situation similar to that seen in the rats on the torula yeast diet can be chemically induced by blocking the electron transport system, and hence the glycolytic pathway, with rotenone (Kather *et al.* 1972b). When phenazine methosulphate is added, to reoxidise the NADPH, the pentose phosphate and the fatty acid synthesis pathways are able to proceed and an even more pronounced reversal of the previous preference for the incorporation of label into fatty acid from the 6 labeled glucose. In section 5.2 the assumption was made that there was no isotope effect and that the results from the isotopically labeled glucose would parallel the metabolism of glucose in the adipocyte. If this is indeed the case the results described above indicate that flux of carbon from glucose through the glycolytic

pathway for fatty acid synthesis is impaired in adipocytes of rats raised on the torula yeast diet which would account for the high rate of pentose phosphate pathway activity seen in these cells.

### **5.4.3 Site of Action of the Material Causing Potentiation of the Adipocyte Assay**

It is important to realise that the GTF extract used in the current study is a crude extract but is similar to that on which Toepfer (1977) *et al.* based many of their conclusions about the properties of GTF. While this fraction has been shown to be far from pure (Chapter 3) any conclusions drawn here about the site of action of GTF's action are valid as the aim is to explain the mechanism by which the effects attributed to GTF fractions are brought about. To explain the mechanism by which these effects are being brought about it is important to locate the site at which the potentiations occur. To do this a biochemical explanation was sought which would explain the major trends in the potentiation of sub optimal insulin action by extract B revealed in Table 5.2. On this basis it can be seen that significant positive potentiations were only achieved in the cases of the conversion of; D-[U- $^{14}\text{C}$ ]-glucose into  $^{14}\text{CO}_2$ ,  $^{14}\text{C}$ -fatty acid and  $^{14}\text{C}$ -total lipid, D-[1- $^{14}\text{C}$ ]-glucose into  $^{14}\text{CO}_2$  and  $^{14}\text{C}$ -fatty acid and  $^{14}\text{C}$ -acetate into  $^{14}\text{C}$ -fatty acids. The potentiation of the conversion of D-[1- $^{14}\text{C}$ ]-glucose into labeled product indicates that the potentiation by the crude yeast extract is of the incorporation of glucose into fatty acids via the pentose phosphate pathway.

The strong positive potentiation of the action of sub optimal insulin in the conversion of  $^{14}\text{C}$  from the tracer quantity of  $^{14}\text{C}$ -acetate into  $^{14}\text{C}$ -fatty acid indicates a potentiation of fatty acid synthesis. The significant negative potentiations of the conversion of  $^{14}\text{C}$  from  $^{14}\text{C}$ -acetate into glycerol,  $\text{CO}_2$  and total lipid confirms that the direction of the potentiation is through the pathway beginning with acetyl-CoA-carboxylase. These results further show that the potentiation of the conversion of acetate to fatty acids is at the expense of other fates of acetate such as the tri carboxylic acid cycle, which would result in  $\text{CO}_2$  evolution, or incorporation of label into the glycerol fraction.

However as discussed below it is impossible to say whether this is a direct potentiation of fatty acid synthesis by GTF.

The fact that GTF does not potentiate the conversion of  $^{14}\text{C}$  from D-[6- $^{14}\text{C}$ ]-glucose to  $^{14}\text{C}$ -fatty acids,  $^{14}\text{C}$ -glycerol or  $^{14}\text{CO}_2$  is significant and indicates that GTF is not potentiating the glycolytic pathway. This is consistent with the observation in section 5.4.2 that the glycolytic pathway appeared to be impaired in the adipocytes of rats raised on the torula yeast diet. This result is supported by the lack of potentiation of the

conversion of  $^{14}\text{C}$  from D-[1- $^{14}\text{C}$ ]-glucose to  $^{14}\text{C}$ -glycerol. Normally label from the one labeled glucose would be converted to labeled glycerol at the same rate as that of D-[6- $^{14}\text{C}$ ]-glucose. However it can be seen from Table 5.2 that there was no significant positive potentiation of either process indicating that there is no potentiation of the glycolytic pathway.

The yeast extract's specific potentiation of the metabolism of D-[U- $^{14}\text{C}$ ]-glucose and D-[1- $^{14}\text{C}$ ]-glucose leads to the conclusion that the potentiation effect of this extract is manifested as a potentiation of the action of the pentose phosphate pathway. This would be consistent with the observations described above that indicate that the metabolism of glucose via glycolysis is impaired and so the pentose phosphate pathway plays an increased role in the metabolism of glucose in these cells. Circumstantial support for this comes from the observations of Czech *et al.* (1977) that pentose phosphate pathway activity is diminished in the cells of older rats that have been fed ad libitum and since it is seen that the effect of GTF diminishes with the age of the rat (Anderson *et al.* 1979) this would indicate that an active pentose phosphate pathway is required to see a potentiation of insulin action in the adipose tissue.

However, in the pentose phosphate pathway carbons other than the one position carbon are reintroduced into glycolytic pathway at the level of fructose-6-phosphate and glyceraldehyde-3-phosphate. While potentiation of the conversion of  $^{14}\text{C}$  from D-[1- $^{14}\text{C}$ ]-glucose to  $^{14}\text{CO}_2$  and  $^{14}\text{C}$ -fatty acids indicates a potentiation of pentose phosphate pathway activity, the lack of potentiation of the conversion of label from D-[6- $^{14}\text{C}$ ]-glucose into  $^{14}\text{CO}_2$  and  $^{14}\text{C}$ -fatty acids indicates that this potentiation does not extend further than the evolution of  $\text{CO}_2$  by 6-phosphogluconate dehydrogenase (see Figure 5.8). The potentiation of the conversion of  $^{14}\text{C}$  from the one carbon in  $^{14}\text{C}$ -fatty acids would then only be possible by a corresponding potentiation of the reincorporation of  $^{14}\text{CO}_2$  at some later metabolic step such as pyruvate carboxylase or acetyl-CoA carboxylase. A scheme outlining how this could be accomplished in the adipocyte is shown in Figure 5.8.

However, while potentiation of the conversion of  $^{14}\text{C}$  from D-[1- $^{14}\text{C}$ ]-glucose to  $^{14}\text{CO}_2$  and  $^{14}\text{C}$ -fatty acids shows that pentose phosphate pathway is potentiated by the yeast extract, it is not necessarily directly potentiating the action of insulin on this pathway. If the pentose phosphate pathway is not being potentiated directly by GTF in adipocytes of rats on the torula yeast diet, then potentiation of the pentose phosphate pathway could conceivably occur indirectly in two ways:

## 1. A POST PENTOSE PHOSPHATE PATHWAY STIMULATION OF FATTY ACID SYNTHESIS

It is possible that GTF is potentiating the action of enzymes that would increase fatty acid synthesis and that act after the pentose phosphate pathway. A possible post pentose phosphate pathway site for an indirect potentiation of fatty acid synthesis by the yeast extract could be at pyruvate dehydrogenase and pyruvate carboxylase. Work by Holdsworth and Appleby (1984) showed that GTF stimulated pyruvate carboxylase and pyruvate dehydrogenase in yeast and other workers have shown that insulin has a direct stimulatory effect on the activity of pyruvate dehydrogenase (Seals and Jarett 1980). These observations suggest a possible mechanism for the potentiation of insulin stimulated fatty acid synthesis that is seen in the absence of any potentiation of the glycolytic pathway. This would involve potentiation of the reincorporation of the CO<sub>2</sub> evolved from the pentose phosphate pathway at the level of pyruvate carboxylase. This enzyme combines CO<sub>2</sub> and pyruvate to produce oxaloacetate which yields acetyl-CoA by the mechanisms of the tri carboxylic acid cycle and so if its action is potentiated then there is likely to be a corresponding increase in the amount of acetyl-CoA available for fatty acid synthesis.

However it is more likely that these results indicate a potentiation of insulin stimulation of the fatty acid synthesis enzyme systems. There is evidence that potentiation of fatty acid synthesis enzymes would concomitantly lead to the observed increase in pentose phosphate pathway activity. For example it has been reported that inhibition of fatty acid synthesis results in a decrease in insulin stimulation of the pentose phosphate pathway and of total glucose utilisation (Fried *et al.* 1981). Further it has been reported by Eggleston and Krebs (1974) that the activity of glucose-6-phosphate dehydrogenase, the first enzyme and a regulatory step in the metabolism of glucose by the pentose phosphate pathway, is dependant on the NADPH/NADP<sup>+</sup> ratio. Their results indicate that stimulation of fatty acid synthesis would lead to a secondary increase in pentose phosphate pathway activity by a lowering of the NADPH concentration. Further, adipocytes of older rats often have a deficient fatty acid synthesis metabolism (Fried *et al.* 1981) and it has been observed by Anderson *et al.* (1979) that while the potentiation of the action of sub optimal concentrations of insulin by GTF extracts only occurs in rats on the torula yeast diet, the potentiation effect is also only noted in rats under a certain age. These rats are likely to have a more active fatty acid synthesis metabolism than the older rats and it indicates that, as well as the requirement of an active pentose phosphate pathway mentioned above, that an active fatty acid synthesis pathway may also be a

requirement for adipocytes to show potentiations of insulin stimulated metabolism in response to GTF extracts.

A likely site of potentiation of fatty acid synthesis pathways is the enzyme acetyl-CoA carboxylase (see Figure 5.8). This is the first committed step in the *de novo* synthesis of fatty acids and is an important regulatory point in fatty acid synthesis (Wakil *et al.* 1983). This makes it a likely site for the potentiation of fatty acid synthesis, and as discussed above this would lead to an activation of glucose-6-phosphate dehydrogenase and hence a potentiation of CO<sub>2</sub> evolution in the pentose phosphate pathway. Acetyl-CoA carboxylase is positively stimulated by citrate and by the dephosphorylation of the enzyme, while phosphorylation of the enzyme or a high acyl-CoA concentration inhibits it (Wakil *et al.* 1983). The enzyme is also effected by a number of compounds including guanine nucleotides and coenzyme-A (Wakil *et al.* 1983) and so it is possible that compounds found in the yeast extract could effect the enzyme. Further, exposure of adipocytes to insulin results in a rapid increase in the activity of acetyl-CoA carboxylase (Wakil *et al.* 1983) thus suggesting that this enzyme would offer a likely site for the potentiation of insulin action caused by the yeast extracts.

## 2. BY POTENTIATION OF GLUCOSE TRANSPORT.

That potentiation of glucose transport into the adipocyte would lead to a potentiation of the activity of the pentose phosphate pathway is indicated by several observations. It has been observed by Kather *et al.* (1972a) that the contribution of the pentose phosphate pathway to the metabolism of glucose in adipocytes increases linearly with increases in glucose transported into the cell and of fatty acids synthesised. It was concluded from this work that the rate limiting step for the metabolism of glucose in the adipocyte was transport. This work is supported by the observations of Taylor *et al.* (1976) who demonstrated that, if glycolysis was blocked using fluoride and if phenazinemethosulphate was added to enable the rapid re oxidation of the NADPH produced by the pentose phosphate pathway then, the rate of <sup>14</sup>CO<sub>2</sub> evolution from D-[1-<sup>14</sup>C]-glucose was linearly related to the rate of glucose transport.

Early experiments by Mertz and co-workers claimed to locate the site of action of chromium, and hence of GTF, as being at the transport of glucose across the plasma membrane. These experiments indicated that low levels of chromium augmented the insulin stimulated transport of glucose (Mertz *et al.* 1961) and of galactose (Mertz & Roginski 1963) in the fat pads of rats raised on a torula yeast diet, whereas the low levels of chromium alone had no effect. Since those reports much work has

concentrated on trying to prove that GTF extracts could act to potentiate insulin in its stimulation of glucose transport (Mertz 1969). However later work by the same group indicated that, while yeast GTF extracts did cause a small potentiation of insulin stimulated transport of 2-deoxyglucose into adipocytes from rats raised on the torula yeast diet, it was insufficient to explain the observed potentiation of the metabolism (Anderson *et al.* 1979). However their results are called into question by observations that using 2-deoxyglucose to measure glucose transport is only valid when using very low glucose concentrations (Gliemann 1985). While the glucose concentration used in Mertz's measurements is not given, if it is assumed that it is the same as that used by Anderson (1978a), then it is far too high for accurate measurement of glucose transport by this method. Further the use of 2-deoxyglucose as a measure of glucose transport may not be valid in tissue other than that from normal animals (Gliemann 1985). This means that the overall conclusion that glucose transport is not stimulated by GTF extracts is suspect. Recent work by Tokuda *et al.* (1987) has shown that a crude GTF extract from brewers yeast had a marked insulin like effect on the transport of 3-O-methylglucose into the isolated epididymal adipocytes of rats fed a normal diet. Basal 3-O-methylglucose transport was stimulated 2.2 fold in the absence of insulin but no such effect was noted in the presence of insulin, indicating a lack of insulin potentiating activity. Overall the results of studies of the effect of GTF on the insulin stimulated uptake of glucose in adipocytes are not conclusive and this would be an important area for future research.

It is currently thought that, in insulin sensitive tissue, the rate of glucose transport into the adipocyte is regulated by the number of glucose transporters present in the plasma membrane (Simpson and Cushman 1986). It is thought that insulin stimulates glucose transport by increasing the production of these transporters in the microsomes and the translocation of these to the plasma membrane (thus increasing  $V_{max}$ ), rather than by increasing the affinity of the transporter for glucose (which would increase  $K_m$ ). It is most likely that if GTF extracts potentiate insulin stimulated glucose transport it would be this process that would be potentiated.

Overall the results presented in this section support the hypothesis that the potentiation by GTF extracts of the action of sub optimal insulin concentrations is caused by a potentiation of either glucose transport or acetyl-CoA carboxylase, both of which would lead to a concomitant potentiation of the pentose phosphate pathway. The lowering of glycolytic contribution to fatty acid synthesis in adipocytes of rats on the torula yeast diet may explain why the potentiation by GTF is only obvious in rats on this and similar diets as stimulation of glucose transport or fatty acid synthesis is only likely to have a direct

stimulatory effect on the relatively unregulated pentose phosphate pathway as discussed above.

#### **5.4.4 Implications of Investigations of the Site of Action of GTF Fractions for the Adipocyte Assay System**

One of the aims of this work is the development of a more specific assay for GTF activity. The data presented here indicates that using D-[1-<sup>14</sup>C]-glucose is at least as sensitive as using D-[U-<sup>14</sup>C]-glucose as a substrate. While earlier workers (Mertz *et al.* 1965b, Roginski *et al.* 1970) used D-[1-<sup>14</sup>C]-glucose as a substrate they don't give any reasons for using this isotope and in later assay procedures described by the same group D-[U-<sup>14</sup>C]-glucose was used with no comment being made as to the reasons for doing this. It is assumed that initial use of D-[1-<sup>14</sup>C]-glucose was purely coincidental and does not reflect any appreciation for the important role of the one labeled glucose in measuring the GTF induced potentiations. More purposeful investigations of the potentiation by GTF of the metabolism of the carbon labeled in the one position are described by Sherriff (1983) although this work did not advocate that D-[1-<sup>14</sup>C]-glucose be used as a regular substrate in the assay.

If as indicated that pentose phosphate pathway activity is stimulated by GTF, then use of D-[1-<sup>14</sup>C]-glucose would be a more specific measure of GTF activity. In further assays it was decided to define GTF activity as the ability to potentiate the insulin stimulated metabolism of both D-[U-<sup>14</sup>C]-glucose and D-[1-<sup>14</sup>C]-glucose into both CO<sub>2</sub> and fatty acids in the rats raised on the torula yeast diet. The part that different diets play in the GTF effect is discussed in the next chapter.

#### **5.4.5 Implications of the Potentiation Activity Shown by the Crude Extract From Torula Yeast**

A potentiation of the action of suboptimal insulin by extracts from brewers yeast is expected and is consistent with other investigations of GTF (Toepfer *et al.* 1977). However the results of potentiation calculations (Table 5.1) clearly show that crude extract from torula yeast, T, also caused significant potentiation in the adipocytes of rats raised on the torula yeast diet and by this definition it too is showing GTF activity. This confirms the activity that this fraction had in the standard yeast assay (Section 3.3.1.2) and this is the first reported case of an extract from torula yeast having activity in the low chromium epididymal adipocyte assay. This result further calls into question the assumption that the substance causing potentiation of insulin action in the adipocyte assay is the same substance which was originally defined as GTF (Mertz 1969). GTF activity

in a fraction was originally defined by the ability of a fraction to reverse the effects that were supposedly caused by a dietary deficiency of GTF. It is claimed that raising rats on the torula yeast based diet produces adipocytes in which potentiation by GTF will be observed and so by definition that diet is deficient in GTF (Anderson *et al.* 1978a). It follows then that as torula yeast is the only source of protein in those diets that torula yeast is itself deficient in GTF. However extracts from torula yeast potentiate the action of sub optimal quantities of insulin in the adipocytes of rats that have been raised on a torula yeast based diet and this indicates that this assay may not be measuring GTF activity as originally defined. This means that it is important to resolve whether the torula yeast is the component of the diet responsible for the properties of the adipocytes of animals raised on that diet. A fuller investigation is required of the importance of diet in the production of adipocytes in which these potentiations can be observed and this is the basis of the next chapter.

## CHAPTER 6

# THE EFFECT OF DIET ON THE RESPONSE OF THE RAT EPIDIDYMAL ASSAY SYSTEM TO GTF

### 6.1 Introduction

The dietary state of animals has always been postulated as being an important determinant of whether the adipocytes obtained from the animals would show a potentiation of the action of sub optimal amounts of insulin in response to the addition of a GTF extract (Section 1.2.2). Early workers used a high sucrose diet which included either casein or torula yeast as a protein source (Mertz 1969) to induce a state where such potentiations could be observed., although later workers have mainly used torula yeast as the protein source (Anderson *et al.* 1978a, Davies *et al.* 1985a) It was suggested that the reason that this diet was required to see the potentiation effect by GTF extracts was that the diet was deficient in GTF and thus the tissue from animals on this diet were more responsive to extracts containing GTF (Mertz 1969). As torula yeast was the sole source of protein in the diet it was assumed that this must be deficient in GTF. However work in chapter 5 showed that an extract from torula yeast exhibited a similar amount of insulin potentiating activity to an extract of brewers yeast, a traditional source of GTF, in adipocytes of rats grown on a torula yeast based diet. This indicates that either the torula yeast is not deficient in GTF as had been assumed or that the assay was not measuring GTF activity as originally defined.

The importance of having a special diet for observing GTF activity has been clouded by several observations. The results of Wooliscroft and Barbosa (1977) indicate that the apparent impairment of glucose tolerance in rats on a torula yeast diet may only be an artifact of the methods used to measure glucose tolerance. Supporting this is the observation by Donaldson *et al.* (1985) that there was no difference in the glucose tolerance between a group of rats fed a low chromium diet and those on a normal stock diet. This could however reflect the use of casein as a protein source rather than torula yeast. Further, Hwang *et al.* (1987) have recently reported obtaining a GTF response in the adipocytes of normal rats. However contradicting this is the work by Sherriff (1983) which indicated that no GTF response could be seen in the adipocytes of normal rats.

This confusion about the importance of diet to the GTF response in the adipocyte assay lead to the investigation described in this chapter of the effect of 4 different diets on the adipocyte assay system.

## **6.2 Methods and Materials**

### **6.2.1 Animals and Diet**

Animals were raised as described in section 5.2.1 except for different diets. The torula yeast diet (TD 83155, Torula Yeast Diet for Chromium Studies) was supplied by Teklad, Madison, Wisconsin, the composition of which is specified by the manufacturer and is shown in appendix 1. The casein and brewers yeast based diets were prepared in our laboratory using the same formula as in appendix 1 but with a different protein source. For the alternative protein source brewers yeast was kindly supplied by Dominion Breweries, Mangatainoka, New Zealand and casein was obtained from the New Zealand Cooperative Dairy Company, Hamilton, New Zealand. To simplify diet preparation, corn oil (AMCO, Auckland, New Zealand) was used in the diet prepared in our laboratory in place of the lard used in the Teklad diet. The source of all other components used is shown in appendix 1. The normal diet used was Harvey Farms Rat Nuts (Harvey Farms, Palmerston North, New Zealand).

Prior to mixing of components, sucrose was powdered using a ceramic ball mill. Diets were prepared by mixing the dry ingredients by shaking in plastic bag. The mixed ingredients were then placed in a large plastic vat and the liquid ingredients were mixed in a minimum volume of ethanol and sprayed in a fine mist onto the surface of the diet. The mixture was then thoroughly mixed using a plastic stirrer. The diets were all stored at 4 °C.

### **6.2.2 Adipocyte Assay**

The adipocyte assay for GTF activity was carried out as described in section 5.2.2. Comparisons were made between the insulin stimulated metabolism of D-[U-<sup>14</sup>C]-glucose, D-[1-<sup>14</sup>C]-glucose and D-[6-<sup>14</sup>C]-glucose, to CO<sub>2</sub>, total lipid, fatty acid and glycerol. For the purposes of testing the potentiation of insulin action by GTF, crude GTF extract B was used at a final concentration of 66 µg/ml, as this had shown significant levels of activity in Chapter 5.

### **6.2.3 Amino Acid Analysis**

Amino acid analysis was carried out as described in section 3.2.5 except for determination of tryptophan. To determine tryptophan a sample of diet was ground using a ceramic mortar and pestle. About 50 mg of this was hydrolysed in a screwtop teflon vial under nitrogen in 4.3 M LiOH at 100 °C for 18 hours. This was then appropriately diluted to give a pH of about 4 and then applied to the amino acid analyser column as previously described.

### **6.2.4 Trace Metal Analysis**

Samples of the diets were ground to a powder in a ceramic mortar and pestle, which had been previously washed in concentrated HNO<sub>3</sub> (BDH analar). Samples were ashed in new beakers which had been washed in the same manner as the mortar and pestle. The sample was first wet ashed in 50:50 concentrated H<sub>2</sub>SO<sub>4</sub>:HNO<sub>3</sub> (both BDH analar) by boiling almost to dryness. The residue was then dry ashed in a muffle furnace at 600 °C for 18 hours. The trace elements were taken up for analysis by soaking the ash in 2 M HCl (BDH analar) overnight. Trace metal analysis was carried out using inductively coupled argon plasma emission spectroscopy as described in section 3.2.6.

## **6.3 Results**

### **6.3.1 Growth Rate of Rats**

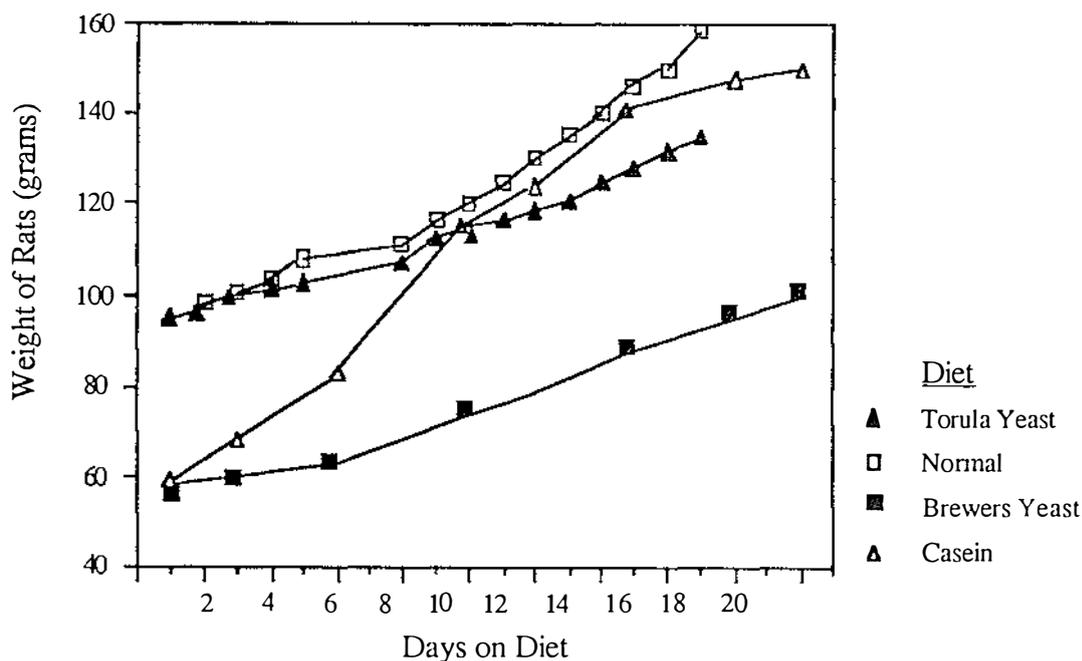
A comparison of growth rates of rats on the 4 different diets is shown in Figure 6.1. It can be seen from these results that rats on the torula yeast diet and on the brewers yeast diets grew at a similar rate but which was lower than that of rats on the normal rat diet (Harvey Farms) and the casein based diet. Rats on these latter two diets grew at approximately the same rate from about 1 week onwards.

### **6.3.2 Adipocyte Assays**

The results of the adipocyte assays on tissue from the rats on the torula yeast diet were shown previously in Figure 5.7. The results of assays on adipocytes of rats from the normal, brewers yeast and casein diets are shown in Figures 6.2-6.4 respectively. The potentiations of the action of the sub optimal insulin concentration, calculated from this data are shown in Table 6.1. The results are the average of at least three determinations and the errors shown indicate the variance of these results. As in Chapter 5, these results indicate some significant potentiations by GTF although the absolute magnitude of the amplification of metabolism involved was again not large. However these results show that the most consistently significant potentiations by GTF were in the tissue of rats raised

on the torula yeast diet. This effect was most pronounced in the potentiation of the conversion of D-[U- $^{14}\text{C}$ ]-glucose and D-[1- $^{14}\text{C}$ ]-glucose to  $\text{CO}_2$  and fatty acids.

Figure 6.1 Comparison of Growth Rates of Rats on Different Diets



### 6.3.3 Amino Acid Analysis

The results of the amino acid analysis of the diets were calculated as  $\mu\text{moles}$  of amino acid per gram dry weight of solid and also as percentage by dry weight of solids and they are shown in Table 6.2. Tryptophan levels were not estimated for the casein diet and the brewers yeast diet as at the time of analysis insufficient amounts of these diets remained.

### 6.3.4 Trace Element Analysis of the Diets

The inductively coupled argon plasma emission spectrometer was calibrated for measuring 23 elements simultaneously. This leads to compromises in optimum detection parameters which in turn leads to decreased sensitivity to some elements such as chromium. This means that levels of chromium below  $0.2 \mu\text{g/g}$  could not be determined reliably. To determine the accuracy of the system for measuring chromium in this type of matrix triplicate samples of a certified standard brewers yeast (National Bureau of Standards, Washington DC, Reference Material 1569) were analysed. This analysis

indicated a chromium composition of 2.02  $\mu\text{g/g}$  as opposed to the certified composition of 2.12  $\mu\text{g/g}$ .

High levels of emission from the high concentrations of phosphorous, sulphur and calcium resulted in the potential for suppression of response from other elements. The method of standard additions was used to correct for this interference, with a 4  $\mu\text{g/ml}$  standard of cadmium being used for this. The results of the analysis are shown in Table 6.3.

Table 6.1 Potentiation, by Crude GTF Fraction B, of the Action of Sub Optimal Insulin Concentration in Adipocytes of Rats Raised on Different Diets

Product	Substrate	Normal Diet	Casein Diet	Brewers Yeast Diet	Torula Yeast Diet
$^{14}\text{CO}_2$	U- $^{14}\text{C}$ -glucose	$-15 \pm 10\%$	$15 \pm 10\%$	$-5 \pm 10\%$	$30 \pm 10\%$
	1- $^{14}\text{C}$ -glucose	$30 \pm 15\%$	$5 \pm 10\%$	$25 \pm 15\%$	$30 \pm 10\%$
	6- $^{14}\text{C}$ -glucose	$-5 \pm 10\%$	$10 \pm 10\%$	$-25 \pm 15\%$	$0 \pm 15\%$
$^{14}\text{C}$ -Total Lipid	U- $^{14}\text{C}$ -glucose	$25 \pm 15\%$	$25 \pm 15\%$	$5 \pm 10\%$	$25 \pm 15\%$
	1- $^{14}\text{C}$ -glucose	$10 \pm 10\%$	$10 \pm 15\%$	$10 \pm 10\%$	$0 \pm 15\%$
	6- $^{14}\text{C}$ -glucose	$5 \pm 10\%$	$-55 \pm 20\%$	$15 \pm 10\%$	$5 \pm 10\%$
$^{14}\text{C}$ -Fatty Acids	U- $^{14}\text{C}$ -glucose	$-10 \pm 15\%$	$-15 \pm 20\%$	$30 \pm 25\%$	$40 \pm 15\%$
	1- $^{14}\text{C}$ -glucose	$10 \pm 10\%$	$-15 \pm 15\%$	$-10 \pm 15\%$	$40 \pm 15\%$
	6- $^{14}\text{C}$ -glucose	$-10 \pm 20\%$	$0 \pm 15\%$	$0 \pm 10\%$	$-50 \pm 20\%$

## 6.4 Discussion

### 6.4.1 Relationship Between Growth Rate and GTF Response

It is very interesting to note that there does not seem to be any necessary correlation between the growth rate of the rats and the ability of the metabolism of the adipocytes of those rats to respond to GTF extracts. This is shown by the fact that while the torula yeast diet results in an impaired growth rate and tissue that responds to GTF, rats on the brewers yeast diet had a rate of growth similar to that of rats on the torula yeast diet but the adipocytes of these rats did not respond as consistently to GTF extracts. Further, the normal diet and the casein diet resulted in rats that grew at a faster rate but the adipocytes

Table 6.2 Amino Acid Analysis of Diets(Results are expressed in  $\mu$ moles of amino acid gram of solid and percentage of solid by weight).

Amino Acid	Normal Diet	Casein Diet	Brewers Yeast Diet	Torula Yeast Diet
Aspartic Acid/ Asparagine	105 (1.35%)	135 (1.8%)	109 (1.4%)	195 (2.1%)
Threonine	50 (0.60%)	91 (1.1%)	60 (0.6%)	95 (1.1%)
Serine	60 (0.65%)	128 (1.3%)	66 (0.7%)	91 (0.9%)
Glutamic Acid/ Glutamine	222 (3.18%)	404 (5.9%)	122 (1.8%)	265 (3.9%)
Proline	102 (1.14%)	242 (2.8%)	47 (0.5%)	55 (0.6%)
Glycine	85 (0.65%)	62 (0.5%)	87 (0.7%)	171 (1.3%)
Tryptophan	8 (0.16%)	-	-	10 (0.2%)
Alanine	76 (1.10%)	87 (0.8%)	103 (0.9%)	154 (2.2%)
Valine	75 (0.80%)	154 (1.8%)	70 (0.8%)	130 (1.5%)
Methionine	20 (0.30%)	54 (0.8%)	23 (0.3%)	55 (0.8%)
Isoleucine	51 (0.65%)	106 (1.4%)	50 (0.6%)	102 (1.3%)
Leucine	115 (1.50%)	190 (2.5%)	75 (1.0%)	149 (1.9%)
Tyrosine	30 (0.55%)	73 (1.3%)	26 (0.5%)	51 (1.0%)
Phenylalanine	45 (0.75%)	79 (1.3%)	33 (0.6%)	70 (1.1%)
Lysine	65 (0.94%)	141 (2.1%)	74 (1.1%)	142 (2.1%)
Histidine	25 (0.40%)	49 (0.7%)	20 (0.3%)	55 (0.8%)
Arginine	35 (0.60%)	60 (1.0%)	58 (1.0%)	80 (1.4%)

Table 6.3 Trace Element Composition of the Diets as Determined by ICP.(Results Expressed as  $\mu$ g/g dry weight)

Element	Normal Diet	Casein Diet	Brewers Yeast Diet	Torula Yeast Diet
Aluminium	51.3	3.6	16.4	13.7
Chromium	0.65	>0.6	>0.3	0.6
Copper	7.0	13.0	13.5	12.1
Iron	20.7	79.4	66.8	130.0
Manganese	31.1	35.0	40.0	5.9
Nickel	1.5	>0.8	0.9	0.2
Tin	0.2	>0.6	0.6	0.2
Zinc	54.8	11.7	11.4	26.7

Notes on Figures 6.2a and 6.2b

Adipocytes were isolated from rats (180-220 g) which had been maintained on the Normal stock diet. Adipocytes were incubated for 90 minutes in pH 7.4, 2% Albumin:30 mM HEPES buffer that contained 0.5 mM glucose and 0.2  $\mu$ Ci of labeled substrate. Incubation temperature was 37°C and the flasks were shaken at 100 rpm. Total incubation volume was 1.5 ml. Results are the mean of triplicate estimations. Bars shown on the graph represent a blank (▨), 10  $\mu$ U/ml Insulin (▩), 100  $\mu$ U/ml Insulin (▧), the sample only (■) and the sample in the presence of 10  $\mu$ U/ml Insulin (▨).

Figure 6.2a Effect of Crude GTF Extract B on the Insulin Stimulated Conversion of D-[U-<sup>14</sup>C]-Glucose, D-[1-<sup>14</sup>C]-Glucose and D-[6-<sup>14</sup>C]-Glucose into <sup>14</sup>CO<sub>2</sub> in Adipocytes Isolated From Rats on a Normal Diet

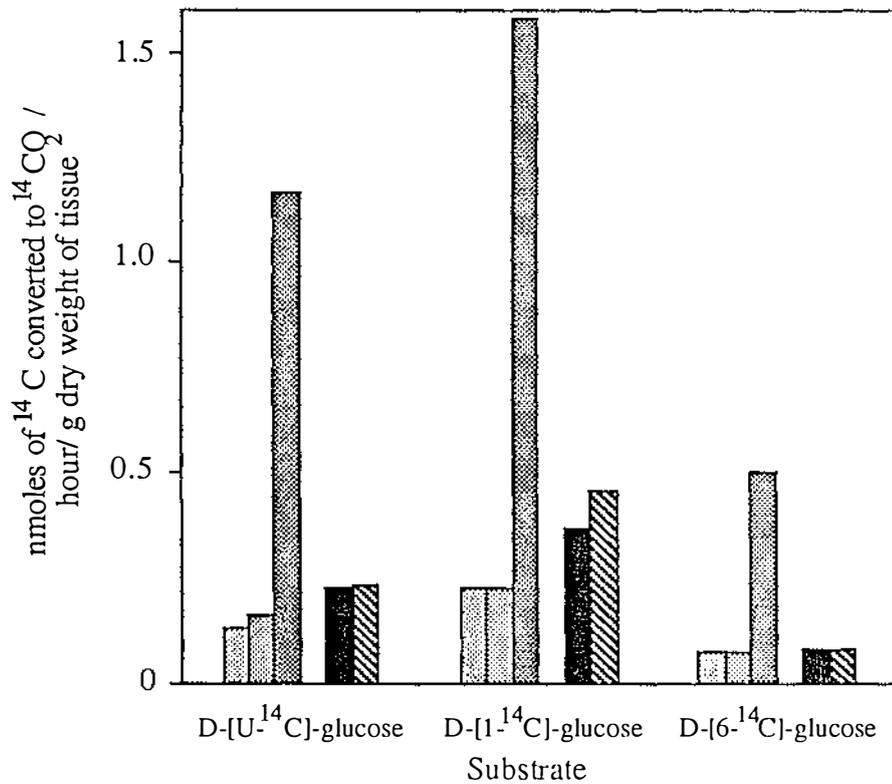
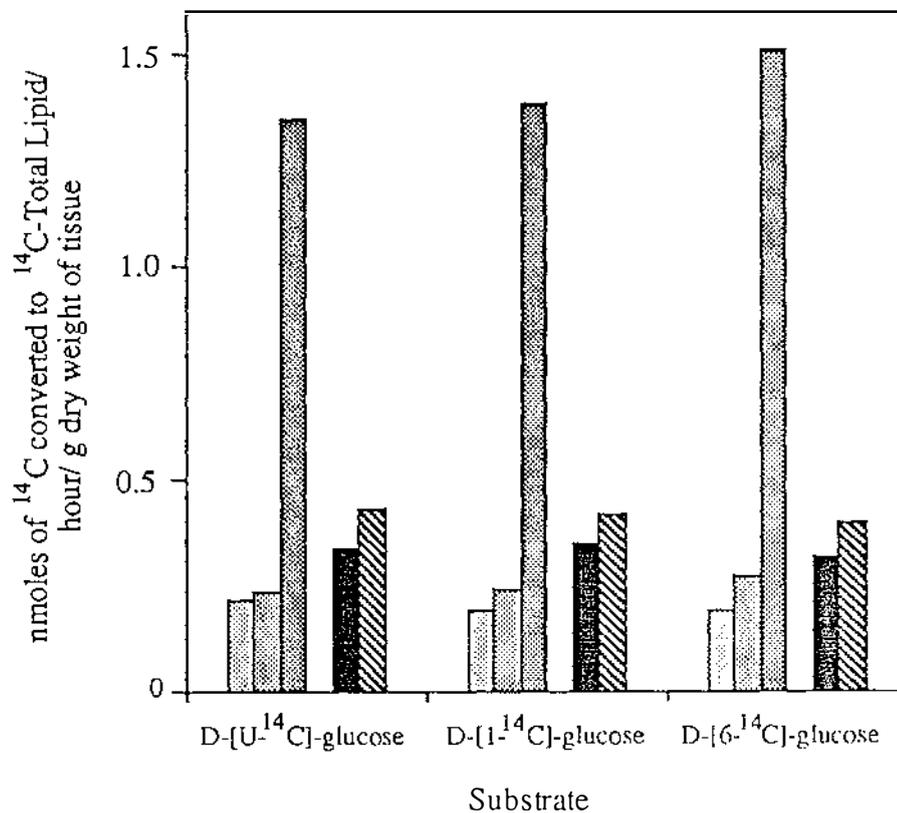


Figure 6.2b Effect of Crude GTF Extract B on the Insulin Stimulated Conversion of D-[U-<sup>14</sup>C]-Glucose, D-[1-<sup>14</sup>C]-Glucose and D-[6-<sup>14</sup>C]-Glucose into <sup>14</sup>C-Total Lipid in Adipocytes Isolated From Rats on a Normal Diet



#### Notes on Figures 6.2c and 6.2d

Adipocytes were isolated from rats (180-220 g) which had been maintained on the Normal stock diet. Adipocytes were incubated for 90 minutes in pH 7.4, 2% Albumin:30 mM HEPES buffer that contained 0.5 mM glucose and 0.2  $\mu$ Ci of labeled substrate. Incubation temperature was 37°C and the flasks were shaken at 100 rpm. Total incubation volume was 1.5 ml. Results are the mean of triplicate estimations. Bars shown on the graph represent a blank (▣), 10  $\mu$ U/ml Insulin (▤), 100  $\mu$ U/ml Insulin (▥), the sample only (▦) and the sample in the presence of 10  $\mu$ U/ml Insulin (▧).

Figure 6.2c Effect of Crude GTF Extract B on the Insulin Stimulated Conversion of D-[U-<sup>14</sup>C]-Glucose, D-[1-<sup>14</sup>C]-Glucose and D-[6-<sup>14</sup>C]-Glucose to <sup>14</sup>C-Fatty Acid in Adipocytes Isolated From Rats on a Normal Diet

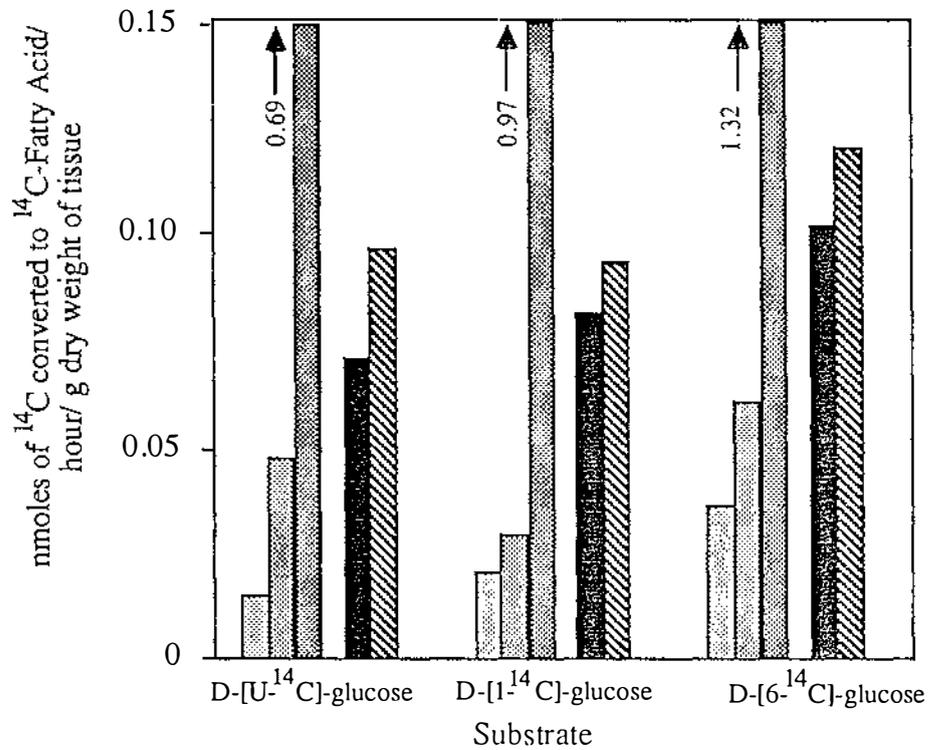
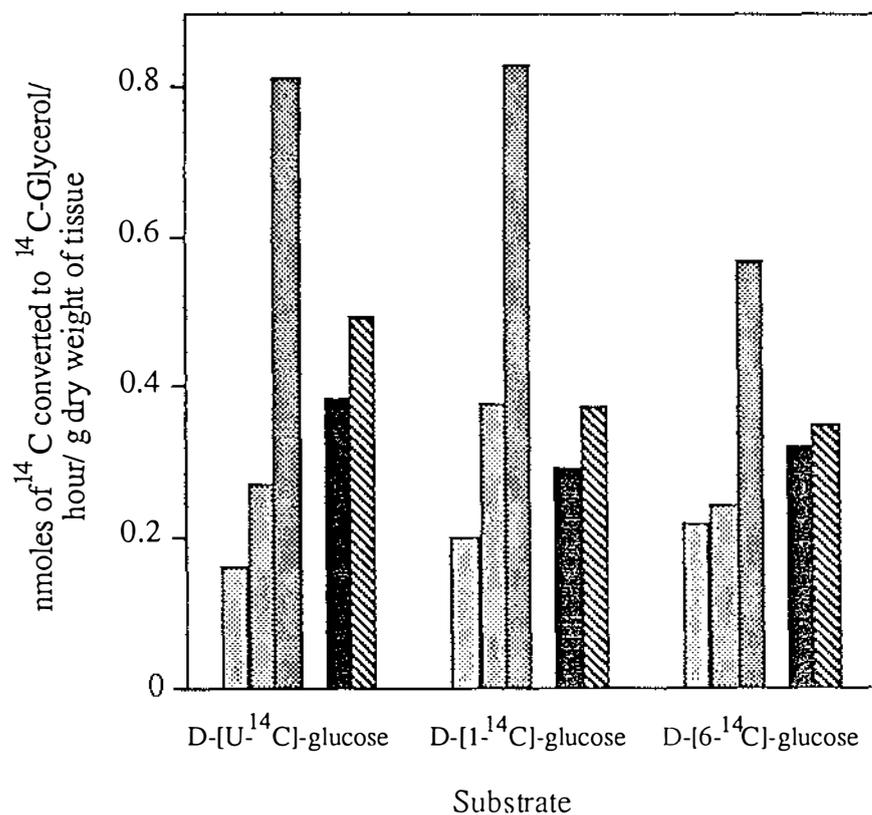


Figure 6.2d Effect of Crude GTF Extract B on the Insulin Stimulated Conversion of D-[U-<sup>14</sup>C]-Glucose, D-[1-<sup>14</sup>C]-Glucose and D-[6-<sup>14</sup>C]-Glucose into <sup>14</sup>C-Glycerol in Adipocytes Isolated From Rats on a Normal Diet



### Notes on Figures 6.3a and 6.3b

Adipocytes were isolated from rats (180-220 g) which had been maintained on the Brewers yeast diet. Adipocytes were incubated for 90 minutes in pH 7.4, 2% Albumin:30 mM HEPES buffer that contained 0.5 mM glucose and 0.2  $\mu$ Ci of labeled substrate. Incubation temperature was 37°C and the flasks were shaken at 100 rpm. Total incubation volume was 1.5 ml. Results are the mean of triplicate estimations. Bars shown on the graph represent a blank (■), 10  $\mu$ U/ml Insulin (■), 100  $\mu$ U/ml Insulin (■), the sample only (■) and the sample in the presence of 10  $\mu$ U/ml Insulin (■).

Figure 6.3a Effect of Crude GTF Extract B on the Insulin Stimulated Conversion of D-[U-<sup>14</sup>C]-Glucose, D-[1-<sup>14</sup>C]-Glucose and D-[6-<sup>14</sup>C]-Glucose to <sup>14</sup>CO<sub>2</sub> in Adipocytes Isolated From Rats on a 30% Brewers Yeast Diet

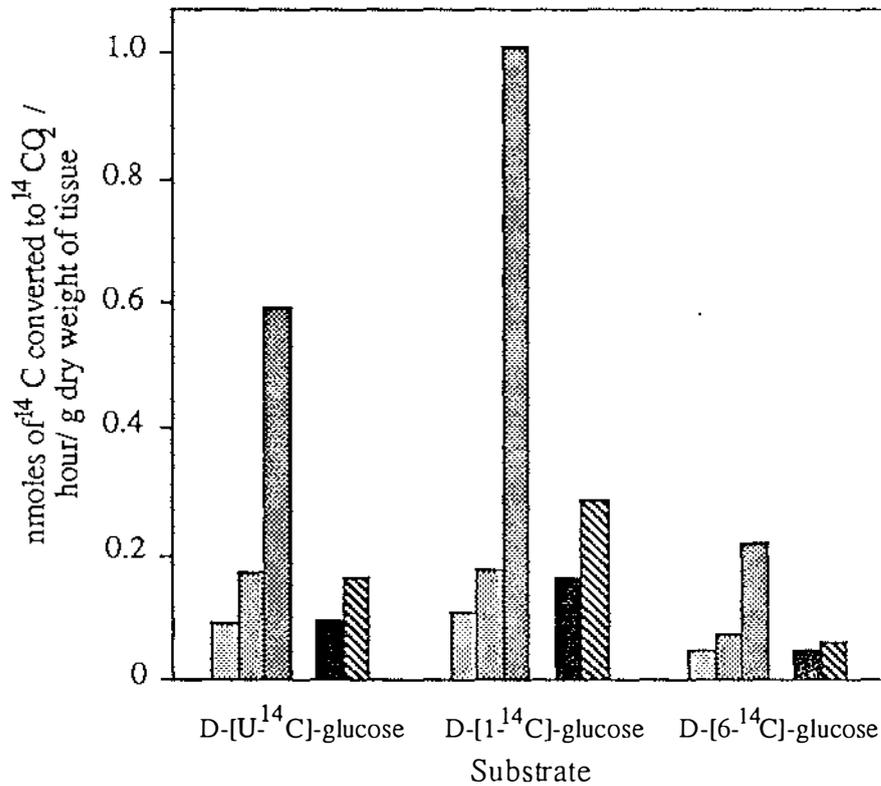
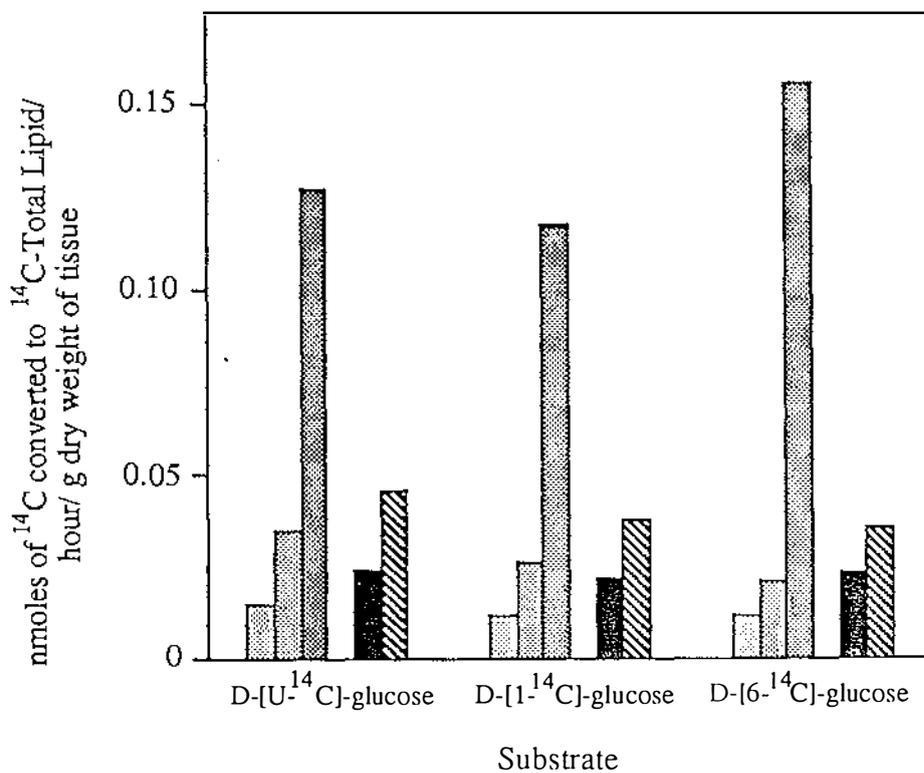


Figure 6.3b Effect of Crude GTF Extract B on the Insulin Stimulated Conversion of D-[U-<sup>14</sup>C]-Glucose, D-[1-<sup>14</sup>C]-Glucose and D-[6-<sup>14</sup>C]-Glucose into <sup>14</sup>C-Total Lipid in Adipocytes Isolated From Rats on a 30% Brewers Yeast Diet



### Notes on Figures 6.3c and 6.3d

Adipocytes were isolated from rats (180-220 g) which had been maintained on the Brewers yeast diet. Adipocytes were incubated for 90 minutes in pH 7.4, 2% Albumin:30 mM HEPES buffer that contained 0.5 mM glucose and 0.2  $\mu$ Ci of labeled substrate. Incubation temperature was 37°C and the flasks were shaken at 100 rpm. Total incubation volume was 1.5 ml. Results are the mean of triplicate estimations. Bars shown on the graph represent a blank (■), 10  $\mu$ U/ml Insulin (▨), 100  $\mu$ U/ml Insulin (▩), the sample only (■) and the sample in the presence of 10  $\mu$ U/ml Insulin (▧).

Figure 6.3c Effect of Crude GTF Extract B on the Insulin Stimulated Conversion of D-[U-<sup>14</sup>C]-Glucose, D-[1-<sup>14</sup>C]-Glucose and D-[6-<sup>14</sup>C]-Glucose into <sup>14</sup>C-Fatty Acid in Adipocytes Isolated From Rats on a 30% Brewers Yeast Diet

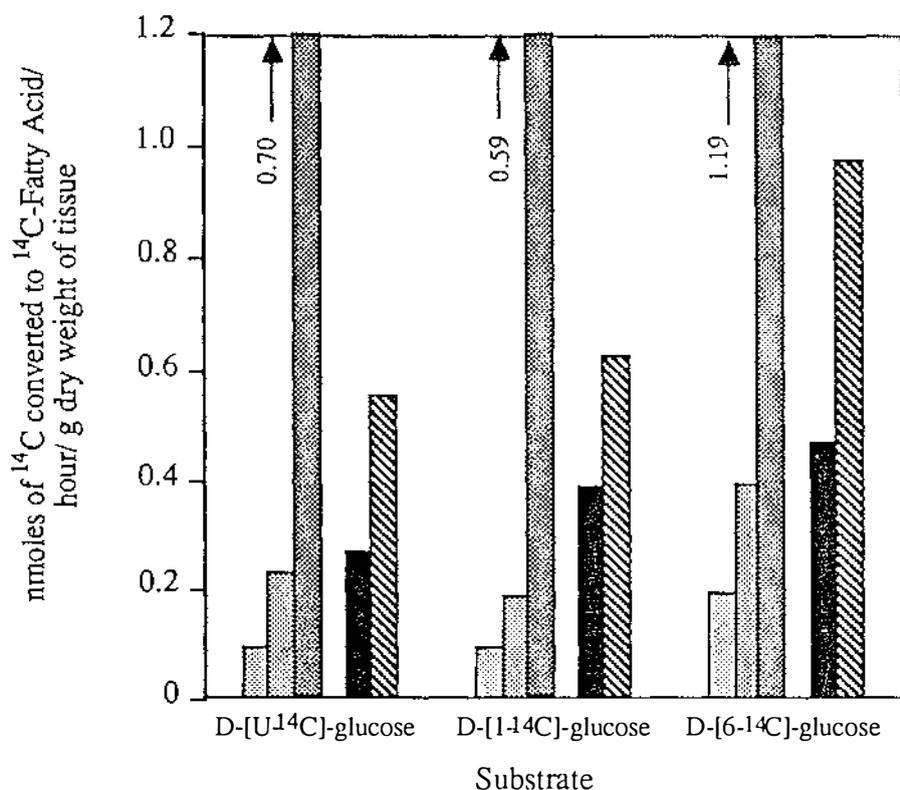
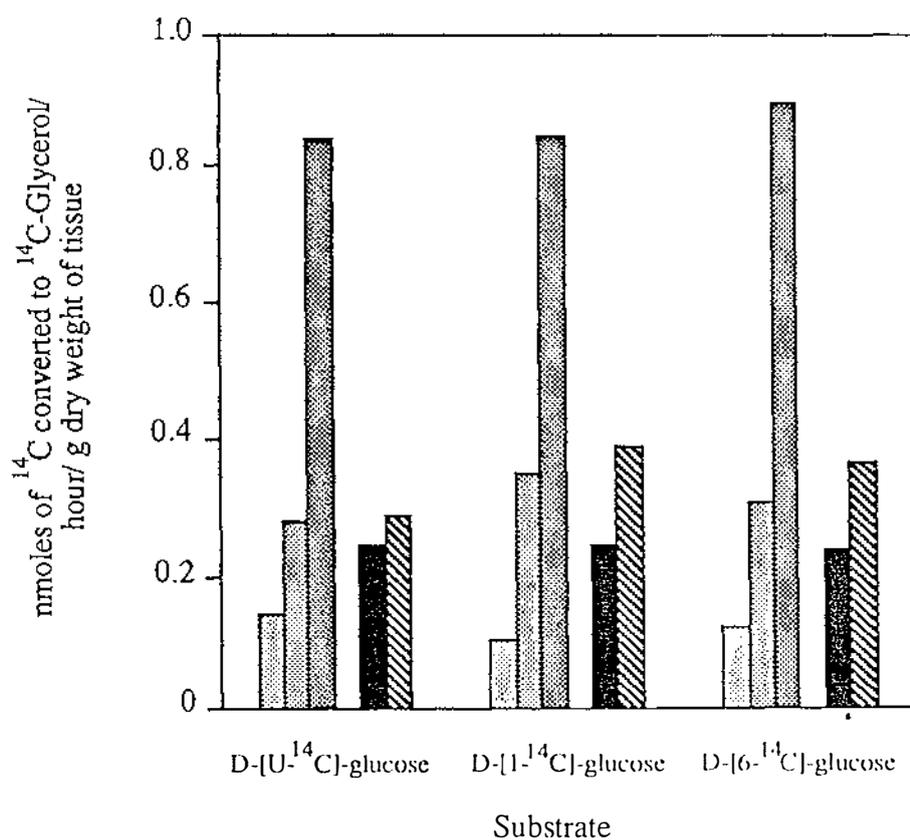


Figure 6.3d Effect of Crude GTF Extract B on the Insulin Stimulated Conversion of D-[U-<sup>14</sup>C]-Glucose, D-[1-<sup>14</sup>C]-Glucose and D-[6-<sup>14</sup>C]-Glucose into <sup>14</sup>C-Glycerol in Adipocytes Isolated From Rats on a 30% Brewers Yeast Diet



### Notes on Figures 6.4a and 6.4b

Adipocytes were isolated from rats (180-220 g) which had been maintained on the Casein based diet. Adipocytes were incubated for 90 minutes in pH 7.4, 2% Albumin:30 mM HEPES buffer that contained 0.5 mM glucose and 0.2  $\mu$ Ci of labeled substrate. Incubation temperature was 37°C and the flasks were shaken at 100 rpm. Total incubation volume was 1.5 ml. Results are the mean of triplicate estimations. Bars shown on the graph represent a blank (▣), 10  $\mu$ U/ml Insulin (▤), 100  $\mu$ U/ml Insulin (▥), the sample only (▦) and the sample in the presence of 10  $\mu$ U/ml Insulin (▧).

Figure 6.4a Effect of Crude GTF Extract B on the Insulin Stimulated Conversion of D-[U-<sup>14</sup>C]-Glucose, D-[1-<sup>14</sup>C]-Glucose and D-[6-<sup>14</sup>C]-Glucose into <sup>14</sup>CO<sub>2</sub> in Adipocytes Isolated From Rats on a 30% Casein Diet

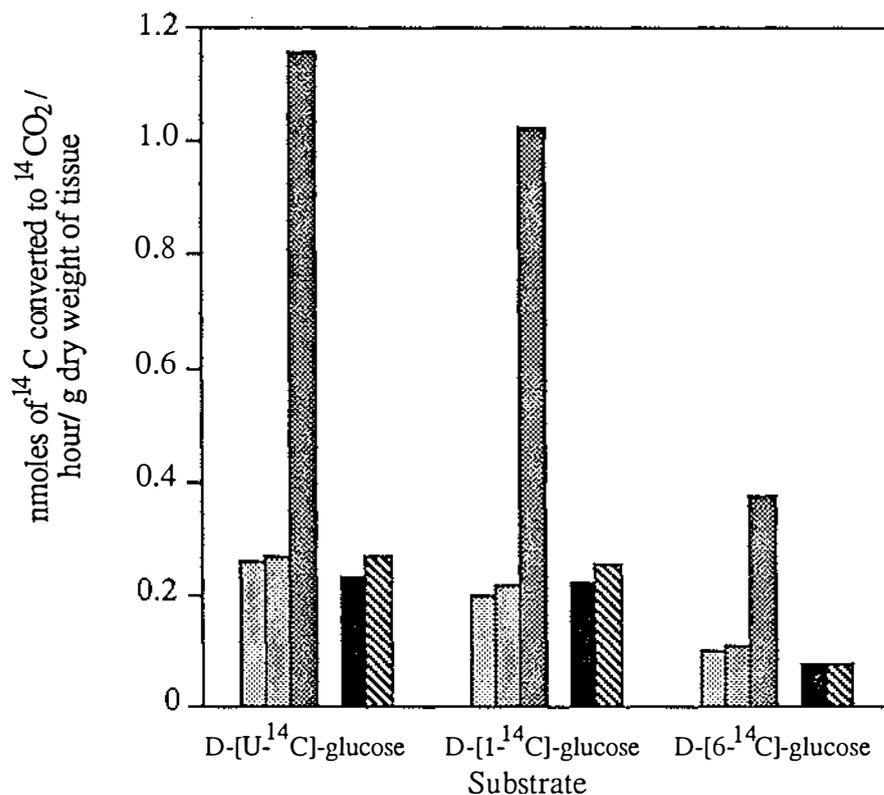
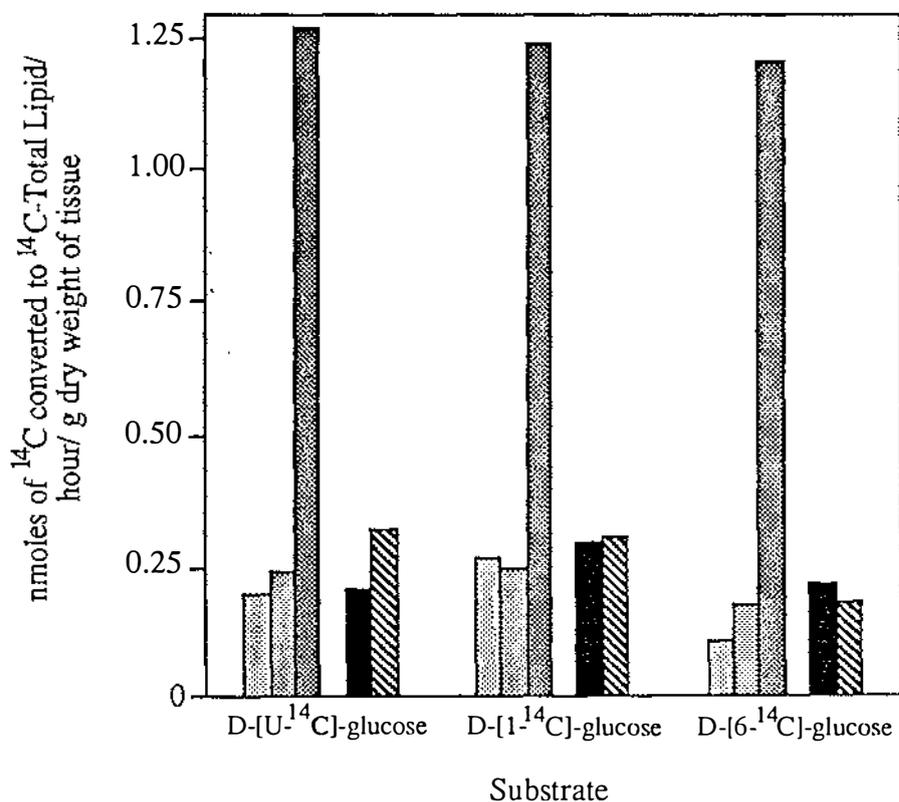


Figure 6.4b Effect of Crude GTF Extract B on the Insulin Stimulated Conversion of D-[U-<sup>14</sup>C]-Glucose, D-[1-<sup>14</sup>C]-Glucose and D-[6-<sup>14</sup>C]-Glucose into <sup>14</sup>C-Total Lipid in Adipocytes Isolated From Rats on a 30% Casein Diet



#### Notes on Figures 6.4c and 6.4d

Adipocytes were isolated from rats (180-220 g) which had been maintained on the Casein based diet. Adipocytes were incubated for 90 minutes in pH 7.4, 2% Albumin:30 mM HEPES buffer that contained 0.5 mM glucose and 0.2  $\mu$ Ci of labeled substrate. Incubation temperature was 37°C and the flasks were shaken at 100 rpm. Total incubation volume was 1.5 ml. Results are the mean of triplicate estimations. Bars shown on the graph represent a blank (▣), 10  $\mu$ U/ml Insulin (▤), 100  $\mu$ U/ml Insulin (▥), the sample only (▦) and the sample in the presence of 10  $\mu$ U/ml Insulin (▧).

Figure 6.4c Potentiation by Crude GTF Extract B of the Insulin Stimulated Conversion of D-[U-<sup>14</sup>C]-Glucose, D-[1-<sup>14</sup>C]-Glucose and D-[6-<sup>14</sup>C]-Glucose into <sup>14</sup>C-Fatty Acid in Adipocytes Isolated From Rats on a 30% Casein Diet.

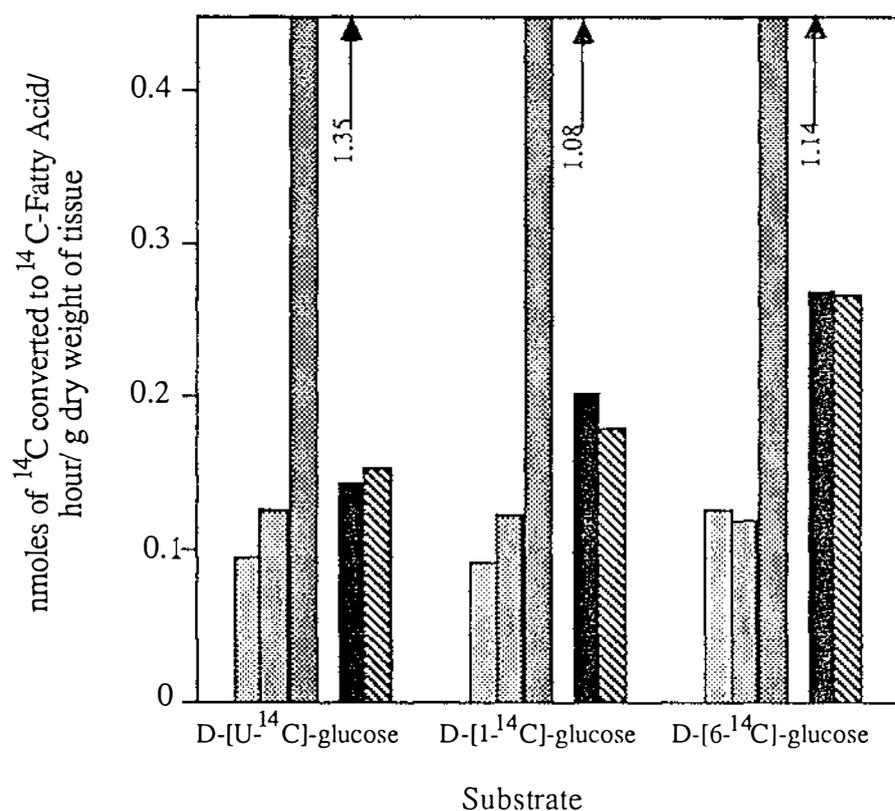
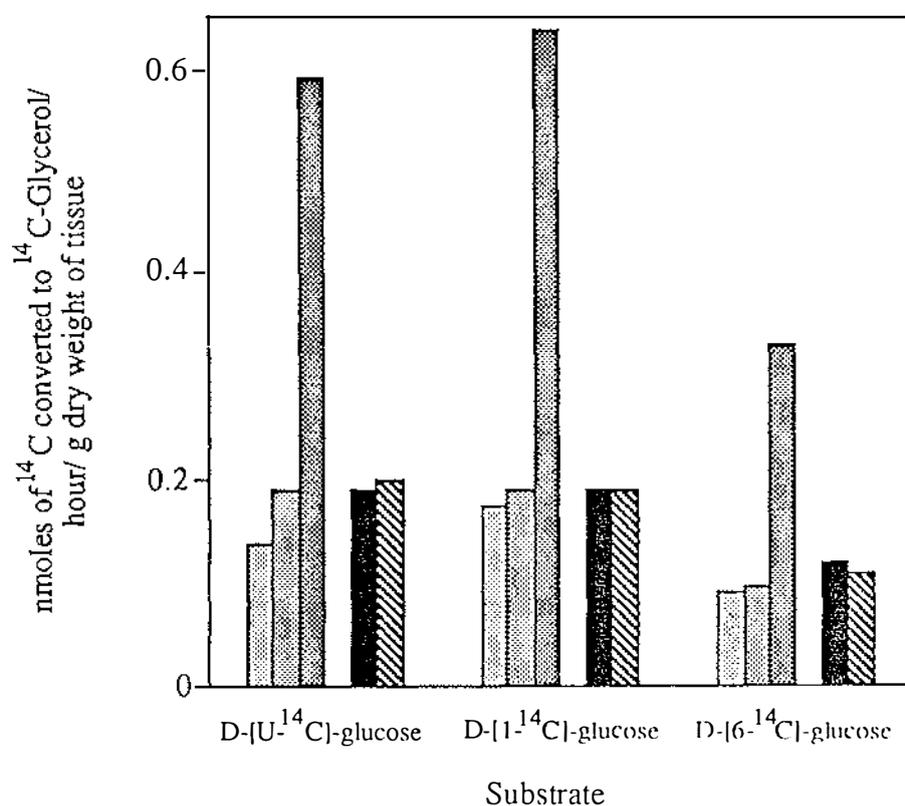


Figure 6.4d Effect of Crude GTF Extract B on the Insulin Stimulated Conversion of D-[U-<sup>14</sup>C]-Glucose, D-[1-<sup>14</sup>C]-Glucose and D-[6-<sup>14</sup>C]-Glucose into <sup>14</sup>C-Glycerol in Adipocytes Isolated From Rats on a 30% Casein Diet



of these were still able to respond to insulin extracts in some cases. This tends to indicate that factors, other than those affecting growth rates, are responsible for determining whether adipose tissue will respond to GTF.

### **6.4.2 Observations About the Metabolism of Adipocytes Isolated from Rats Raised on Different Diets**

As in chapter 5 it is important to look firstly at differences in the basic metabolism of adipocytes from rats raised on different diets. From comparisons of the results presented in Figures 5.7 and 6.2 to 6.4 an interesting observation about differences in the insulin stimulated metabolism of the adipocytes can be made. Comparisons of the maximally insulin stimulated conversion of label from the three different types glucose substrate into the labeled products measured reveals a similar rates for all diets except for conversion of label into fatty acid. As previously discussed in section 5.4.2 the maximally insulin stimulated rates of conversion of D-[1-<sup>14</sup>C]-glucose and D-[6-<sup>14</sup>C]-glucose into fatty acid are very similar in the adipocytes of the animals on the torula yeast diet (Figure 5.7c). This is also seen to a lesser extent in the animals on the casein diet (Figure 6.4c). This is consistent with the observations of Sherriff (1983) where a similar effect was noticed in adipocytes of rats on a torula yeast diet. However in adipocytes of rats raised on the normal diet and the brewers yeast diet the maximally insulin stimulated rate of conversion of D-[6-<sup>14</sup>C]-glucose into labeled fatty acid was approximately twice as much as that seen from D-[1-<sup>14</sup>C]-glucose and this is consistent with the results of other workers who have investigated the adipocytes of rats fed diets presumed to be sufficient in all nutrients (Milstein 1951, Winegrad and Renold 1958, Kather *et al.* 1972a & b, Fried *et al.* 1981). The higher rate of conversion of label from D-[6-<sup>14</sup>C]-glucose into fatty acids in the adipocytes of rats raised on the brewers yeast and stock diets confirms the conclusion in section 5.4 that this was the major area of difference between normal adipocytes and those from rats on the torula yeast diet.

It is interesting to note that this depression of the insulin stimulated conversion of label from D-[6-<sup>14</sup>C]-glucose into labeled fatty acid seen in the rats on the torula yeast diet was also seen to a lesser extent in the rats fed the casein based diet. This is interesting as other workers have used similar high sucrose diets with casein as a protein source to induce a state very similar to that seen by the more usual torula yeast based diet (Schwarz & Mertz 1957, Yi-Ching *et al.* 1981 and Donaldson *et al.* 1985). The results shown here indicate a common physiological effect of these diets which could explain their suitability as diets for producing tissue responsive to GTF extracts. The mechanism of this depression is

not known but it is probably related to an inhibition of the glycolytic pathway in the rats on these diets.

### **6.4.3 Importance of Different Diets to Ability to Observe Potentiations by GTF Extracts**

Analysis of potentiations of insulin metabolism in adipocytes of rats raised on different diets (Table 6.1) does not conclusively show that a deficient dietary state is required to observe a potentiation by a GTF extract as other workers have claimed (Anderson *et al.* 1978a, Davies *et al.* 1985a). It can be seen in Table 6.1 that positive potentiations of insulin action were seen in adipocytes from all the dietary states however it is true that the potentiations observed in the adipocytes of rats raised on the torula yeast diet were consistently the highest. This trend was particularly evident in the conversion of label from D-[1-<sup>14</sup>C]-glucose into <sup>14</sup>CO<sub>2</sub> and <sup>14</sup>C-fatty acids which were identified as the most likely sites of potentiation of insulin action by GTF extracts (section 5.4.3). This indicates that there is indeed something about the metabolism of these cells which lends itself to the observation of these potentiations. The possibility that this is that these cells have a reduced glycolytic pathway, thus increasing the relative role of the pentose phosphate pathway in these cells, is discussed in chapter 5 and the results discussed in section 6.4.2 support this theory.

### **6.4.4 Dietary Explanations for the Different Responses Seen in Different Tissues**

It has been a central premise of GTF research that the chief reason for the development of the glucose intolerance associated with a GTF deficient state is the low level of chromium seen in the diets used to raise these rats. However no in depth analysis of these special diets appears to have been published and few efforts have been made to correlate the observed condition with any condition other than chromium deficiency. The possibility exists that some other dietary deficiency or combination of deficiencies is responsible for the observed effects. Possible causes are:

**Sucrose Content of the Diet** The torula yeast diet generally used in studies of GTF has a 60% sucrose content and other diets used in similar studies have also had high levels of sucrose (Yi-Ching *et al.* 1985, Donaldson *et al.* 1985). High sucrose diets have been shown to have a diabetogenic effect in humans (Beck-Nielsen *et al.* 1982) and to lead to the development of hyperglycemia in rats (Matsuo *et al.* 1984). It must be likely therefore that the high sucrose content of the diet used here plays at least some part in inducing impairments of the glucose metabolism of the rats on the diet. However, while three of the four diets used had very high levels of sucrose, only the adipose tissue from

the rats on the torula yeast diet was able to consistently show a significant potentiation of insulin action by GTF. This indicates that the sucrose content of the diet is not an important determinant of GTF response in adipose tissue of animals on the diet.

**Fibre Content of the Diet** It has become apparent in recent years that dietary fibre has a significant effect on glucose tolerance (Kritchevsky 1988). It is therefore a possibility that the fibre content of the diet may have some relationship to the glucose intolerant state seen in animals that respond to GTF. The initial effect of fibre appears to be a slowing of the absorption of metabolites from the gut, resulting in a flattening of the glucose response curve although longer term benefits for glucose tolerance have also been noted (Kritchevsky 1988). While no determinations were made of the fibre content of diets used in the current work, it is safe to assume that in the synthetic diets used here that the only source of fibre would be the protein source which comprised 30% of the diet (Appendix 1). Using the figures tabulated by the National Academy of Science (1972) some estimation of the likely dietary fibre content can be made. These results are shown in Table 6.4. From these results it is interesting to note that the two diets used to obtain the deficiency syndrome required for GTF investigations are the two which have the lowest dietary fibre contents. The significance of this is unknown but it is worth further investigation as at least part of the deficiency symptoms may be alleviated by higher amounts of dietary fibre.

Table 6.4 Estimations of Fibre Content of Rat Diets

Diet / Protein	Source Supplier	Fibre Content of Protein Source <sup>1</sup>	Total Fibre Content of the Diet
Normal	Harvey Farms	NA.	3.2 % <sup>2</sup>
Torula Yeast	Teklad	2.2 %	0.7 %
Brewers Yeast	-	3.2 %	1.1 %
Casein	-	0.0 %	0.0 %

Notes: 1. Crude Fibre figures given in "Nutrient requirements for Laboratory Animals", National Academy of Science, 1972.

2. Modified Acid Detergent Fibre as determined by manufacturer.

### **Trace Element Composition of the Diet**

It has been held that the disturbances in glucose metabolism seen in rats fed specially purified diets were attributable to the low chromium content of those diets. However deficiencies of trace elements other than chromium may account for disturbances of the glucose metabolism and some elements have a direct effect on insulin stimulated

parameters in the rat adipocyte (section 1.5.3). The concentration of trace elements in the diets was determined and is summarised in Table 6.3. The occurrence in the diets of those elements that were known to have effects on the glucose metabolism is discussed below:

**Chromium** It can be seen that while chromium is low in all the diets it was at levels too low for the ICP analysis to detect in the case of the casein and the torula yeast diets. Efforts to more accurately analyse the chromium content of the diets using the more sensitive graphite furnace atomic absorption spectrometry proved fruitless due to high levels of chromium contamination in all batches of the furnaces that were tested. However the ICP analysis indicates very low levels of chromium in the diet and this is consistent with the results of earlier workers who claimed that diets inducing impairment of glucose tolerance had particularly low levels of chromium (see Chapter 1).

**Zinc** A deficiency of zinc in the torula yeast diet was initially suspected as no zinc is added to that diet during preparation (Appendix 1). However the analysis reveals that in all the diets the levels of zinc are up to the 12  $\mu\text{g/g}$  level suggested for diets for laboratory rats (National Academy of Science 1972). It is thus unlikely that zinc levels in the diet are a major contributing factor to the difference in condition between rats fed the torula diet and those fed the normal diet.

**Manganese** The level of manganese in all the diets is below the level of 50  $\mu\text{g/g}$  recommended by the National Academy of Science (1972). The level in the torula yeast diet is particularly low, approximately six fold lower than in the other diets, and is barely more than the 4  $\mu\text{g/g}$  that was provided in the diet from the mineral mix (Appendix 1). This manganese deficiency may contribute to condition of the animals on the torula yeast diet.

**Nickel** Nickel is similar to chromium in that it appears in much lower levels in the casein and torula yeast diets than it does in the brewers yeast and Harvey Farms diets. While no recommendation for dietary nickel levels is made in the National Academy of Science (1972) guidelines, it is possible that this too may have an effect on the observed condition of the animals on deficient diets.

**Others** There were no variations in the concentrations of the other elements analysed (Al, Cu, Fe, Sn) that would explain the different conditions seen in rats fed the different diets.

### **Amino Acid Composition of the Protein**

It can be seen from the results in Table 6.2 that all diets have at least the levels of amino acids recommended by the National Academy of Science (1972), with the casein diet having particularly high levels of all amino acids. This data indicates that amino acid

deficiency is not a likely to give rise to the physiological condition state of animals that are seen to respond to GTF.

Overall this comparison of constituents of the four diets studied revealed that diets giving rise to adipose tissue that responded to GTF had several features in common. It was found that levels of nickel, chromium and manganese as well as dietary fibre levels were very low in the torula yeast diet while chromium, nickel and fibre levels were also low in the casein diet. Other factors that were suspected of being involved in the glucose metabolism of adipose tissue included the high sucrose content of the diets, the amino acid content of the diet and levels of other trace elements, particularly zinc, however none of these seemed to be important factors in the production of adipose tissue that responded to GTF. Previously it was assumed that a low chromium concentration was the major contributory factor in the diet (Mertz 1969) and while the results presented here are consistent with this assumption they also indicate that there may also be other factors responsible for the observed effects.

#### **6.4.5 Conclusions**

The results of Chapters 5 and 6 indicate that the torula yeast diet is not essential for the production of adipocytes in which the potentiation of insulin action by GTF extracts can be observed. However this diet is more effective than the other diets studied in producing adipocytes that will respond to the GTF extracts. The effect of GTF extracts in these cells is most evident in the potentiation of the insulin stimulated conversion of D-[1-<sup>14</sup>C]-glucose to <sup>14</sup>CO<sub>2</sub> and <sup>14</sup>C-fatty acids. These results indicate that there must be some unique feature of adipocytes from rats on the torula yeast rats which leads to these cells responding to GTF extracts. The experiments in chapter 6 confirm that the major metabolic difference between the adipocytes of rats raised on the torula yeast diet and those from rats raised on other diets was the reduced ability to convert D-[6-<sup>14</sup>C]-glucose to <sup>14</sup>C-fatty acid in the adipocytes of rats on the torula yeast diet. This was taken to be due to a deficiency of the glycolytic pathway which meant that the pentose phosphate pathway took a more prominent role in the metabolism of these cells. As the evidence suggested that the GTF extracts were potentiating the conversion of <sup>14</sup>C from D-[1-<sup>14</sup>C]-glucose into fatty acids via the pentose phosphate pathway this gave a possible explanation as to why the potentiations were more obvious in the adipocytes from torula yeast rats. However the results in chapter 6 do not resolve the questions raised in chapter 5 about whether the assay was in fact measuring GTF activity as originally defined by Mertz (1969).

Overall the results of Chapter 5 and 6 confirm that diet has an important, although not absolutely critical role, in determining whether adipocytes will respond to GTF extracts. However the results also raise a number of serious questions about the validity of the assumption that the material causing the potentiation of the action of sub optimal levels of insulin in adipocytes represents GTF activity as originally defined. The potentiation of the action of insulin is still a very interesting occurrence and the nature of the material responsible for this could still have important implications for medical conditions relating to glucose intolerance.

## 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

In Chapter 3 an attempt was made to identify the nature of the substance causing the activity in the yeast assay that had been attributed to GTF. This work began with the crude GTF extract described by Toepfer *et al.* (1977) and followed the most active fractions at each purification step. An anionic fraction containing adenine and a small amount of glutamic acid and glycine was identified as the most active subfraction although none of those components was able to individually account for the observed activity. A modified yeast assay (Chapter 4) identified a more definitive stimulation of yeast cell metabolism by the crude GTF extract but this activity was not seen in the active anionic fraction described above. This suggested that the active anionic fraction did not contain material that would conform with the properties defined for GTF. However the validity of the results from the modified yeast assay has not been confirmed and so it was necessary to compare the results of these assays with those from a more definitive assay system for GTF activity. The most commonly accepted assay for GTF activity is the low chromium rat epididymal adipocyte assay system but investigations of this assay system (Chapter 5 and 6) raised the possibility that this assay is also not measuring GTF as originally defined. However the potentiations of the action of insulin by GTF extracts that are seen in these assays definitely exists and is in itself an interesting phenomenon which may have beneficial implications for medical conditions resulting from glucose intolerance. The GTF extract used in Chapter 5 and 6 was impure and so if the material responsible for the potentiations was to be isolated and characterised, a purification scheme similar to that described in Chapter 3 would be required. Using this scheme would also allow the results of the adipocyte assays of these fractions to be compared with the results of assays of similar fractions in the yeast assay systems. This would confirm whether or not the material responsible for activity in the yeast assay systems was

also responsible for potentiations of insulin action seen in the adipocyte assay was measuring.

## **7.2 Methods and Materials**

### **7.2.1 Isolation of Crude GTF Extract**

Crude GTF extracts were obtained from yeast using the methods based on those of Toepfer *et al.* (1977) which are described in Section 3.2.1.

### **7.2.2 Fractionation of Crude GTF Extracts**

Fractions of the crude GTF extracts were initially separated using high voltage paper electrophoresis as described in Section 3.2.2 and eluted from the paper using the methods described in Section 3.2.3. Fractions showing significant activity in the low chromium rat epididymal adipocyte assay system were further separated using HPLC. The procedure for this was the same as described in Section 3.2.4 except that a different buffer system was used. In this separation the buffer gradient began with 0.1% trifluoroacetic acid: 5% acetonitrile: 94.9% water and changed to 0.1% trifluoroacetic acid: 80% acetonitrile: 19.9% water

### **7.2.3 Amino Acid, Chromium and UV/visible Spectral Analysis of Fractions**

Fractions were analysed for amino acid content and trace metal composition as described in section 3.2.5 and 3.2.6. The nature of the purified active fractions was investigated using UV/visible spectroscopy, as described in section 3.2.8.

### **7.2.4 Mass Spectrometric Analysis of Fractions.**

Mass spectroscopy was carried out by the Department of Scientific and Industrial Research, Palmerston North, New Zealand using a VG 70S magnetic sector mass spectrometer. Measurements were made using liquid SIMS, a variation of the fast atom bombardment source (FAB) excitation technique that employs caesium ions in place of argon. As well as a normal analysis, the metastable analysis technique was also used to gain further information on the fragments of the molecules present. This technique focuses on the ion path of the major molecular ion to gain information on the components of the molecule. A collision cell containing helium is placed in the ion path of the major ionised molecule and collision of this molecule with the helium results in fragmentation. The mass of the fragments are measured and this gives information on the likely components of the molecule.

### **7.2.5 Determination of GTF Activity**

GTF activity was determined using the low chromium epididymal rat adipocyte assay as described in section 5.2. The ability of samples to potentiate the action of suboptimal concentrations of insulin, in the conversion of  $^{14}\text{C}$  from D-[U- $^{14}\text{C}$ ]-glucose and D-[1- $^{14}\text{C}$ ]-glucose to  $^{14}\text{CO}_2$  and  $^{14}\text{C}$ -fatty acids, was investigated. As discussed in section 6.4, only fractions able to potentiate the insulin action on all four of these parameters were considered active. Because of the inter assay variations, the activity of an internal standard of crude GTF extract B was determined in all assays although there were still some variations between levels of activity seen for the crude extract B in these assays and those reported previously for B in Chapter 5. This highlights that comparisons of levels of activity should only be made between results obtained in the same assay.

## **7.3 Results**

### **7.3.1 Electrophoresis Fractions**

When subject to high voltage paper electrophoresis the components of the crude extract from brewers yeast separated in a very similar manner to that described in Chapter 3 with the same 15 bands of material being revealed by ninhydrin staining as those shown in Figure 3.1. However the number of electrophoresis bands that could be assayed concurrently in the adipocyte assay was restricted although as discussed in section 7.2.5 it was important to assay fractions simultaneously to gain accurate comparisons of activity. It was impractical to assay all 15 bands simultaneously so to counter this the material from the electrophoresis bands was pooled into a total of six fractions based on similarities in the composition of the fractions found in Chapter 3. Samples were pooled as follows; **B-A-I** (B-A-1, B-A-2, B-A-3), **B-A-II** (B-A-4, B-A-5), **B-A-III** (B-A-6, B-A-7), **B-N-I** (B-N-1, B-N-2), **B-B-I** (B-B-1, B-B-2, B-B-3), **B-B-II** (B-B-4, B-B-5, B-B-6). These pooled fractions were assayed and the results are shown in Figures 7.1 and 7.2 and the percentage enhancement of insulin action is tabulated in Table 7.1. From these results it was concluded that fraction B-B-I consistently showed the most potentiation and therefore was considered to have the most GTF activity. This fraction was selected for further purification.

### **7.3.2 HPLC of Fraction B-B-I**

The HPLC profile of the separation of B-B-I is shown in Figure 7.3a. The various peaks were pooled into 5 fractions labeled B-B-I-1 to B-B-I-5 as shown in in Figure 7.3a. This was firstly to ensure that there was enough of each fraction for assay, and secondly to ensure that all the fractions could be assayed concurrently. A total of 27 mg of B-B-I was applied to the HPLC column in 5 separate runs. From this 15.8 mg was eluted in

Figure 7.1 Effect of the Electrophoresis Fractions of Crude GTF Extract B, on the Insulin Stimulated Conversion of D-[U-<sup>14</sup>C]-glucose to <sup>14</sup>CO<sub>2</sub>

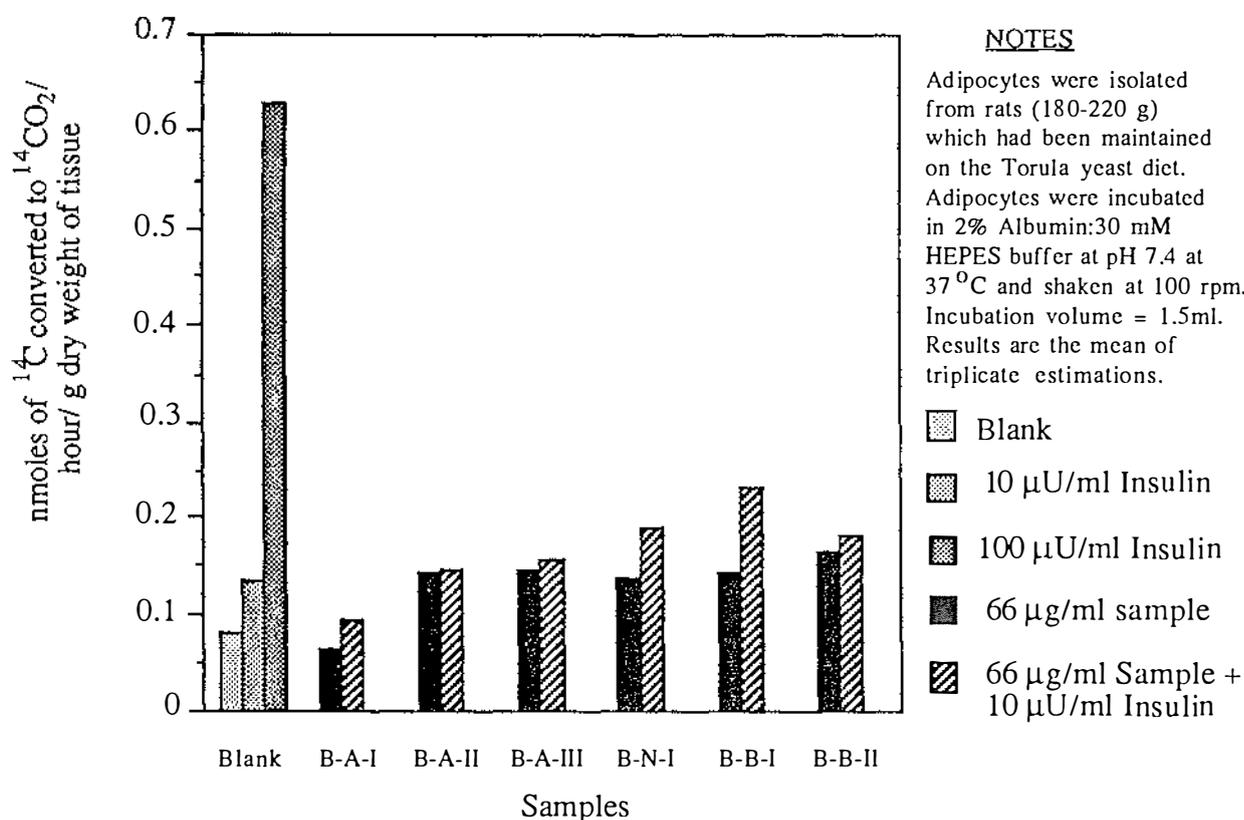


Table 7.1 Potentiation of the Action of Sub Optimal Concentrations of Insulin, by Electrophoresis Fractions of Crude GTF Extract B.

Sample	Concentration (μg/ml)	Potentiation for D-[U- <sup>14</sup> C]-Glucose to CO <sub>2</sub>	Potentiation for D-[1- <sup>14</sup> C]-Glucose to CO <sub>2</sub>	Potentiation for D-[1- <sup>14</sup> C]-Glucose to Fatty Acid
Crude B	100	28%	2%	140%
B-A-I	66	39%	-32%	4%
B-A-II	66	-34%	-22%	25%
B-A-III	66	-10%	-5%	0%
B-N-I	66	79%	-44%	-10%
B-B-I	66	65%	28%	4%
B-B-II	66	-13%	8%	-16%

### Notes on Figures 7.2a and 7.2b

Adipocytes were isolated from rats (180-220 g) which had been maintained on the Torula yeast diet. Adipocytes were incubated for 90 minutes in pH 7.4, 2% Albumin:30 mM HEPES buffer that contained 0.5 mM glucose and 0.2  $\mu$ Ci of labeled substrate. Incubation temperature was 37°C and the flasks were shaken at 100 rpm. Total incubation volume was 1.5 ml. Results are the mean of triplicate estimations. Bars shown on the graph represent a blank (▣), 10  $\mu$ U/ml Insulin (▤), 100  $\mu$ U/ml Insulin (▥), the sample only (▦) and the sample in the presence of 10  $\mu$ U/ml Insulin (▧).

Figure 7.2a Effect of the Electrophoresis Fractions of Crude GTF Extract B<sub>1</sub> on the Insulin Stimulated Conversion of D-[1-<sup>14</sup>C]-glucose to <sup>14</sup>CO<sub>2</sub>

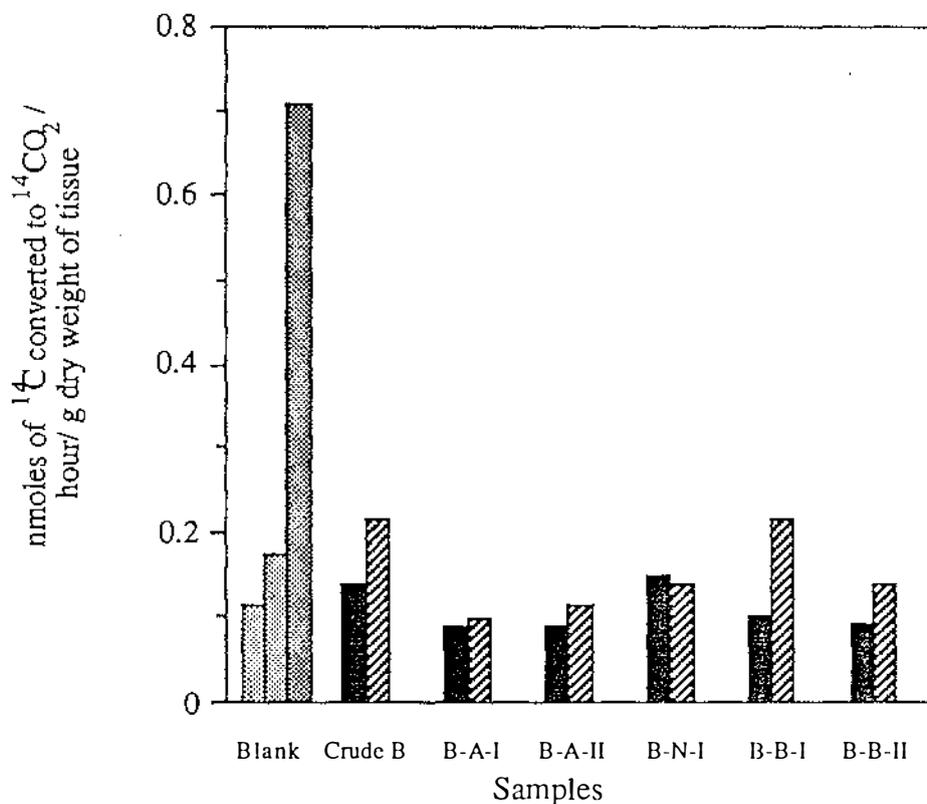
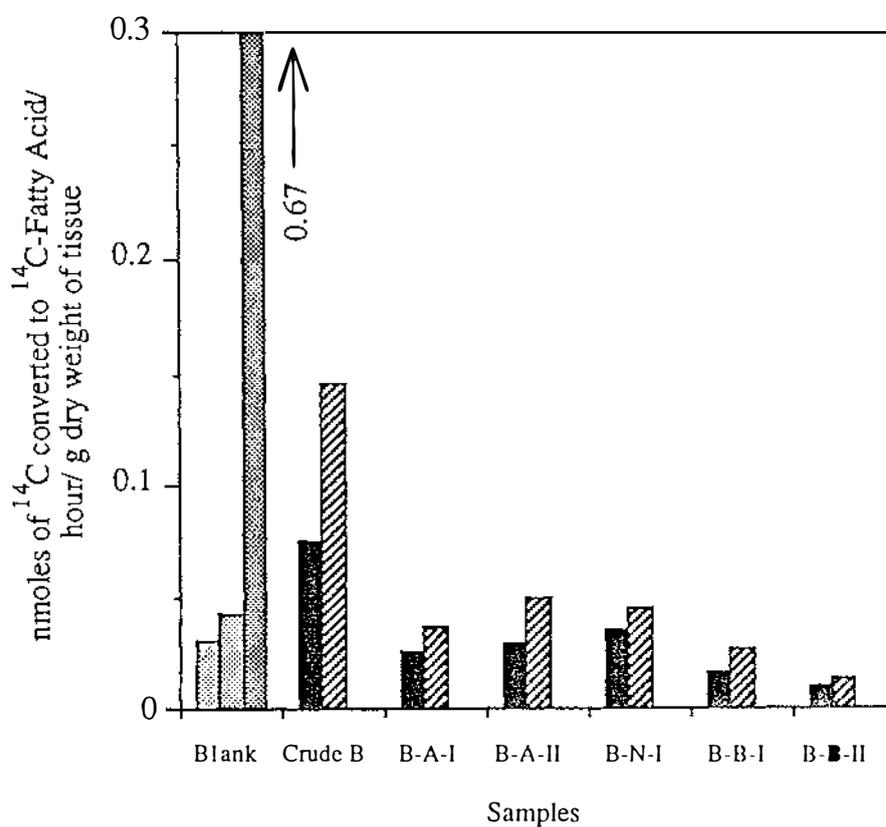


Figure 7.2b Effect of Electrophoresis Fractions of Crude GTF Extract B<sub>1</sub> on the Insulin Stimulated Conversion of D-[1-<sup>14</sup>C]-glucose to <sup>14</sup>C-Fatty Acids



fraction B-B-I-1, 5.0 mg in B-B-I-2, 3.0 mg in B-B-I-3, 0.5 mg in B-B-I-4 and 1.1 mg in B-B-I-5. The fractions isolated were assayed as in section 7.3.1 with the results of these assays being shown in Figures 7.4 and 7.5 and the percentage enhancement of insulin action is tabulated in Table 7.2. Of these fractions, fraction B-B-I-1 showed the most consistent ability to bring about high levels of potentiation of insulin action across the range of potentiations measured. On this basis it was selected for further purification.

The HPLC elution profile of B-B-I (Figure 7.3a) shows that of all the fractions this one contained the least number of peaks, indicating it was the most homogeneous. However it was still not pure and further separation was required. As B-B-I-1 was the first material to be eluted from the HPLC it was obviously the most polar. To gain further separation of this polar material it was run on the same column as before but using only water to elute the column and the elution profile is shown in Figure 7.3b. Three peaks could be identified although the major peak had a significant shoulder indicating that it was still not pure. The three peaks identified were labeled B-B-I-1-1 to B-B-I-1-3 as indicated in Figure 7.3b. Over 80% of the B-B-I-1 material applied to the column was eluted in the B-B-I-1-2 peak. There was insufficient material to assay fractions B-B-I-1-1 and B-B-I-1-3 but there was sufficient material to assay fraction B-B-I-1-2 and the results of this assay are shown in Figures 7.7 and 7.8. The potentiations calculated from this data is shown in Table 7.3. These results show that the fraction B-B-I-1-2 is not as active as the crude GTF extract.

Table 7.2 Potentiation of the Action of Sub Optimal Concentrations of Insulin by HPLC Fractions of B-B-I

Sample	Concentration ( $\mu\text{g/ml}$ )	Potentiation for D-[U- $^{14}\text{C}$ ]-glucose to $\text{CO}_2$	Potentiation for D-[U- $^{14}\text{C}$ ]-glucose to Fatty Acids	Potentiation for D-[1- $^{14}\text{C}$ ]-glucose to $\text{CO}_2$	Potentiation for D-[1- $^{14}\text{C}$ ]-glucose to Fatty Acids
Crude B	66	225%	100%	155%	65%
B-B-I-1	66	225%	110%	121%	20%
B-B-I-2	66	80%	- 50%	- 20%	40%
B-B-I-3	66	140%	60%	10%	0%
B-B-I-4	20	- 20%	20%	70%	- 5%
B-B-I-5	33	180%	5%	10%	60%

### 7.3.3 Amino Acid Analysis of Purified Fractions

For amino acid analysis several mg of each fraction analysed was freeze dried from a solution of the fraction and was analysed as described previously. Amino acid analysis of fractions B-B-I, B-B-I-1 and B-B-I-1-2 revealed that the total amino acid content of each

Figure 7.3a HPLC Profile of Preparative Separation of B-B-I

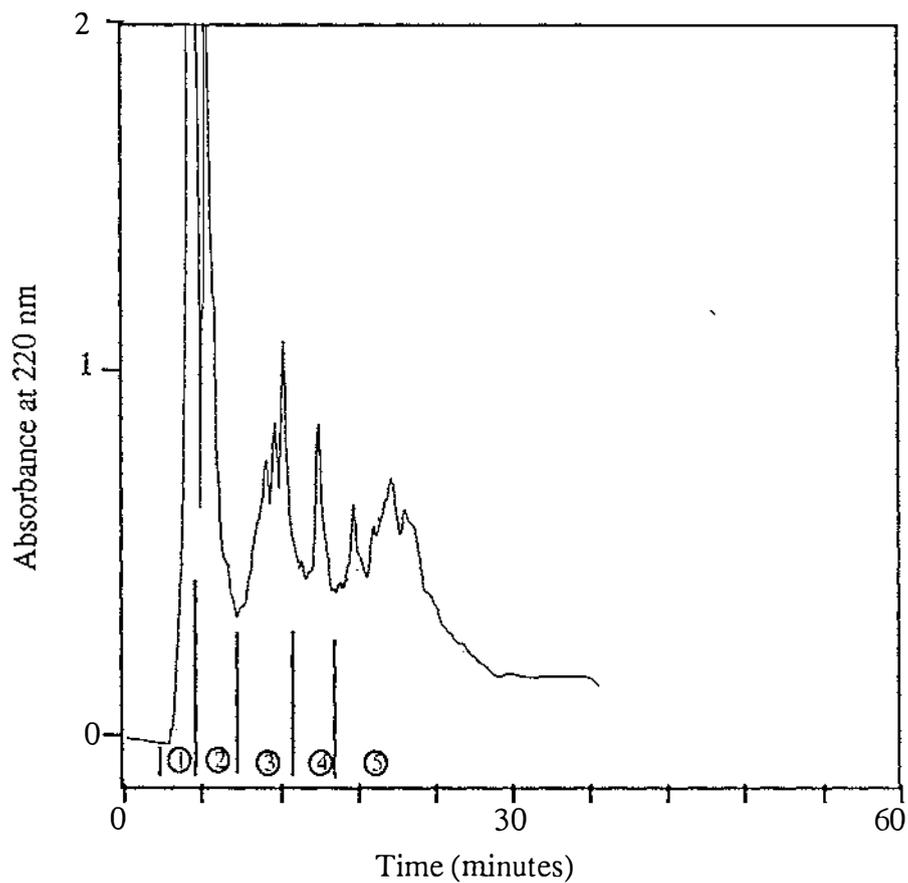
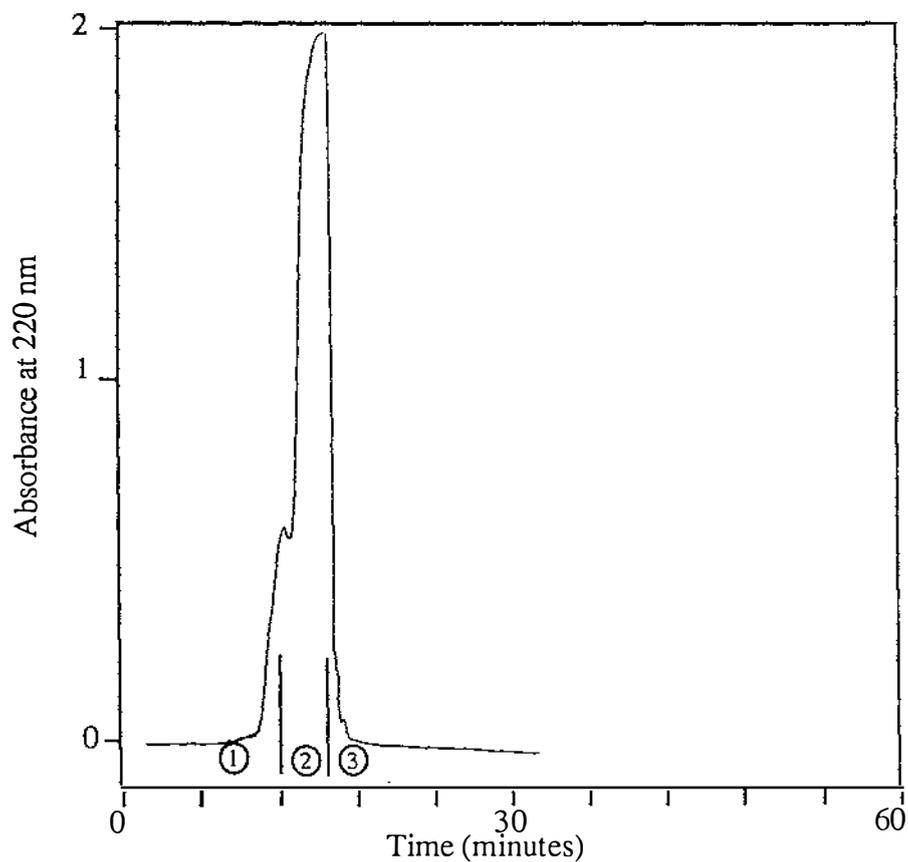


Figure 7.3b HPLC Profile of Separation of B-B-I-1



#### Notes on Figures 7.4a and 7.4b

Adipocytes were isolated from rats (180-220 g) which had been maintained on the Torula yeast diet. Adipocytes were incubated for 90 minutes in pH 7.4, 2% Albumin:30 mM HEPES buffer that contained 0.5 mM glucose and 0.2  $\mu$ Ci of labeled substrate. Incubation temperature was 37°C and the flasks were shaken at 100 rpm. Total incubation volume was 1.5 ml. Results are the mean of triplicate estimations. Bars shown on the graph represent a blank (▨), 10  $\mu$ U/ml Insulin (▩), 100  $\mu$ U/ml Insulin (▧), the sample only (■) and the sample in the presence of 10  $\mu$ U/ml Insulin (▨).

Figure 7.4a Effects of HPLC Fractions of B-B-I, on the Insulin Stimulated Conversion of D-[U- $^{14}$ C]-glucose to  $^{14}$ CO $_2$

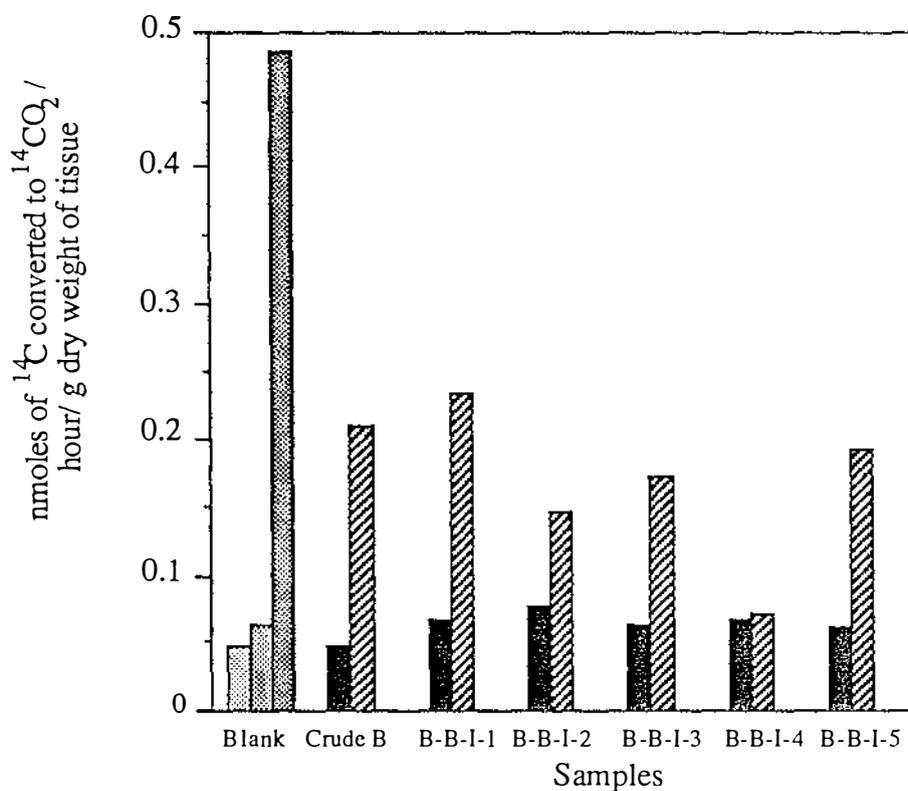
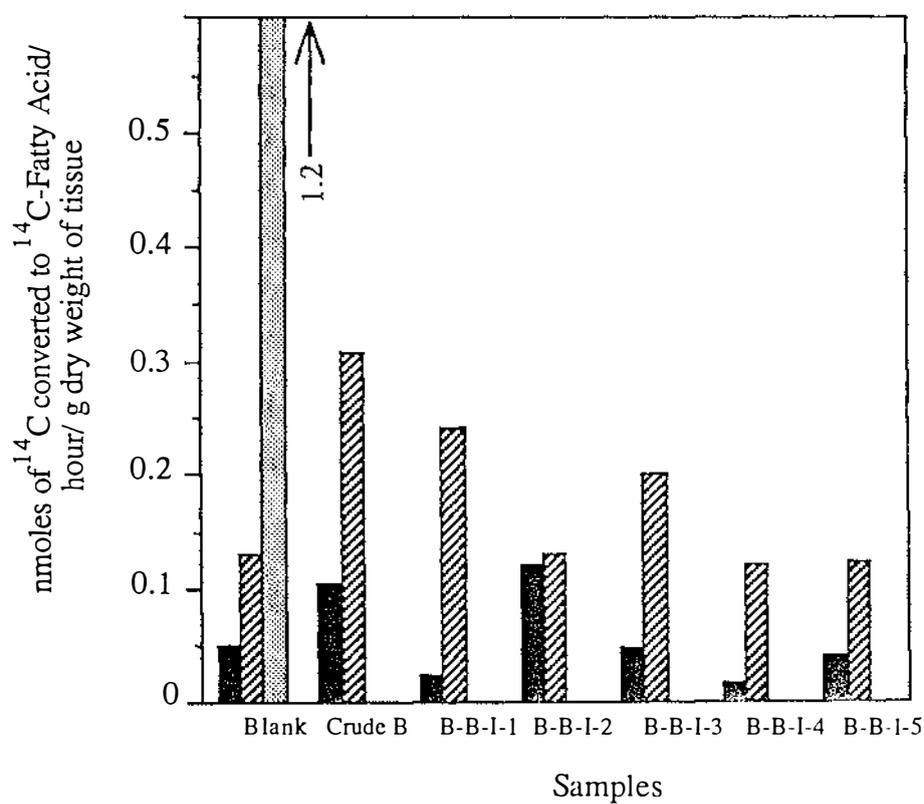


Figure 7.4b Effects of HPLC Fractions of B-B-I, on the Insulin Stimulated Conversion of D-[U- $^{14}$ C]-glucose to  $^{14}$ C-Fatty Acids



### Notes on Figures 7.5a and 7.5b

Adipocytes were isolated from rats (180-220 g) which had been maintained on the Torula yeast diet. Adipocytes were incubated for 90 minutes in pH 7.4, 2% Albumin:30 mM HEPES buffer that contained 0.5 mM glucose and 0.2  $\mu$ Ci of labeled substrate. Incubation temperature was 37°C and the flasks were shaken at 100 rpm. Total incubation volume was 1.5 ml. Results are the mean of triplicate estimations. Bars shown on the graph represent a blank (▨), 10  $\mu$ U/ml Insulin (▩), 100  $\mu$ U/ml Insulin (▧), the sample only (▦) and the sample in the presence of 10  $\mu$ U/ml Insulin (▨).

Figure 7.5a Effects of HPLC Fractions of B-B-I, on the Insulin Stimulated Conversion of D-[1-<sup>14</sup>C]-glucose to <sup>14</sup>CO<sub>2</sub>

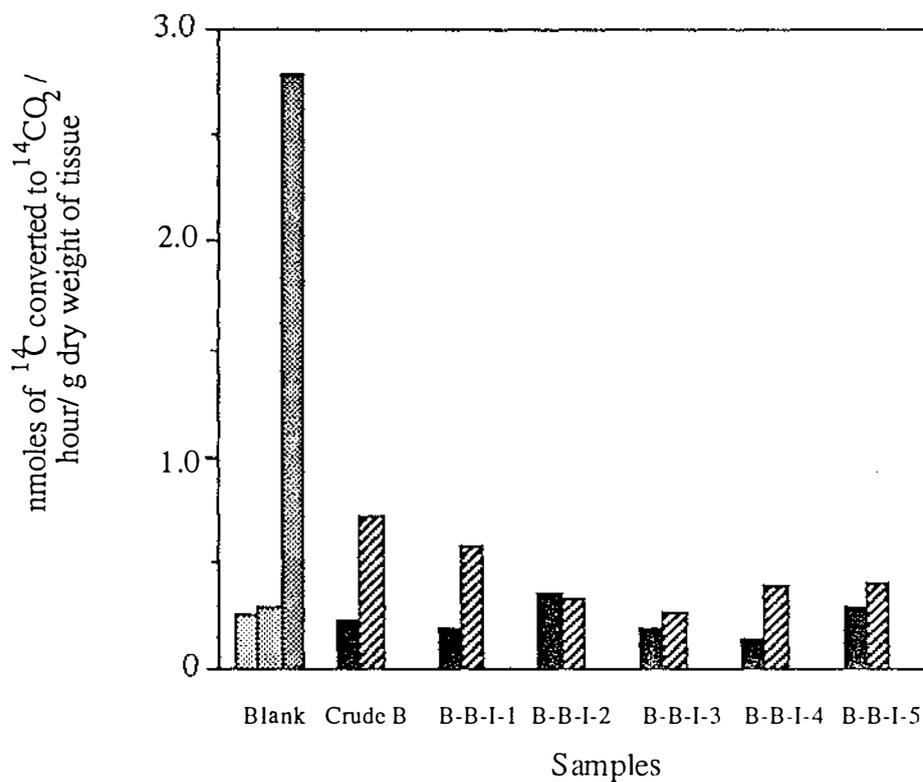
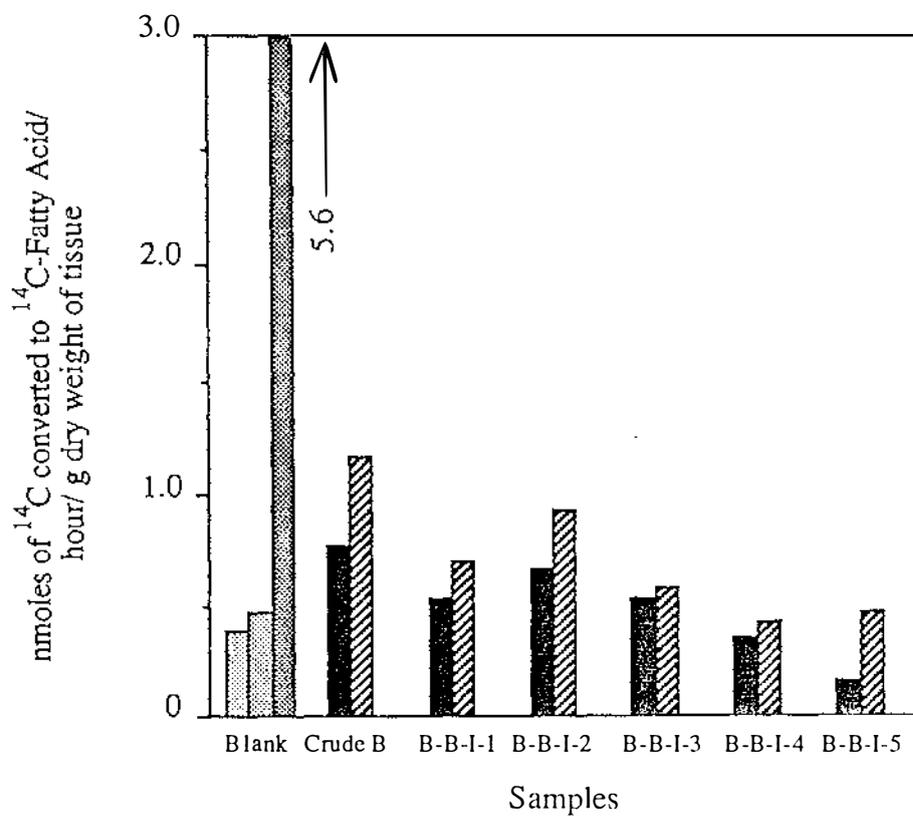


Figure 7.5b Effects of HPLC Fractions of B-B-I, on the Insulin Stimulated Conversion of D-[1-<sup>14</sup>C]-glucose to <sup>14</sup>C-Fatty Acids



of these fractions was less than 1% by weight and thus formed an insignificant part of the material.

### 7.3.4 Chromium Analysis of Purified Fractions

The graphite furnace atomic absorption analysis for chromium revealed chromium concentrations of 4.0  $\mu\text{g/g}$  in fraction B-I, 4.6  $\mu\text{g/g}$  in fraction B-I-1 and 3.3  $\mu\text{g/g}$  in fraction B-I-1-2.

Table 7.3 Potentiation of the Action of Sub Optimal Concentrations of Insulin by Fraction B-B-I-1-2

Substance	Concentration $\mu\text{g/ml}$	Substrate	Product Incorporated Into	
			CO <sub>2</sub>	Fatty Acids
B	66	D-[U- <sup>14</sup> C]-glucose	85%	155%
B-B-I-1-2	66	D-[U- <sup>14</sup> C]-glucose	10%	70%
	66	D-[1- <sup>14</sup> C]-glucose	5%	70%

### 7.3.5 UV/Visible Spectrum of Purified Fractions

The UV/visible spectra of B-I (Figure 7.9a) shows a slight shoulder at about 260 nm, indicating the possible presence of a small amount of aromatic compounds, and a further shoulder peak is seen at about 215 nm. Fraction B-B-I-1 shows even less UV absorbance (Figure 7.9b) with no peak at all at 260 nm and a diminished shoulder at about 215 nm. This confirms the results in 7.3.3 which indicated low levels of amino acid in these fractions.

### 7.3.6 Mass Spectral Analysis of Purified Fractions

Mass spectral analysis of the most purified fraction, B-B-I-1-2 was unsuccessful due to the small amount of compound available for analysis. However HPLC profiles showed that B-B-I-1 was mainly made up of the material in B-B-I-1-2 (Figure 7.3b) and so the former fraction was investigated using the mass spectroscopic techniques described in section 7.2.3. The results of this analysis are shown in Figure 7.6. The SIMS analysis indicated a molecular mass of 428. Further analysis by metastable analysis revealed 2 fragments, each of molecular mass 92, coming off the original molecule, leaving a protonated fragment of molecular mass 245. However due to the low resolution of the analysis and the lack of obvious correlations with the spectra of known materials definitive identification of the material in the sample could not be made.

Figure 7.6a Mass Spectrum of B-B-I-1 Using Liquid SIMS Source

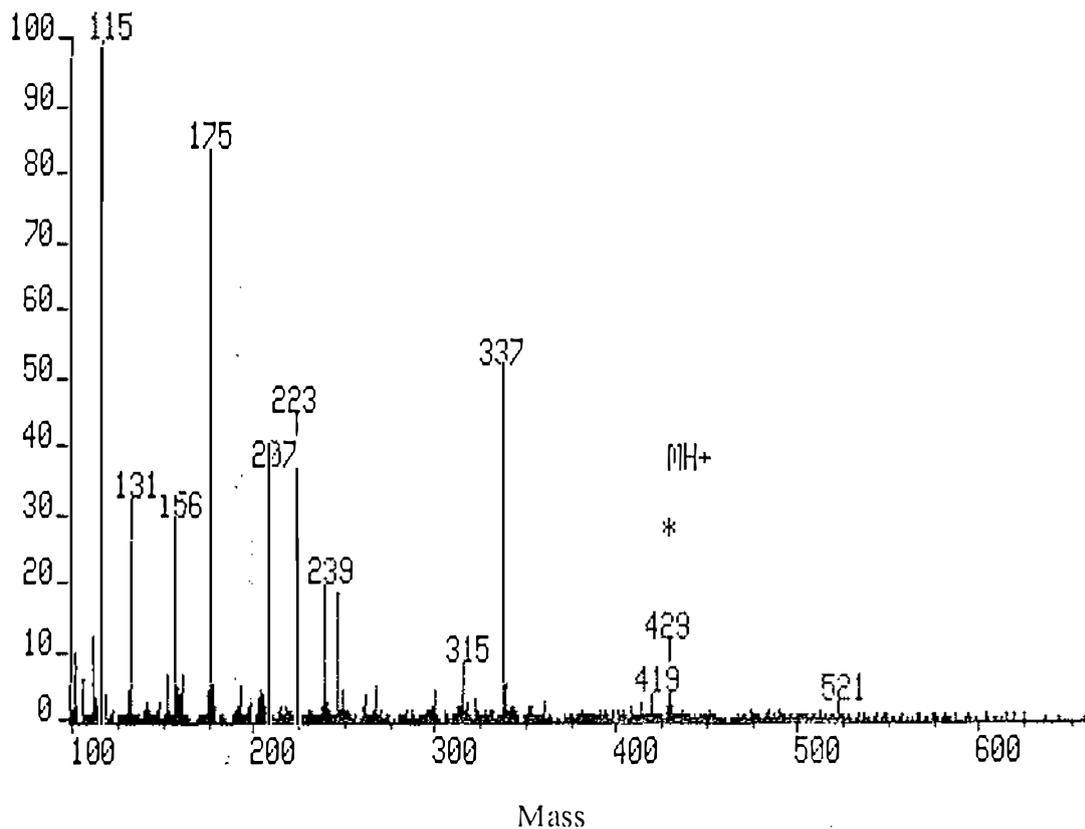
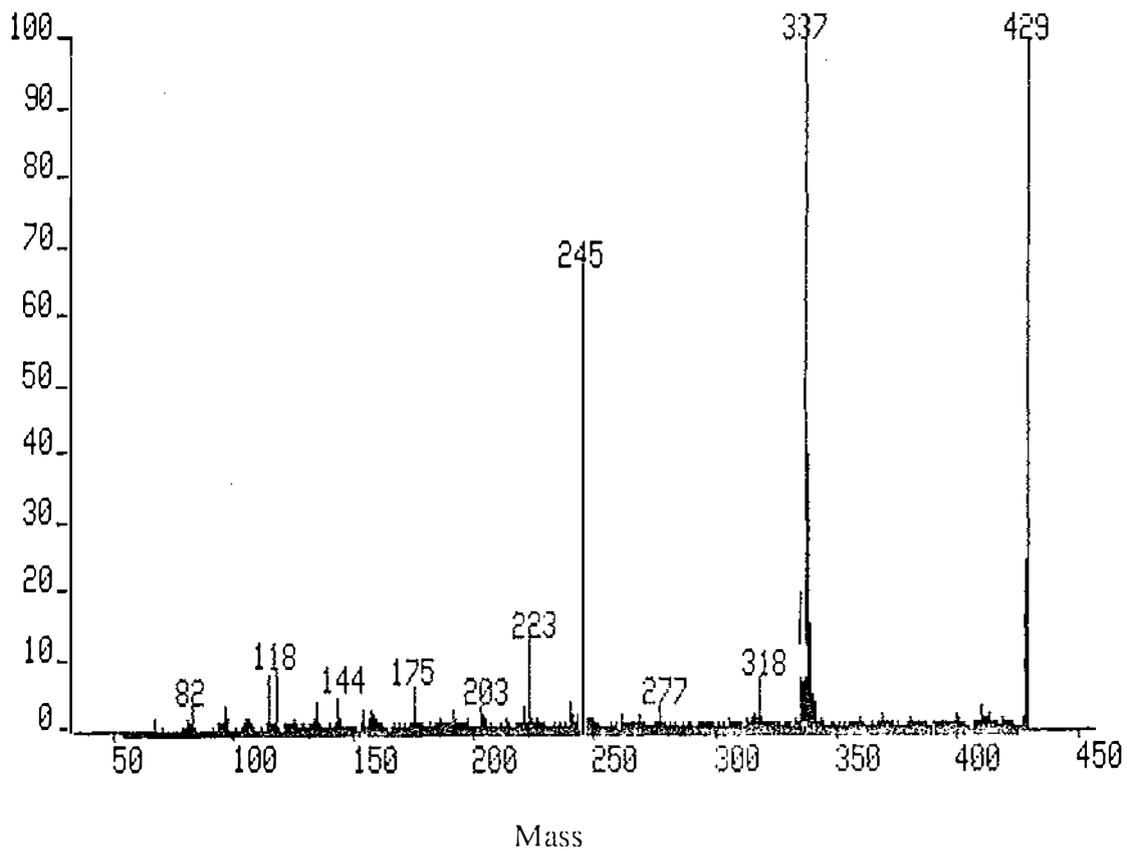


Figure 7.6b Mass Spectrum from Metastable Analysis of B-B-I-1  
(With Glycerol Spectrum Removed)



Notes on Figures 7.7a and 7.7b

Adipocytes were isolated from rats (180-220 g) which had been maintained on the Torula yeast diet. Adipocytes were incubated for 90 minutes in pH 7.4, 2% Albumin:30 mM HEPES buffer that contained 0.5 mM glucose and 0.2  $\mu$ Ci of labeled substrate. Incubation temperature was 37°C and the flasks were shaken at 100 rpm. Total incubation volume was 1.5 ml. Results are the mean of triplicate estimations. Bars shown on the graph represent a blank (▤), 10  $\mu$ U/ml Insulin (▥), 100  $\mu$ U/ml Insulin (▦), the sample only (▧) and the sample in the presence of 10  $\mu$ U/ml Insulin (▨).

Figure 7.7a Effect of Fraction of B-B-I-1-2, on the Insulin Stimulated Conversion of D-[U- $^{14}$ C]-glucose to  $^{14}$ CO $_2$

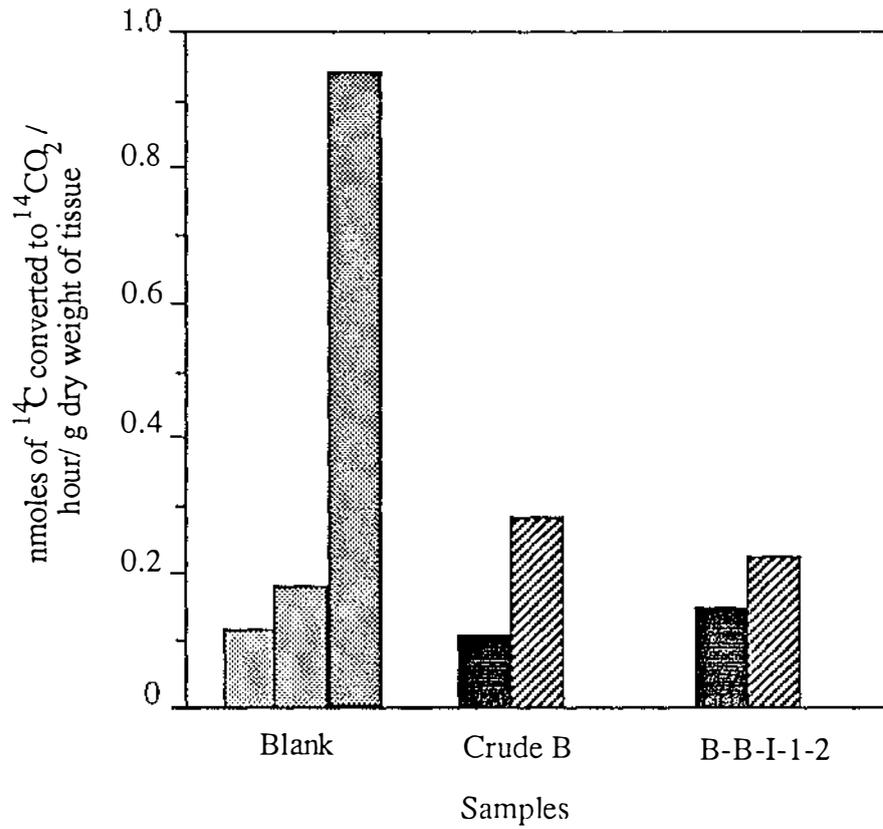
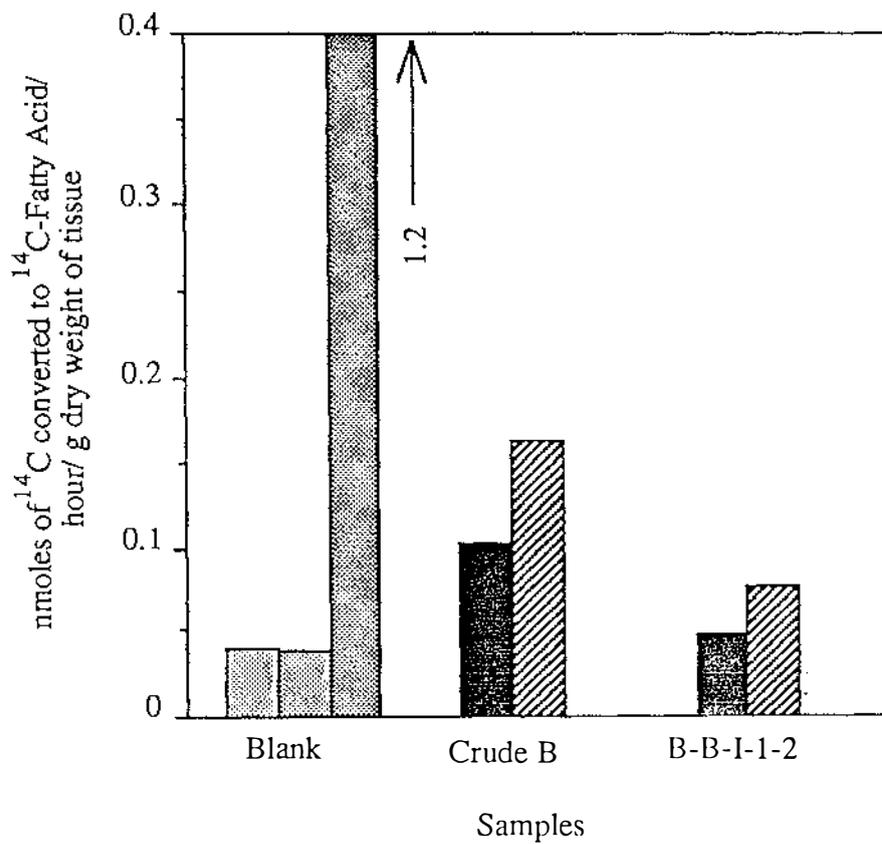


Figure 7.7b Effect of Fraction of B-B-I-1-2, on the Insulin Stimulated Conversion of D-[U- $^{14}$ C]-glucose to  $^{14}$ C-Fatty Acids



### Notes on Figures 7.8a and 7.8b

Adipocytes were isolated from rats (180-220 g) which had been maintained on the Torula yeast diet. Adipocytes were incubated for 90 minutes in pH 7.4, 2% Albumin:30 mM HEPES buffer that contained 0.5 mM glucose and 0.2  $\mu$ Ci of labeled substrate. Incubation temperature was 37°C and the flasks were shaken at 100 rpm. Total incubation volume was 1.5 ml. Results are the mean of triplicate estimations. Bars shown on the graph represent a blank (▨), 10  $\mu$ U/ml Insulin (▩), 100  $\mu$ U/ml Insulin (▧), the sample only (■) and the sample in the presence of 10  $\mu$ U/ml Insulin (▨).

Figure 7.8a Effect of Fraction of B-B-I-1-2, on the Insulin Stimulated Conversion of D-[1-<sup>14</sup>C]-glucose to <sup>14</sup>CO<sub>2</sub>

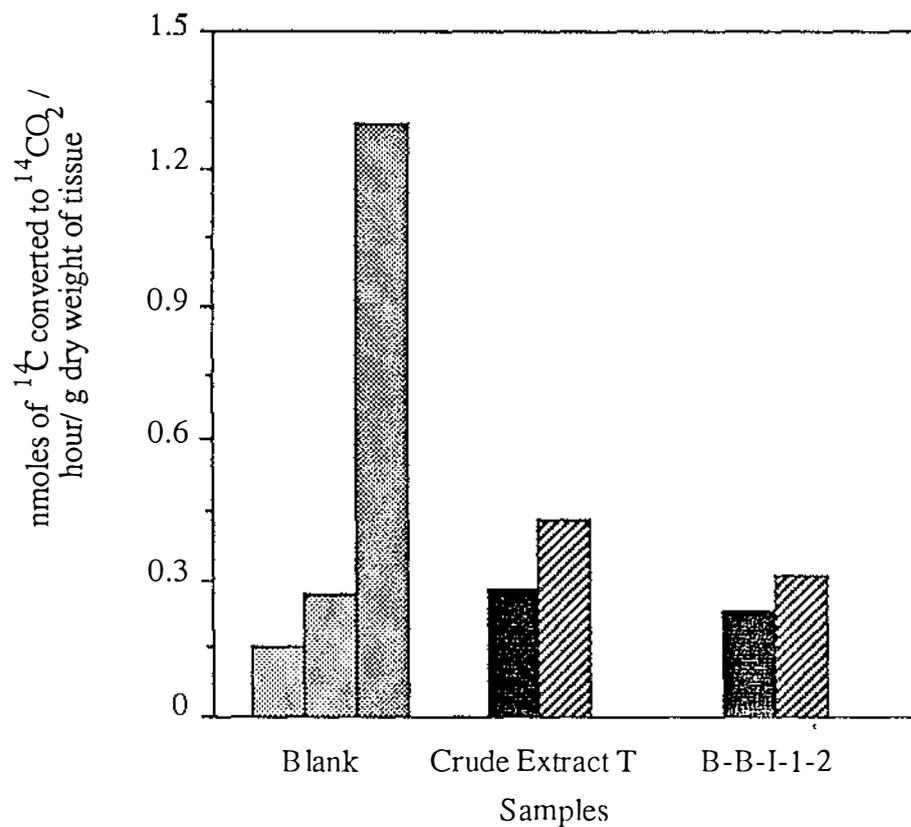


Figure 7.8b Effect of Fraction of B-B-I-1-2, on the Insulin Stimulated Conversion of D-[1-<sup>14</sup>C]-glucose to <sup>14</sup>C-Fatty Acids

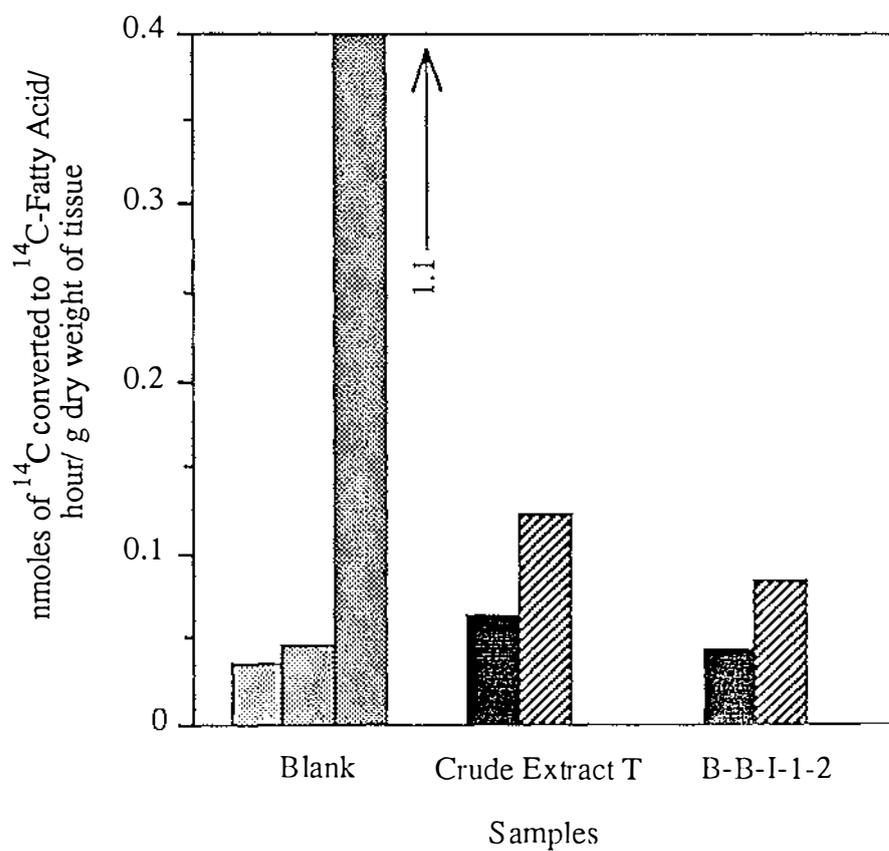


Fig 7.9a UV/Vis Spectrum of B-B-I

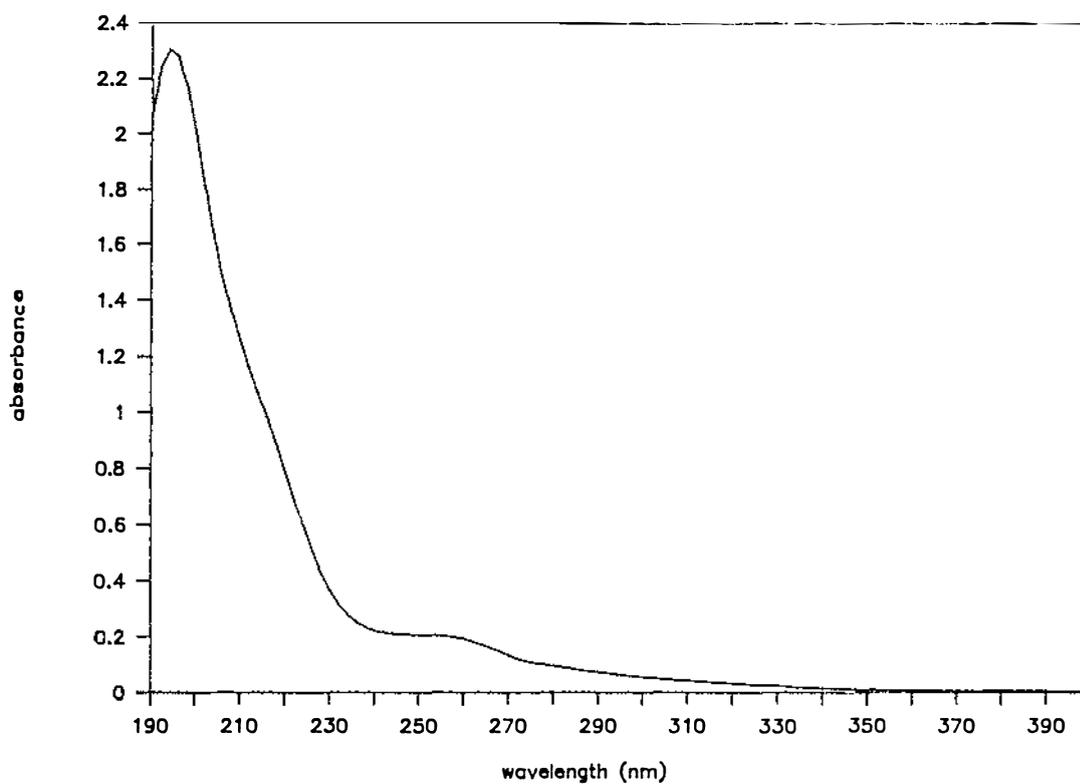
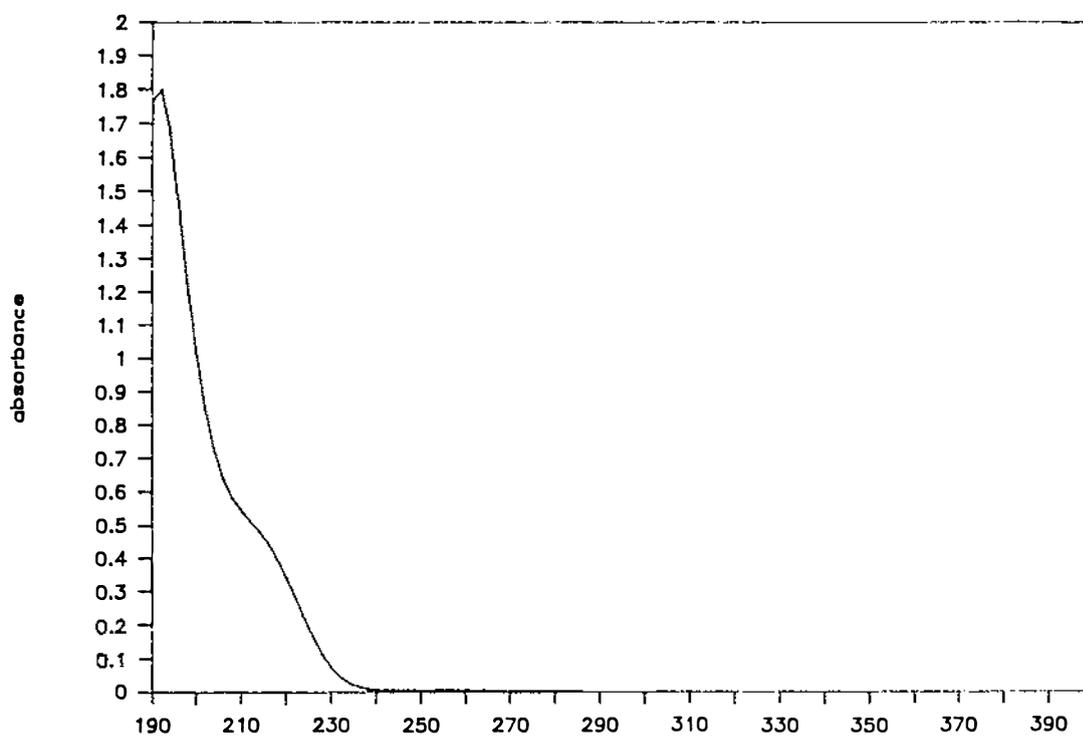


Fig 7.9b UV/Vis Spectrum of B-B-I-1



## 7.4 Discussion

The purification of the crude GTF extract B described in this chapter is very similar to those described in Chapter 3, where the standard yeast assay was used to determine the activity of the samples. The results of the low chromium adipocyte assays of the purified fractions, shown in Tables 7.1, 7.2 and 7.3, show the value of the expanded definition of GTF activity defined in section 6.4. It can be seen that in Table 7.1 that while several electrophoresis fractions, crude GTF extract B showed the most consistent ability to bring about high levels of potentiation of insulin action across the range of potentiations measured. This was held to indicate that, of the the electrophoresis fractions, fraction B-B-I showed the most GTF activity. This meant that the low chromium epididymal adipocyte assay was able to more clearly distinguish between different fractions showing apparent GTF activity. An inability to differentiate between a number of fractions showing apparent GTF activity was one of the major failings of the standard yeast assay for GTF described in Chapter 3.

Fraction B-B-I did not show any more activity than B and in fact it generally exhibited lower levels of activity than the crude extract (Table 7.1). However it was decided to further purify this fraction as one of the aims is to investigate the substances responsible for causing the effects attributed to GTF by seeking the most active purified fractions of the original GTF extract. Further purification using HPLC led to the separation of 5 fractions from fraction B-B-I, these being labeled B-B-I-1 to B-B-I-5. Of these, fraction B-B-I-1 showed the most activity in the adipocyte assay where it was almost as active as the crude GTF extract (Table 7.2). Fractions B-B-I-3 and B-B-I-5 also showed significant GTF activity but it was decided to further purify the most active fraction, which in this case was fraction B-B-I-1. Fraction B-B-I-1 was analysed by mass spectroscopy and was found to mainly contain a molecule of a molecular mass of 428 although the low resolution of the analysis didn't allow accurate determination of the material present. Other analysis showed that the molecule contains negligible amounts of amino acid and absorbs poorly in the UV region of the spectrum.

Further purification of B-B-I-1 was attempted using HPLC although limited amounts of fraction B-B-I-1 meant that only one of the 3 bands seen on the HPLC trace could be recovered in a large enough quantity to analyse. However this fraction, labeled B-B-I-1-2, showed only limited GTF activity in the adipocyte assay (Table 7.3) and mass spectrometric analysis proved unsuccessful.

The chromium concentration of fraction B-I was low compared with other fractions isolated from B (Table 3.7). The chromium concentration of B-I was consistent with the previously observed chromium content of the fractions which made up B-I (B-B-1 to B-B-3, see Table 3.7). Further purification of B-I did not lead to a concomitant increase in the chromium content of the most active sub fractions with fractions B-I-1 and B-I-1-2 having approximately the same chromium content as that of B-I. This shows that the most active fraction in the low chromium epididymal rat adipocyte assay was not a chromium complex. If it were then a chromium concentration several orders of magnitude higher would be expected in the purified fractions and it would be expected that there would be a consistent increase in chromium concentration with purification and this was not observed. This situation closely parallels the results described in Chapter 3 where an active anionic fraction was purified. It was found that the purification steps did not increase the chromium concentration of the most active purified fractions indicating that the substance responsible for the observed activity was also not a chromium complex.

The results presented here reveal two major trends when trying to purify the fraction giving the most GTF activity:

1. While some purified samples show high levels of activity in the low chromium rat epididymal adipocyte assay, purification of the sample does not seem to increase the specific activity above that of the crude GTF extract B, as can be seen in Tables 7.2 and 7.3.
2. The GTF activity is not concentrated in one fraction but is spread through several of the fractions. For example in Table 7.2 it can be seen that while B-B-I-1 was the most active of the fractions, both B-B-I-3 and B-B-I-5 showed indications of having significant GTF activity.

Again these trends are very similar to the pattern seen when the purification of GTF was attempted in Chapter 3 using the simple yeast assay for GTF to monitor activity. These results seem to confirm that both the yeast assay system and the low chromium epididymal adipocyte system are measuring similar properties in the purified GTF extracts.

In summary the separation procedures described here were unable to concentrate a single factor responsible for the activity attributed to GTF in the low chromium epididymal adipocyte assay. This is unusual as if GTF existed it would be expected that some amount of increased specific activity could be brought about by purification. The fact that several fractions contained activity may indicate that the overall effect of crude GTF extracts could be due to an additive effect of several components of the extract and that factors other than GTF may be responsible for this activity. A number of substances

likely to be found in yeast extracts have been shown to possess similar effects *in vitro* to those of GTF extracts (see section 1.5) and it may be that the observed activity of the extract is in fact due to an additive effect of these. Unfortunately the nature of the material responsible for the potentiation in the low chromium epididymal adipocyte system was not determined. This was also the experience of other workers who have described detailed purification schemes of crude GTF yeast extracts (Haylock *et al.* 1983a, Davies *et al.* 1985a) and this inability to positively identify a substance responsible for the noted effects remains a major enigma in this area of research. While the adipocyte assay system may not be measuring GTF as originally defined (Section 6.4) it can be concluded that no single factor exists to explain the the potentiation of the action of insulin in the adipocytes of rats that have been raised on a torula yeast diet. Further it appears that any involvement of chromium in the fractions that do cause potentiation is only artifactual and that a chromium complex is not responsible for these potentiations.

## CHAPTER 8

# OVERVIEW

The major problem with GTF research to date is that the very substance which this research seeks to identify lacks a suitable definition. GTF was originally defined as the substance able to repair an impairment in glucose tolerance seen in animals on a certain deficient diet. This definition has been expanded although GTF still only exists as a list of criteria (see section 1.1) and no single compound has ever been isolated which fulfils this criteria. The definition of GTF is particularly loose as; a) it is now known that a very large number of dietary factors affect glucose metabolism and b) the extracts initially claimed to contain GTF have since been shown to contain a wide range of substances. Despite this workers have proceeded to attempt to identify a unique substance that could be described as GTF and to attempt to isolate this compound they have developed assay systems that are claimed to measure GTF activity. However these assay systems are only related to the original definition of GTF by the fact that some substances that showed GTF activity by the original loose definition were also active in the new assay systems. This was held to indicate that the new assay systems were measuring the same substance as the original system.

Two of these assay systems, the GTF yeast assay and the low chromium rat epididymal adipocyte assay, were used in this investigation in an attempt to determine GTF activity in fractions of yeast. However the current study indicates that neither of these assay systems are measuring GTF activity as originally defined. Firstly the yeast assay has been shown to respond to substances likely to be found in crude GTF extracts such as amino acids (Jackson 1985) and ammonia (Chapter 2). In Chapter 4 an attempt was made to differentiate those samples acting as mere fermentable substrates from those that were causing a genuine stimulation of metabolism as would be expected for GTF. The results of this were inconclusive as while the crude extract was active in this modified yeast assay system, the purified fraction of this crude extract which had shown the most activity in the standard yeast assay was not active in the modified assay system. This indicated that the standard yeast assay system was not measuring GTF activity as originally defined.

Secondly, the fact that extracts prepared from torula yeast had significant activity in both the GTF yeast assay and the low chromium rat epididymal assay (Chapters 3 and 5) suggests that these two assay systems are not measuring GTF activity as originally defined. Since torula yeast the sole protein source in the diet, that is used to raise the rats whose adipocytes respond maximally to GTF fractions in the assay, it has been assumed

that torula yeast is deficient in GTF. If the adipocyte assay was measuring only GTF activity then extracts from the torula yeast would not be expected to possess activity in that assay system. Therefore the fact that torula yeast extracts are very active in the adipocyte assay indicates that it is likely that this assay system is not measuring GTF activity as originally defined. The conclusion that neither of the assay systems is measuring GTF activity as originally defined is supported by the fact that on further purification of the crude GTF extract, the activity from that fraction is dispersed amongst several sub fractions. If the assay was measuring a unique GTF compound present in the crude extract then the activity would be expected to concentrate in one fraction.

While it doesn't appear that the assay systems are necessarily measuring GTF activity as it was originally postulated it is important to explain the very real effects caused by the yeast extracts observed in the current study. The most important of these is the potentiations of insulin metabolism in the adipocytes of rats on a special torula yeast diet. The results presented here (Chapters 5 & 6) concur with the observations of other workers (Mertz 1969, Anderson *et al.* 1978a, Davies *et al.* 1985a) who also found that dietary regimes are important in determining whether the action of sub optimal quantities of insulin in adipocytes of rats will be potentiated by GTF extracts. As found by these other workers the effects are most pronounced in the adipocytes of rats fed a torula yeast/sucrose diet low in chromium. In the current work it was found that the key difference between the metabolism of the adipocytes of animals on normal diets and those on the torula yeast diet was that in those of rats on the torula yeast diet there was an impairment of fatty acid synthesis via the glycolytic pathway. This increased the relative importance of the pentose phosphate pathway in the synthesis of fatty acids in these cells. It was found that the most important potentiation of the actions of sub optimal concentrations of insulin by the GTF extract was obvious in the products of the pentose phosphate pathway. It was postulated that this was either due to a potentiation of glucose transport into the cell or due to a potentiation of the post pentose phosphate pathway fatty acid synthesis mechanisms, either of which was likely to lead to a concomitant increase in pentose phosphate pathway. The strong potentiation of the metabolism of acetate to fatty acids suggested the latter mechanism and the enzyme acetyl-CoA carboxylase was postulated as a possible site for this potentiation. However ultimately there is insufficient evidence to decide between these two mechanisms and resolving this would be an important area for future research.

The nature of the fractions causing the activity attributed to GTF in these assays was investigated beginning with the GTF extract of Toepfer *et al.* (1977). This fraction was thought to be contain GTF of the type postulated by Mertz *et al.* (1974) and which supposedly conformed with the original definition of GTF. The investigations here show

that this material was indeed very active in both the yeast assay and the low chromium epididymal adipocyte assay. A comparison of the patterns of activity seen for the various subfractions of the crude yeast extract obtained in the current study shows that there is a high degree of correlation between the results of the standard yeast assay (Tables 3.4 and 3.8) and those of the low chromium epididymal adipocyte assay (Tables 7.1, 7.2 and 7.3). However, the conclusion that this material represented a reasonably pure form of GTF (Toepfer *et al.* 1977) has been shown to be false as investigations described here show the material was far from pure. Further, on purification the activity found in the original extract dispersed into the subfractions rather than concentrating in a single fraction as would be expected if the sample contained a single unique GTF. This is consistent with the results of other workers who have identified a wide range of fractions showing activity attributed to GTF (Haylock *et al.* 1983a & b, Held *et al.* 1984, Davies *et al.* 1985a & b). Therefore the results presented in the current study show that it is unlikely that a single substance exists to explain the effects attributed to GTF. The composition of the fractions that were isolated in the current study and which displayed activity attributed to GTF show little relationship to the composition for GTF postulated by Mertz *et al.* (1974) and this is further support for the postulate that no unique substance to explain the effects attributed to GTF exists.

The fact that the current study shows that further purification of crude GTF extracts confirms the presence of several active sub fractions highlights the dangers of accepting conclusions made by other workers who followed the chromium content of fractions as an indicator of GTF material present. These workers often only subsequently assayed those fractions and therefore even if such an assay showed activity it is likely that other fractions that were not assayed would also have shown activity.

The occurrence of chromium in crude GTF extracts has long been held to indicate the presence of a unique chromium complex in these extracts, which it was assumed was responsible for the effects attributed to GTF. In the current study the yeasts used to obtain these extracts had a chromium concentration of the order of several  $\mu\text{g/g}$  and yet the crude extract had a concentration of  $44 \mu\text{g/g}$  (Table 3.2). It appears however that this concentration of chromium is not indicative of a unique chromium complex which is responsible for the effects attributed to GTF, as on purification of the crude GTF extract the chromium is spread amongst many fractions (Table 3.7 and section 7.3.4) and the chromium levels in these fractions bear no relationship to activity in the yeast assay (Table 3.4) or the low chromium epididymal adipocyte assay (Tables 7.1, 7.2 and 7.3). It thus appears that the chromium is merely an artifact of the isolation procedure. There are two

plausible explanations for the appearance of the artifactual chromium in the crude GTF extract:

1. It has been shown that chromium binds to yeast cell components (Kumpulainen *et al.* 1978, Haylock *et al.* 1983b and Hwang *et al.* 1987). Since the low molecular weight materials which bind chromium well are exactly the type of material isolated in the initial extraction steps (Chapter 3), chromium would be fortuitously separated with these.
2. Isolation of the crude GTF extract involves the treatment of the extracted yeast cell suspension with activated charcoal at pH 3.5 followed by elution of the charcoal under highly basic conditions. Since chromium binds to charcoal at pH 3.5 and is eluted at a high pH (Huang and Wu, 1977), this step may well concentrate the chromium and explain the level of 44  $\mu\text{g/g}$  chromium described above.

It therefore is simplistic to assume that the higher levels of chromium present in the crude extract indicate that GTF is a chromium complex. On the contrary, on the basis of the results presented here it seems likely that the material responsible for the *in vivo* effects attributed to GTF is not a chromium complex. This does not preclude a biologically active form of chromium (BAC) from having some *in vivo* role in metabolism but it is unlikely that it is responsible for the effects attributed to GTF.

In summary it appears that the *in vitro* assay systems for GTF activity are not measuring GTF as originally defined and so properties attributed to GTF on the basis of these assays seem invalid. Further it appears that there is not a single unique compound responsible for the activity that these *in vitro* assay systems reveal. In the absence of a unique compound the most active fractions in each of the assay systems were investigated however the exact nature of these fractions remained elusive although it is apparent that they are not chromium complexes and that they bear little resemblance to the composition postulated for GTF. This should not detract from the fact that any substances showing potentiation of the action of insulin in adipocytes which had an impaired glucose metabolism are potentially interesting as they could have pharmacological significance for the treatment of conditions relating to impairments of the glucose metabolism. Thus even though there appears to be no relationship between these substances and the glucose tolerance factor as originally defined, their effects should be investigated in tissues such as muscle which play a more significant role in the maintenance of glucose homeostasis.

## List of Abbreviations Used

Ala	Alanine
Arg	Arginine
Asn	Asparagine
Asp	Aspartic Acid
B	Crude yeast extract from brewers yeast
Cys	Cysteine
CoA	Co-enzyme A
GFAA	Graphite Furnace Atomic Absorption Spectroscopy
Gln	Glutamine
Gly	Glycine
Glu	Glutamic Acid
GTF	Glucose Tolerance Factor
HPLC	High Performance Liquid Chromatography
H #	The H Number
ICP	Inductively Coupled Argon Plasma Emission Spectroscopy
Ile	Isoleucine
Leu	Leucine
Lys	Lysine
Phe	Phenylalanine
Ser	Serine
Thr	Threonine
Trp	Tryptophan
T	Crude yeast extract from torula yeast
UV	Ultra Violet
Val	Valine

**APPENDIX 1**Composition of Diet for Rat Studies

Note: The suppliers indicated for the chemical components of the diet are those of the components prepared in our laboratory. Those of the Teklad diet were not specified.

<u>Component</u>	<u>Supplier</u>	<u>Concentration g/kg</u>
Protein		300.00
DL-methionine	Fluka	3.00
Sucrose	Ajax	603.1587
Fat		50.00
Hubbell-Mendel-Wakeman Mineral Mix (see below)		40.00
Manganese Sulphate	Ajax	0.12
Sodium Selenite	May and Baker	0.0003
Inositol	Sigma	1.00
Para Amino Benzoic Acid	Sigma	0.3
Niacin	BDH	0.1
Calcium Pantothenate	BDH	0.06
Thiamin Hydrochloride	BDH	0.012
Riboflavin (Vitamin B <sub>2</sub> )	BDH	0.012
Pyridoxine Hydrochloride	BDH	0.005
Folic Acid	BDH	0.002
Biotin	BDH	0.0003
Vitamin B <sub>12</sub>	BDH	0.0002
Choline Chloride	BDH	2.00
Menandione (Vitamin K <sub>3</sub> )	BDH	0.002
DL-Tocopherol Acetate (Vitamin E)	BDH	200 U/kg
Vitamin A	BDH	12500 U/kg
Vitamin D <sub>2</sub>	BDH	2500 U/kg

Hubbell-Mendel-Wakeman Mineral Mix

<u>Component</u>	<u>Supplier</u>	<u>Concentration g/kg</u>
Calcium Carbonate	May and Baker	543.0
Magnesium Carbonate	BDH	25.0
Magnesium Sulphate	Riedal Hahn	16.0
Sodium Chloride	BDH	69.0
Potassium Chloride	BDH	112.0
Potassium Dihydrogen Phosphate	Ajax	212.0
Ferric Pyrophosphate (we used Ferric Sulphate)	BDH	20.5 (33.0)
Potassium Iodide	Ajax	0.08
Manganese Sulphate Monohydrate	Ajax	0.35
Sodium Fluoride	Aldrich	1.0
Aluminium Potassium Sulphate. 12 H <sub>2</sub> O	May and Baker	0.17
Cupric Sulphate	May and Baker	0.90

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## ERRATUM

All results for Figures 5.7, 6.2, 6.3 and 6.4 should be multiplied by a factor of 15.