GTF FROM BREWERS' YEAST

by

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Diana de Davies

9/3/1983

PREFACE AND ACKNOWLEDGEMENTS

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ABSTRACT

The nutritional deficiency of chromium has been linked with impaired glucose tolerance, and more recently has been implicated in cardiovascular disease. It was believed that a biologically potent form of chromium existed as a coordination complex with nicotinic acid and various amino acids. The biologically active chromium complex was termed glucose tolerance factor (GTF) due to the curative effect on rats raised on a chromium deficient diet resulting in impaired glucose tolerance. The most studied source of GTF has been brewers' yeast although little has been learnt regarding the structure of GTF. The aim of this thesis was to isolate and characterize GTF.

An existing *in vitro* assay system for GTF has been developed and is reported in this thesis. The assay was based on the anaerobic oxidation of ^{14}C-glucose by yeast (*Saccharomyces cerevisiae*) and its stimulation by GTF.

Brewers' yeast was used as a starting material for the isolation of GTF. A defined synthetic medium was eventually used for yeast culture in order to establish that GTF was produced by yeast and not derived from culture medium components. GTF was isolated from yeast grown in synthetic medium and in higher yield when chromium was present. Low levels of chromium were found to inhibit yeast growth.

A biologically active cationic fraction extracted from yeast was purified by chromatography. Nuclear Magnetic Resonance spectroscopy and other instrumental techniques indicated that the isolated material was a small lysine peptide devoid of chromium which could be likened to a class of basic hypoglycemic agents.

It was concluded that, as recent clinical trials reported in the literature indicated a division between the effect of chromium supplementation, and supplementation with high chromium yeast, GTF isolated from yeast grown in presence of chromium represents an interesting hypoglycemic agent.

CONTENTS

CHAPTER 1

INTRODUCTION

1.1 PROLOGUE

Many enzymes, particularly those involved in oxidation-reduction reactions, contain certain metal ions of the first and second transition series as prosthetic groups. Chromium, a transition metal in subgroup VI B of the periodic table, was first implicated as being an essential nutrient in the 1950's. In humans, chromium deficiency has been linked with impaired glucose metabolism (Mertz 1981), raised cholesterol levels and cardiovascular disease (Newman *et al* 1978). As well as human studies, chromium supplementation studies have been conducted on rats (Schwarz and Mertz 1959), rabbits (Abraham *et al* 1982), turkeys (Steele and Rosebrough 1978), swine (Steele *et al* 1977), trout (Tacon and Beveridge 1982), hens (Jensen *et al* 1978), squirrel monkeys (Davidson *et al* 1967) and microorganisms (Gutierez *et al* 1974, Burkeholder and Mertz 1966).

Supplementation with brewers' yeast was reported first in 1927 by Glaser and Halpern, when it was found that yeast extract potentiated insulin action. The protective factor in yeast extract was later described as glucose tolerance factor (GTF) by Mertz and Schwarz (1957), the activity of which was attributed to the chromium content of the yeast.

McCarty (1980) has speculated on the possible therapeutic applications of GTF derived from yeast. As the action of insulin was reported to be enhanced by GTF, the predictions were made by considering the wide range of physiological effects attributed to insulin. The most

obvious application was seen to be the treatment of either maturity onset diabetes or juvenile diabetes mellitus as some studies have indicated therapeutic results with yeast or yeast extract (Liu *et al* 1977). GTF was seen to have a role in cancer therapy as insulin injections, anti-glycolytic and anti-gluconeogenic agents have been found to retard tumour growth. GTF administration was seen as a possible alternative, with less likelihood of toxicity posing a problem. High doses of GTF were proposed as a possible hunger suppressant during low calorie dieting as insulin has been found to stimulate the hypothalamic satiety centre. The anabolic effect of insulin on muscle was seen as indicating that GTF supplementation may have value in the management of anorexia nervosa, neoplastic cachexia, cystic fibrosis and protein malabsorption. Tryptophan (or tyrosine) therapy of depression was proposed as another area in which GTF supplementation could be beneficial along with migraine and insomnia treatment. As insulin enhances brain dopa uptake, increased GTF levels were expected to produce a similar effect and may thus be useful in the dopa treatment of Parkinsonism. The reduction in total serum cholesterol and triglyceride levels, accompanied by an enhancement of HDL levels with yeast supplements was seen as having value in preventative health measures and special value in the therapy of various hyperlipidemias.

It can be seen that GTF has been implicated in the treatment of many disorders, yet little is known of the nature of the material.

1.2 THE INITIAL ESTABLISHMENT OF CHROMIUM AS A MICRONUTRIENT

Mertz and Schwarz (1954) reported severe hypoglycemia and inability to utilize injected glucose in rats dying from necrotic liver degeneration when raised on a torula yeast based diet.

Supplementation of the diet with debittered brewers' yeast or defatted pork kidney powder protected against death from necrotic liver degeneration, a syndrome later described as being caused by a combined vitamin E-selenium deficiency. Brewers' yeast and pork kidney powder supplementation was found to correct the impaired glucose tolerance detected in rats raised on the torula yeast diet several weeks before the onset of the terminal phase.

The prophylactic factor was termed factor 3 (Mertz and Schwarz 1955). Crude extracts of brewers' yeast and acid hydrolysates of defatted pork kidney powder were also found to be effective, however, with progressive purification the factor protecting against necrotic liver degeneration was clearly separated from a factor that prevented the defect in glucose metabolism which was designated as glucose tolerance factor (GTF) (Mertz and Schwarz 1957). Ashing of active preparations did not destroy the protective ability of GTF although the activity was reduced, indicating a mineral content. Chromium was implicated as being the active ingredient by a spot test of the ashed sample. In order to establish the identity of the active element, complexes of forty different elements were given by stomach tube to rats fed on a diet containing 30% torula yeast (Mertz and Schwarz 1959). Only chromium III complexes were found to consistently improve glucose tolerance, thus chromium was regarded as the essential element of GTF.

Commercial pelletized diets as well as semi-purified torula yeast based diets were compared with natural diets (Mertz and Schwarz 1959). Rats fed the commercial diets developed impaired glucose tolerance whereas the rats fed on a natural diet did not. Impaired glucose tolerance was corrected within 18hours by giving GTF containing fractions from brewers' yeast or from pork kidney powder.

Yeastamin, an enzymatic digest of brewers' yeast, was also found to be a source of GTF as dietary supplementation prevented the development of low glucose removal rates. GTF concentrates were successfully applied by stomach tube; purified GTF was also satisfactorily administered intraperitoneally and intravenously (Mertz and Schwarz 1959).

As conversion to fat was regarded as the major metabolic pathway for excess sugar, Mertz, Roginski and Schwarz (1961) investigated glucose uptake by peripheral tissue - specifically, epididymal fat pads - in order to define the site of action of GTF. Various chromium salts were found to stimulate the oxidation of glucose *in vitro* by fat pad pieces as did those of manganese although to a lesser degree. The effect was found only in presence of exogenous insulin and observed best with sub-optimal insulin levels.

Mertz and Roginski (1963) investigated the effect of inorganic chromium on galactose uptake of epididymal fat pads from chromium deficient rats. Galactose is poorly metabolised by peripheral tissue *in vitro* with entry into the cell being insulin regulated. Significant increases in the rate of entry of galactose were found with low levels of chromium (0.01-0.1μg). The increase was only found with tissue from chromium deficient rats. It was concluded that the site of sugar entry into the cell was effected by chromium III and that chromium may be an essential cofactor for insulin action, although other sites were known to be effected by higher chromium levels (Curran 1954 - cholesterol synthesis).

1.3 CHROMIUM-INSULIN INTERACTION

The inhibition of the effect of insulin on sugar transport by a thiol blocking agent (Cadenas *et al* 1961) and later the release of bound insulin from epididymal tissue (Fong *et al* 1962) suggested that a disulphide bridge may bind insulin to its receptor. Polarographic studies of chromium-insulin-mitochondria interactions undertaken in specific and non-physiological conditions found that a polarographic wave, appearing to originate from the mitochondrial membrane, was shifted to a more positive potential when insulin was added, suggesting an interaction between the mitochondrial sulphydryl group and insulin (Christian *et al* 1963). A similar effect was produced by chromium. Insulin and chromium together produced an effect threefold greater than the sum of the individual shifts. The results were interpreted as demonstrating that chromium initiates the formation of the new disulphide links between the intrachain of insulin and membrane sulphydryls by participating in a ternary complex in which chromium would coordinate to four sulphur atoms. The result of the possible coordination was thought to be a weakening of the original intrachain disulphide of the A chain of insulin, leading to the formation of a new insulin-membrane disulphide bridge. Mertz *et al* (1974) published a diagram illustrating the hypothetical ternary complex (Figure 1.1).

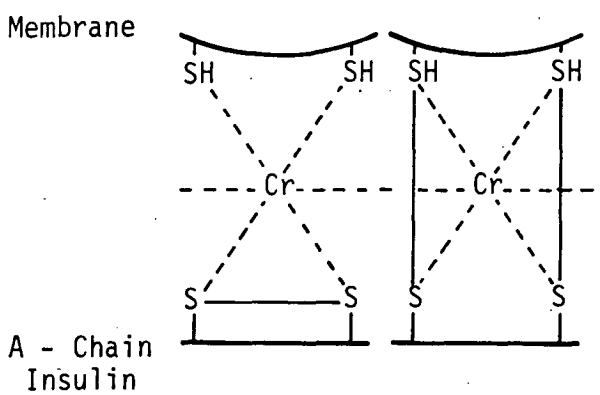


FIGURE 1.1 Hypothetical ternary complex of chromium at site of action (Mertz *et al* 1974).

Excessive non-physiological quantities of insulin as used in the early binding experiments can bind nonfunctionally and insulin can be re-extracted intact as would seem to have been the case with Fong *et al* (1962). Campbell and Mertz (1963) isolated liver mitochondria and measured the swelling of the mitochondria by the change in absorbance at 520nm in the presence of small quantities of insulin and chromium. Swelling was found to occur at concentrations of chromium and insulin at which each were ineffective, further implicating a chromium-insulin-receptor effect.

Mertz (1969) reviewed aspects against the sulphydryl ternary complex model at the receptor and pointed out that carboxyl groups have a high affinity as ligands for chromium compared to the thiol group and would thus seem to discount the ternary complex model. In view of more recent findings, a chromium-insulin interaction rather than one involving the structure of the receptor, which is not known, would seem to be more likely. Despite extensive investigations, the coupling system between the insulin receptor complex and the influence of the hormone on enzymes and membranes has not been identified (Walaas and Horn 1981).

Belusko (1977) has demonstrated by circular dichroism and front gel chromatography, a dissociative effect of chromium III on zinc added to insulin, preventing aggregation that would otherwise occur. Anderson (1978) reports that synthetic labelled chromium complexes bind tightly to insulin *in vitro* thus some chromium-insulin interactions would seem to exist. Anderson (1979) later demonstrated that a chromium-nicotinic acid-glutathione complex (Section 1.9.3) had a minimal effect on glucose transport in isolated adipocytes and implied a primary effect after entry of glucose into the cell, whereas, the chromium-insulin-receptor complex had been thought to favour increased glucose

transport. The binding of chromium to insulin and the effect of chromium on glucose oxidation may thus be unrelated.

1.4 BODY STORES OF CHROMIUM

Chromium is present in large amounts in the brain and liver (Nath *et al* 1979) and high levels of chromium have been associated with inhibition of insulin secretion from the pancreas of the rat (Ghafghazi *et al* 1979). Studies of chromium exchange in animals using ^{51}chromium, show that chromium is rapidly cleared by urinary excretion and tissue uptake (Hopkins 1965, Mertz *et al* 1965, Onkelinx 1977).

Jain *et al* (1981), using ^{51}chromium of high specific activity, studied tissue chromium exchange in both chromium deficient and supplemented rats. Chromium deprivation of a moderate degree reduced serum chromium and tissue-exchangeable chromium pools but did not change total tissue chromium, ^{51}chromium distribution or glucose tolerance. Chromium supplementation and chromium overload increased serum chromium and total, exchangeable and non-exchangeable tissue chromium pools but not ^{51}chromium distribution. A simplified flameless atomic absorption technique was developed by Jain *et al* and used in conjunction with the isotope technique but has yet to be published.

1.5 HUMAN STUDIES

1.5.1 Inorganic chromium in clinical trials and recommended dietary chromium

Chromium deficiency in human subjects cannot yet be reliably diagnosed by chromium analysis of body fluids or tissues (Mertz 1981). Difficulties in obtaining true chromium levels in biological material has

invalidated earlier work on determining chromium balances, body chromium pools and chromium status of individuals (Section 1.10).

It has been implied that the recognition of chromium deficiency depends on diagnosis of impaired glucose tolerance in the presence of normal levels of insulin (Mertz 1976). Recent work by Nath *et al* (1979) and Riales (1979) has demonstrated that dietary chromium may affect not only glucose homeostasis but also lipid metabolism.

Jeejeebhoy (1977) and Freund (1979) reported independently that patients sustained by administration of intravenous fluids developed glucose intolerance and insulin resistance. The disorders were normalized after administration of chromium chloride for seven days but not by the administration of insulin. 20µg per day of chromium was found to be sufficient to maintain the well being of the patient. Sherman (1968) treated normal subjects and diabetes mellitus sufferers with placebos and 150µg per day of chromium chloride in a double blind investigation of glucose tolerance and hyperglycemia. It was concluded that chromium had not improved glucose tolerance after sixteen weeks treatment and that chromium is not essential for normal glucose tolerance in man. Mertz (1977) responded to the findings of Sherman by indicating that a number of factors affect glucose removal rates and thus chromium deficiency should not be construed as the sole criterion for impaired glucose tolerance.

As a result of clinical trials, a recommended daily allowance (RDA) of 50-200µg per day of trivalent chromium was proposed (Mertz 1980). An average chromium intake for healthy males in the United States, for comparison, was reported to be 78±43µg per day by Kumpulainen *et al* (1979).

Regional variations resulted in some diets being reported as marginal i.e. less that 50µg per day. Kumpulainen (1980) later reported that the mean chromium intake per capita for Finnish people is the lowest known being 29µg per day.

It would thus seem that adequate chromium intake is not always supplied by common diets in the United States or in countries with low chromium soils such as Finland or where basic malnutrition problems exist. Until it becomes possible to test for chromium deficiency in humans, the extent of chromium deficiency cannot be appreciated (Mertz 1969).

1.5.2 The case for biologically active chromium

Mertz (1977) suggested that with advancing age the ability to convert inorganic chromium to a biologically active form decreases and that some insulin requiring diabetics may also have this defect (Anderson and Mertz 1977).

Carbohydrate abnormalities in malnourished children were found to respond to inorganic chromium within sixteen hours (Hopkins 1965). Improvement of glucose tolerance was also reported in marasmic Turkish children fed 250µg of inorganic chromium sixteen hours before testing (Gurson 1971). Elderly subjects however, fed up to 100µg per day, required one to three months to respond. One third to one half of the elderly subjects failed to respond (Mertz 1977, Glinsmann 1966). Due to the disappointing response of the elderly people to inorganic chromium, investigations with brewers' yeast were pursued.

Doisy *et al* (1976) studied the effect of brewers' yeast extract on subjects over the age of sixty five with impaired glucose tolerance. Half of the subjects showed improved glucose tolerance. Liu *et al* (1977) also reported improved tolerance to glucose in women with hyperglycemia after supplementation with yeast extract. A recent controlled study by Offenbacher and Pi-Sunyer (1980) of a group of subjects with a mean age of 78 years and including some mild diabetics, were fed chromium rich brewers' yeast with other members of the group being fed low chromium torula yeast. The group receiving brewers' yeast had improved glucose tolerance, insulin sensitivity and cholesterol levels thus establishing the effectiveness of brewers' yeast supplementation.

Differences in absorption rates between inorganic chromium and chromium in yeast GTF, imply that chromium may be more nutritionally important as the GTF complex. Chromic chloride was found to be poorly absorbed in the gut with 0.5-1.5% absorption being reported in animals and man (Donaldson 1966, Anderson 1980, Levine 1968). This is presumed to be because of the tendency of chromium to olate at physiological pH (Mertz 1969). In animals, 10-25% of the organically complexed chromium in brewers' yeast was absorbed and appeared to be immediately available since the treatment led to the rapid improvement in glucose tolerance (Mertz 1969, Mertz and Roginski 1971).

In conclusion, Anderson (1981), reviewing the nutritional role of chromium in light of improved analytical techniques, points out that the form of dietary chromium does not appear to be as important as had been postulated previously and that it is total chromium intake that is important for normal individuals.

1.6 CHROMIUM, GTF AND YEAST METABOLISM

1.6.1 Inorganic chromium and yeast metabolism

Burkeholder and Mertz (1966) investigated the effects of chromium on yeast metabolism using radio-chromium (^{51}Cr) and the yeast strain *Saccharomyces carlsbergensis* (brewers' yeast, now *Sacch. cerevisiae*) in Sabouraud's liquid medium. ^{51}chromium incorporation was found to be independent of cell growth but was stimulated by the addition of glucose. Glucose oxidation was stimulated by low levels of chromium (0.1ppm) relative to chromium-free cells while higher levels (10ppm) were found to depress fermentation. Generally, a lag time of several hours was found before increased oxidation, and this was attributed to the tissue required to form a chromium complex (GTF) which could then exert its influence on glucose metabolism.

Polansky and Anderson (1977), while investigating extractive techniques for GTF, found a slow chromium uptake followed by rapid increase in uptake after eight days culture of *Sacch. carlsbergensis* in synthetic medium. Glucose additions at five and nine days growth decreased the amount of incorporated chromium label contrary to the findings of Burkeholder and Mertz (1966) using peptone based medium (Sabouraud's Liquid Medium). Anderson *et al* (1978), using ^{51}chromium, found incorporation to be lower in the synthetic culture medium of Toepfer *et al* (1970) compared with a peptone based medium. An extract of yeast grown on the synthetic medium had comparatively little biological activity and may indicate that the GTF originated from the crude medium.

Kumpulainen *et al* (1978) found that the incorporation of chromium from Sabouraud's medium correlated directly with the metabolic activity of the yeast and that the incorporation was highest with anaerobic conditions. A slight reduction in growth occurred with 1.0ppm chromium, the effect being more marked at 4.0ppm. Haylock *et al* (1982) reported that chromium exerted a slight inhibitory effect on fermentation rate in phosphate buffer and a defined synthetic medium, but not on cell growth. These findings are similar to those reported by Mirsky, Weiss and Dori (1980). Haylock *et al* (1982) concluded from preincubation experiments with simple chromium III salts, that there was no evidence that brewers' yeast can synthesize a biologically potent chromium complex. Furthermore, response to GTF fractions was found regardless of whether the yeast was replete or depleted of chromium.

Burkeholder and Mertz (1966) found no difference in chromium uptake over the concentration range of 0.001-1.0ppm contrary to the findings of Kumpulainen (1978). As the chromium content of the cells was always less than the medium and correlated positively with the concentration of the medium, Kumpulainen *et al* (1978) attributed chromium uptake to diffusion and ionic concentration balance. The nature of the ligands of added chromium complexes was found to have little influence on the rate and extent of chromium absorption in yeast (Anderson *et al* 1977).

1.6.2 Toxicity of chromium

Hartelius (1956) found chromium to be toxic at 200ppm and that toxicity is related to the nature of the medium. High levels of chromium in malt wort had no effect on cell growth, whereas the same

concentration in defined synthetic medium, inhibited totally. Ono and Weng (1982) have found that the structure gene for homocitrate dehydrase is involved in the detoxification of chromium in the wild type strains of yeast.

The accumulation or metabolism of a metal does not establish physiological need but may be a detoxification mechanism (Harrison, Hoare, 1980). It may be that low levels of chromium diffusing into the yeast cells are toxic, resulting in the slight reduction in fermentation rates previously noted.

In media containing peptone, it may also be added that chromium is bound by polypeptides and thus not able to diffuse into the yeast cell. Defined synthetic medium lacking peptone could not provide the same protective mechanism. Part of this thesis will examine the possibility that GTF is produced in response to chromium III.

1.6.3 Yeast as an assay system for GTF

Burkholder and Mertz (1966) found that the response to GTF by brewers' yeast was immediate and dependant on glucose utilization, thus the yeast system was proposed as a biological test for GTF. Mirsky *et al* (1980) were not able to produce an immediate response to GTF unless the cells were preincubated with glucose before the addition of GTF, however, after preincubation, significant increases in carbon dioxide production resulted in response to fractions derived from yeast extract. The yeast assay system was utilized successfully by Haylock *et al* (1982) for rapid screening for GTF activity in yeast and fractions from other sources. No separate preincubation period was used,

however, the fermentation time of 300minutes allowed sufficient time to overcome a lag phase of 100minutes.

Burkeholder and Mertz (1967) report that yeast fractions active by the criteria of the yeast assay system were tested and found to affect glucose oxidation of epididymal fat tissue in presence of insulin only, thus establishing a link between the yeast assay system and the fat pad assay system. The effect of GTF in both systems was thought to involve a common effect on transport, however, Anderson (1979) subsequently demonstrated that the action of GTF in adipocytes was after the transport step.

1.6.4 Yeast Metabolism and GTF

Burkeholder and Mertz (1966) have reported enhanced glucose utilization by yeast in presence of GTF and that the repsonse is dose dependent. Mirsky *et al* (1981) reported the same effect for *Sacch. carlsbergensis*, *Sacch. cerevisiae* and *Sacch. ellipsoideus* and for not only glucose but fructose and mannose. GTF enhanced galactose metabolism, suggesting that GTF may influence the transport of sugar into the cell since the steps preceding fructose 1,6 diphosphate in the intracellular metabolism of galactose differ from that of glucose (Jaspers *et al* 1976). Further evidence for the transport hypothesis was found in the lack of stimulation of GTF on cell free extract and thus the hypothesis that GTF enhances fermentation rates by increasing the reaction velocity, was put forward by Mirsky *et al* (1980). Mirsky *et al* reasoned that the rate-determining step in the utilization of sugar was its transport, thus increased reaction velocity is due to increased transport. Uptake of deoxyglucose was found to be greatest when the cells were preincubated with GTF and glucose, thus Mirsky *et al* concluded that metabolic activity is required for triggering the action of GTF.

1.7 CRITERIA FOR GTF ACTIVITY

1.7.1 Peripheral tissue

Improvement in glucose tolerance of torula yeast fed rats after supplementation with GTF was the original criterion for GTF deficiency. Extrapolating to the site of glucose removal, the action of GTF was investigated *in vitro* in peripheral tissue.

Fat tissue from animals supplemented with chromium chloride was found to have a 67% higher glucose uptake *in vitro* as compared to unsupplemented control animals. Increases of approximately 90% of glucose oxidation *in vitro* by fat tissue from deficient animals were observed when chromium complexes (chrome alum neutralized) were added to the tissue in presence of small quantities of insulin (Mertz *et al* 1961). Of thirteen elements tested, only manganese in addition to chromium III stimulated glucose oxidation. Roginski (1974) reports that nicotinic acid also stimulated glucose oxidation by fat pad pieces. The use of fat pad pieces thus became an assay system for GTF until it was demonstrated that an adipocyte preparation allowed more samples to be tested per animal with less interference from compounds such as nicotinic acid.

Anderson (1978) reported that adipocytes isolated from epididymal fat tissue of chromium deficient rats can be used as a rapid and sensitive technique to detect and quantitate biologically active chromium. The method, an adaptation of the Robdell (1964) adipocyte preparation, is similar to the fat pad assay in that increased glucose oxidation with GTF added is only observed in presence of sup-optimal levels of insulin. The effects of inorganic chromium and nicotinic acid were insignificant on isolated adipocytes although sulphydryl compounds produced a GTF type of response (Anderson 1979).

As a number of compounds are known to mimic insulin (Czeck 1977) and divalent ions (Cu^{2+}, Co^{2+}, Mn^{2+} , Ni^{2+}) have been reported to mimic the effect of insulin on isolated fat cells (Saggerson *et al* 1976), care should be taken in establishing the presence of GTF. Copper deficiency in rats results in impaired glucose tolerance (Hassel 1982) and copper in presence of thiols can mimic insulin effects (May and Contoreggi 1982).

The main advantage of the adipocyte assay is that a larger number of samples can be screened per animal (Anderson and Bratner 1978).

1.7.2 Absorption

Mertz and Roginski (1971) have demonstrated that chromium in its biologically active form is absorbed and retained in rats to a much greater degree than chromium chloride. As absorption of radio-chromium poses less problems experimentally, various workers have chosen to use absorption or bioavailability in preference to glucose tolerance tests for *in vivo* evaluation of GTF (Votava 1973, Huffman *et al* 1973). Anderson *et al* (1980) reports, however, that a compound well absorbed from the gastrointestinal tracts of animals, had previously been found to be inactive in the fat pad assay (Mertz 1969) and thus bioavailability may not necessarily imply bioactivity.

1.7.3 Other indicators

Yeast as an assay system has been discussed (Section 1.6.3). Gutierrez *et al* (1974) report the use of *Flavobacterium rhenanum* as a microbiological assay for GTF. The microorganism, a contaminant of a wild yeast culture, gave an increased dose response on addition of

GTF-containing fractions to the growth medium. A linear correlation between the response of *F. rhenanum* and the rat epididymal fat bioassay was reported. Anderson (1981) comments that the assay was discarded as it was not specific but does not elaborate further.

Placental transport of ^{51}chromium and ^{51}chromium labelled GTF extracted from brewers' yeast provided a means of distinguishing the effect of GTF from that of chromium *in vivo* (Mertz 1969), and has later been suggested by Mertz (1974) as a definitive metabolic property of GTF but was not developed as an assay system.

Nath and Sidhu (1979) compared the effect of GTF preparations on insulin sensitive diaphram tissue with hepatic tissue, as a means of establishing GTF activity. Unfortunately, the complex was not entirely separated from free glutathione or nicotinic acid. There was little difference in the incorporation of glucose into glycogen in liver or diaphram in the control rats and rats receiving insulin alone or a chromium-glutathione-nicotinic acid preparation alone. No glutathione or nicotinic acid controls were reported. The test group receiving both insulin and the chromium preparation showed a 24-fold increase in glycogen synthesis in diaphram but no effect on liver glycogen. Glycogen synthesis in liver is not sensitive to insulin and, as it was found to be unaffected by a chromium-containing preparation, it would seem that the assay system of Nath *et al* implicates the site of action *in vitro* to be glucose transport. The use of diaphram tissue had originally been reported (abstract only) by Mertz and Thurman (1968) as an assay system for GTF.

In summary, the most commonly used bioassays for GTF reported in the literature are:

i) Epididymal fat pad assay.

ii) Adipocyte assay

iii) Glucose tolerance tests on deficient animals

iv) Absorptivity

v) Microbiological assay

vi) Diaphram tissue

1.8 THE EXTRACTION AND STRUCTURE OF GTF

1.8.1 Sources of GTF

The first reported sources of GTF were defatted pork kidney powder and brewers' yeast (Mertz and Schwarz 1957). Toepfer *et al* (1973) in surveying the potential GTF content of various foods, found no correlation between total chromium and biological activity as determined by the fat pad assay but were able to correlate the chromium content of ethanol extracts with activity. Brewers' yeast was found to have the highest biological activity followed by black pepper, calfs' liver, cheese and wheat germ.

Anderson (1982) determined the chromium levels in selected beers and found a range of 0.48 to 56ppb. He reported that the chromium was absorbed by humans similarly to chromium occurring endogenously in goods and thus it may be significant nutritionally. Chromium extracted from beer was found to be low in *in vitro* insulin potentiating activity.

Mirsky *et al* (1980) and Haylock *et al* (1982) used commercially available yeast extract powder as a source of GTF. Haylock *et al* (1982) also extracted active material from sage, black peppercorns, molasses, wheat bran and pork kidney powder. Sage was found to be a poor source of GTF activity in agreement with the general observation that leafy vegetables were devoid of insulin potentiating activity *in vitro* (Mertz *et al* 1974).

1.8.2 GTF from higher plants

Blincoe (1974) separated a ^{51}chromium containing anionic material with a molecular weight of 2900 daltons from lucerne. Starich and Blincoe (1982) report a chromium complex isolated from alfalfa, also anionic, had a high affinity for gel column packing giving false estimates of molecular weight. The complex was similar to other chromium containing complexes isolated from beans and wheat (Huffman and Allaway 1973). The absorption of the complex was compared with the absorption of inorganic chromium in marginally chromium deficient rats and it was found that less than 0.5% was absorbed in either case. Studies on the uptake of ^{51}chromium by plants demonstrated that most of the chromium was retained in the roots and was thus unavailable to grazing animals.

1.8.3 Extraction and structure of GTF

Schwarz and Mertz (1959) found that extracts containing GTF, from brewers' yeast or hydrolyzates of pork kidney powder lost their activity on storage at 4°C and that the loss in activity was generally associated with the formation of a brown precipitate. They identified chromium III as the active ingredient by glucose tolerance tests on rats raised on a torula yeast based diet and dosed by stomach tube with salts of forty different elements. Only chromium III improved glucose tolerance. GTF was reported to be water soluble, extractable with phenol and isobutanol and absorbable on cation exchange resin and charcoal. Burkeholder and Mertz (1966) disintegrated yeast cells to yield two low molecular weight intracellular fractions containing chromium, and which were active in the yeast assay system. Chromium was found to be present as an organic complex that was dialyzable.

Thurman and Mertz (1969) batchwise extracted yeast grown on 10-100ppm chromium and used ethanol and trichloroacetic acid to extract GTF. Mertz deduced that not all of the chromium extracted was associated with biological activity as some was retained by the ion exchange resin (presumably the Dowex 50W cation exchange resin reported earlier) and was thus free chromium (Schwarz and Mertz 1959). Alcoholic extracts of brewers' yeast labelled with ^{51}chromium were fractionated by gel filtration (details not given) and the resultant fractions were monitored by chromium content and stimulation of glucose oxidation in the fat pad assay. Although biological activity and chromium content were found to be closely associated, non-chromium containing material eluted from the column was found to have activity. The nature of the active material not associated with chromium was not further investigated. The molecular weight of the isolated chromium complex was later reported to be 300-500 daltons (Mertz 1974). The optimal dose reported by Mertz (1969) in the fat pad assay was 200μg GTF containing 0.2ng of chromium. Assuming a molecular weight of 400 daltons, the chromium content can be calculated to be 0.4ng of chromium per mole of GTF. The amount of chromium would thus seem to be inadequate if chromium is the central coordinated atom in the GTF complex.

Votava, Hahn and Evans (1973) extracted yeast cultures grown on Sabouraud's medium containing low levels of ^{51}chromium (4μg/ℓ) with high specific activity. They were able to extract 100% of the label with butanol and water; 90% of the chromium remained in the aqueous phase. Fractionation by ion exchange chromatography and gel filtration yielded an anionic fraction with molecular weight 400-600 daltons. Purification on unbuffered Sephadex G25, resulted in a higher molecular weight and this was attributed to reversible aggregation. The isolated material was found to be a chromium-binding peptide consisting of leucine,

isoleucine, proline, valine, alanine, serine and either glutamic or aspartic acid. The biological activity of the material was implied by its greater absorption in the rat, as determined by whole body counting, in comparison to the absorption of inorganic ^{51}chromium.

Mertz (1974) reported the extraction of dry brewers' yeast with 50% ethanol. The extract was absorbed on charcoal and eluted with ammonia and ether. Further purification was by ion exchange chromatography and gel filtration. Paper chromatography yielded material with a similar Rf to nicotinic acid. Mass spectrometry demonstrated the presence of nicotinic acid but not chromium. Chromium was said to have been determined to be present by infra red and atomic absorption spectrometry, however, the results have yet to be published. The insulin potentiating activity was measured in the fat pad assay. Free nicotinic acid which would almost certainly have been present from the spectral data presented, also stimulates glucose oxidation by the fat pad (Roginski 1974). The material was reported to be a glutamic acid, glycine, a sulphur containing amino acid and nicotinic acid complex (Toepfer 1974, Mertz 1974) on the basis of mass spectrometry and amino acid analysis. No chromium levels were reported although more than half of the total chromium was lost during purification.

Toepfer *et al* (1977) after extracting brewers' yeast with ethanol (50%) and eluting from charcoal, hydrolyzed the product for 18hours with 5N hydrochloric acid. No explanation is given for this step which would surely dissociate peptides. Tuman *et al* (1978) found hydrolysis reactivated samples of GTF which had lost activity through storage although, in this instance, the hydrolysis was less severe. Mertz (personal communication 1981) confirms that mild hydrolysis reactivates GTF containing fractions. During fractionation on Dowex and other ion exchange materials (details not available),

the ratio of chromium to dry weight of material increased, but the biological activity did not, an unexpected result if chromium is an essential component. A number of chromium complexes present in the original extract were reported to have less or no biological activity while the biologically active fraction comprised 11% of the total chromium in the starting material. The published ultra violet absorption spectra indicated, assuming a molecular weight of 400 daltons, that the yeast concentrate contained approximately 98% free nicotinic acid. The authors argue that the "shoulders" either side of the 262nm maximum (B band) are reduced in magnitude and indicate complexation, however, the side bands are pH dependent and are not observed at low pH (less than 4.5).

Polansky and Anderson (1977) extracted 80% of labelled chromium with boiling water from yeast grown on a synthetic medium. Less chromium was extracted by ethanol:water, butanol:water or extended sonication. Purification by liquid chromatography included high performance liquid chromatography (HPLC) on a C18 column, yielding at least three chromium containing compounds. The biological activities were not reported. After comparing the rate of chromium uptake from synthetic (hydrolyzed casein) and peptone based medium and finding considerably higher uptake in the peptone medium, Anderson (1978) examined various means of extracting the incorporated ^{51}chromium. Approximately 80% of the label could be extracted at a pH greater than 8.0 with little being extracted at pH 4.5 (similar pH to culture medium). Ethanol (50%) extracted considerably less chromium but a high proportion of the active material was extracted. Teichozyme Y and sonication released 80% of the label but inhibitory material was also released as biological activity was low and decreased even further with increased dose.

Kumpulainen *et al* (1978) isolated both cationic and anionic chromium complexes from yeast and from the spent medium. An anionic fraction not found in fresh medium was isolated. As Evans *et al* (1973) has reported that GTF binds to insulin, Kumpulainen concluded that none of the materials isolated were GTF as they would not bind to insulin. It was unfortunate that they used this criteria for GTF since the binding studies of Evans *et al* (1973) are somewhat ambiguous as only labelled insulin was used and no attempt was made to establish where chromium eluted from the gel column. The estimated molecular weights for cationic fractions from yeast were from 200-500 daltons with the material from the spent medium being 700-1100 daltons. The polarity of all of the complexes was found to be greater than most amino acids.

Mirsky *et al* (1980) obtained crude GTF containing fractions from yeast extract by dialysis and ion exchange chromatography. Haylock *et al*, initially using yeast extract as a source of GTF, devised a system for isolating the various chromium compounds occurring in the extract by elution from a series of ion exchange resins using phosphate gradients. Eleven distinct chromium containing complexes were isolated with varying biological activity in the yeast assay, all of the cationic fractions being active. A cationic fraction from pork kidney powder was found to have a different elution profile to those from yeast. Cationic fractions from peppercorns, molasses, wheat bran, sage and split peas were all found to elute at different pH and are thus of different chemical forms. Extraction was with distilled water at 70°C. Haylock *et al* (1982) report that anionic chromium containing compounds constitute the bulk of isolated chromium containing compounds and also give evidence for the formation of an octahedral chromium-glucose complex which would be expected to be anionic. Most of the complexes were a result of direct reaction between chromium and medium components.

They concluded that material with GTF activity in the yeast assay was not a chromium complex.

Gonzalez-Vergera *et al* (1982) report recently that they have isolated a fraction from brewers' yeast containing all of the chromium originally present in the yeast. The chromium was not associated with nicotinic acid, glycine, cysteine or glutamate, however, a tryptophan-like compound was reported to be associated on the basis of spectral evidence. No details are available at present as to the extraction technique or biological activity.

In summary, a wide variety of chromium containing complexes have been isolated from both yeast and from the medium in which it is grown. There is a general consensus that the material having biological activity is cationic, is of low molecular weight and binds strongly to the cation exchange resin Dowex 50W. Aqueous extraction is generally favoured.

1.9 PREPARATION OF SYNTHETIC GTF

1.9.1 Chromium

Chromium has been found to occur in biological material in the trivalent state (Feldman 1968), the most stable oxidation state for chromium. Chromium III complexes, like those of cobalt III have low rates of ligand interchange and, in fact, many analogous complexes are known. The complexes of chromium III in aqueous solution are susceptible to hydrolysis and formation of polynuclear complexes by olation, more so than those of cobalt III (Rollinson 1973). As chromium III is not subject to high spin, low spin transitions and has a low rate of ligand exchange, chromium III complexes would seem unsuitable as a metal cofactor for the active site of an enzyme.

Mertz (1969) suggests that the relative inertness of the chromium ligand bond would be compatible with a structural function.

1.9.2 Simple complexes

Chromium chloride and chromium acetate were found by Anderson *et al* (1978) to inhibit glucose oxidation in isolated adipocytes. Mertz (1969) reports that neutralized chrome alum and chromium chloride were active in the fat pad assay. Further contradiction between the assay systems lies in the observation by Mertz (1969) that very stable chromium containing complexes, specifically the acetylacetonate or bis-ethylenediamine complexes, did not improve the impaired glucose tolerance of chromium deficient rats whereas a range of anionic, cationic and neutral compounds with varying degrees of hydrolysis (chrome alum, chromium perchlorate) were effective. Anderson (1980) compared the rate of absorption of chromium chloride with chromium acetylacetonate and found the chromium chloride, in agreement with other investigators, was absorbed by only 1% whereas the acetylacetonate was 40% absorbed. The acetylacetonate was also effective in improving the blood glucose levels of diabetic mice and hence was biologically active, contrary to the findings of Mertz. Riley (1980) continued investigating complexes that would not olate in the intestinal tract and successfully synthesized a range of labelled chromium III porphyrin complexes, however, results of biological testing are not yet available.

Chen (1973) measured chromium transport in rat intestine in both *in vivo* and *in vitro* and compared the effect of four chelating agents. Oxalate significantly increased transport while phytate caused a decrease. Citrate and EDTA did not affect transport. Interestingly, ^{51}chromium EDTA has also been used as a soluble rumen marker due to

its lack of absorption (Downes and McDonald 1964). Gonzalez-Vergera *et al* (1981) found whole body retention of chromium EDTA to be 0.88% in normal rats, less than chromium III hydrate (1.96%) but higher than attempts to prepare GTF analogues containing nicotinic acid (0.19%).

1.9.3 Chromium GTF

Toepfer (1974) and Mertz *et al* (1974) having found a close correlation between absorption due to nicotinic acid at 262nm and biological activity, reacted nicotinic acid with chromic chloride at low pH. A fraction was obtained from fractionation of the reaction mixture on Dowex cation exchange resin that was reasonably stable and potentiated the effect of insulin on fat tissue from chromium deficient rats (Roginski 1974). The product was described as a tetraaquodinicotinatochromium complex (Figure 1.2) purely by analogy with a cobalt II-nicotinate tetrahydrate, the structure of which has been determined crystallographically (Anagostopoulos *et al* 1969).

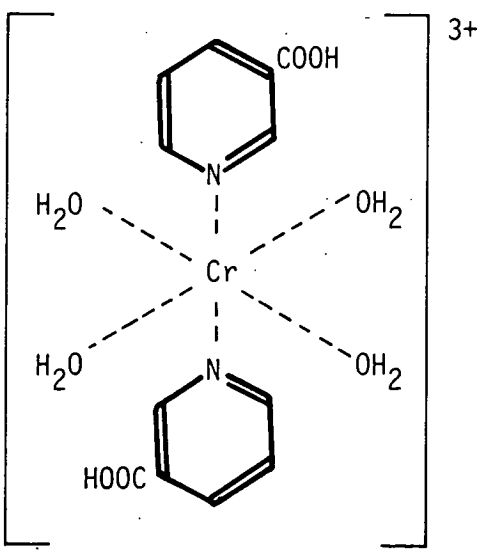


FIGURE 1.2 Proposed coordination of nicotinic acid to chromium (Mertz *et al* 1974).

Gonzalez-Vergera *et al* (1980) have prepared a pyridoxylidene-glycylglycine-chromium III-dinicotinate complex and proposed an axial nicotinic acid configuration. They concluded that coordination through the heterocyclic nitrogen confers stability on the complex but there was no compelling evidence that nicotinic acid is an essential feature of GTF. A trinuclear chromium III nicotinic acid complex coordinated through the carboxyl groups rather than the nitrogen was crystallized by Gonzalez-Vergera *et al* (1982). The complex was virtually insoluble at neutral pH thus precluding its testing in biological systems. The complex characterized by crystallography, was found to be vastly different from the structure proposed by Mertz.

Mertz *et al* (1974) prepared the chromium nicotinate in the presence of ethanol and amino acid ligands to compete with hydroxyl ions in order to reduce the tendency to olate. Although glycine, glutamic acid and cysteine were added to the reaction mixture, glutamic acid did not occur in the purified complex. The complex was found to be active in the fat pad assay despite the absence of glutamate which was thought to be present in yeast GTF. Molar ratios of 2 nicotinic acid:2 glycine:1 cysteine per mole of chromium were reported.

Anderson and Brantner (1977) and Anderson, Polansky, Brantner and Roginski (1977) claimed to have synthesised a chromium-glutathione-nicotinic acid complex which was found after fractionation by HPLC to be active *in vitro*. Anderson and Mertz (1977) report later that the use of HPLC was of limited success because of the instability of the complexes and peculiar binding properties with column packing materials. The compounds have not be isolated as yet because of dissociation during purification (Anderson 1981).

Anderson (1977) incubated labelled chromium-glutathione-nicotinate with labelled insulin and fractionated the solution by gel chromatography. A fraction of the complex was found to bind to insulin, however, it was ambiguous as no control with inorganic chromium and insulin was presented and free chromium (or dissociated) has been shown to bind to insulin (McDonald and Belusko 1976).

Toepfer *et al* (1977) compared the absorption spectra of a complex with molar ratios of 2 nicotinic acid:2 glycine:1 glutamic acid:1 cysteine per mole of chromium with material isolated from brewers' yeast and concluded that the axial nicotinic acid configuration may be the essential GTF configuration with the amino acid moities conferring solubility and being of lesser importance for biological activity.

Anderson *et al* (1978) compared the *in vitro* activity of a chromium-nicotinic acid complex with a chromium-nicotinic acid-glutathione complex and a yeast preparation. The two synthetic complexes had comparable activity but high levels of chromium (50µg/ml) were required to produce a response in comparison with 0.06ng/ml of chromium as yeast GTF. The activity of a chromium-nicotinic acid-glycine complex prepared (no details published) implies that the thiol moiety of cysteine is not necessary to form a biologically active complex.

Kienle *et al* (1979), having followed the synthesis of Toepfer, attempted to purify the active material from the reaction mixture by thin layer chromatography. They found appreciable amounts of unreacted components, diverse compounds that could not be separated and chromium containing compounds that could not be eluted from columns without destruction of the complexes. Nath *et al* (1979) using the method of Toepfer with glutathione rather than individual amino acids,

used an anion exchange resin followed by a Sephadex G25 gel column to purify active material. A molar ratio for the purified complex of 2 nicotinic acid:1 glutathione per mole of chromium was reported. Biological testing by the adipocyte assay was unsuccessful because of a poor response to basal insulin stimulation, however, increased glycogen synthesis was reported in rat diaphram tissue. The control rats were not dosed with an equivalent amount of a simple chromium salt, nicotinic acid or glutathione. Nath claims that the complex has an identical composition to GTF from brewers' yeast.

In summarizing the preparation of synthetic GTF, it would seem that a large number of by-products are produced by the method published by Toepfer (1977) and that elucidation of structure has been prevented by instability of the chromium complexes. Much higher levels of chromium are required to produce a response in biological systems both *in vitro* and *in vivo* compared to the chromium levels in brewers' yeast fractions and this might imply that the yeast GTF may not contain chromium and thus comparison may not be necessary. Tuman *et al* (1978), using non-deficient diabetic mice, found a small effect on nonfasting plasma glucose concentration with synthetic GTF, and Anderson reports activity in the adipocyte assay.

1.9.4 Cobalt GTF

Bertrand and Macheborf (1926, 1934) reported the hypoglycemic action of cobalt salts and their accumulation in the pancreas. Silio (1977) examined the evidence that GTF from pig kidney was chromium containing and, on finding inadequate evidence, he hypothesized that cobalt would be more likely to be the active principle of GTF in higher animals.

Silio (1980) prepared a cobalt-dinicotinamide-glutathione complex via a cobalt II intermediate (Figure 1.3), the crystal structure of which has been determined (Vegas *et al* 1981). Reaction of the intermediate with reduced glutathione resulted in a brown water

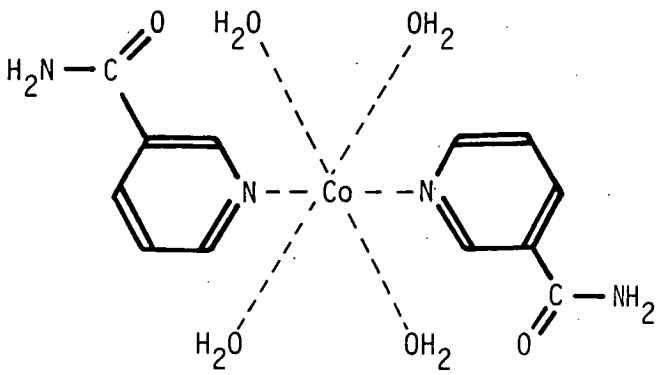


FIGURE 1.3 Trans-tetraaqua-bis-(nicotinamide)-cobalt II-dichloride dihydrate (Vegas *et al* 1981).

soluble complex having a molecular weight of 1500 daltons. The complex was active in the adipocyte assay at a concentration of 5×10^{-7}M (0.03µg/ml), considerably lower than equivalent chromium complexes, and was only active in the presence of insulin. Silio (personal communication 1982) attributes the action of high levels of chromium complexes in the adipocyte assay to the inhibition of pyruvate carboxylase.

1.9.5 Conclusion

The status of synthetic analogues is made difficult to assess since different methods have been used to test biological activity. Anderson *et al* (1978) have shown that thiol groups are not necessary for activity; Gonzalez-Vergera (1981) disputes the necessity for the axial nicotinic acid structure. Glutathione-nicotinic acid-chromium complexes,

prepared for their stability rather than the amino acid mixture of Toepfer (1977), were found to precipitate from aqueous solution (Nath *et al* 1979). Silio (1980) has prepared active material containing cobalt and not chromium. Underlying the differences in the active complexes is the fact that yeast preparations, active in the biological assay, may have low or no chromium and raises the question of the importance of chromium.

Haylock *et al* (1982) report that chromium is present as an impurity in GTF containing fractions derived from yeast. From the data presented by Tuman *et al* (1978) and Mertz (1969), the low chromium levels in purified GTF would seem to indicate that chromium is not part of the GTF complex (Section 1.8.3). It may be that the purified product was still grossly impure, however, the material reportedly had high biological activity indicating a degree of purification.

1.10 ANALYSIS OF CHROMIUM IN BIOLOGICAL MATERIAL

Urinary chromium levels, blood and hair chromium levels have been used as criteria for chromium deficiency hence, the analysis of chromium in biological material has become important. Anderson (1981) has reviewed the variety of techniques available for determining the concentration of chromium in biological material and the expectations of each method. Chromium levels in certain matrices have changed radically with improved methodology. Figure 1.4 depicts the change in reported chromium levels in blood as a function of time (Horwitz 1982), showing a dramatic decrease in chromium levels with the introduction of the graphite furnace in the 1970's and reference materials for chromium. Almost every paper reviewed by Horwitz provided a linear calibration curve and good recovery values for added chromium and even for ^{51}chromium yet, immense variability of several orders of magnitude exists. Much

variability has resulted from contamination, particularly from sampling devices such as syringes and stainless steel needles (Guthrie 1981).

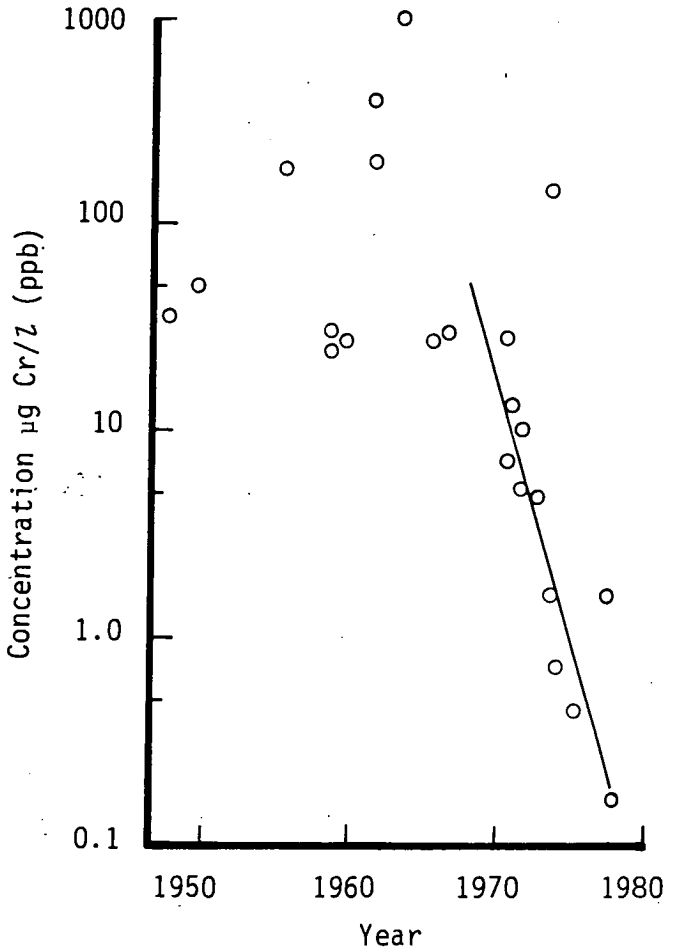


FIGURE 1.4 Chromium concentration in blood as a function of reporting date (Horwitz 1982).

As the level of chromium reported appeared dependent on the nature of the matrix (Kayne 1979), the US National Bureau of Standards issued a range of materials certified for chromium, including brewers' yeast, liver and orchard leaves. Shapcott *et al* (1977), Versieck (1978), Koirtyohann (1976), Kumpulainen (1977) and Tuman *et al* (1978) report losses of chromium during the ashing cycle of flameless atomic absorption atomizers which would appear to reflect the difficulties experienced generally with background correction. Thompson (1980) found that by comparing with a uranium line at 358.5nm, 0.6nm from the chromium line most commonly used, substantially improved background

correction was obtained. Kumpulainen *et al* (1982), using a tungsten iodide lamp, were also able to improve on the deuterium continuum due to increased intensity at 359nm. Guthrie, Wolf and Veillon (1978) stress the problems in determining chromium levels in urine and conclude that deuterium background correction is inadequate.

The purge gas and pyrolitic coating of carbon tube furnaces also considerably influenced the final result. Routh (1980) was able to reduce the non-specific absorption to an acceptable level by using a hydrogen diffusion flame as a supplement to the inert gas sheathing the graphite furnace. Veillon *et al* (1981) report direct urine analysis with added standards prepared in 1M hydrochloric acid, however, Matsusaki (1980) reports chloride interference in determination of chromium. Shapcott (1977) found contaminating chromium in acid used to digest samples which was not reported by Davidson (1972).

Dry heating at greater than 500°C resulted in losses of "volatile chromium". Tuman (1978) reports that digestion with nitric acid and peroxide converts chromium to a non-volatile form and hence, by adopting the following formula, was able to measure volatile chromium which was equated to biological activity.

Total Cr (acid pretreated) - Non-volatile Cr (no pretreatment)
= volatile Cr

Although corrections were made for chromium contamination in acid used for digestion, there is a change in matrix which would account for the differences (Kayne 1979). Seeling (1979), using the procedure of Tuman but with a tantalum cup, found no differences or indications of volatile chromium.

Gorsuch (1959) and McClendon (1978) report losses associated with wet ashing of material with perchloric acid. This is almost certainly due to the formation of chloroxy-chromium compounds which would sublime, resulting in losses (Kumpulainen 1979). Kumpulainen experienced difficulty with NBS brewers' yeast SRM-1569 due to acid insoluble silicates strongly absorbing the analyte from the digestion but found no volatility during dry ashing.

Kumpulainen (1980) stresses the importance of avoiding contamination during sampling of urine and human milk. As doubts have been raised concerning the significance of the trace element content of hair as an indicator of nutritional status (Bradfield and Hambridge 1980, Assarian and Oberleas 1977, Spivey-Fox and Tao 1981), Kumpulainen *et al* (1982) and Salmela *et al* (1981) used a variety of solvents and detergents to remove contaminating chromium from samples prior to analysis. Success with washing with lauryl sulphate may lead to further attempts to determine hair chromium with a view to establishing chromium deficiency. Mild cleaning procedures before chromium determination by neutron activation analysis, resulted in a small difference being found in the chromium content of scalp hair from normal and diabetic individuals (Tiefenbach 1979).

Concluding this section, Iyengar *et al* (1982) find that there are still serious methodological problems in the determination of chromium in biological materials. The measure of volatile chromium has been shown to be an artefact of the methods employed.

1.11 CONCLUSION

Although chromium supplementation in humans may not fulfill the rather optimistic hypothesis of McCarty (1980), there appears to be correlation between chromium and carbohydrate metabolism (Anderson 1981) and cardiovascular disease (Newman *et al* 1978). Mertz (1979) has stated that, "Although we have de-emphasised the nutritional importance of GTF, we are still convinced of the biochemical importance of GTF whatever it may be within the organism. So I think this will be one major key for the solution of our problems".

The aim of this thesis is to investigate the chemical nature of GTF as it occurs in brewers' yeast and in active chromium coordination compounds. A yeast assay system has been developed for detecting GTF in fractions, however, when possible, the fractions have been submitted to Ms J. Sherriff to determine activity in the adipocyte assay.

CHAPTER TWO

ANALYTICAL METHODS

2.1 INTRODUCTION

2.1.1 Chromium determination and chromium deficiency

Chromium deficient rats were raised by Ms J. Sherriff to provide tissue for the adipocyte assay for GTF activity (Chapter 5). As glucose tolerance tests to establish the chromium deficiency of the animals were unsuccessful (Sherriff, M.Sc. Thesis, 1983), the collection and analysis of rat urine for chromium determination was attempted. In a further attempt to establish chromium deficiency, whole body chromium determinations were carried out on the carcasses of rats killed after glucose tolerance tests. Although Jain *et al* (1981) found no difference in total tissue chromium (Chapter 1.4), it was hoped that the whole body determination may indicate some degree of deficiency when compared with chromium replete rats. The chromium levels in two batches of "low chromium" food were determined and compared with the food supplied to normal colony rats.

The problems associated with the determination of chromium by flameless atomic absorption spectrometry (AAS) has been discussed in Chapter 1.10. Samples requiring preconcentration, such as yeast medium, or desalting, were solvent extracted by an extraction procedure adapted from effluent techniques (Varian, 1975). As chromium does not readily form chelates suitable for solvent extraction (Stary, 1964), samples and standards were heated with the chelation agent prior to extraction.

The instrumentation and principles of operation of AAS has been well documented and will not be described (e.g. L'vov, 1970).

2.1.2 Chemiluminescence

Seitz *et al* (1972) and Chang *et al* (1980) report the determination of chromium III by chemiluminescence with a detection limit of 0.2ppb. The method was sensitive and specific for chromium III. The catalysis by metal ions of the oxidation of luminol by hydrogen peroxide is utilized by the method. In presence of excess reagents, the intensity of light emission is proportional to the metal concentration. The method reported by Seitz *et al* (1972) utilizes the kinetically slow rate of formation of Cr (EDTA) to remove interference by the metal ions which are rapidly complexed and hence not available as catalysts.

2.2 METHODS

2.2.1 Atomic absorption spectrophotometer

The atomic absorption spectrophotometer (AAS) used for chromium determination was a single beam Varian Techtron Model 1000. A carbon rod atomizer (CRA 63) workhead had been incorporated beside the flame workhead in the instrument to facilitate changeover from one technique to the other. A deuterium square wave modulated background corrector (made by J. Jordan) was used with both flame and furnace atomization. The limit of correction was 0.9 absorbance units (AU). The hollow cathode lamp used for chromium determination was a multi-element lamp (Varian) and was generally operated at 8-10mA. The chromium line at 357.9nm was used in preference to the more sensitive line at 425.4nm to improve background correction and

reduce incandescent noise (Section 2.2.1.1). A CRA mask was used along with aluminium foil placed in front of the photomultiplier in an attempt to further reduce incandescent noise. A larger than normal spectral bandwidth was necessary to permit the adjustment of the background corrector (SBW 1.0nm).

The tube furnaces used in the CRA were coated with pyrolitic graphite. The tubes were plain thus restricting the maximum sample volume to 10μl. Frequent replacement of the tube furnaces was necessary due to the high atomization temperatures used (2 500°C). "High Purity" nitrogen with a flow rate of 1.0μl/min was used as the sheath gas. Sample injection was achieved manually with a SGE micro-syringe (0-15μl) having disposable plastic capillary tips.

The original nebulizer in the instrument had been replaced by a Varian "adjustable tantalum nebulizer". Maximum sensitivity with an air/acetylene flame was achieved by adjusting the flame so that it was rich in acetylene. Aspiration rates were adjusted to suit the chromium level and volume of the sample being analysed.

The signal from the AAS was recorded by a fast response Hitachi chart recorder (QPD 33) and a peak height detector (made by J. Jordan). The limits of detection using flame atomization were 0.05 to 10.0 ppm for aqueous standards. Detection limits of 1.25×10^{-10} to 2.5×10^{-9}g for chromium were found using the CRA furnace. The operating temperatures of the furnace were determined by reference to standard curves supplied by the manufacturer.

2.2.1.1 Technical problems associated with deuterium background correction and incandescent noise

Continuous background correction is the preferred method for elements with analytical lines below 350nm. The hydrogen lamp intensity at greater than 350nm is low (Figure 2.1) thus a high gain was required resulting in increased noise. In order to zero the instrument with the corrector operating, a large spectral band width (SBW) was used to compensate for the low intensity of the hydrogen lamp. The use of high gain and large SBW is not conducive to maintaining a high signal ratio to unmodulated light originating from the furnace walls when operating at the high temperatures required for refractory elements. The overall result was increased baseline noise when using correction and a troublesome spike due to incandescent noise.

2.2.1.2 Preparation of standards and glassware

'A' grade volumetric flasks and bulb pipettes were used for preparation of standards and samples. The glassware was acid soaked in 10% nitric acid (AR, Univar), rinsed with deionized water three times and finally rinsed with two washings of deionized distilled water. All solutions were prepared with distilled deionized water and standard solutions were prepared by serial dilution of BDH chromium nitrate standard for AAS (1mg/ml).

2.2.2 Digestion of samples

A 10ℓ yeast culture was grown in liquid yeast medium containing 4.0ppm chromium (Chapter 4.2.2) and 2.5mCi of ^{51}chromium (New England Nuclear). The yeast was harvested by centrifugation, air dried and

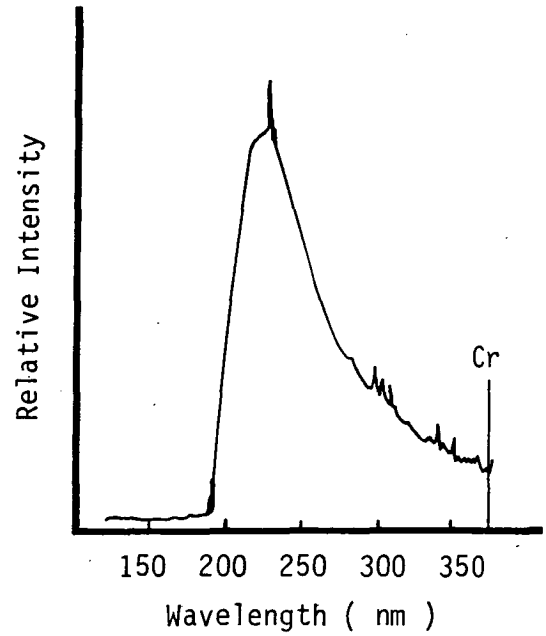


FIGURE 2.1 The hydrogen lamp spectrum and wavelength of the chromium line used. (a), (b) are examples of typical recorded outputs for chromium determination in an organic matrix by CRA-AAS with and without continuous correction.

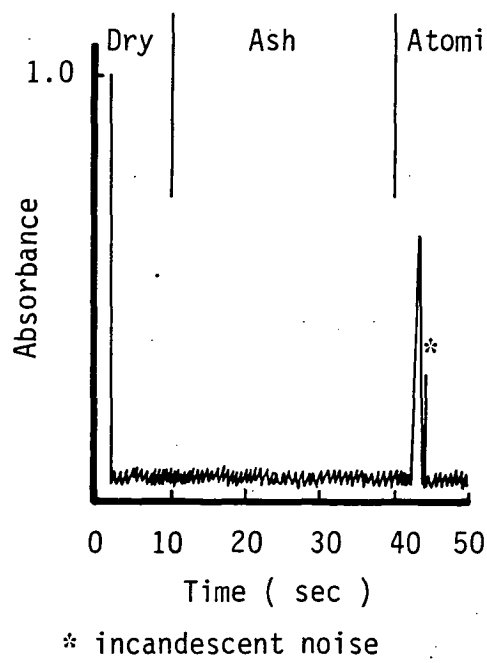


FIGURE 2.1 (a) corrected

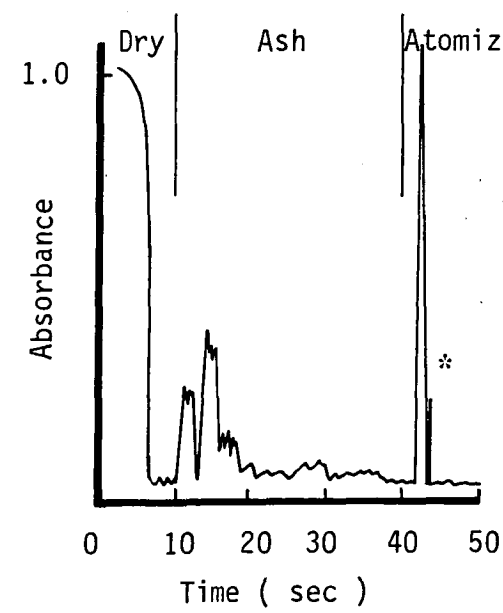


FIGURE 2.1 (b) uncorrected

ground to a powder. The yeast was then used to assess the efficiency of wet digestion as compared to dry ashing at 500°C with sulphuric acid and hydrogen peroxide as described by Kumpulainen *et al* (1979).

2.2.2.1 Dry ashing

3 samples of ^{51}Cr yeast of approximately 0.5 and 1.0g were weighed to the nearest 0.1mg into vitreosil crucibles. The crucibles (with lids) were heated to 500°C overnight in a clean muffle furnace (Analite Pty. Ltd.). 25µl of concentrated sulphuric acid (Aristar, BDH) and 25µl of hydrogen peroxide (Merck) were added to the ash which was then taken to dryness on a hot plate before returning the crucibles to the furnace for a further 2hrs. The ash was rinsed from the crucibles with 1.0N nitric acid (Aristar, BDH) into polyethylene γ-counting tubes so that the final total volume was 2.0ml. The number of counts per minute (cpm) arising from the ^{51}Cr were determined in a NaI well γ-counter (Kontron MR 252). The results are presented in Table 2.3.

2.2.2.2 Wet ashing

Triplicate samples of 0.07 to 0.5g of ^{51}Cr yeast were weighed to the nearest 0.1mg into acid washed 10ml Kjeldahl flasks. 1.0ml of concentrated nitric acid was added to each flask along with acid washed boiling chips. The flasks were allowed to stand overnight at room temperature with the tops covered. The flasks were heated in a Kjeldahl digestor (Electrothermal) in a clean fume hood with a small filter funnel sitting in the neck of each flask to prevent loss of contents and contamination. An extra 1.0ml of nitric acid was added to each flask and digestion continued until the evolution of fumes of nitrogen dioxide ceased and the volume of the contents

was reduced by half. The contents of the flask were diluted to 5.0ml and 2.0ml aliquots were counted. The results are presented in Table 2.1.

2.2.2.3 γ-counting of digested samples

In order to determine the % recovery of the isotope after digestion, equivalent amounts of the dry ^{51}Cr yeast were weighed into γ-counting vials and a linear calibration curve of cpm vs grams of yeast was plotted. To determine the extent of self absorption, 100μl of ^{51}Cr (30 000cpm) was pipetted onto 0.5g of dry yeast (without isotope) in a counting vial and the cpm compared with 100μl of aqueous ^{51}Cr. The vials were prepared in triplicate.

2.2.3 Chromium determination in rat urine

Rat urine was collected over a period of 24hrs from both "chromium deficient" and replete rats housed in plastic lined metabolic cages. Contamination of the urine with faeces and food were problems that were not overcome.

5μl aliquots of urine were injected into the CRA tube furnace with the following settings:

Cycle	Duration	Temperature (°C)
dry	35 sec	80
ash	50 sec	900
atomize	2 sec	2500

The non-atomic absorption was determined using the deuterium lamp only and was found to be greater than could be corrected for (0.9AU) by the instrument.

2.0ml aliquots of rat urine were digested with 2.0ml of nitric acid (Aristar, BDH). The digestions were diluted to 5.0ml and chromium determined by standard addition using the CRA furnace. Reduction of non-atomic absorption was achieved but a high reagent blank (0.048g/ml) was found due to the chromium content of the BDH nitric acid. The analysis of chromium in urine was abandoned due to the collection problems and, for further analysis, Merck "suprapur" nitric acid was obtained (0.002ppm Cr).

2.2.4 Whole body chromium determination

The total body chromium of "chromium deficient" and replete rats was determined on eight rat carcasses provided by Ms J. Sherriff in a double blind study. The rats had been killed and intestinal tract removed. The bodies were dried in vitreosil evaporating dishes in a clean vented oven at 90°C over two days. The dried bodies were then ashed at 500°C in a clean muffle furnace (Analite Pty. Ltd.). The ash was finely ground and the total weight determined to the nearest 0.1g. The ashes were stored in polyethylene containers. As salt levels of digested ash were high, the following extraction and preconcentration was used.

Duplicate homogeneous samples of ash (0.6g to nearest 0.1mg) from each ashed rat were taken and digested into 10ml of nitric acid (Merck). The digest was diluted to approximately 10ml. 2.5ml of freshly prepared 1% ammonium pyrrolidine dithiocarbamate (APDC) was added and the pH adjusted to 6.0 with 1N ammonium hydroxide (AR, Univar). Chromium standards were treated in the same manner. The samples and standards were heated at 80°C for 2hrs to aid complexation. After cooling the flasks, 2.5ml of water saturated methyl-isobutylketone, Merck (MIBK) was added and shaken for 1min before withdrawing the solvent.

The MIBK was aspirated into a pre-mix air/acetylene flame and the corrected signal recorded. The total chromium was calculated from the linear calibration curve (Table 2.2).

2.2.5 Determination of chromium in rat food

The chromium levels in two batches of Teklad "low chromium" rat food and pelletized rat food (supplied by Gibsons) were determined with NBS brewers' yeast SRM 1569 as reference material. The finely ground samples were air dried at 80°C. Triplicate samples of approximately 0.8g (to the nearest 0.1mg) were wet ashed in acid washed Kjeldahl flasks. On completion of digestion, the flask contents were diluted to 2.0ml. Standards and samples were aspirated and total chromium content calculated on a dry weight basis (Table 2.3).

2.2.6 Determination of chromium in yeast medium

The chromium level of freshly prepared liquid yeast medium and synthetic medium (Chapter 4.2) was determined by chelation with APDC and solvent extraction before the addition of chromium salts. The chromium levels of yeast culture medium after addition of chromium were sufficiently high (1.0-4.0ppm) to allow direct reading by AAS.

The pH of 50ml of medium was adjusted to 6.0 with 1N ammonium hydroxide (AR, Univar). 2.5ml of freshly prepared 1% APDC was added. Chromium standards were prepared and treated in the same manner. The samples and standards were heated at 80°C for 2hrs and, on cooling, were extracted with 5.0ml of water saturated MIBK. 10µl of the MIBK extract was injected slowly into the CRA with the drying cycle operating in order to prevent losses due to solvent creep. The following settings were used.

Cycle	Duration	Temperature (°C)
dry	50 sec	50
ash	20 sec	900
atomize	2 sec	2500

The results were calculated from the calibration curve (Table 2.4).

2.2.7 Determination of chromium in GTF fractions

The chromium content of concentrated fractions from yeast was determined by injection of 2-10μl directly into the CRA furnace. The samples were not pretreated and were diluted so that the chromium level was within the detection limits given in Section 2.2.1. Generally, two different dilutions were analyzed. Continuous background correction was used and was found to be adequate. The operating conditions were as follows.

Cycle	Duration	Temperature (°C)
dry	50 sec	90
ash	50 sec	900
atomize	2 sec	2500

2.2.8 The luminescence biometer and chromium determination

A DuPont 760 luminescence biometer specifically designed for the measurement of ATP (adenosine triphosphate) by the firefly reaction was used in the determination of chromium III by chemiluminescence. The maximum light output of the sample over three seconds was displayed as a digital readout. The maximum volume of the reactants was restricted to 800μl. Activation of the instrument was

automatic on injection of a reactant through a septum into the reaction vial.

2.2.8.1 Reagents

(i) 0.2M EDTA (ethylenediaminetetraacetic acid, AR, Univar) as stock solution.

(ii) 0.1M KOH-H_3BO_3 buffer. 6.18g of H_3BO_3 (Merck) was dissolved in 500ml of distilled water and the pH adjusted to 10.6 with 1.0N KOH (AR, Univar). 100ml of stock EDTA was added to the buffer and the total volume adjusted to 1.0ℓ.

(iii) Luminol. 18.0g of crystalline luminol (5-amino-2,3-dihydro-1,4-phthalazinedione, Sigma) was dissolved in 100ml of buffer.

(iv) Peroxide. To 0.34ml of 30% hydrogen peroxide (Merck) was added 1.0ml of stock EDTA solution and the final volume adjusted to 10.0ml with distilled water.

2.2.8.2 Chemiluminescence assay

500μℓ of luminol solution, 10μℓ of EDTA and 250μℓ of sample were pipetted (Oxford automatic pipettes) into acid washed reaction vials. A vial was placed in the biometer and 40μℓ of peroxide was injected with the syringe supplied with the instrument.

Duplicate standards were pipetted into vials containing 10μℓ of EDTA. The vials were heated in a convection oven at 80°C for 60min and cooled before adding 500μℓ of luminol reagent. The

bioluminescence was determined as described above (Figure 2.2).

2.2.9 Determination of radioisotope activity

2.2.9.1 ^{51}chromium and ^{14}carbon

The preparation of individual counting standards will be described in sections of the thesis where the isotopes were used. Samples were generally counted for 10.0min and standards as well as blank vials, containing ACS II scintillation cocktail (Amersham) in the case of β-counting, were routinely determined with the samples. The final concentration was determined by ratio. 15ml of scintillation cocktail was routinely used so that the counting efficiency was the same for all samples.

Samples and standards were prepared so that the upper limits of each counter were not exceeded over a period of 10.0min.

(i) γ-counter

Kontron MR 252 with 1.75" NaI crystal

Window preset for ^{51}chromium

Maximum counts 0.8×10^6

% efficiency for γ-radiation = 10.6

(ii) β-counter

Beckman liquid scintillation system

Maximum counts 1×10^6

% efficiency for β-radiation = 87

Determination of the amount of label in dual isotope preparations was by the method of Olson (1977). The samples were recounted after a known period of time in excess of 7 days.

2.2.9.2 Detection of isotopes on chromatograms

(i) Spark chamber

Both ^{51}Cr and ^{14}C were detected on chromatograms using a Birchover Instruments spark chamber. 10% methane in argon was used as the counting gas and a high voltage of 1900V was applied to the coiled wires. The sparks were photographed by exposure to Polaroid film. At least 1 400dpm were required for the radioactivity to be detected. To aid in orientation of the photograph, 5µl spots of ^{14}C in benzoic acid were made on the corners of the chromatograms.

(ii) X-Ray film

Chromatograms which had been dried and not treated with visualization reagents (e.g. ninhydrin) were sandwiched with Kodak Medical X-Ray film (30 x 38cm) in X-Ray cassettes. Exposure time was varied according to the activity of the material on the chromatogram. The film was developed according to the manufacturers' instructions.

2.3 RESULTS

2.3.1 Ashing technique

	Dry wt. sample (mg)	Total cpm ^{51}Cr	% Response ± standard error of estimate
Wet ash - nitric [1]	70	39 600	102 ± 3%
"	140	70 250	102 ± 2.5%
"	370	191 700	102 ± 2%
"	450	233 270	103 ± 2%
Dry ash - 500°C [2]	500	235 870	91 ± 2%
"	1000	455 390	80 ± 2%

[1] 2.0ml of nitric acid. [2] 500°C for 18hr before addition of sulphuric acid.

TABLE 2.1 The recovery of ^{51}Cr after ashing of labelled brewers' yeast.

2.3.2 Self absorption

100µl aqueous standard ^{51}Cr = 30 236cpm ± 4%

100µl standard + 0.5g dry yeast = 31 041cpm ± 4%

2.3.3 Whole body chromium determination and rat food

Sample No.	Cr Replete Total Cr (µg)	Sample No.	Cr Deficient Total Cr (µg)
B	14.3	W	21.4
C	31.5	X	11.0
D	1.5	Y	10.8
E	2.3	Z	3.0

TABLE 2.2 The determination of whole body chromium in rats fed Teklad "low chromium" diet and the diet + chromium acetate. B, C, W, X had been cannulated; the cannula plug was stainless steel.

Sample	µg Cr/g dry wt.
Teklad batch 1	0.46 ± 0.05
Teklad batch 2	0.78 ± 0.05
Gibsons	0.82 ± 0.05
NBS yeast SRM 1569	2.10 ± 0.05

TABLE 2.3 Chromium determination of Teklad "low chromium" diet and a normal colony rat diet (Gibsons).

2.3.4 Chromium levels in yeast medium

Culture medium	Cr (µg/ml)
100ml synthetic	0.002
5.0ℓ "	0.001
100ml liquid yeast medium	0.020
10.0ℓ "	0.020

TABLE 2.4 The chromium concentration in synthetic yeast medium (Table 4.4 & 4.7) and liquid yeast medium (Table 4.3).

2.3.5 Chemiluminescence standard curve for chromium III determination

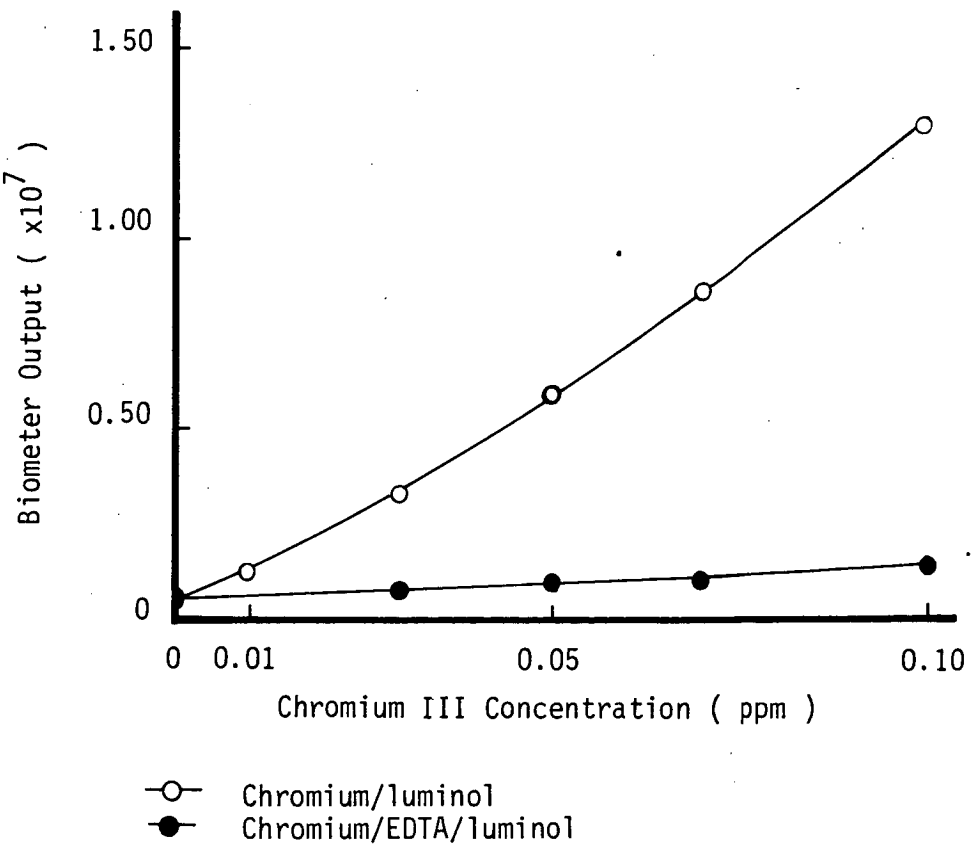


FIGURE 2.2 The chemiluminescence assay for chromium III.

2.4 DISCUSSION

2.4.1 Digestion of samples

The results of wet and dry ashing are presented in Table 2.1. Losses of ^{51}Cr occurred with the dry ashing technique due to the sulphuric acid "creeping" out of the crucible rather than drying. No losses were found on wet ashing of ^{51}Cr yeast with nitric acid, thus wet ashing was used routinely. No significant counting errors due to self absorption of the isotope were found (Section 2.3.2). The practice of allowing the samples to stand overnight with acid before digestion, considerably reduced the viscosity of the samples and reduced charring and sputtering. Nitric acid was diluted by 50% for digestion of the Teklad "low chromium" diet as the high sugar content caused excessive foaming.

It became apparent during an attempt to analyze rat urine, that low chromium acids should be used and the volume kept to a minimum for all digestions to avoid contamination.

National Bureau of Standards reference material SRM 1569 (a kind gift from the Cenovis Health Food Company) was analyzed in conjunction with rat food (Table 2.3). The mean result of three analyses was marginally less than the NBS certified value. Kumpulainen *et al* (1978) also reports values lower than the certified value for NBS brewers' yeast and attributed the problem to chromium being strongly bound to insoluble silicates not digested.

2.4.2 Chromium deficiency

The analysis of rat urine was abandoned as a measure of chromium deficiency due to insurmountable difficulties in collecting urine without contamination from food or faeces (Section 2.2.3). Direct injection of urine into the CRA furnace was not an appropriate method for the AAS unit used since background correction was insufficient (Section 2.2.3) (Veillon *et al* 1981, Guthrie *et al* 1978). Had sufficient sample been available, digestion followed by desalting and concentration may have been successful.

The analysis of the "low chromium" rat food revealed a higher level of chromium than calculated by the manufacturers (0.12ppm) and was actually little different to the diet supplied to normal colony rats, thus casting doubt on the chromium deficiency of the animals. Whole body chromium determination of replete rats (50ppm chromium added to Teklad diet) was found to be no different to that of deficient rats (Table 2.3.3), however, within the eight rat carcasses ashed, four had been cannulated and showed significantly higher body chromium levels, most likely due to contamination from the stainless steel cannula plug.

2.4.3 Chromium in yeast culture medium

The extraction procedure described in Section 2.2.6 using APDC and MIBK was successful as a preconcentration step in the analysis of freshly prepared culture medium. The chromium levels in the synthetic yeast medium were found to be 2ppb, thus the yeast subcultured in synthetic medium could be regarded as being chromium deficient. Chromium levels in the liquid yeast medium were found to be 0.02ppm or 20ng/ml.

2.4.4 Chromium determination by chemiluminescence

The initial results obtained with the DuPont biometer (Figure 2.2) appeared promising with aqueous chromium III standards. Unfortunately, the instrument was not available for further work thus no data on samples was obtained. The method is reported as an alternative analytical method to AAS and is potentially interesting for GTF studies as it is specific for chromium III. The adaption of the method of Seitz *et al* (1972) was successful, however, the detection limit was not as low as was expected. Recrystallization of the luminol as reported by Chang *et al*(1980) and the replacement of EDTA by DTPA (diethylenetriaminepentaacetic acid) were considered as possible improvements to the method reported in Section 2.2.8.

CHAPTER THREE

CHROMATOGRAPHY

3.1 INTRODUCTION

Ion exchange chromatography (separation by charge) was first reported as a means of purifying GTF by Schwarz and Mertz (1959). The cation exchange resin was neutralized with ammonia to displace the active material. Mirsky *et al* (1980) report the fractionation of GTF by using an anion exchange cellulose and then proceeding with cation exchange chromatography. Haylock *et al* (1982) report the use of a phosphate pH gradient to improve the separation of fractions from cation exchange chromatography.

As multiple column chromatography involves concentration of the column eluant, volatile solvent systems were used throughout to prevent the concentration of large amounts of salts which would have affected analysis, biological activity and been difficult to remove, as desalting by dialysis was not possible due to the low reported molecular weight of GTF (300-500 daltons, Saner, 1980). A range of volatile buffer systems has been reviewed by Dawson *et al* (1969).

The use of gel filtration chromatography has been reported in the fractionation of GTF and the determination of molecular weight (Kumpulainen *et al* 1978, Toepfer *et al* 1977, Polansky *et al* 1977). Votava *et al* (1973) reported an aggregation process for GTF on the basis of the change in elution volume from Sephadex (dextran) with buffers of different ionic strength.

The use of high performance liquid chromatography (HPLC) as a means of fractionating GTF has been reported by Polansky and Anderson (1977) using a reverse phase system (C-18) but no details have been published. An evaluation of the reverse phase chromatography of peptides by HPLC has been published recently by Wehr *et al* (1982) and the use of the ion pairing reagent trifluoroacetate in the separation of peptides has been reported by Bishop *et al* (1981) and Isied *et al* (1982). The technique and theory of HPLC has been well documented (Snyder *et al* 1974).

The use of paper chromatography and associated techniques has been well documented by Smith (1969). Paper chromatography was used by Toepfer *et al* (1977) to qualitatively compare fractions. The use of paper chromatography in this chapter includes the use of preparative chromatography for the purification of GTF.

3.2 METHODS

3.2.1 Column chromatography - detection and fraction collection

The column eluate was passed through the 5mm absorbance cell of a LKB Uvicord II scanner (253nm) which was routinely adjusted to give 100% transmittance with the buffer used. 10ml samples were collected by a LKB Ultrorac fraction collector. The transmittance was recorded with a Heathkit chart recorder.

Detection of ^{51}chromium was achieved by collecting fractions in γ-counting vials and then transferring the vials to the Kontron MR 252 γ-counter. Where chromium concentration was high (chromium complex preparation), a small portion of each fraction was aspirated into an atomic absorption spectrophotometer and the relative chromium content plotted.

3.2.2 Ion exchange chromatography

(i) Dowex 50W-X8 (H^+): a cross linked polystyrene with sulphonyl functional groups (100-200 mesh, Bio Rad) was used batchwise, as described in Chapter 5.2.4, as it has been demonstrated that active material is sorbed by Dowex and can be displaced at high pH. When used for column chromatography, the resin was pre-swollen in distilled water before pouring into the column taking care to exclude air bubbles. The column was equilibrated at 4°C by eluting with three column volumes of water (gravity feed) before loading the sample at a pH of 3.9. The active material was eluted with either ammonia or triethylamine/ acetate buffer (TEA). Where a linear gradient is reported, the gradient was generated by a Gradipore gradient mixer. Dowex resin was regenerated after use with 10% hydrochloric acid according to the manufacturer's instructions.

(ii) Dowex 50W-X2 formate: was prepared by the technique reviewed by Smith (1969) for quantitation of iodoamino acids. The resin (100-200 mesh) was washed with 1.0N ammonia (Univar) and then equilibrated with 0.2M ammonium formate buffer, pH 3.9. Elution by increasing ionic strength was unsuccessful but a gradient from 1.0N formate to 0.5N ammonia, resulted in fractionation (Figure 5.5.1). The ammonium formate was removed from fractions by lypholization.

(iii) Anion exchange cellulose DEAE 22 (Whatman) was thoroughly washed with 0.1N ammonia and 0.1N hydrochloric acid. The column was transferred to 4°C and washed with distilled water until no further absorbance at 253nm was detected. The column size was 50mm x 400mm. A sample was loaded onto the DEAE 22 cellulose and eluted with water. The water eluant was passed directly onto a Dowex 50W-X8 (H^+)

column which was connected in series with the DEAE cellulose column. The two columns were then disconnected and the strongly sorbed material was eluted from the DEAE cellulose with 1N hydrochloric acid and the eluant lypholized. The now separate Dowex column was eluted with ammonia as described in Chapter 5.2.4.

Amphoteric material was fractionated with DEAE Sephadex (Pharmacia), a beaded ion exchange cellulose. The cellulose was supplied pre-swollen. The column was poured taking care to exclude air bubbles and was then equilibrated at 4°C with 0.25N ammonia before loading the sample. The fractions were eluted with a linear gradient from 0.25N ammonia to 1M acetic acid. Tightly sorbed material was displaced by 0.1N hydrochloric acid.

3.2.3 Gel filtration

Bio-Gel P, porous acrylamide beads (100 - 200 wet mesh, Bio Rad) columns were prepared, with different exclusion limit gels and different column sizes being prepared in the same manner. The sample volume used was generally 5% of the total volume of the column. The gel was suspended in buffer (generally, 50mM ammonia), swirled and allowed to stand at room temperature for four hours. The swollen gel was then deaerated by aspiration and poured into a glass column which contained 25% of the total column volume of buffer. A plunger with filter was inserted into the column so that the filter was touching the gel. The column was then transferred to a 4°C room where it was equilibrated under operating conditions (gravity feed, head height 900mm) and stored to discourage bacterial growth.

Bio-Gel P6 (25mm x 400mm) was used in initial investigations for molecular weight determination. The void volume was determined with cytochrome c (2mg) and the column was calibrated with glucagon (5mg), vitamin B_{12} (1mg) and glutathione (5mg) which was detected by the addition of 5 drops of 2% sodium nitroprusside (Merck). The calibration was satisfactory using 50mM ammonia which also ensured that the samples which were ammonia soluble did not precipitate.

A Bio-Gel P4 column (25mm x 900mm) was packed in an attempt to increase resolution and the amount of material which could be loaded. When volumes of material exceeded 5% of the column volume, the sample was divided and duplicate column runs were made. Calibration for molecular weight determination was only achieved at high triethylamine acetate (TEA) buffer concentrations (Table 3.1) and, as 50mM ammonia produced good resolution and was easily removed by lypholization compared with TEA, ammonia was used routinely and molecular weight determination discontinued.

The use of Bio-Gel P2 was considered as a possible replacement for P4 but band spread was excessive (Table 3.2). Phenyl Sepharose Cl-4B was considered, as separation by hydrophobic interaction may have been possible. The use of 0.05N ammonia and later, 1.0M TEA pH 6.3 gradient to 0.05N ammonia, were both unsuccessful with little sample retention occurring.

3.2.4 Paper chromatography

Descending paper chromatography was used as a technique to qualitatively assess the purity of fractions after column chromatography.

The chromatography tanks used were glass 210mm x 550mm and 550mm high with tightly fitting ground glass lids. The solvent reservoirs (200ml) were supported in the top of the tank. 50ml of solvent was poured into the bottom of the tank and allowed to equilibrate for several hours before suspending the paper from the reservoir and allowing the paper to equilibrate prior to filling the reservoir. The chromatography paper used was Whatman 3 MM medium flow, 0.3mm thick and 455 x 570mm in area.

The paper was prepared for development by placing 5µl spots of sample every 20mm across the paper 100mm from the bottom edge (reservoir edge). The edge to which the solvent travels was trimmed with pinking shears to encourage even solvent flow. Paper for preparative chromatography was thoroughly washed with 0.2N ammonia and air dried before applying 0.5ml of sample across the origin as a streak (100mm from the reservoir edge) and drying.

The chromatograms were removed from the tanks after elution for 18hrs and suspended in a vented oven at 40°C.

When the papers were dry, they were examined for absorption or fluorescence with UV light (254nm, 360nm) or radioactivity (Chapter 2.2.9.2). Detection with ninhydrin (0.2% in acetone applied as a spray) was satisfactory and used routinely. Spray reagents were used on a thin strip cut from each edge of preparative chromatograms.

The preparative chromatograms were cut into strips, each strip containing a separated compound. The strips were placed into a descending chromatography tank and eluted with 0.1N ammonia. The eluant was collected directly into freeze drying pots and the samples then lypholized.

Solvent systems

(i)	n-butanol	200ml
	glacial acetic acid	50ml
	distilled water	250ml

The two phase system was prepared by shaking the solvents in a separating funnel and allowing the layers to equilibrate and separate over one hour before discarding the aqueous phase.

(ii)	iso-propanol	140ml
	ammonia	40ml
	water	10ml

3.2.5 High performance liquid chromatography

Chromatography was performed on a system consisting of two Water Associates M-6000 A solvent delivery pumps with Model 660 solvent programmer and U6K injector. The column used was a radially compressed reverse phase column - 5μm C-18 packing in a RCM 100 compression unit. Detection was at 260nm and 220nm with a Model 440 absorbance detector and a variable wavelength detector made by Mr J. Jordan of the Biochemistry Department. Refractive index was also recorded after optical measurement with a Waters differential refractometer.

Initial investigation using relatively impure fractions from a 10ℓ culture indicated that a 10μm reverse phase column with a 70:30 methanol/water mobile phase (isocratic) may be satisfactory. Nicotinic acid and nicotinamide were used as model substances to develop the system.

With purification of sample 2^e (Figure 5.5.3) it became apparent that no pyridine ring structure was present (lack of UV absorbance) and the model was changed to that of the separation of peptides as 2^e was ninhydrin positive. Glu-gly, cys-gly (Serva), glutathione (Sigma) and oxidized glutathione were prepared (1mg/ml) and 1µl of each mixed in a syringe (10µl, pressure Lok) and injected. The mixture was resolved with a radially compressed 5µm C-18 column with an aqueous phase containing 0.3% trifluoroacetate (BDH) ion-pairing reagent. Fraction 2^e when injected into the system, was not retained and was eluted at the void volume. Manipulation of the mobile phase by addition of methanol, acetonitrile, acetic acid and ammonia did not improve retention.

3.3 RESULTS

The molecular weights of active fractions 3 and 4 (Figure 5.3) were found to be 1180 and 960 daltons respectively by using a Bio-Gel P4 column and 50mM ammonia. The initial use of HPLC with a reverse phase C-18 packing and 70:30 methanol/water mobile phase indicated a high degree of impurity with at least eight different peaks being detected.

Calibration compound	MW	$\frac{V_e}{V_o}$ 50mM NH_3	$\frac{V_e}{V_o}$ 260mM TEA pH 7.5
flavin mononucleotide	478	0.95	-
NAD	663	1.11	2.58
riboflavin	375	1.65	-
vitamin B_{12}	1 355	2.42	2.20
nicotinamide	122	3.62	3.42

TABLE 3.1 The ratio of elution volume to void volume of compounds used as calibration markers on Bio-Gel P4 (25mm x 900mm) in buffers of differing ionic strength. Flow rate = 30ml/hr.

Bio-Gel	No. of theoretical plates	Column volume (ml)	Exclusion limit (daltons)
P2	105	470	1 800
P4	657	375	3 600
P6	573	177	4 600

TABLE 3.2 The number of theoretical plates calculated from the bandspread of 1mg of vitamin B_{12} in 50mM ammonia.

Results of column chromatography and paper chromatography as used in the separation of GTF are presented along with extraction data in Chapter 5. The Rf values of various substances in the systems finally used are presented in Table 3.3.

Solvent system	Compound	Rf
butanol/acetic	glycine	0.23
"	tyramine	0.64
"	nicotinic acid	0.75
"	glu-gly	0.26
"	(ox.) cys-gly	0.10
iso-propanol/NH_3	glycine	0.15
"	nicotinic acid	0.47
"	n-methyl-adenosine	0.58

TABLE 3.3 The Rf values of known compounds on paper chromatograms in the solvent systems indicated.

The use of absorption and fluorescence under UV light and the use of ninhydrin was found to be satisfactory for visualization of developed chromatograms. Spraying with o-phthalaldehyde followed by inspection at 254nm (Benson *et al* 1975), for the detection of primary amines, yielded no additional information although the method was found to be more sensitive than ninhydrin. The use of p-anisidine/phthalic

acid (sugar detection), Ehrlichs' reagent (indoles) and benzidine reagent (sugars) did not give a positive result with active material. Compounds on chromatograms were detected non-specifically by heating the paper to 230°C, insufficient to char the paper, and then examining at 360nm (Alperin, 1982). No additional compounds were found by this method which proved to be sensitive. An example of a developed chromatogram is given in Figure 3.1.

3.4 DISCUSSION

The use of an ammonium formate pH gradient was found to increase the number of fractions eluted from yeast extract sorbed to cation exchange resin (Figure 5.5.1). Active material was strongly sorbed to cation exchange resin in agreement with Mertz and Schwarz (1959) and difficult to displace, implying that GTF is a highly charged cationic complex.

Bonilla (1969) and Khym (1974) reported excellent separation of basic proteins, ribonucleotides, ribonucleosides and purine and pyrimidine bases with low exclusion limit polyacrylamide gels when a low ionic strength alkaline buffer was used. Separation was by sorption rather than difference in migration rate. Schwartz *et al* (1965) report the use of Bio-Gel P2 for the separation of yeast ribonucleic acid. The same phenomena was observed using Bio-Gel P4 for fractionation of yeast extract. High concentrations of triethylamine acetate were necessary to obtain a linear calibration plot of Ve/Vo vs $\log_{10}$MW. As the active complex appeared to be highly charged, it was uncertain that the buffer concentration was high enough to suppress the sorption effect. Dilute ammonia was used, thus utilizing the sorption effect for fractionation rather than trying to suppress it.

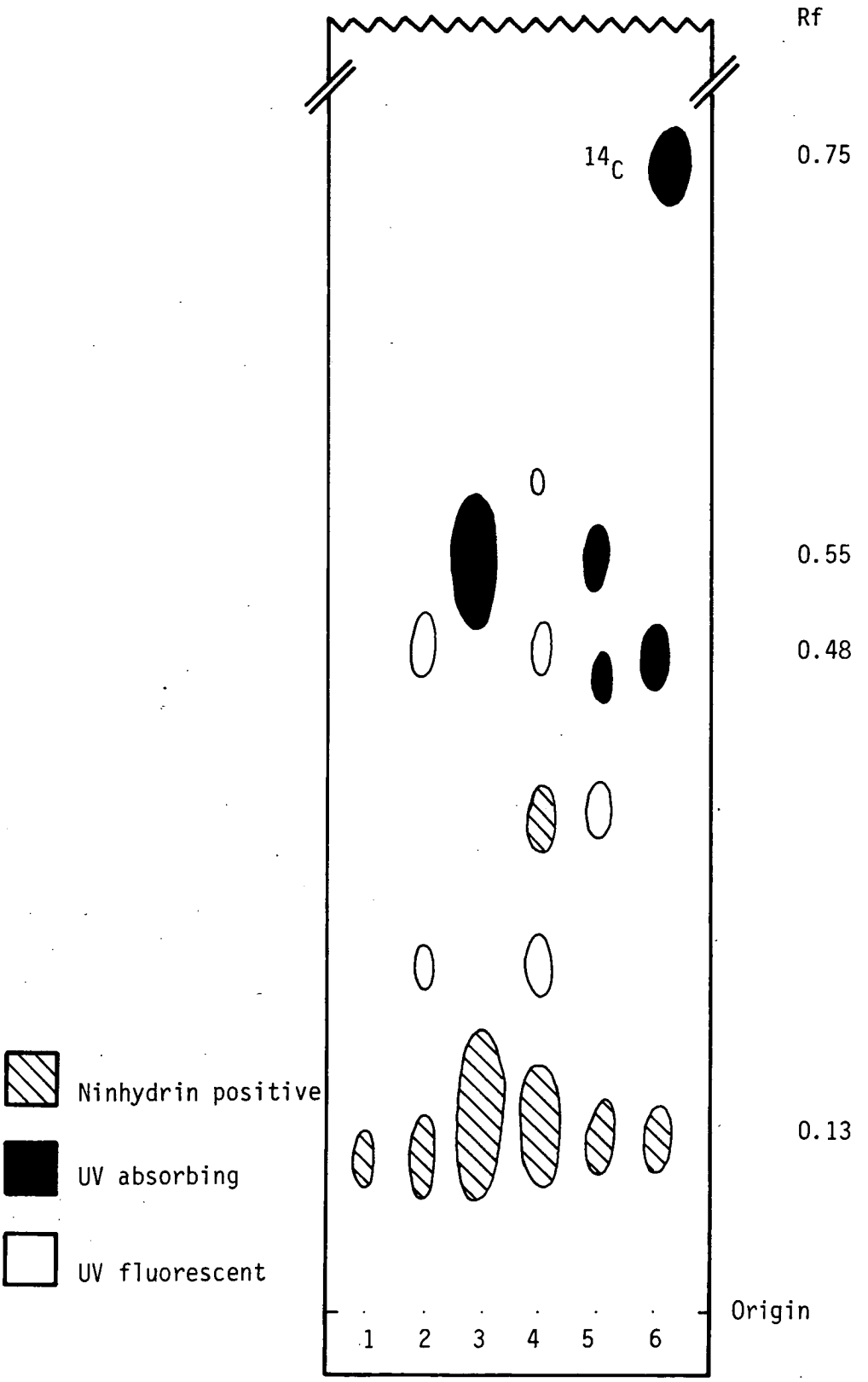


FIGURE 3.1 An example of a quantitative paper chromatogram developed in butanol/acetic acid. Samples 1-6 were from Bio-Gel P4, 3 and 4 were biologically active. Free nicotinic acid with an Rf of 0.75 was detected by absorbance and presence of radioactivity.

The aggregation effect reported by Votava *et al* (1973) would seem to be a sorption effect and not "reversible aggregation" as reported, since Khym (1974) has reported similar sorption effects with modified dextrans such as the Sephadex used. Starich and Blincoe (1982) report an anionic chromium containing complex with a high affinity for column packings (Chapter 1.8.2).

Fractionation by HPLC was unsuccessful as no retention of the active material on the C-18 column was achieved, even with high concentrations of ion-pairing reagent. Close examination of the results reported by Bishop *et al* (1981), who used a similar system, revealed that, of the peptides reported, gly-gly-lys tri-peptide eluted at 2.3ml which appeared to be the void volume of the system. The basic amino acid, lysine, would seem to have been responsible for the loss of retention as all other peptides reported were either neutral or acidic. Another mechanism other than hydrophobicity may be involved as Iseid (1982) reports the separation of positively charged cobalt III peptide complexes using trifluoroacetate counter ions.

Preparative chromatography was used for the final fractionation of yeast extract due to the lack of success with high performance liquid chromatography and was found to be satisfactory. The Rf value of nicotinic acid was found to be 0.75 in butanol/acetic acid and was well separated from other components. The active material of Toepfer *et al* (1977), common to both the synthetic preparation and the yeast preparation would almost certainly be free nicotinic acid with a reported Rf of 0.78 and activity in the adipocyte assay (Sherriff, MSc thesis, 1983).

3.5 CONCLUSION

GTF was fractionated by column and paper chromatography. The methods are reported in this chapter with the fractionation of yeast extract being reported in Chapter 5.

CHAPTER FOUR

YEAST MAINTENANCE AND GTF ASSAY

4.1 INTRODUCTION

4.1.1 Taxonomy

Various yeasts were referred to in Chapter 1 as brewers' yeast. Torula yeast was found by Schwarz (1951) not to contain the prophylactic factor 3 present in debittered brewers' yeast. Mirsky *et al* (1980) report the response of *Saccharomyces cerevisiae*, *Sacch. ellipsoideus*, and *Sacch. carlsbergensis* to GTF. Recent changes in taxonomy have occured due to advances in the knowledge of microfungi and a brief discussion is warranted to clarify the different names used in the literature.

Torula yeast used as rat feed in GTF deficient diets was most likely to be *Torulopis utilis*, now *Candida utilis* (Lodder 1970). *C. utilis* has been used as a foodstuff on a commercial scale as it is undiscriminating in its nutritional demands and grows rapidly. Although *C. utilis* has a fermatative ability, it is not as strong as genus *Saccharomyces* (Cook 1954).

Until the end of the 19th century, surplus brewers' yeast was used to leaven bread dough. *Sacch. cerevisiae* and *Sacch. carlsbergensis* have long been recognized as two brewing species where the former includes top fermenting or ale producing strains and the latter are bottom fermenting or lager producing strains. *Sacch. uvarum* became the correct name for all strains previously known as *Sacch. carlsbergensis* and *Sacch. uvarum*. Recently, all strains of *Sacch. uvarum* are now strains of *Sacch. cerevisiae* (Barnett 1979).

Metabolic differences between genera *Candida* and *Saccharomyces* are most pronounced in glucose metabolism with 0-30% glucose metabolized aerobically via the hexose monophosphate pathway by *Sacch. cerevisiae* and 30-50% by *C. utilis* Chang and Peterson (1949) showed that *C. utilis* neither required or accumulated biotin whereas, *Sacch. cerevisiae* accumulated biotin and would not grow unless 0.2ppm was present in the medium.

For purposes of the yeast assay system discussed in this chapter, a pure strain of *Sacch. uvarum* catalogue number NCYC 529 was subcultured, the original slope was kindly provided by the Australian Brewers' Association. Large cultures grown for extraction were inoculated with wort freshly obtained from Cascade Breweries. A pure strain of *Sacch. cerevisiae* (formerly *Sacch. ellipsoideus*) was a kind gift from Dr L. Blackwell (Massey University, Palmerston North, New Zealand). To avoid confusion, yeast strains referred to from the literature will be named as reported by the authors.

4.1.2 Fermentation

The anaerobic metabolism of hexoses by living yeast can be expressed by the Gay-Lussac equation first put forward in 1815 and accounts for approximately 95% of glucose metabolised.

$$C_6H_{12}O_6 \longrightarrow 2\ C_2H_5OH + 2\ CO_2$$

The remaining 5% is converted mainly into cell carbohydrates and succinic acid. Most efficient conversion to carbon dioxide takes place at pH 5.8 to 6.0 (Cook 1958). If the pH is raised into the alkaline region, ethanol, acetic acid, glycerol and carbon dioxide are produced.

The steps of glycolysis in yeast leading to pyruvate, are the same as those in muscle, however, yeast contains pyruvate decarboxylase and alcohol dehydrogenase but lacks lactate dehydrogenase.

It was been observed in fermentation experiments, that the yield of carbon dioxide can be appreciably less than theoretical. Liener and Buchanan (1951) have shown by isotopic techniques that growing cells can obtain up to 5% of their carbon requirements by assimilating carbon dioxide. Yeast can also grow aerobically in media containing ethanol or acetate as the sole carbon source.

4.1.3 Yeast Assay

The yeast assay to be described is derived from the observations of Burkeholder and Mertz (1966) and Mirsky, Weiss and Dori (1980). Burkeholder and Mertz report a doubling of carbon dioxide production after four fours in presence of 100ng chromium III per ml but depressed fermentation with 10 000ng. Chromium deficient cells (*Saccb. carlsbergensis*) were used in fresh medium. A nitrogen atmosphere was used with 1 x 10^8 cells in 2ml of phosphate butter at 25°C and pH 3.5. Glucose in the Warburg flask was 2.8mM. Little difference was seen between the control and chromium containing flasks without a 16hour incubation prior to the experiment. GTF containing fractions produced a response in the first 10-20minutes without preincubation, the greatest effect reported being 46% increase in carbon dioxide production.

Mirsky *et al* (1980) used 3 x 10^8 cells/ml, a nitrogen atmosphere and 0.1M phosphate buffer at pH 5.7 and 30°C, with 20mM glucose. The cells were preincubated at room temperature before each fermentation experiment for 30min to ensure consumption of endogenous

substrates. Anaerobic preincubation of the cells with glucose was found to be required to produce an immediate effect of GTF. GTF stimulated the production of carbon dioxide. Vitamins that may have been present in GTF containing fractions, had no effect on fermentation. Simple chromium salts were also found to be ineffective. At 120min fermentation, a 111% increase over control was reported for *Sacch. carlsbergensis* being a total production of 11.4μM carbon dioxide/3 x 10^8 cells with 20.2μM/3 x 10^7 cells being produced by *Sacch. ellipsoideus*.

Haylock *et al* (1982), when optimizing the standard yeast assay for screening fractions for GTF activity, found cell number, conductivity, pH and glucose concentration could be varied to increase the response to GTF. Furthermore, Mirsky *et al* (1980) presented data showing different responses of various strains of yeast to yeast GTF fractions. A summary of published conditions is tabulated below (Table 4.1).

Author	Strain	Cell No. (cells in test vol.)	Temp. (°C)	pH	Glucose (μmoles in test vol.)	CO_2/2hr Control (μmoles)
Burkeholder & Mertz (1966)	*S. carlsbergensis*	1 x 10^8	25	3.5	11.1	33
Mirsky *et al* (1980)	*S. carlsbergensis*	3 x 10^8	30	5.7	50	5.4
"	*S. cerevisiae*	6 x 10^8	30	5.7	50	21.8
"	*S. ellipsoideus*	3 x 10^7	30	5.7	12.5	9.76
Haylock *et al* (1982)	*S. cerevisiae*	1.5 x 10^8	30	5.75	55.6	2.2

TABLE 4.1 Yeast fermentation conditions for detection of GTF active fractions (all anaerobic).

Based on these previously published assays, it was decided to use U-^{14}C labelled glucose as the substrate to measure $^{14}CO_2$ produced radiometrically and also to see whether stoichometric amounts of ethanol (measured enzymatically) were produced. The development of such an assay is the subject of Section 4.3.

4.2 METHODS

4.2.1 Yeast culture

A pure strain of *Saccharomyces cerevisiae (Sacch. carlsbergensis)* catalogue No. NCYC 529, provided by the Australian Brewers' Association, was maintained on a solid yeast morphology medium (Table 4.2). For purposes of the yeast assay, the pure strain was serial subcultured aseptically in 100ml of sterile chromium-free medium (Table 4.4). Where possible, all chemicals were analytical grade. Cultures were inoculated with 6 x 10^8 cells per 100ml culture and grown aerobically at 18°C. Two days growth were sufficient to provide cells for the GTF assay. The strain *Sacch. ellipsoideus* was maintained in the same way.

Yeast for growth of a large culture (200ℓ) was obtained from Cascade Breweries as three day old wort. The wort was centrifuged (2 000xg - MSE Mistral 6L) and the supernatant discarded. The remaining yeast was washed twice with distilled water and centrifuged (500xg) to remove hop residues, then at 2 000xg for 10min. The washed cells were resuspended and used to inoculate preparative cultures (Section 4.2.3). Cascade yeast was also serial subcultured in liquid yeast medium (Table 4.3).

Malt Extract (Difco)	1.0g
Yeast Extract Powder (Oxoid)	0.2g
Bactopeptone (Difco)	1.0g
Maltose (Difco)	1.0g
Glycerol (Sigma)	0.2g
Potassium dihydrogenphosphate (Univar, AR)	50mg
Ammonium sulphate (Univar, AR)	50mg

The above dissolved in 100ml distilled water pH adjusted to 5.0, then

Agar (Difco)	1.5g

Sterilized by heat.

TABLE 4.2 Solid medium for maintenance.

Bactopeptone (Difco)	5.0g
Yeast Extract (Oxoid)	2.0g
Glucose (Ajax, AR - anhydrous)	40.0g
Potassium dihydrogenphosphate (May & Baker, AR)	0.2g
Ammonium sulphate (Univar, AR)	1.0g

Final pH adjusted to 5.5 after dissolving in 1.0ℓ deionized water.

TABLE 4.3 Liquid yeast medium.

Asparagine (anhydrous, Sigma)	5.0g
Casamino acids (Difco, acid hydrolysed casein)	0.2g
Glucose (anhydrous, Ajax, AR)	10.0g
Magnesium sulphate (Univar, AR)	1.0g
Ammonium phosphate (Univar, AR)	2.0g
Potassium dihydrogenphosphate (May & Baker, AR)	0.5g
Calcium chloride (Univar, AR)	0.2g

The above dissolved in 1.0ℓ of distilled water, pH adjusted to 5.5 and autoclaved. Then,

Sterile Vitamin Mix (Table 4.5)	1.0ml
Iron citrate (0.9% w/v)	0.1ml
Trace metal mix when required (Table 4.6)	1.0ml

TABLE 4.4 Chromium deficient synthetic medium (based on Difco morphological medium).

Inositol (meso, BDH)	10mg
Calcium pantothenate (Kock-Light)	10mg
Biotin (D-cryst., Kock-Light)	2mg
Thiamine hydrochloride (Kock-Light)	10mg
Pyridoxine hydrochloride (BDH)	10mg
Nicotinamide (Sigma)	10mg

The above dissolved in 100ml and sterilized by autoclave.

TABLE 4.5 Vitamin mix.

Copper sulphate hydrate (Univar, AR)	19.6mg
Zinc sulphate hydrate (Univar, AR)	1570.0mg
Cobaltous chloride hydrate (Sigma)	20.0mg
Manganous chloride hydrate (Univar, AR)	360.0mg
Sodium molybdate (BDH)	12.6mg

The above dissolved in 1.0ℓ and sterilized by autoclave. 1ml of trace metal mix was added to 1.0ℓ of chromium deficient synthetic medium and, where indicated, 0.1ml was added to 100ml of phosphate buffer.

TABLE 4.6 Trace metal mix

(A)	Ammonium sulphate (Univar, AR)	90.0g
	Asparagine (anhydrous, Sigma)	5.0g
	Glucose (anhydrous, Ajax, AR)	200.0g
	Casamino acids (acid hydrolysed casein, Difco)	1.0g
	Cysteine hydrochloride (Ajax)	0.1g
	Potassium chloride (BDH, AR)	2.0g
	Sodium chloride (BDH, AR)	2.0g
	Magnesium sulphate (Univar, AR)	10.0g
	Calcium chloride (Univar, AR)	0.2g
	Potassium dihydrogenphosphate (May & Baker, AR)	20.0g

The above made to approximately 15ℓ and pH adjusted to 5.5 and sterilized by autoclave.

(B)	Trace elements	
	Boric acid (Univar, AR)	100mg
	Copper sulphate hydrate (Univar, AR)	8mg
	Ferric chloride	40mg
	Manganous sulphate hydrate (Univar, AR)	80mg
	*Sodium molybdate (BDH)	40mg
	Zinc sulphate (Univar, AR)	80mg
	*Potassium iodide (Univar, AR)	20mg

*added separately, the remaining salts were dissolved in 100ml and added to (A).

(C)	Vitamins (autoclaved separately)	
	Biotin (D-cryst., Kock-Light)	1mg
	Calcium pantothenate (Kock-Light)	80mg
	Inositol (meso, BDH)	400mg
	Thiamine hydrochloride (Kock-Light)	80mg
	Pyridoxine hydrochloride (BDH)	80mg
	Riboflavin (BDH)	40mg
	Folic acid (BDH)	1mg

The above are dissolved in 500ml, sterilized by autoclave and added to (A) aseptically. The total volume of the culture medium is 20ℓ.

TABLE 4.7 Synthetic medium for large scale yeast cultures (5-200ℓ).

4.2.2 Growth of Cascade brewers' yeast in liquid yeast medium

The growth of only one culture will be reported as the method was essentially the same for all grown on the bactopeptone based liquid yeast medium. Where differences exist between cultures extracted, the differences will be indicated in Chapter 5.

9.0ℓ of liquid yeast medium (Table 4.3) were prepared and sterilized by autoclave in a 10ℓ flat bottomed pyrex flask containing a 75mm stirring bar. When the flask had cooled, it was fitted with a sterilized brewers' air trap. The flask was placed on top of a "Magnetic 9x9" stirrer (Coleman Industries) and the stirring rate adjusted to approximately 200rpm. A three day old culture of chromium deficient yeast grown in liquid yeast medium (3.8×10^8 cells) was used to inoculate the 9.0ℓ of medium. The culture was grown at 18°C in a constant temperature cabinet.

^{51}chromium acetate was prepared by dissolving 44.0mg of chromium acetate (Hopkin and Williams) in 10.0ml of water containing 10mCi of carrier free ^{51}chromium (New England Nuclear). 9.0ml of the preparation was added to the growing culture. The chromium concentration in the medium was calculated to be 1mg/ℓ and the activity of the isotope was 1μCi/μg chromium. ^{51}chromium acetate standard for γ-counting was prepared by diluting 100μℓ to 100ml with 0.3N acetic acid. A 100μℓ aliquot of the dilution was sealed in a polycarbonate counting tube (Disposable Products Australia) and was used as a standard.

After three days growth, 100g of anhydrous glucose (Univar, AR) and 100mg each of cysteine hydrochloride (Ajax) and reduced glutathione (crystalline, Sigma) were added to the culture. The same

additions were made on day 5. The culture was harvested when the cell number reached 2.20 x 10^7 cells/ml. The culture was harvested by pouring the culture into 1.5ℓ polyethylene centrifuge pots and separating the cells by centrifugation at 2000xg (MSE Mistral 6L). The wet weight of the yeast pellet was determined by difference.

4.2.3 The growth of a 200ℓ culture

200ℓ of synthetic culture medium was prepared as shown in Table 4.7 omitting sterilization, as the volume was too great. The medium was contained in a fibreglass container (400mm dia. x 930mm) with a close fitting lid having a brewers' air trap. The vessel was sterilized by irradiation with a "Germicide" UV light for 16hrs and the salts were dissolved in deionized water sterilized by filtration through a 0.2μm Millipore filter. The container was supported over a "Magnetic 9x9" stirrer (Coleman Industries). The preparation of the yeast that was used to inoculate the culture has been described in Section 4.2. The culture was grown at room temperature (20-25°C). The cell concentration after inoculation was 0.7 x 10^6 cells/ml. The stirring rate was adjusted so that the cells were evenly suspended in the medium.

Both ^{51}chromium and ^{14}C-nicotinic acid were added to the culture. 880.0mg of chromium acetate was dissolved in 20.0ml of distilled water containing 0.4ml of ^{51}chromium (1000μCi, carrier free, New England Nuclear). 0.1ml of the ^{51}chromium acetate was reserved as a standard for γ-counting. The final ^{51}chromium concentration of the culture was 1.0mg/ℓ with the activity of the isotope being 0.005μCi/μg Cr.

The ^{14}C-nicotinic acid, labelled in the carboxyl group, was prepared as follows. 400.0mg of nicotinic acid (anhydrous, Sigma) were dissolved in 20.0ml of water containing 250μCi of carrier free ^{14}C-nicotinic acid (New England Nuclear) and pH adjusted to 5.5 with 1M TRIS (Ultrol). A β-counting standard was prepared by diluting 25μl of the 20.0ml solution to 5.0ml. The remainder of the solution was added to the culture.

Samples were withdrawn during the growth of the culture and extra glucose additions were made when required as determined by testing the supernatant with Labstix (Ames, lower limit of detection 0.25%). The results of cell determinations and the additions to the culture are tabulated in Section 4.4.1.

Prior to harvesting (8 days growth), stirring was stopped and the cells were allowed to settle. Cells still in suspension were removed from the supernatant by centrifugation at 6000xg with a continuous flow head fitted to a Sorvall SS40 centrifuge. The supernatant was pumped through the centrifuge head with a peristaltic pump at approximately 1l/min. 20l of the spent medium was set aside for extraction with activated charcoal (Chapter 5). The cell slurry remaining in the bottom of the tank was harvested by centrifugation in 1.5l pots at 2000xg (MSE Mistral 6L). The wet weight of the pellet was determined by difference.

4.3 YEAST ASSAY

Two day old chromium deficient yeast grown as described in Section 4.2 were centrifuged at 2000xg (GS 200 bench centrifuge, Clements) for 5min, the medium discarded and the cells resuspended in

an equivalent volume of 0.05M potassium phosphate buffer, pH 5.5. The cells were shaken overnight at room temperature (aerobic), at 100rpm by a reciprocating shaker (Paton Industries) to deplete the cells of endogenous substrates. The yeast cells were evenly suspended and an aliquot taken and diluted 1ml in 200ml of saline (0.9% sodium chloride, AR). The cell number in the dilution was determined by counting 0.5ml by Coulter Counter (Coulter Electronics) having an aperture of 100μm and impedance matched to the saline used (20kV). The particle count due to the saline was subtracted from the cell count and the total number of cells calculated. The cells were centrifuged (2000xg for 5min) and resuspended in a volume of phosphate buffer (0.05M, pH 5.5) such that the cell concentration was 2×10^7 cells/ml. The cells were continually stirred with a magnetic stirrer while 1.0ml aliquots were pipetted (Excalibur 0.2-1.0ml) into 25ml glass scintillation vials (Australian Pharmaglass).

Fractions to be tested were pipetted (Oxford Sampler 10μl) into the vials containing the yeast and allowed to stand at room temperature for a preincubation period of 1hour before adding labelled glucose. Duplicates of all test vials were prepared along with control vials without sample. During the preincubation, the vials were fitted with Clinbritic rubbers having a plastic centre well, a 1.10 x 38mm disposable hypodermic needle to introduce nitrogen, and a 1.23 x 13mm needle serving both as an outlet for nitrogen and access for the syringe needle used to introduce glucose (Figure 4.1). A 15 x 15mm square of Whatman 3MM chromatography paper was placed in each centre well with 100μl of 5.0N sodium hydroxide to absorb carbon dioxide produced during fermentation.

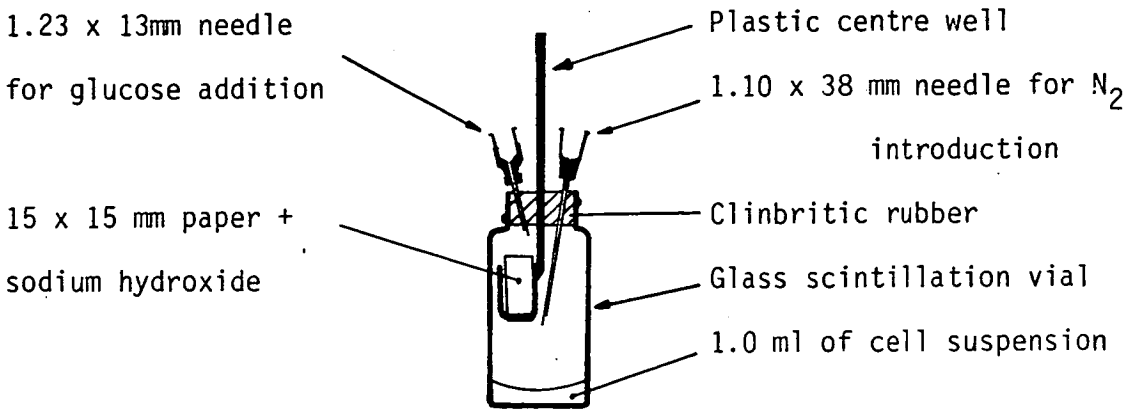


FIGURE 4.1 A fermentation vial for collection of carbon dioxide generated during the yeast assay.

The vials were placed in a Metabolyte water bath/shaker (New Brunswick Scientific) at 30°C and shaken at 100rpm. "High Purity" nitrogen, water saturated by bubbling through a 250ml drechsel bottle, was distributed to the vials via a manifold fitted with 250mm lengths of silicone tubing fitted with polyethylene connectors. Approximately 100ml/min/vial of nitrogen was introduced for 10min to purge oxygen from the vial and displace dissolved oxygen in the cell suspension.

To each vial, 100µl of universally labelled ^{14}C-glucose (100µmoles, 0.3µCi/100µl, New England Nuclear) was introduced with a 100µl SGE syringe taking care to avoid the centre well. The 13mm needle was withdrawn from the stopper and then the 38mm needle. The fermentation was stopped after 120min by introducing 0.2ml of 3.0N sulphuric acid to the cell suspension by injecting through the stopper with a 1.23 x 13mm needle and 1.0ml glass syringe. As approximately 80% of carbon dioxide evolved during fermentation was absorbed, the cells were shaken for a further hour to allow complete absorption.

The vials were opened and the paper removed from the wells with forceps, and placed into plastic scintillation vials (Packard)

containing 1.0ml of distilled water. A further 0.5ml of water was used to rinse the wells, the washings being transferred to the scintillation vials by pasteur pipette. The papers were allowed to stand for approximately 1hour before adding 15ml of ACS II aqueous scintillation cocktail (Amersham). The scintillation vials were shaken well before measuring β-radiation in a Beckman Liquid Scintillation counter. Generally, 48hours were required for the phosphorescence of the blank vial (paper, 100μl 5.0N NaOH, 1.5ml water, 15ml scintillation fluid) to fall to background levels.

To determine glucose and ethanol levels in the supernatant, the cell suspension was transferred to 15ml conical centrifuge tubes, the vials were rinsed with 1.0ml of phosphate buffer, and the cells were centrifuged at 2000xg for 5min. The supernatant was poured off and neutralized with 0.8ml of 1M TRIS (Ultrol, Calbiochem). The final volume of supernatant was adjusted to 3.0ml. Aliquots were taken for ethanol determination by alcohol dehydrogenase assay (Boehringer 1970) and glucose determination by glucose oxidase method (Boehringer 1970).

^{14}Carbon fixed in the cells was determined after washing twice with phosphate buffer by resuspending the yeast pellets in 5.0ml of ACS II scintillation fluid and transferring into plastic scintillation vials with a further 10.0ml of ACS II.

4.3.1 Calculation of results from the yeast assay

To enable the comparison of activities of different fractions, the results from each fraction were expressed as a percentage of a known active fraction which was used as a GTF control along with the

control vials containing cells and glucose. The following formula was used to calculate the % of GTF control.

$$\frac{\text{average sample cpm - average control cpm}}{\text{average GTF control cpm - average control cpm}} \times 100$$

A short program was written to calculate the results and present them in such a way that the printout was a record of the experiment when attached to a work sheet detailing the fractions tested in each vial. The computer system used was a Kontron PSI 80 system interfaced with a Star DP8480 printer.

Percentage response of a sample in relation to the control vials was calculated when required by the following formula:

$$\frac{\text{average sample cpm - average control cpm}}{\text{average control cpm - background cpm}} \times 100$$

Calculation of carbon dioxide collected in terms of μmoles per 2×10^7 cells was by the ratio of cpm with background subtracted, with the ratio of glucose cpm having known concentration. It was assumed that the ^{14}C-carbon dioxide represented all of the carbon dioxide derived from the ^{14}C-glucose as the cells had been starved to deplete them of endogenous substrates.

```
:LIST

10 PRINT CHR$(17);"DID YOU ACTIVATE $MX80 #3?";CHR$(17)
20 N1=3
30 T=0
40 INPUT "The date of assay";A$
50 PRINT #3: TAB (30);"YEAST ASSAY ";A$
60 INPUT "CO2 blank value";B
70 INPUT "Control values";C,D
80 E=((C+D)/2)-B
90 INPUT "GTF controls";C,D
100 G=((C+D)/2)-B
110 PRINT #3:"CONTROL=";E
120 PRINT #3:"GTF CONTROL=";G
125 PRINT #3:
130 PRINT #3:"MEAN CPM-BG","% OF GTF CONTROL","RANGE","TEST NO."
140 F=(1/(G-E))*100
150 INPUT "Sample results";C,D
160 P=((C+D)/2)-B
170 P1=(P-E)*F
180 S=((C-D)/2)*(1/P)*100
190 PRINT #3:P,
200 PRINT #3:USING"###.#",P1,
210 PRINT #3:USING"###.#",S,
220 N1=N1+2
230 N2=N1+1
240 PRINT #3: TAB (60);N1;",";N2
250 INPUT "ERROR (if error type 1)";T
260 IF T=1 THEN 270 ELSE 150
270 N1=N1-2
280 N2=N2-2
290 GOTO 150
300 END

Ready
```

YEAST ASSAY 10/12/82

CONTROL= 14839
GTF CONTROL= 24537

MEAN CPM-BG	% OF GTF CONTROL	RANGE	TEST NO.
21714	70.9	0.2	5, 6
35357	211.6	0.0	7, 8
15838	10.3	0.6	9, 10
21000	63.5	0.2	11, 12

SECTION 4.3.1 The program used to calculate yeast assay results and an example of the final results presentation.

4.4 RESULTS

4.4.1 Geometry of the assay system

The final geometry of the yeast assay evolved from a series of prototypes. The needles selected allow unrestricted flow of nitrogen. Blockages resulting in vials not being purged of oxygen had previously been a problem in pilot systems. The size of the paper in the centre well was such that 100µl of sodium hydroxide was absorbed and would not splash out of the well. Agitation sufficient to keep the cells suspended, and cell numbers were kept constant to enable the comparison of day to day results. The Clinbritic rubbers were found to self heal sufficiently well to allow reuse several times before discarding. Vials and centre wells were soaked in a solution of Pyroneg (Diversy) and rinsed with deionized water prior to use.

4.4.2 Collection of carbon dioxide

In order to assess the efficiency of the collection of carbon dioxide, vials were set up for a yeast assay but containing 1.0ml of water instead of yeast suspension and 100µmoles and 200µmoles of ^{14}C-sodium bicarbonate in place of glucose, and the assay proceeded with as described in Section 4.3.

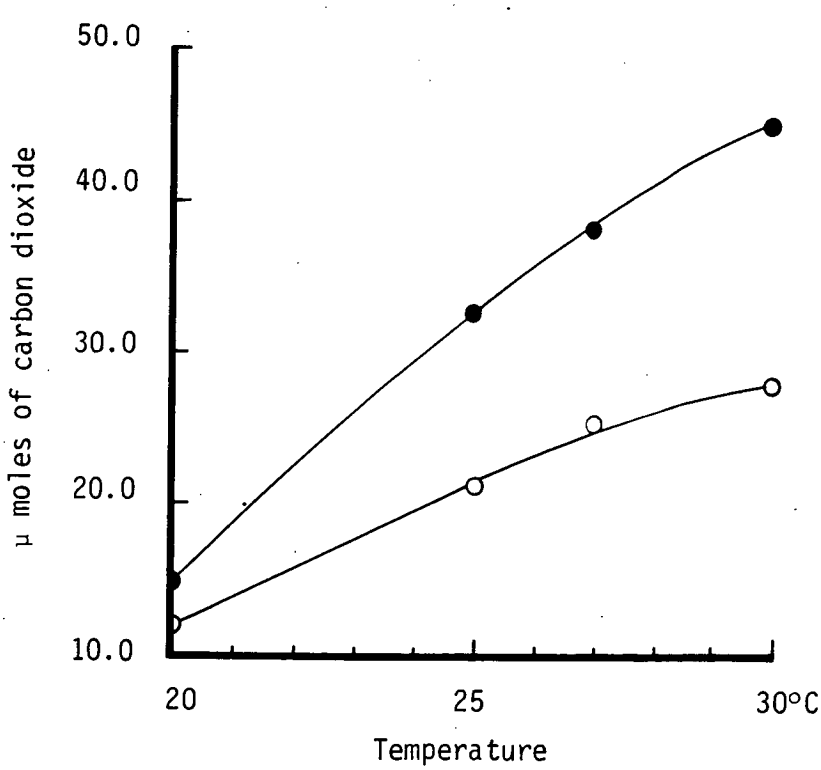
µmoles HCO_3^-	cpm added	mean cpm recovered	No. of vials
100	138 596 + 0.3%	138 034 + 0.2%	4
200	277 100 + 0.2%	276 712 + 0.2%	4

TABLE 4.8 Recovery of ^{14}C-carbon dioxide generated during the yeast assay.

Sodium hydroxide was found to be most suitable for absorbing carbon dioxide. Hyamine absorber (Sigma) containing methanol had been used in early experiments, however, the vapour was adversely affecting the yeast cells. The results indicate no losses of carbon dioxide (Table 4.8).

4.4.3 Temperature

Results at different temperatures for the control vials and vials containing the active fraction Y10 Seph. 7-23 (Chapter 5) were compiled and show that the greatest difference and hence greatest sensitivity was at 30°C (Figure 4.2). As condensation around the necks of the vials occurred at higher temperature, to avoid losses of ^{14}C-ethanol or contamination of the sodium hydroxide, temperatures higher than 30°C were not considered. 30°C was used routinely.



○ 2 x 10^7 cells + 40mM glucose.

● 2 x 10^7 cells + 40mM glucose + GTF

FIGURE 4.2 The effect of temperature on anaerobic fermentation.

4.4.4 Preincubation

Chromium deficient yeasts were depleted and then incubated with and without glucose for 0-60min to determine the effect of preincubation on fermentation. The results are presented in Table 4.9.

As the best response to the active fraction with preincubation was without glucose, and for 60min, the preincubation was used routinely.

Time	Preincubation without glucose		Preincubation with 8µmoles glucose	
(min)	µmoles ethanol	% increase	µmoles ethanol	% increase
Control	4.46	-	3.53	-
0	5.42	21.5	7.16	103
30	8.66	48.5	7.59	115
60	14.00	213.9	9.38	166

TABLE 4.9 The effect of preincubation on fermentation at 25°C , 10µmoles U-^{14}C-glucose added to vials at the end of preincubation.

4.4.5 Decline in yeast assay response and trace metal addition

The glucose concentration had initially been 40mM for the yeast assay, hence the following results were not obtained using 100mM glucose as outlined in Section 4.3. A gradual decline in response to control GTF containing fractions over a period of more than a month was found from accumulated results (Figure 4.3). The loss of response did not correlate with the basal fermentation rate of control cells. As it had been reported that active fractions lose activity on storage (Chapter 1.8.3), the decline in response was attributed to loss of sample activity. The amount of GTF control (Sephacel 7-23) routinely used was

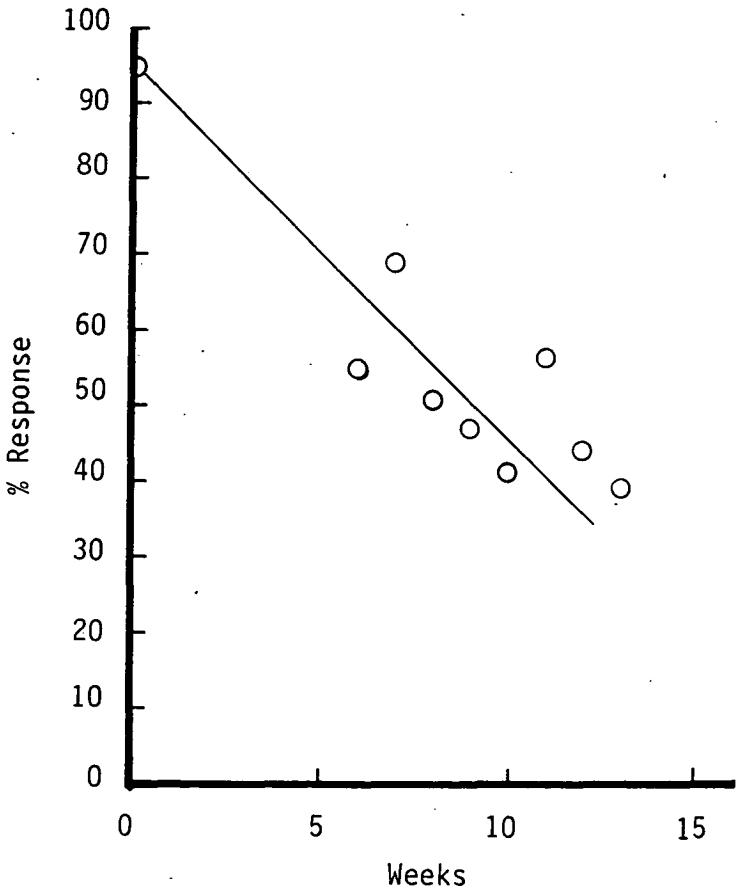


FIGURE 4.3 The decline in the yeast assay response (40mM glucose, 25°C) to the GTF control fraction with time.

considerably more than required to produce a maximum effect (Figure 4.4). It seemed that if the fraction was deteriorating, the concentration of GTF was being diminished and thus the saturation effect would not be observed. A 1 in 2 diultion was found to produce the same effect as the concentrate when the overall response of the yeast assay was reduced to 50% of the original after eight weeks, implying that the activity of the fraction had not diminished. It was thus thought that the yeast may have become insensitive to GTF due to a deficiency in the medium, and that the fractions were not deteriorating.

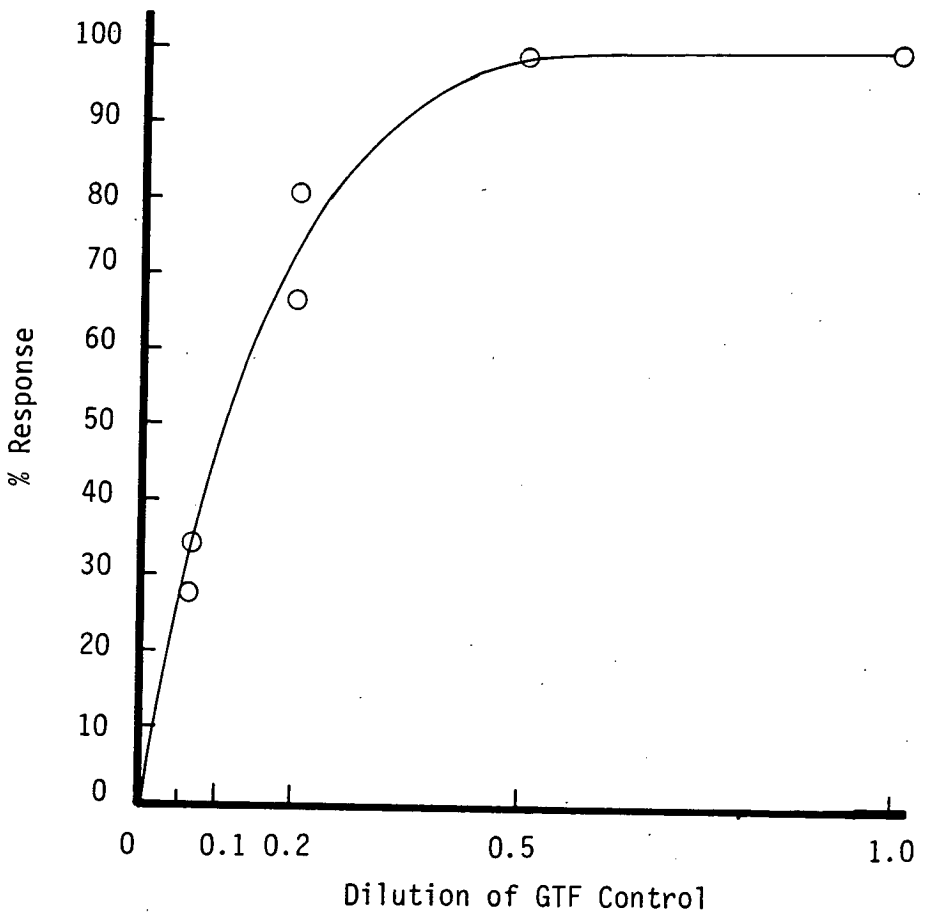


FIGURE 4.4 The dose response of the yeast assay to the GTF control fraction. 1.0 = 10µl of fraction.

As zinc is known to be an essential cofactor of many enzymes involved in yeast metabolism (e.g. pyruvate carboxylase, alcohol dehydrogenase) and, as it was absent from the synthetic medium, a trace

metal mix was prepared and added to the synthetic medium (Table 4.6). Yeast from a slope rather than serial subcultured yeast, were used to innoculate the trace metal replete medium. An increase of 20% in response to the control GTF fraction resulted (Table 4.10).

Temp. (°C)	Trace metal addition	Glucose (mM)	% Response	Duration of assay	CO_2 control vials (µmoles)
30	-	40	61.2	2hr	30.9
30	added	40	79.7	2hr	33.2
25	"	40	64.5	2hr	33.8
25	"	100	98.0	2hr	56.5

TABLE 4.10 Enhancement of carbon dioxide production by GTF with variations in glucose concentration and trace metal addition.

Although it was not conclusive that the trace metal mix was responsible for the increased response, and as no loss of sensitivity had occurred, the use of the trace metal mix was continued, providing the yeast with a more comprehensive medium. A beneficial result of trace metal addition was an increase in the total yield of cells per culture.

4.4.6 Glucose concentration

A glucose concentration of 40mM had been selected after pilot studies (with 8mM) had indicated that no glucose remained in the supernatant when an active sample was assayed. 40mM glucose was found to result in low supernatant glucose levels (4mM) in vials containing GTF after the response of the assay was increased by altering parameters which have been discussed.

The increase in glucose to 100mM resulted in an increased response (Table 4.10). When glucose levels remained high during the assay, no imbalances were found between the glucose used and carbon dioxide produced, and no indication was found that fermentable substrates that might be present in samples were interferring since, in the absence of U-^{14}C-glucose, the samples produced negligible $^{14}CO_2$.

4.4.7 Specificity of the assay for GTF

In order to establish that the fractions found to be active by the yeast assay did not contain an established compound that would cause a response, a range of compounds were tested for GTF activity (Table 4.11), including the vitamin mix used in the growth of large scale cultures. Of the compounds tested, ammonium chloride produced a small response. Ammonia produced a large response due to the increased pH of the cell suspension. It was thus apparent that care should be taken to ensure that all residual ammonia has been removed from ammonia eluted fractions concentrated by lypholization.

Compound	Concentration	% Response over control
Ammonia*	7.0mM	60
Glycine	5.8mM	nil
Thiamine hydrochloride	0.1mM	inhibitory
Ammonium chloride	1.0mM	18
poly-L-lysine	0.05mM	inhibitory
poly-DL-lysine	0.05mM	inhibitory
DL-Carnitine	0.06mM	nil
N-methyladenosine	0.2mM	slightly inhibitory
Vitamin mix	†10µl	nil
Nicotinic acid	0.1mM	nil
Chromium	60mg/ml	nil

* increase in pH of cell suspension to 6.75.

† The composition of the vitamin mix is presented in Tabel 4.7 (C).

TABLE 4.11 The response of the yeast assay to various compounds that may have been present in fractions tested.

4.4.8 *Sacch. ellipsoideus* and yeast assay response

The variety of yeast donated by Dr L. Blackwell was compared with the named strain described in Section 4.2. To avoid confusion, the variety will be referred to as *Sacch. ellipsoideus*.

Coulter counting The sizing display on the Coulter Counter (Coulter Electronics) showed that *Sacch. ellipsoideus* had a narrow size distribution compared to the yeast used for the yeast assay.

Microscope examination The cells were seen to be spheroidal in contrast to the *Sacch. uvarum* which were generally irregular and elliptical in shape with a broader size distribution.

Mean diameter of *Sacch. ellipsoideus* = 5.0×10^{-3}mm $\pm 0.8 \times 10^{-3}$, n = 40

Mean diameter of *Sacch. uvarum* = 6.7×10^{-3}mm $\pm 1.1 \times 10^{-3}$, n = 30

	Yeast	Control (µmoles CO_2)	GTF Control (µmoles CO_2)	% Response
Week 1	*Sacch. uvarum*	45.7	70.2	53.6
	Sacch. ellipsoideus	14.0	24.4	74.3
Week 2	*Sacch. uvarum*	57.9	82.9	43.2
	Sacch. ellipsoideus	19.7	32.8	66.5

TABLE 4.12 The fermentation of the two varieties of yeast in consecutive assays. Both cultures were trace metal replete and chromium free.

The greater response of the different strain to the control GTF fraction (Table 4.12) may be due to the fact that the cells had not been serial subcultured to the same extent as the cells with which they were compared. Bearing this in mind, it would seem that the *Sacch. ellipsoideus* responds to the same extent as the yeast usually

used in the assay. The most apparent difference was the lower rate of fermentation with basal fermentation being one third of the standard yeast fermentation. A contributing factor to low fermentation rates may have been the tendancy of the strain to clump together during the yeast assay. Tight clumping may have reduced the availability of glucose to the cell.

4.5 DISCUSSION

Yeast assay systems for GTF previously described (Section 4.1.3) relied on manometric techniques to measure the response of yeast to GTF. In such systems, appreciable quantities of carbon dioxide would remain dissolved in the medium. The GTF assay reported in this thesis (Section 4.3) utilises the quantitative collection of ^{14}carbon dioxide produced as a result of the oxidation of U-^{14}C-glucose, a previously unpublished approach. The addition of acid to stop fermentation ensured the release of dissolved carbon dioxide from the buffer, which was then efficiently collected by absorption (Section 4.4.2). The response obtained to GTF after 2hrs fermentation is comparable to that obtained by Mirsky *et al* (1980) and Haylock *et al* (1982) over the same time interval.

The concentration of ethanol produced in the medium was also found to reflect GTF activity (Table 4.9) and hence the use of labelled glucose could, if required, be dispensed with by determining the ethanol concentration in the medium. As results obtained from the collection of ^{14}carbon dioxide alone were found to be adequate and reproducible, the oxidation of labelled glucose was routinely used without ethanol determination.

The stoichiometric increase in carbon dioxide and ethanol production from an exogenous labelled source of glucose proves that GTF effects the anaerobic metabolism of glucose by yeast. The previously reported manometric techniques measured endogenous as well as exogenous carbon dioxide. The use of variously labelled glucoses has been further investigated by Appleby and Holdsworth (to be published) in order to determine the site of action of GTF.

The most effective preincubation (Section 4.4.4) was found to be without glucose, contrary to the findings of Mirsky *et al* (1980) where they added a small amount of glucose to their anaerobic preincubation, which would, in any case, have all been consumed over the preincubation period.

In agreement with Mirsky *et al* (1980), no response was found to vitamins that may be extracted from yeast. In agreement with Haylock *et al* (1982), Mirsky *et al* (1980) and contrary to the finding of Burkeholder and Mertz (1966), no response was found to low levels of chromium, even though the cells were grown in deficient medium (2ppb Cr, Chapter 2).

The apparent lack of difference in the % enhancement of carbon dioxide produced in response to GTF by different yeasts was contrary to the differences reported by Mirsky *et al* (1980), (Section 4.4.8). The lack of difference may reflect some difficulties with yeast taxonomy, i.e. *Saccb. uvarum* routinely used in this thesis, may not have been identical to the *Saccb. carlsbergensis* reported. The differences in carbon dioxide production of control cells would seem to support this but differences in % enhancement due to GTF may have been associated with the manometric technique, as vastly different quantities of carbon dioxide were produced.

4.6 CONCLUSION

A rapid and reproducible assay for GTF activity has been developed based on the oxidation of labelled glucose by yeast cells under defined conditions.

Conditions are described for the growth of yeast labelled with ^{51}Cr and ^{14}C-nicotinic acid, and the processing of this yeast is described in Chapter 5.

CHAPTER FIVE

THE ISOLATION OF GTF

5.1 INTRODUCTION

A review of the extraction of GTF has been discussed in Chapter 1.8.3. The extraction of GTF for the purpose of this thesis was initially based on the extraction of ^{51}chromium from whole brewers' yeast. Based on this information, the assumption was made that extracted chromium represented extractable GTF. The extraction technique of Anderson *et al* (1978) was used as they had demonstrated that 70-87% of ^{51}chromium in brewers' yeast could be extracted at high pH with correspondingly high "relative bio-activity".

A cationic chromium co-ordination complex was sought that would stimulate anaerobic glucose oxidation by yeast cells (Chapter 4.3) and would potentiate the effect of insulin on glucose oxidation by adipocytes in the assay developed by Anderson (1978). When possible, samples were assayed for biological activity in the adipocyte assay by Ms J. Sherriff (MSc thesis, 1983). The adipocytes were isolated from the fat pads of Hooded Wistar rats that had been raised on Teklad torula yeast, "low chromium diet" (Chapter 2.4.2). The adipocytes were prepared, essentially by the method of Robdell (1964), using modifications described by Ms Sherriff. The effect of samples on glucose oxidation by adipocytes to carbon dioxide was measured in the presence of sub-optimal levels of insulin. The basal level activity of samples was determined in the assay without exogenous insulin to establish that the effect produced was not an insulin mimicing effect such as produced by oxidized glutathione, kinetin riboside or hydrogen peroxide generated

by divalent ions such as Cu^{2+} (May and Contoreggi 1982).

It should be stressed that the availability of the adipocyte assay for testing yeast extract fractions was dependent on the availability of 150-180g rats raised on Teklad diet. Delays in obtaining the diet resulted in many weeks interruption in the husbandry of the animals and thus lack of data for some fractions. Crude fractions were found to contain inhibitory material thus obscuring the response of the adipocytes. For this reason, testing by the adipocyte assay was reserved for samples with a higher degree of purity.

The question of the amount of sample required for activity in both assay systems became difficult as the importance of chromium in yeast fractions diminished. Initially, sample volumes resulting in ~60ng/ml Cr in the assay system was thought to be adequate however, decreasing chromium levels resulted in testing on a dry weight basis.

5.2 RESULTS

5.2.1 Extraction of ^{51}Cr

The extraction of yeast using dilute ammonia generally resulted in the extraction of 70% of incorporated ^{51}chromium, slightly less than reported by Anderson *et al* (1978). The accumulated results of pilot studies of extraction of ^{51}Cr (Table 5.1) were typical of all ten cultures grown. Butanol was added to the extraction to increase the proportion of ruptured cells.

Wet weight of cells (g)	Medium Cr (ppm)	Total Cr in yeast	Extraction (0.1N NH_3)	% Cr in 1st extraction	Total % Cr extracted
108	1.0	0.28	3 x 200ml	35	55*
220	1.5	1.47	2 x 500ml	60	71
105	4.0	4.31	3 x 200ml	49	70

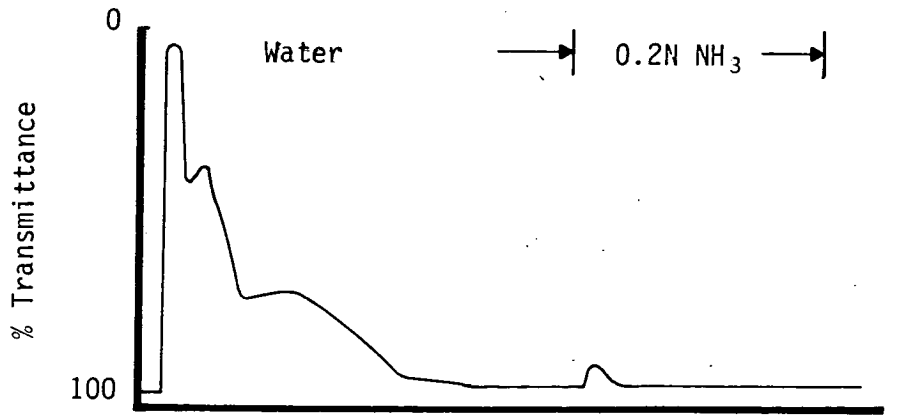
* A fourth extraction with 200ml NH_3 and 50ml n-butanol yielded a further 3% of extracted chromium with 90% of cells being disrupted.

TABLE 5.1 The extraction efficiency of ^{51}chromium from yeast cells grown in liquid yeast medium.

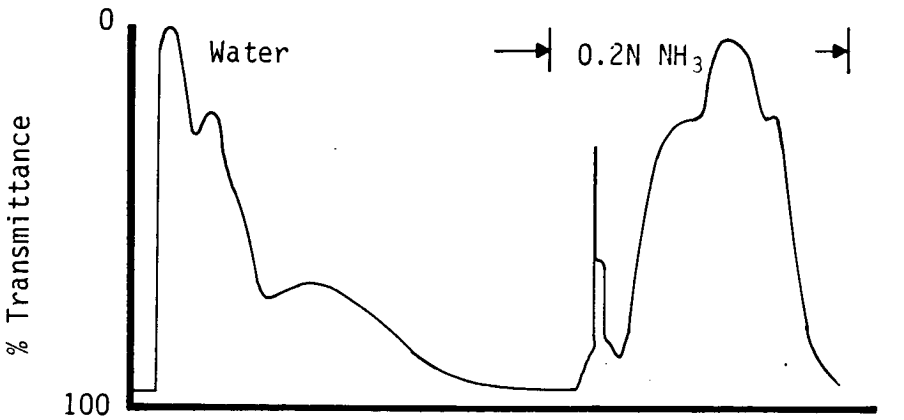
5.2.2 The extraction of yeast grown with and without chromium

Two 5ℓ cultures of *Sacch. cerevisiae* were grown in synthetic medium (Table 4.7) under identical conditions except that 2.0ppm Cr was added to one flask, and were inoculated with 4 x 10^8 cells from the same subculture. The growth rate of the two cultures was vastly different. The chromium deficient culture grew vigorously and was harvested after 5 days growth to yield a 27.0g wet pellet of yeast. The slow growing chromium replete culture was harvested after 8 days growth yielding 18.5g of wet cells. Counting of the cells using a Coulter counter (Coulter Electronics) was inaccurate due to branching and clumping of the chromium replete cells.

The two pellets were extracted twice with 0.1N ammonium hydroxide (8ml NH_3/g wet cells), centrifuged (7 700xg, Sorval RC2-B), and the supernatants lypholised. The dry solids were resuspended in 5.0ml and loaded onto Dowex 50W-X8 (H^+) resin and eluted as shown in Figure 5.2. The water eluted and ammonia eluted materials were lypholised and resuspended so that the concentration was 560µg/10µℓ. The biological activity was determined by yeast assay (Table 5.2).



(i) The elution of ammonia extract of chromium deficient yeast from Dowex 50W-X8, 15mm x 250mm.



(ii) The elution of ammonia extract of chromium replete (1.80pm) from Dowex 50W-X8, 15mm x 250mm.

FIGURE 5.1 The extraction of 5ℓ synthetic yeast cultures grown with and without chromium.

	Dowex 50W-X8 Eluant	Total wt. (mg)	Biological Activity Yeast Assay (%) (560µg)
Cr+	Water	51.0	53.8
	Ammonia	20.9	18.6
Cr-	Water	44.0	89.0
	Ammonia	1.0	44.7

TABLE 5.2 The yield and biological activity of material eluted from Dowex in Figure 5.1.

5.2.3 The extraction of a 10ℓ yeast culture grown in liquid yeast medium

The growth of yeast in liquid yeast medium has been described in Chapter 4.2 and the growth rate of the culture is depicted in Figure 5.2. The yield of wet cells was 108g.

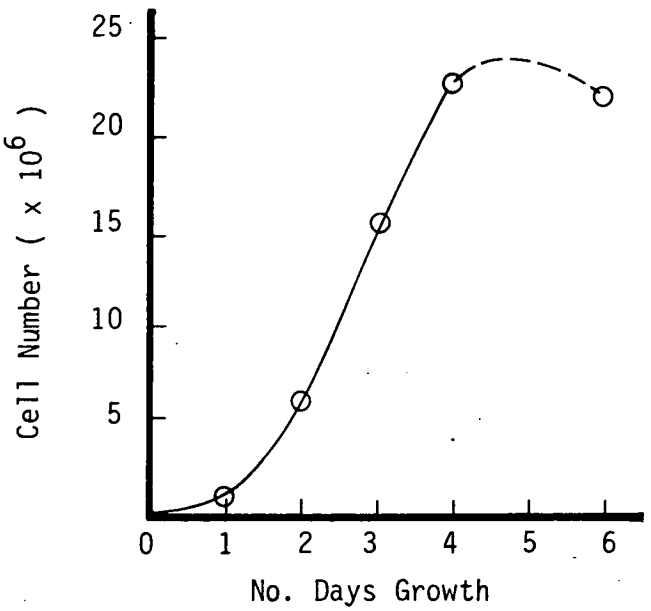


FIGURE 5.2 The growth of a 10ℓ culture of brewers' yeast in liquid yeast medium.

The cells were extracted with 2 x 200ml of 0.1N ammonia (AR, Univar) and the extract centrifuged at 7 700xg (Sorval RC2-B). The extract was lypholized and resuspended in 475ml of distilled water prior to ultrafiltration at 4°C under nitrogen (100mm membrane, 10 000 MW cut off, Amicon). The ultrafiltrate was concentrated to 20ml and the pH adjusted to 3.9 before loading onto a Dowex 50W-X8 (H^+) column (25mm x 400mm). The active material (Table 5.3.1) was eluted with 0.2N ammonia, lypholized and resuspended in 6.0ml of distilled water before loading onto a Bio Gel P6 column (Bio Rad) with dimensions 25mm x 400mm.

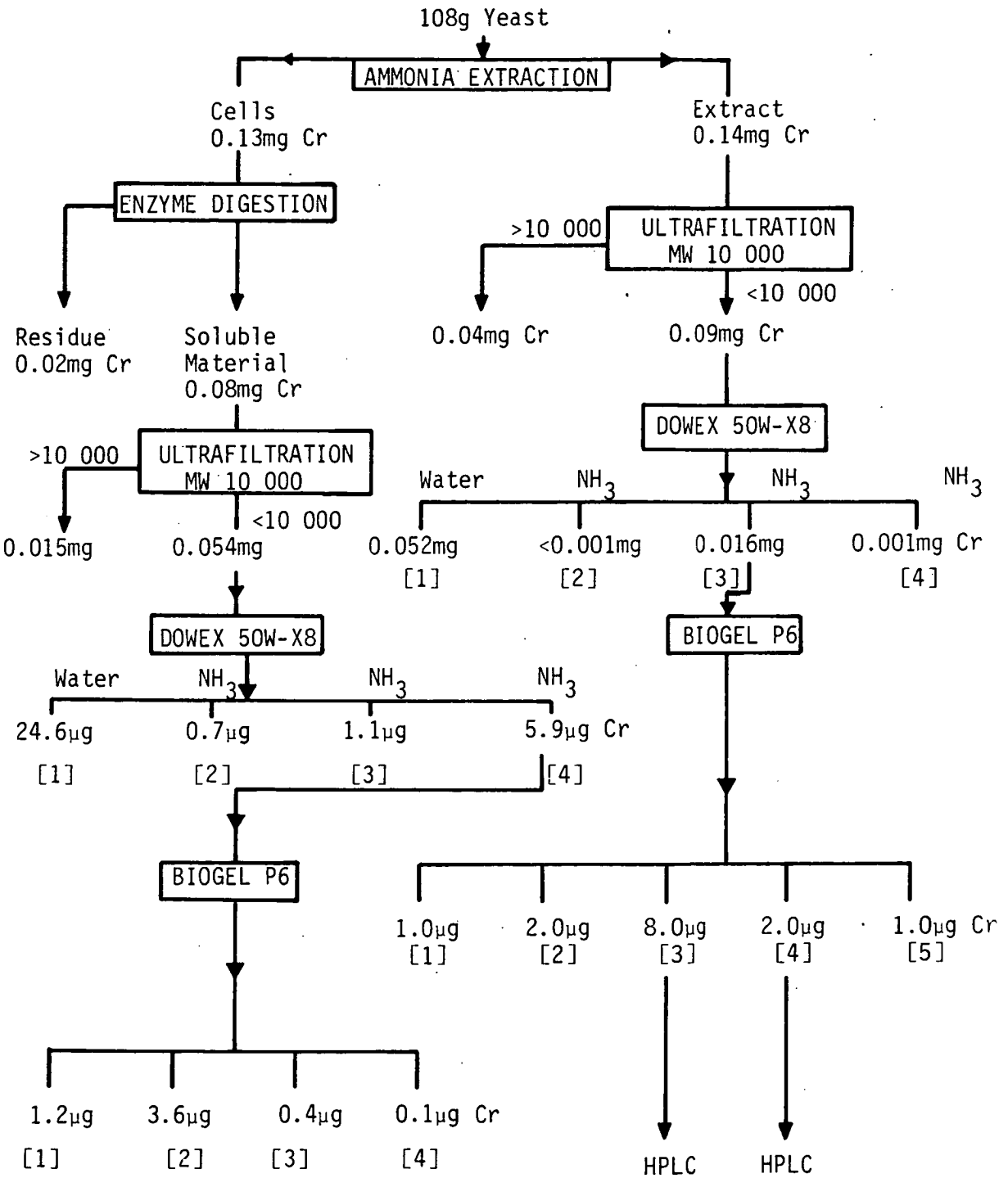
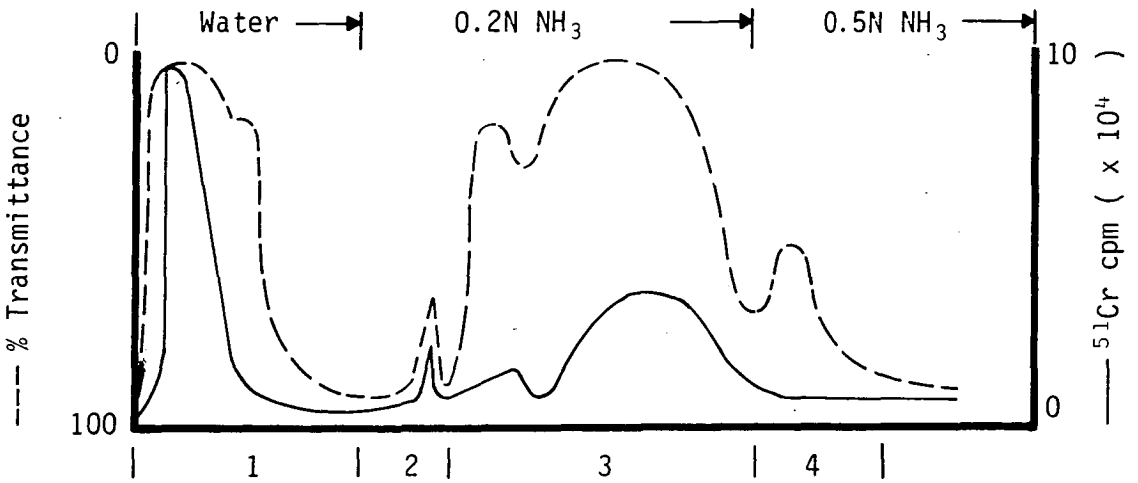
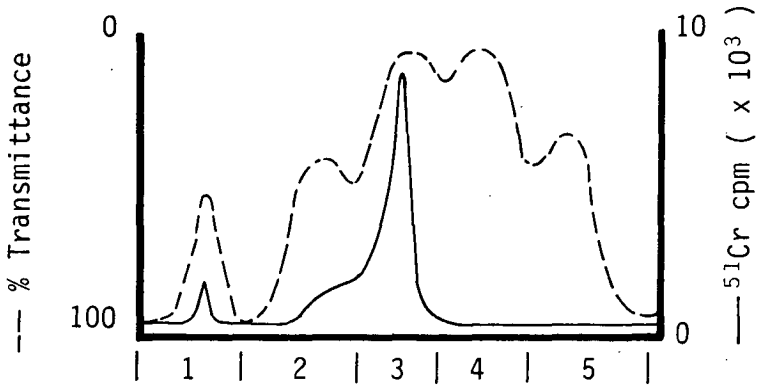


FIGURE 5.3 The distribution of ^{51}chromium after fractionation of yeast culture extract. The 10% culture was grown in liquid yeast medium (Table 4.2.2) with 1.0ppm Cr.



(i) Dowex 50W-X8, 25mm x 420mm. Elution profile of NH_3 extract ultrafiltrate from Figure 5.3.



(ii) Bio Gel P6, 25mm x 420mm, eluted with 50mM NH_3. Elution profile of active Dowex fraction [3] from above.

FIGURE 5.3.1 The elution profiles of active material extracted from 108g of yeast. See flow chart Figure 5.3 and Table 5.1.

The residue remaining after extraction of the yeast was suspended in 160ml of distilled water and digested at 30°C over two days by 250mg of "Zymase" and 50mg of DNA-ase at pH 7.4, releasing 60% of the chromium in the residue. The digestion was ultrafiltered as before and the ultrafiltrate treated in the same manner as the ammonia extract.

Fraction (Figure 5.1)		ng Cr in assay	Biological Activity	
			Yeast	Adipocyte
NH_3 Extract, Dowex	1	65	18%	-
" "	2	<1	nil	-
" "	3	47	76%	- *
" "	4	<1	nil	-
" Bio Gel P6	1	56	25%	-
" "	2	71	24%	-
" "	3	44	111%	nil
" "	4	45	52%	active
" "	5	45	33%	-
Enzyme Digest, Dowex	1	35	15%	-
" " "	4	51	24%	-
" " Bio Gel P6	1	70	4%	-
" " "	2	94	11%	-
" " "	3	88	19%	-
" " "	4	60	nil	-

* not tested until further fractionated on Bio Gel P6.

TABLE 5.3.1 The biological activity of fractions from brewers' yeast shown in Figure 5.3 and 5.3.1.

5.2.4 200ℓ yeast culture - synthetic medium

Details of the growth and harvesting of the 200ℓ culture are to be found in Chapter 4.2. To establish the presence or otherwise of active material in spent culture medium, a charcoal extract of 20ℓ of medium was purified to the stage where fractions could be assayed by the adipocyte assay.

5.2.4.1 Spent medium

20ℓ of spent medium were stirred overnight with 500g of coarse activated charcoal (Norit) after adjusting the pH to less than 3.0 with concentrated hydrochloric acid. The charcoal was filtered and washed with distilled water in a buchner funnel. The charcoal was twice extracted with 500ml of 0.2N NH_3/50% ethanol. The extract was reduced *in vacuo* at 40°C with a Rotavapour (Büchi) and then taken to dryness by lypholization (-70°C, 0.05 torr). The solids were resuspended in 5ml of distilled water and the pH adjusted to 3.9 with 0.1N HCl before loading onto a Dowex 50W-X8 (H^+) column (25mm x 400mm) (See Figure 5.4 and Table 5.4).

5.2.4.2 Extraction of the yeast cells

The yeast pellet was extracted twice by shaking with 5.0ℓ of 0.2N NH_3/14% butanol for 16hr. The cell residue was spun down in a MSE Mistral 6L centrifuge (2 000xg) and the supernatant lypholized. The solids were redissolved with distilled water and the insoluble residue removed by centrifugation at 10 000xg (Beckman L2-65). The supernatant was sorbed batchwise onto 1 000g of Dowex 50W-X8 (H^+).

Batchwise extraction was necessary as white solids precipitated from the supernatant on the lowering of pH to 3.9 by the resin, and would have blocked the flow if packed in a column. The resin was washed thoroughly with 5 equivalent volumes of distilled water before revising the pH of the resin suspension with 1.0N ammonia to displace the active material. The resin was washed again with two equivalent volumes of distilled water, and the washings and ammonia eluate were concentrated by lypholization before resuspending in 140ml of distilled water. This material was loaded onto a DEAE Sephadex (Cl^-) (50mm x 400mm) and water-eluted material passed directly to a Dowex 50W-X8 (H^+) column connected in series (25mm x 400mm). The Dowex column was disconnected and then eluted with 0.2N ammonia. The eluant was lypholized and redissolved in 175ml of distilled water (fraction C1, Figure 5.2). The remaining details of fractionation are presented in Figure 5.4, 5.5.1 - 5.5.3).

5.2.4.3 Amphoteric material

A brown material had been retained by the anion exchange gel (DEAE Sephadex) which was eluted with 1.0N hydrochloric acid and repeatedly lypholized to reduce the acid concentration. The final volume of the material (Figure 5.4 [A]) was 22.0ml, a 2ml portion of which was fractionated further (Figure 5.6) by both DEAE Sephacel and Dowex 50W-X8 followed by preparative chromatography. The biological activities of some of the fractions is shown in Table 5.6.1.

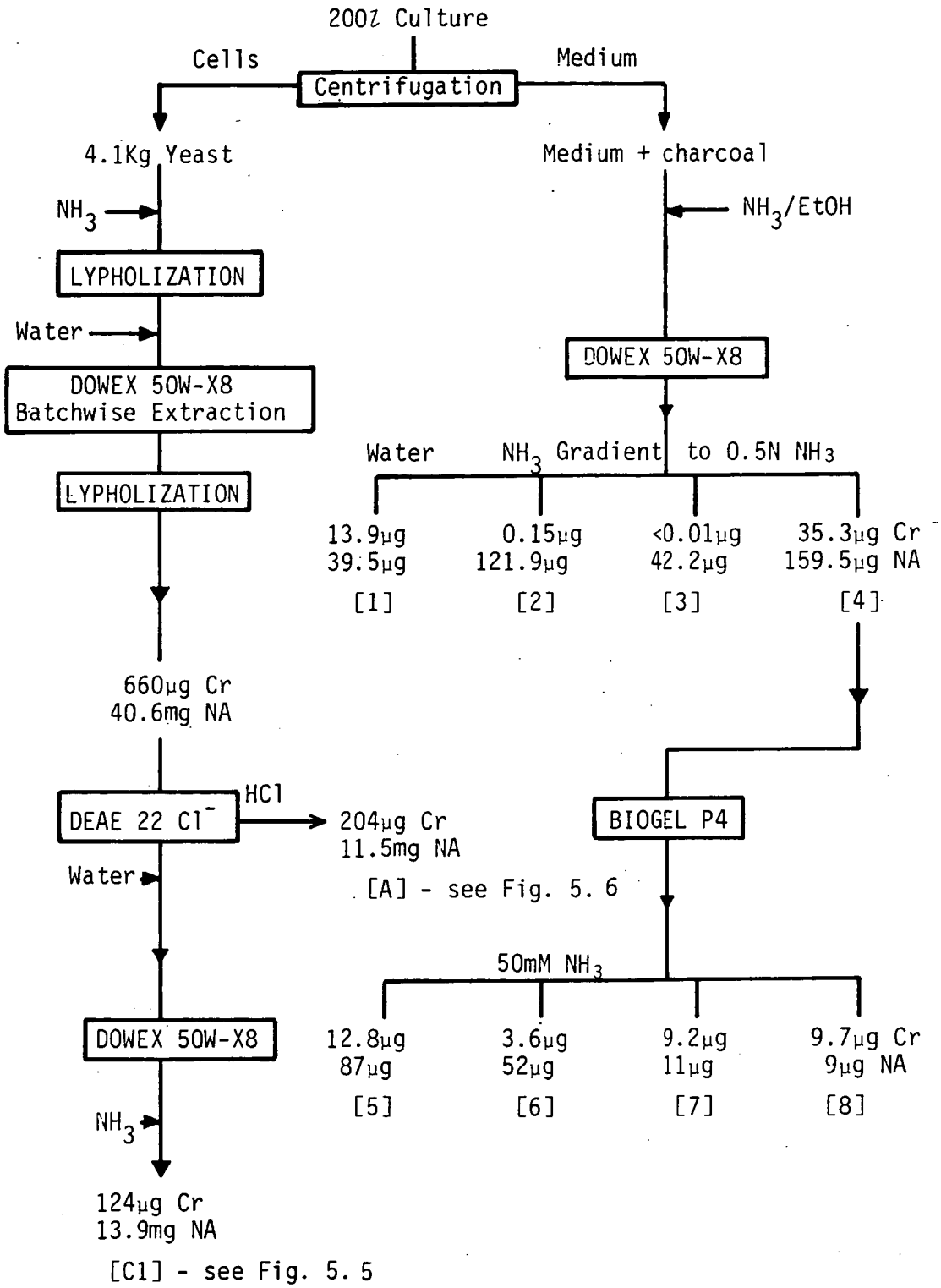
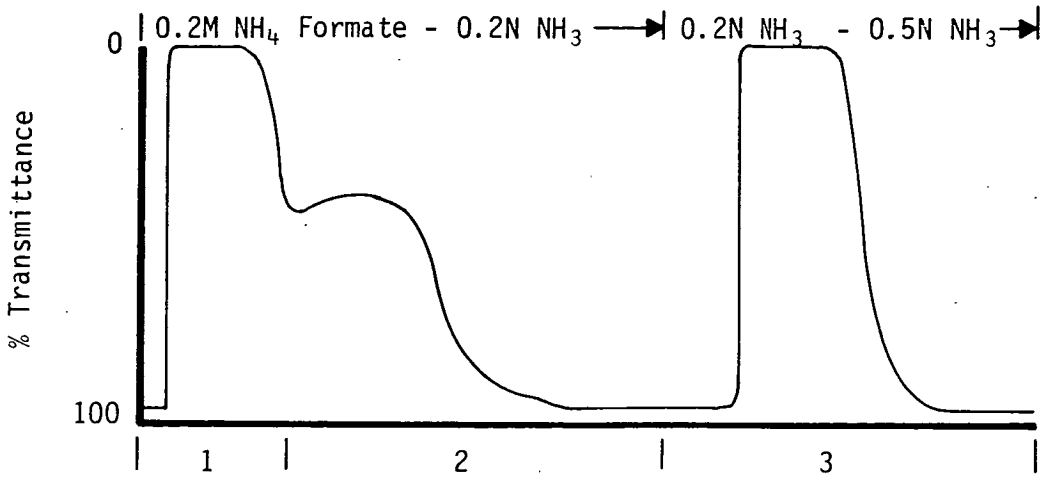


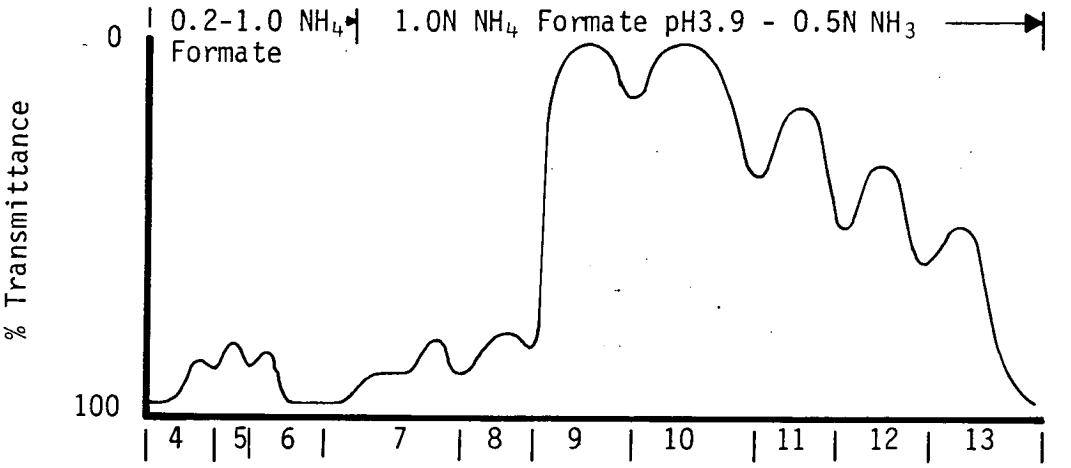
FIGURE 5.4 The initial fractionation of a 200ℓ (1.0ppm Cr) culture grown in synthetic medium and the fractionation of GTF extracted from 20ℓ of spent culture medium by absorption onto activated charcoal (Section 5.2.3.2). Biogel P4 = 25mm x 900mm.

Fraction (Figure 5.4)		Levels of Cr, NA in assay		Biological Activity	
		ng Cr	ng NA	% Yeast Assay	Adipocyte Assay
Dowex	1	46	132	8	inhibitory
	2	0.5	420	7	nil
	3	0.5	222	2	nil
	4	147	663	69	slight
Bio Gel P4	5	44	300	2	active
	6	24	347	13	active
	7	61	75	16	nil
	8	61	58	12	active

TABLE 5.4 The biological activity of fractions derived from 20ℓ of spent synthetic medium. The fractionation is outlined in Figure 5.4.



(i) The initial fractionation with Dowex 50W-X2 50mm x 400mm equilibrated in 0.2N ammonium formate pH 3.9 of fraction C1 (Figure 5.4).



(ii) The active fraction (yeast assay) [3], from (i) above, eluted from Dowex 50W-X2/Formate.

FIGURE 5.5.1 Elution profiles of cationic material from Dowex X2 formate (Figure 5.5).

Fraction		Biological Activity	
		(%) Yeast Assay (400µg)	Adipocyte Assay (80µg)
Dowex X2 Formate	7	54	nil
"	8	51	nil
	9	38	slightly active
	10	33	active
	11	12	active
	12	0	most active
	13	28	active

TABLE 5.5.1 Biological activity of the major fractions from Figure 5.5.

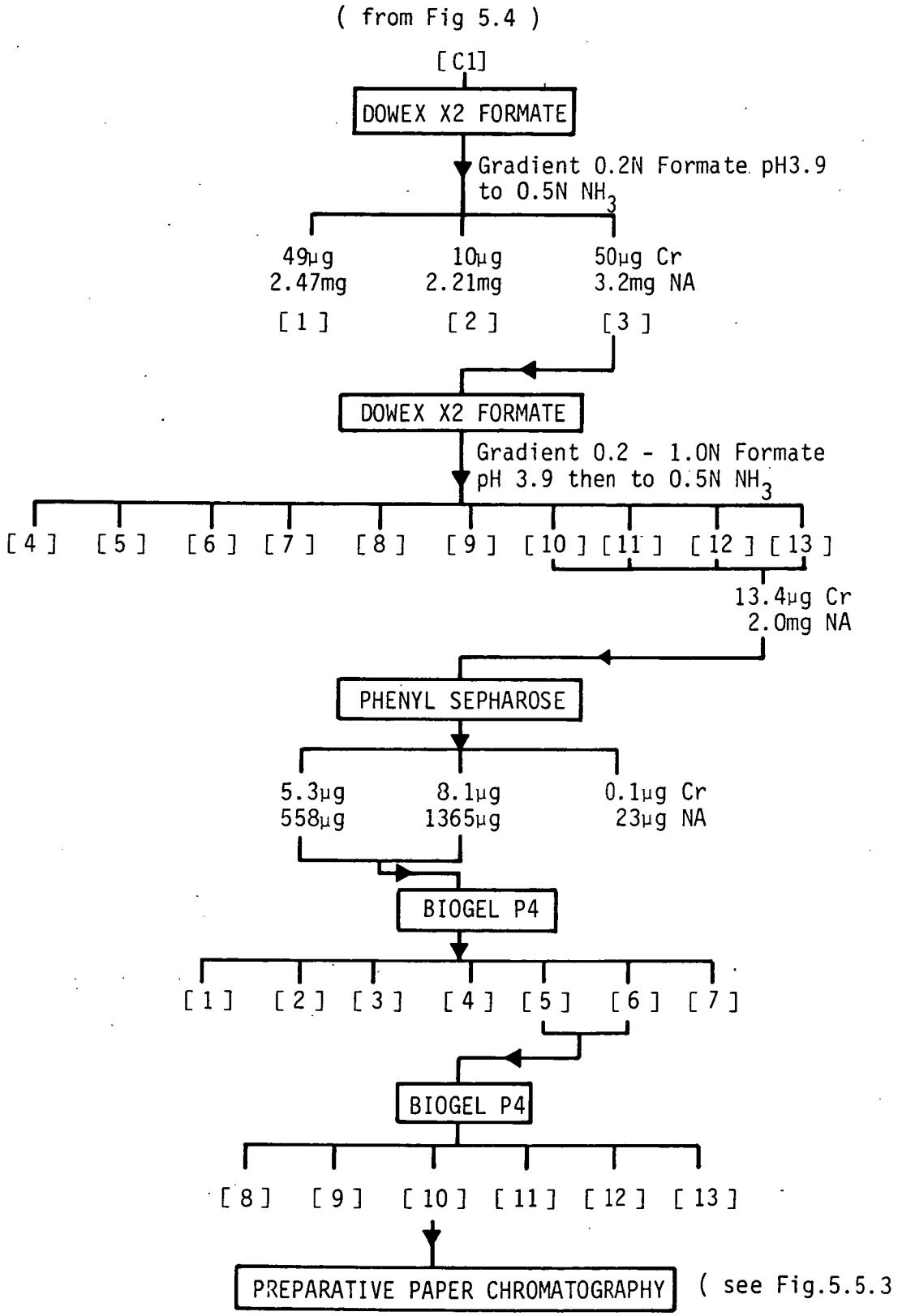
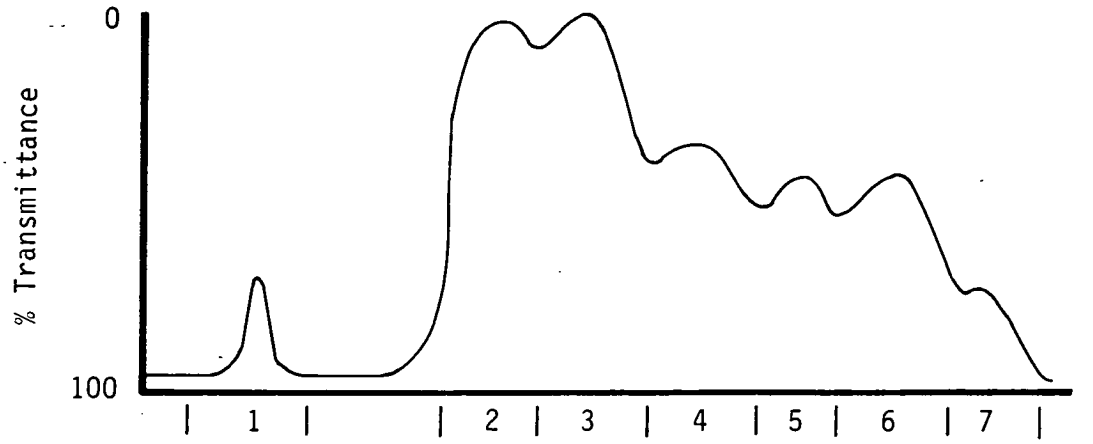
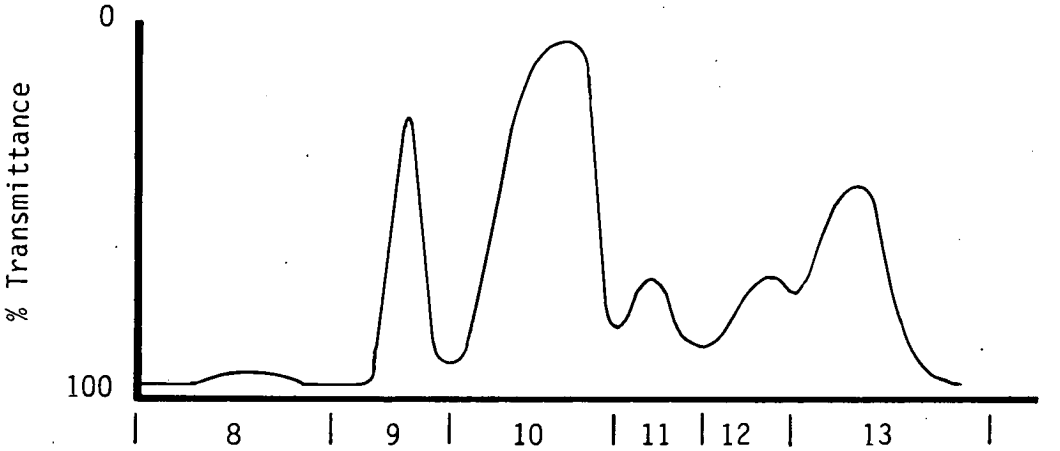


FIGURE 5.5 Fractionation of cationic material [C1] extracted from 4.1Kg of yeast.



(i) Bio Gel P4, 25mm x 900mm, eluant 50mM NH_3. Initial gel fraction of fractions from phenyl sepharose (Figure 5.5).



(ii) Elution of active fractions 5, 6 from (i) above. Same conditions as (i). Fraction [10] was then fractionated by paper chromatography (Figure 5.5.3).

FIGURE 5.5.2 Elution profiles of cationic material from unbuffered Bio Gel P4 (See Figure 5.3).

Fraction	Biological Activity (%) Yeast Assay (400μg)	Fraction	Biological Activity (%) Yeast Assay (400μg)
1	inhibitory	8	22
2	16	9	13
3	13	10	71
4	20	11	91
5	64	12	22
6	111	13	13
7	42		

FIGURE 5.5.2 Biological activity of fractions from Figure 5.3.

PREPARATIVE PAPER CHROMATOGRAPHY

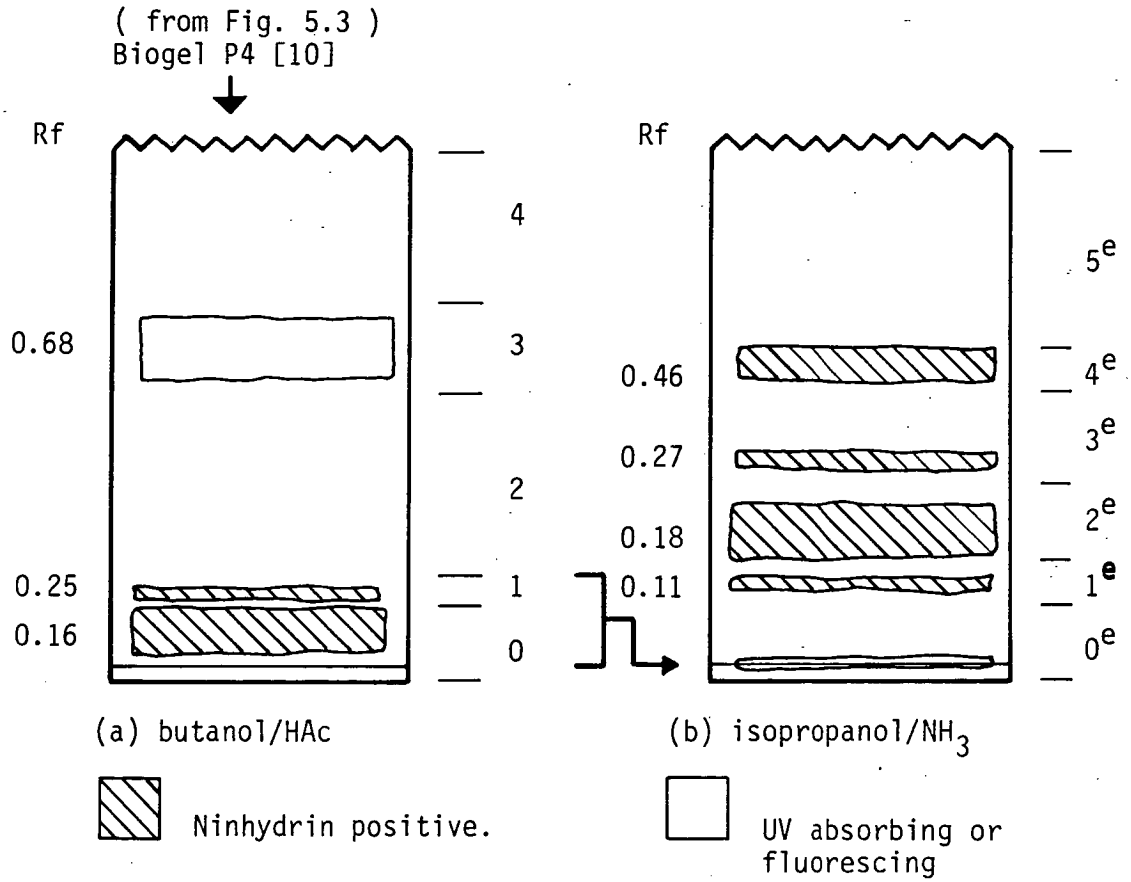


FIGURE 5.5.3 The final fractionation of cationic material by paper chromatography to yield the active fraction 2^e on which structural information was obtained. For paper chromatography details, see Chapter 3.

Fraction		Biological Activity	
		% Yeast Assay (200µg tested)	Adipocyte Assay (80µg)
butanol/HAc	0	199	-
	1	99	-
	2	60	-
	3	67	inhibitory
	4	63	-
isopropanol/NH_3	0^e	75	-
	1^e	81	slightly active
	2^e	53	active (max.)
	3^e	44	active
	4^e	23	inhibitory
	5^e	nil	basal activity

TABLE 5.5.3 Biological activity of cationic material from Figure 5.5.3 above.

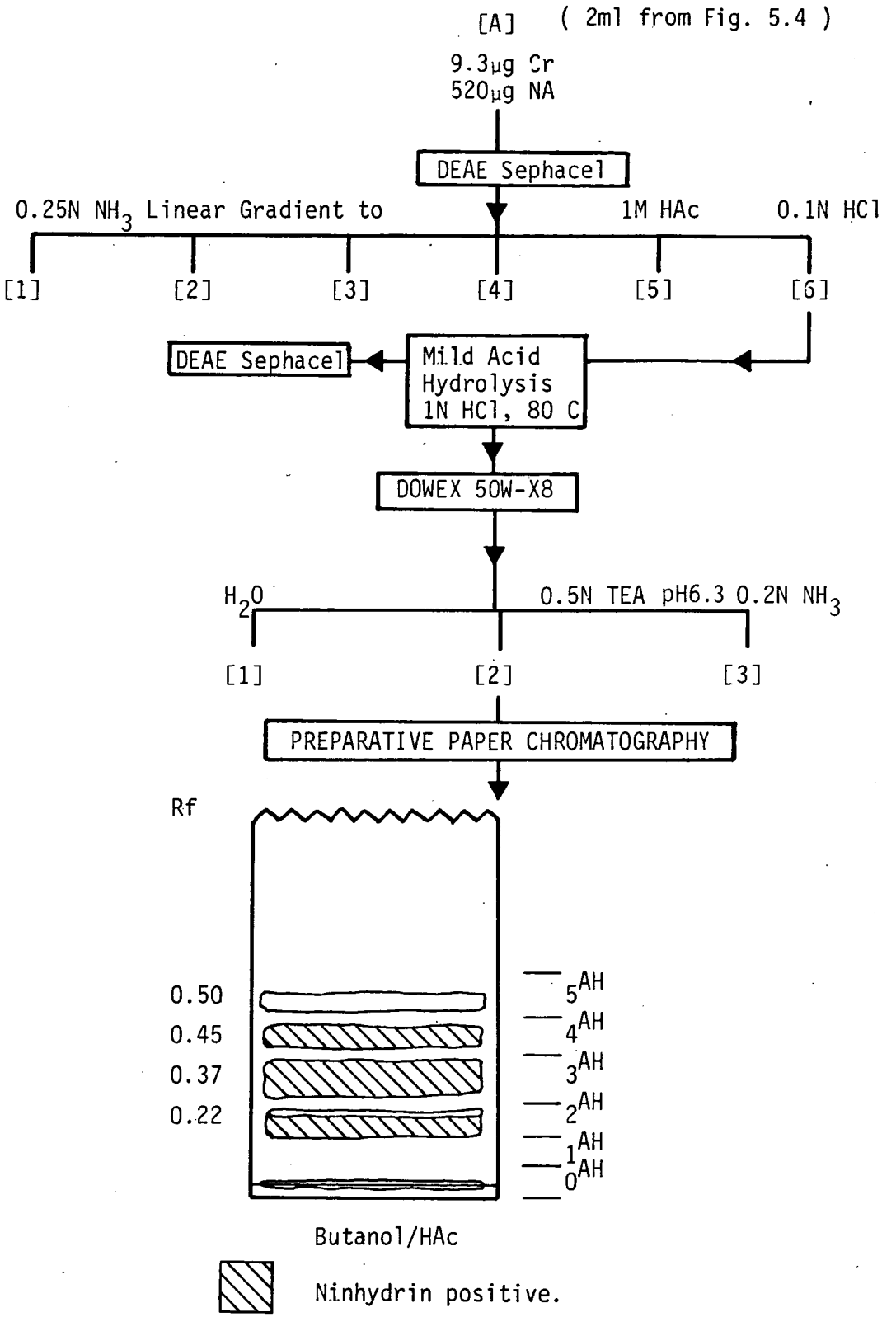
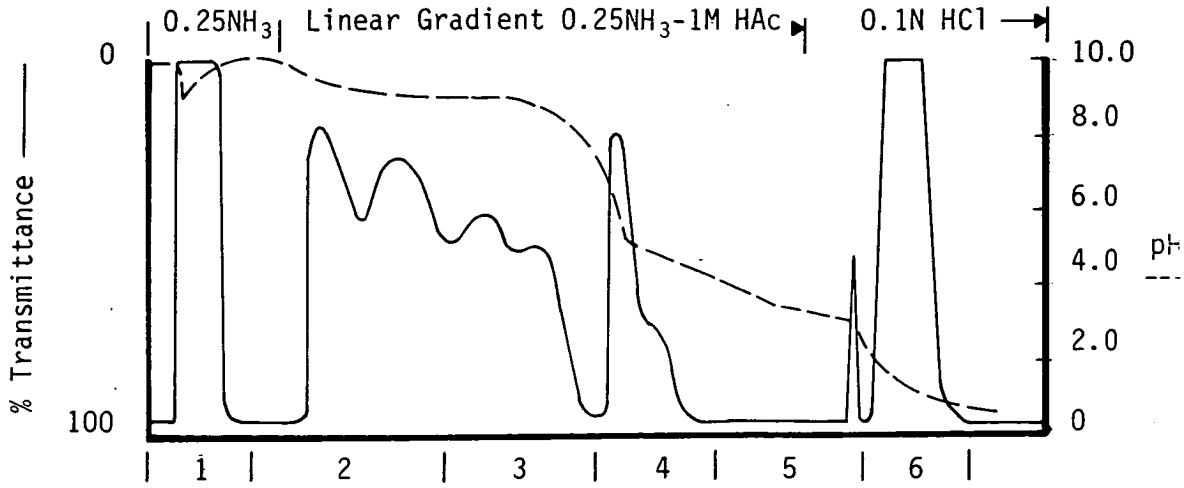
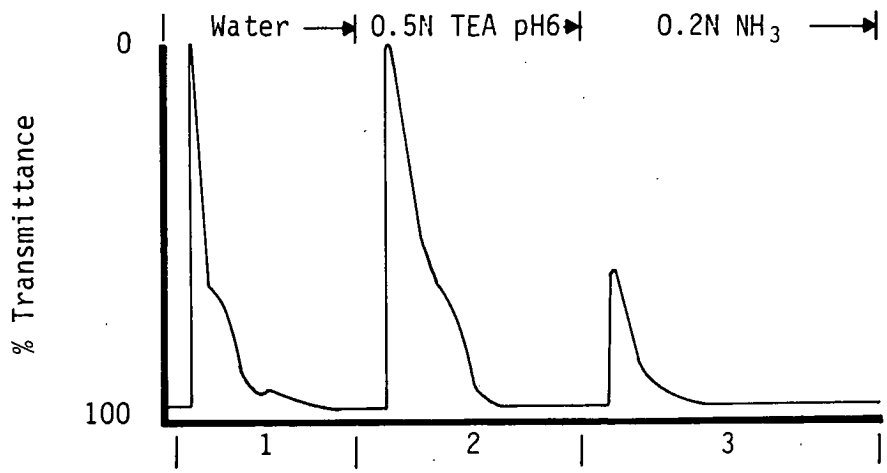


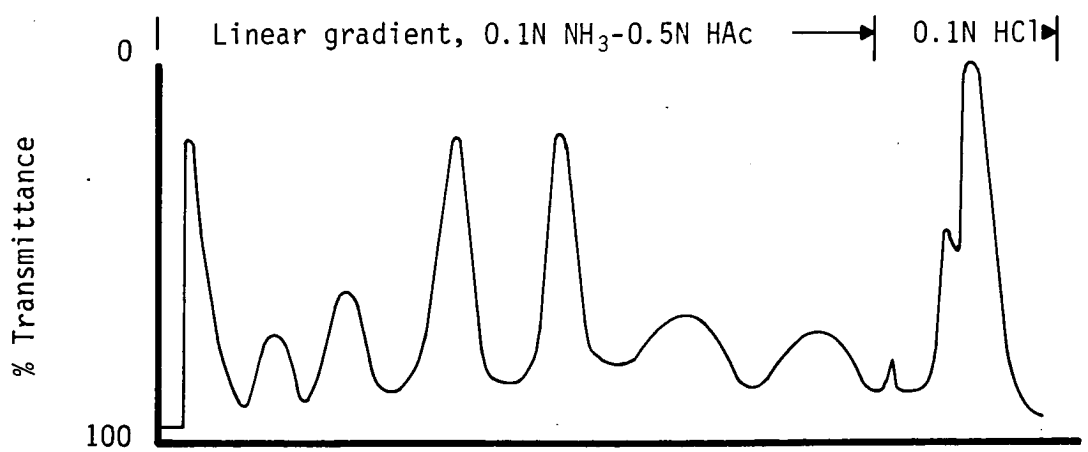
FIGURE 5.6 The fractionation of the amphoteric material [A] from brewers' yeast extract (Figure 5.4) to yield active fraction 3^{AH}. For paper chromatography details see Chapter 3.



(i) The elution of 2ml of [A](from Figure 5.4) from DEAE Sephacel, 15mm x 250mm.



(ii) The elution of [6] from (i) above, after mild acid hydrolysis, from Dowex 50W-X8, 15mm x 250mm.



(iii) The elution from DEAE Sephacel of [6] from (i) above, after mild acid hydrolysis, 15mm x 250mm.

FIGURE 5.6.1 Elution profiles of fractions shown in Figure 5.6.

Fraction (Figure 5.6)		ng Cr/ml in assay	Biological Activity	
			Yeast (%)	Adipocyte
DEAE Sephacel	1	100	106	nil
	2	100	97	-
	3	10	68	-
	4	10	72	-
	5	10	23	-
	6	320	80	-
Dowex 50W-X8	1	200	37	-
	2	180	111	nil
	3	160	56	-
Chromatogram	0^{AH}	10	46	inhibitory
	1^{AH}	10	19	-
	2^{AH}	10	76	-
	3^{AH}	5	92	active
	4^{AH}	10	60	-
	5^{AH}	10	38	-

FIGURE 5.6.1 The biological activity of fractions derived from amphoteric material as outlined by Figure 5.6. No entry for the adipocyte assay indicated that the assay was not available.

5.3 DISCUSSION

In order to determine the effect of chromium on the production of GTF, the chromium deplete and replete cultures described in Section 5.2.2 were grown. Synthetic medium was used in preference to liquid yeast medium due to the considerably lower chromium content, thus ensuring that the deplete culture was actually deficient. Pilot studies had previously shown satisfactory growth of yeast in synthetic medium. Cultures grown as pilot studies with 4.0ppm Cr in liquid yeast medium containing peptone and yeast extract were apparently not affected by the chromium present, yet the yield of cells from the chromium deplete and replete (2.0ppm Cr) cultures demonstrates a toxic effect. Kumpulainen *et al* (1978) report a slight inhibition of growth at 1.0ppm Cr, yet Burkeholder and Mertz (1966) report no effect up to 10ppm Cr.

The result of GTF extraction from the two completely synthetic cultures indicates yeast can synthesize a biologically active cationic material in the absence of chromium (Table 5.2). The material was not tested in the adipocyte assay as, from previous experience, a higher degree of purification would have been required before a response could be observed and the yield of 1mg, consumed by the yeast assay, was insufficient to allow further purification. As there was some suggestion that a higher total yield of GTF may result from the addition of chromium, 1.0ppm Cr was added to the cultures grown for GTF extraction. Furthermore, GTF reported to be active in the adipocyte assay, has historically been extracted from chromium containing material. As the use of the adipocyte assay in conjunction with the yeast assay as criteria for GTF has only been reported in one other instance (Burkeholder and Mertz 1967) and GTF is reported to be a chromium containing material, the addition of chromium to the medium was a precaution in the event that chromium may be needed to produce material that was active in

the adipocyte assay.

The enzyme digestion of cell residue (Figure 5.3) after ammonia extraction yielded very little material with biological activity (Table 5.3.1), thus the residue was discarded in later experiments. High molecular weight material (ultrafiltration retentate) was also discarded as GTF is reported to have a molecular weight of less than 10 000 daltons, the exclusion limit of the membrane used. Chromium containing material eluted from Dowex cation exchange resin with water had little biological activity in relation to ammonia eluted material, thus only the ammonia eluted material was further fractionated in all cases. The water eluted material may have been equivalent to the anionic material reported by Votava *et al* (1973) which was called GTF on the basis of absorption from the gastrointestinal tract of rats (Chapter 1.7). Kumpulainen *et al* (1978) also report anionic material extracted from yeast as do Mirsky *et al* (1980) who report no activity with material eluted from anion exchange cellulose.

The fractionation of the active ammonia eluate on Bio Gel P6 (Figure 5.3.1) resulted in fraction 3 (ii) containing chromium and fraction 4 with no significant chromium. Fraction 3 was found to be active by the yeast assay but not by the adipocyte assay in which fraction 4 was active, casting doubt on the importance of chromium and confusion as to which fraction represented GTF. As an insulin potentiating compound was sought, the adipocyte assay was decided on as the final criteron for GTF.

The yield of active material was low (less than 3mg) and still impure, hence a large culture was grown to provide more active material. As Mirsky *et al* (1980) and Haylock *et al* (1982) had extracted GTF from

yeast extract, and as yeast extract and bactopeptone were components of the liquid yeast medium, we decided to grow a 200ℓ culture using synthetic medium in order to establish that GTF extracted from the cells was produced by the cells and not derived from the medium. A 10ℓ pilot study indicated that growth in synthetic medium was feasible. The chromium concentration was 1.0ppm chromium to lessen growth inhibition observed with 2.0ppm Cr in the 5.0ℓ cultures. The use of synthetic medium also allowed investigation of the possibility that GTF may diffuse from the cells into the medium. Kumpulainen *et al* (1978) reported the formation of anionic chromium containing material in spent yeast medium but was unable to establish its biological activity (Chapter 1.8.3).

^{51}chromium was added to the large culture to facilitate analysis in the early stages of extraction and, as nicotinic acid was deemed to be an essential component of GTF, ^{14}C-nicotinic acid was added to the culture. The labelled nicotinic acid was taken up by the yeast and was presented in the extracted material but, when present in detectable levels in active fractions, it was seen on chromatograms as free nicotinic acid (Rf 0.75, butanol/acetic acid, Chapter 3) and was not found incorporated in a complex.

Amphoteric material arising from the yeast extract was tightly bound to anion exchange gel and could only be displaced by hydrochloric acid. It was found that active material was present after elution with hydrochloric acid and lypholization. Elution from DEAE Sephadex with acid followed by lypholization appears to have caused partial hydrolysis of the material to yield active material which would no longer sorb to anion exchange cellulose (fraction [1], Figure 5.6.1). Strongly sorbed active material was deliberately acid hydrolysed under mild conditions and was found to yield a range of fractions when reloaded onto

DEAE Sephacel and an active fraction which was sorbed by cation exchange resin. After further but incomplete purification on chromatography paper (Figure 5.6) by elution with butanol/acetic acid, a fraction was found (3^{AH}) that was active in both the adipocyte assay (with some basal activity) and the yeast assay and contained negligible quantities of chromium. The Rf value in butanol/acetic acid of the 3^{AH} shows that it is not the same as the final cationic fraction 2^{e}.

As a cationic Cr co-ordination complex was sought, most effort was concentrated on the cationic material C1 (Figure 5.5). After fractionation on Dowex cation exchange resin with ammonium formate, a division of activity was found between the yeast assay and adipocyte assay and again after fractionation by preparative chromatography. The adipocyte assay with no basal activity (2^{e}, Table 5.5.3) had only slight activity in the yeast assay. The structure of this material which contained insignificant chromium was investigated due to its outstanding activity in the adipocyte assay when performed with isolated adipocytes from rats fed on Teklad diet, a torula yeast based diet generally used as chromium deficient food, but which was inactive with adipocytes from colony rats.

Details of fraction 2^{e} are presented in Chapter 7 which deals with structure.

5.4 CONCLUSION

Fractions having GTF activity have been isolated from yeast and synthetic medium proving that GTF is produced by yeast and that GTF can diffuse into the medium. GTF active in the yeast assay can be isolated from yeast grown in the absence of chromium. The fractions found by the adipocyte assay contained insignificant quantities of chromium thus chromium is not essential for activity and the structure of GTF.

CHAPTER SIX

SYNTHETIC GTF

6.1 INTRODUCTION

The synthesis of the active chromium complex of Toepfer *et al* (1977) has been discussed in Chapter 1.9.3. Appreciable amounts of unreacted components and diverse compounds resulted from the technique (Kienle *et al* 1979). Anderson *et al* (1978) report an active chromium-glycine-nicotinic acid complex but give no details of the preparation or structure. As no sulphydryl groups would be present in such a preparation, unlike the Toepfer *et al* (1977) preparation, the investigation of the glycine complex seemed more appropriate as the adipocyte assay has been found to respond to sulphydryl groups.

The preparation of the chromium complexes reported was based on the method of Toepfer *et al* (1977) which was a preparation in solution rather than a solid state reaction (Oki *et al* 1976). The preparation of cobalt GTF was by the patented method of Silio (1980). The tendency of chromium to form polynuclear complexes, and examples of isolated and characterized complexes have been reported by Rollinson (1973).

The purpose of this chapter was to gain some understanding of the nature of the chromium GTF complex and the very different cobalt GTF described in the literature (Chapter 1).

6.2 METHODS

6.2.1 Preparation of cobalt GTF

(i) Trans-tetraaquabis(nicotinamide)cobalt II dichloride dihydrate

1.5g (5m moles) of cobalt II acetate hydrate (Sigma) were dissolved in 2.0ml of distilled water at 35°C in a "Reactivial" conical vial with a teflon cap. 1.25g (10m moles) of nicotinamide (Sigma) were added to the solution. 2.0M potassium hydroxide (AR, Univar) was added to increase the pH to 6.5. The capped vial was heated to 80°C for two hours. On adjusting the pH to 4.4, large prismatic crystals formed. The crystals were filtered from the mother liquor and blotted dry. A saturated solution of the crystals was made at 80°C (pH 4.4) and allowed to cool slowly yielding a well crystallized product. The crystals were stored in air-tight containers.

(ii) Cobalt-nicotinamide-glutathione

A fresh solution of reduced glutathione (crystalline, Sigma) consisting of 0.66g (2m moles) in 2ml of distilled water was prepared. The pH was adjusted to 4.7 after adding 1.0g (2m moles) of the cobalt nicotinamide crystals. The reactants were cooled to 4°C to slow the formation of an intense brown colour. After one hour, the solution was frozen and lypholized to produce an amorphous brown solid.

6.2.2 Preparation of chromium acetylacetonate

2.3g of chromium III acetate (10m moles, Hopkins and Williams) were dissolved in 3ml of distilled water and 3.1ml of acetylacetonate (30mM, Merck) were added. The pH was adjusted to 7.0 with 2N potassium hydroxide. A greenish precipitate was filtered from the solution before heating at 80°C for four hours. On cooling, purple needle like crystals

formed slowly. The crystals were filtered from the mother liquor and blotted dry with filter paper. The crystals were stored in an air tight bottle.

6.2.3 Reaction of ^{51}chromium/^{14}C-glycine/nicotinic acid/ethanol

46mg of anhydrous chromium III nitrate (193μmoles) were dissolved in 2.0ml of distilled water. 100μl of ^{51}chromium chloride with an activity of 1mCi (carrier free, New England Nuclear) were added and allowed to equilibrate at room temperature for 1hour before adding 47.3mg (384μmoles) of nicotinic acid (Sigma). After heating at 80°C for 18hours, 57.7mg of glycine and 1.3mg of 2-^{14}C-glycine (785μmoles, 0.1mCi, Amersham) were added and the volume adjusted to 2.00ml. 500μl were removed from the vial as a counting standard for the two isotopes. 500μl of absolute ethanol (AR, Univar) were added to the remaining 1.5ml, capped, and heated for 18hrs at 80°C. The contents of the vial were allowed to evaporate to 1.0ml after which an additional 1.0ml of ethanol was added and the process repeated with 2.0ml of ethanol. The pH was adjusted to 5.8 with 1N ammonia. The final volume was adjusted to 2.00ml.

The solution was loaded onto a well washed Dowex 50W-X8 (H^+, 100-200 mesh, Bio-Rad) column with dimensions 15mm x 270mm. The column was eluted with water and then with a 0.25M triethylamine acetate linear gradient from pH 7.5 to 10.5. γ-radiation in the fractions was measured and plotted with the recorded % transmittance at 260-280nm. Portions of the pooled fractions were lypholized before suspending in scintillation fluid and counting at intervals of several weeks to enable the calculation of the amount of β-radiation by the method of Olson (1977) based on the rapid decay of ^{51}chromium in comparison with ^{14}carbon.

6.2.4 Chromium-glycine preparation with and without ethanol reflux

2.38mg (10μmoles) of chromium nitrate were dissolved in 1.0ml of distilled water. 3.00mg (40μmoles) of glycine (AR, Univar) were added to the vial containing the chromium solution. A duplicate vial was prepared and 1.0ml of absolute ethanol (AR, Univar) was added. The pH of each vial was adjusted to 6.0 with 5N ammonia before capping and heating for 18hours at 80°C. The final volumes were accurately diluted to 2.0ml. A further vial was prepared containing ethanol, glycine and ammonia in the same concentrations. The contents of the vials were tested for biological activity in the adipocyte assay.

6.2.5 Chromium-glycine-^{14}C ethanol

92mg (387μmoles) of chromium nitrate were dissolved in 1ml of distilled water and 122mg (1.63μmoles) of glycine were added and the solution heated at 80°C for four hours before adjusting the pH of the solution to 6.0 with 1N ammonium hydroxide. 1ml of 1-^{14}C-ethanol (0.1mCi/5.0ml ethanol, New England Nuclear) was added, the vial capped and heated at 80°C for 18hours. The volume was allowed to evaporate to 1.0ml before adding a further 2ml of labelled ethanol and repeating the process.

The contents of the vial were loaded onto a well washed Dowex 50W-X2 (H^+, 100-200 mesh, Bio-Rad) column and eluted with a linear gradient of water to 0.25N ammonia. Three distinct fractions were collected, lypholized and dry weights determined to the nearest 0.1mg. Portions of the extremely soluble solids were dissolved and analysed by atomic absorption spectroscopy for chromium. A known weight of each fraction was dissolved in a small amount of water in scintillation pots before adding scintillation fluid (ACSII, Amersham) and counting the β-disintegrations.

6.3 RESULTS

6.3.1 Cobalt

(i) Orange crystalline intermediate

Cobalt GTF was prepared according to the patented method of Silio (1980). The orange crystalline intermediate compound was found to lose water at relatively low temperature (90°C, with complete loss at 120°C) to form an amorphous purple solid. Differential thermal gravimetric analysis (Stanton and Redcroft, TG750) with an argon atmosphere and a heating rate of $5°Cmin^{-1}$ to 970°C indicated a total of 6 molecules of water and a molecular weight of 490daltons, arrived at by assuming the final product was Co_3O_4. The value compared closely with 482daltons calculated from the published crystal structure (Vegas *et al* 1981). Reflectance spectra of the orange crystals (Beckman DK2 spectrophotometer) and solution spectra (Cary 219, Varian) indicated that the complex was a cobalt II complex. The molar extinction coefficient (ε_{510} = 5.7) indicated that the complex was most likely to be a trans-isomer with octahedral geometry in agreement with the published structure (Vegas *et al* 1981). The structural evidence would seem to indicate that the intermediate of Silio (1980) had been successfully prepared.

(ii) Cobalt GTF

The change in colour and intensity reported in Section 6.3.1 was accompanied by spectral shifts in the visible region from 510nm to 485nm and 383nm and were consistent with a change in oxidation state to cobalt III. Furthermore, the material formed sorbed strongly to Dowex cation exchange resin indicating that it was not anionic as reported by Silio. No activity was found in the yeast assay at a concentration of 5×10^{-7}M, 5×10^{-6}M or 5×10^{-5}M. As the patent described a cobalt II nicotinamide-glutathione anionic complex, it was concluded that the complex prepared was not the same as the patented complex.

Silio later described (personal communication, 1982) the spontaneous oxidation of Co^{2+} to Co^{3+} during the preparation of the complex and cautions that the complex is light sensitive. A generous gift of cobalt GTF (Dr F. Silio, Instituto "G. Maranon, Madrid) was found to be inactive in the yeast assay (5×10^{-7}, 5×10^{-5}M) but active when tested in the adipocyte assay (Sherriff, MSc thesis, 1983) at $5 \times 10^{-7}$to 1×10^{-5}M. The solution spectrum of cobalt GTF (0.5μmoles/2.9ml) was consistent with a cobalt III complex, absorbing at 455, 383 and 257nm with a molar extinction coefficient of ε_{383}nm = 3 538 in contrast with the cobalt II intermediate with an absorption maximum at 510nm and ε_{510} = 5.7. The high extinction coefficent suggests a cis-isomer.

In light of the additional information, it would appear that the attempt to prepare active cobalt GTF may have been successful but activity is only to be found with the adipocyte assay.

6.3.2 Chromium acetylacetonate

The complex was found to have a low solubility in water and had similar physical properties to those expected of the structure elucidated by Morosin (1965). An insoluble chromium hydroxide formed in the aqueous phase on increasing the pH during the preparation of the complex and was thought to arise from the use of what appeared to be a basic chromium acetate, rather than chromium acetate as described by the supplier. The basic chromium acetate was difficult to dissolve, thus anhydrous chromium nitrate was prepared from the acetate with hot nitric acid (AR, Univar) and the product dehydrated *in vacuo*. The anhydrous chromium nitrate was extremely water soluble and was used for subsequent synthesis.

Chromium acetylacetonate was found to be inactive when tested at 200ng Cr/ml in the yeast assay and 200 and 20ng Cr/ml in the adipocyte assay in agreement with Mertz (1969).

6.3.3 Active chromium glycine preparations

The elution profile of a preparation consisting of ^{51}chromium, ^{14}C-glycine, nicotinic acid and ethanol is shown in Figure 6.1. The molar ratio of glycine to chromium was found to be 3.7 glycine to 1 chromium (Table 6.1). As there appeared to be a background level of glycine in fraction 4 of 0.4M, the value was subtracted giving a rounded value of 3 glycine/chromium. UV absorption spectra detected free nicotinic acid in the water eluted fractions 1-3 with most free nicotinic ac eluting in fraction 5. As nicotinic acid is active in the adipocyte assay and there was no evidence that nicotinic acid was coordinated to chromium, a pilot preparation similar to that of Section 6.2.3 was undertaken and initial results indicated that the presence of nicotinic acid in the preparation was not essential for activity in the adipocyte assay. Furthermore, no activity was found until the preparation had been refluxed with ethanol. As a result of these findings, two preparations of chromium glycine complexes were made under identical conditions with the exception of the ethanol reflux step (Section 6.2.4). The chromium glycine complex was inactive in the adipocyte assay (0.52µg/ml Cr) as was the glycine, ethanol, ammonia control. Only the ethanol refluxed complex was found to be active. Fine purple crystals precipitating from the ethanol refluxed preparation were found to have no biological activity in the adipocyte assay (0.5µg/1ml Cr). UV-visible solution spectra of the reaction mixtures showed an absorbance shift of 12nm from the 564nm absorbance maximum of aged chromium nitrate solution, to 552nm for chromium glycine and 540nm for the ethanol refluxed complex.

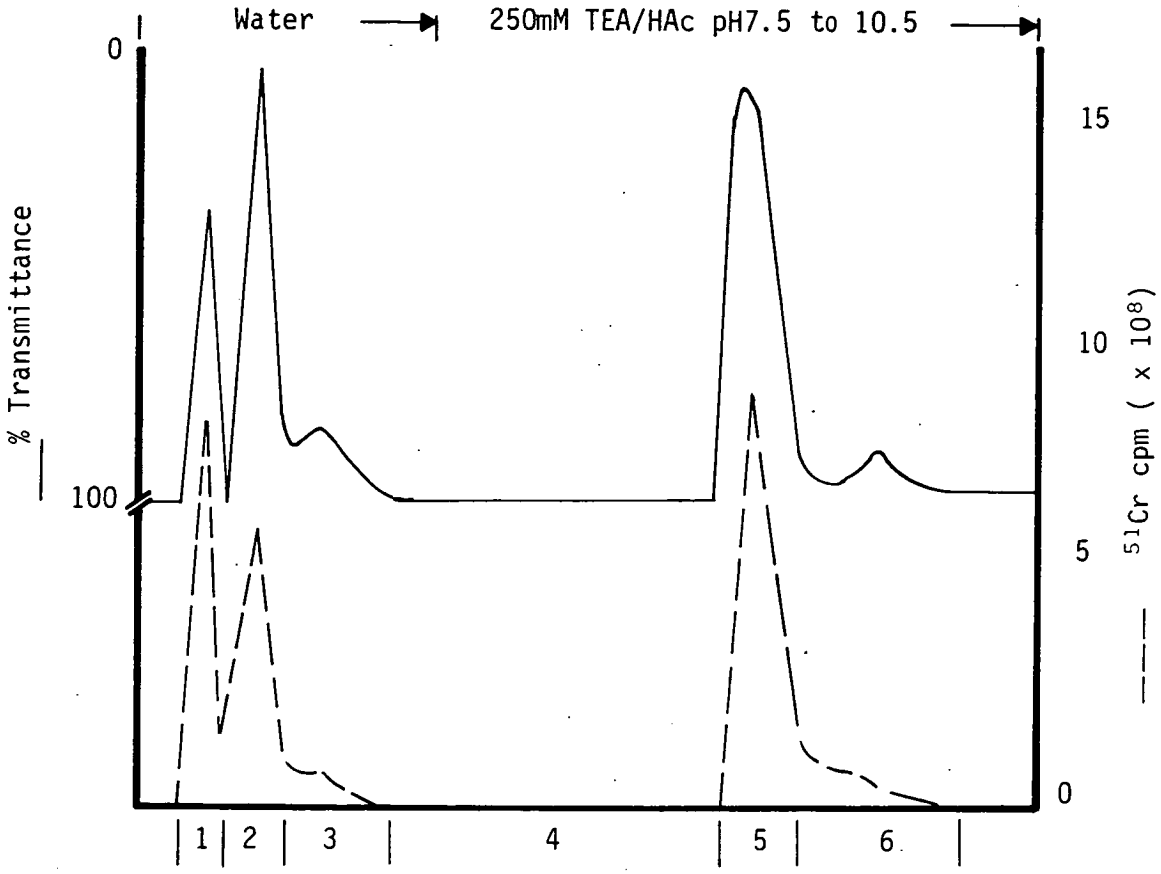


FIGURE 6.1 The elution of a ^{51}chromium/nicotinic acid/ ^{14}C glycine/ethanol preparation from Dowex 50W-X8 resin (Section 6.2.3), column size 15mm x 270mm, flow rate 90ml/hr.

Fraction (From Figure 6.1)	M glycine per 1M Cr	Biological Activity (Adipocyte, 0.5µg Cr)
1	0.56	nil
2	0.66	nil
3	7.8	nil
4	0.4	nil
5	3.7	active
6	0.5	active

TABLE 6.1 The biological activity and ratio of glycine to chromium determined for fractions eluted from cation exchange resin shown in Figure 6.1.

Further investigation of the role of ethanol in the complex involved the use of labelled ethanol (Section 6.2.5). The complex was eluted from Dowex as shown in Figure 6.2. Further attempts to purify fraction 3 (Figure 6.2) by preparative paper chromatography in a butanol/acetic acid solvent system (Toepfer *et al* 1977) resulted in dissociation of the complex. The final product (fraction 3) was lypholized to avoid the loss of activity due to precipitation (Nath *et al* 1979) and ensure the removal of free ^{14}C-ethanol. The freeze dried solids were extremely water soluble.

Differential thermal gravimetric analysis (DTA) of the active cationic fraction 3 from Dowex indicated a molecular weight of 524 if it assumed that the complex decomposes to Cr_2O_3. The sample was heated in argon at a rate of 3°C/min. The program was stopped at 700°C as the sample had reached 14% of its initial weight. The residue was found to be a green-black solid similar in appearance to Cr_2O_3. The decomposition of the complex is shown in Figure 6.3 with indicated possible decomposition products. If the complex is assumed to be mononuclear, a MW of 524 can be calculated or if binuclear, a MW of 1 022.

The results of micro-analysis by the Australian Analytical Services (AMDEL) were as follows:

%C	=	30.39
%N	=	16.48
%H	=	6.20
%Cr	=	9.92
%O by difference	=	37.01

The chromium analysis was by AAS as described in Section 6.2.5.

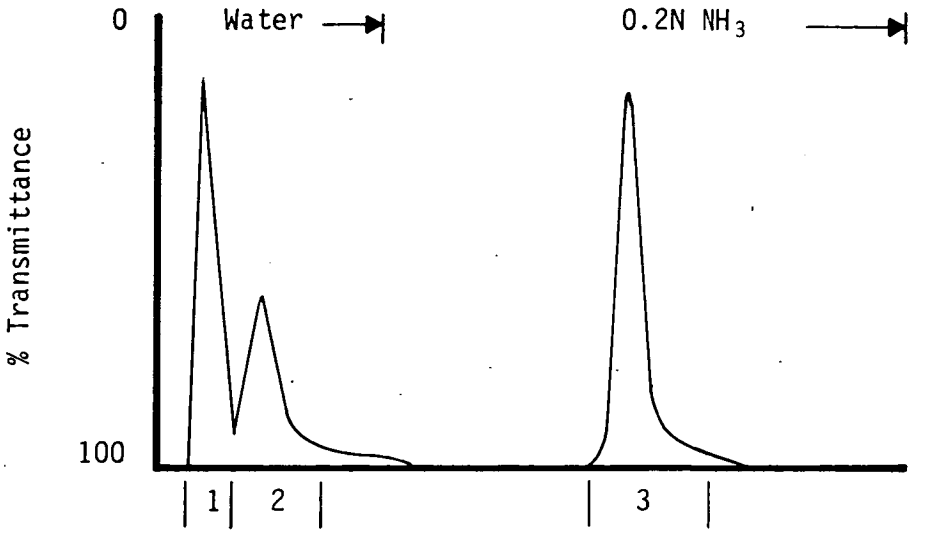


FIGURE 6.2 The elution of a chromium/glycine/^{14}C ethanol preparation from Dowex 50W-X8. The fractions indicated were lypholized.

Fraction	Yield Dry wt. (mg)	M ethanol/1M Cr
1	40.3	3.5
2	14.1	5.6
3	102.4	4.3

TABLE 6.2 The chromium to ^{14}C ethanol ratio of fractions eluted as shown above (Figure 6.2).

Fraction	Biological Activity	
	Yeast* (1.5µg Cr)	Adipocyte (50µg of complex)
1	nil	nil
2	nil	nil
3	30%	active

* % enhancement

TABLE 6.3 The *in vitro* activity of fractions eluted as shown in Figure 6.2.

The %Cr was found to be in agreement with the calculated value from the DTA curve shown in Figure 6.3. The micro-analytical results indicate approximately 13 two carbon molecules per 2 chromium atoms with 12 nitrogen atoms per 2 chromium atoms. As ^{14}C ethanol counting indicated that there were 8.6 ethanol molecules per 2 chromium atoms (Table 6.2) and that 9 ethanol molecules could be assigned to the decomposition curve (Figure 6.3), the remaining imbalance of 6 atoms of nitrogen per 2 atoms of chromium may be attributed to associated ammonia.

Infra red spectra were obtained on a Digilab FTS 20E spectrometer by Mr J. Bignall of the Central Science Laboratory, University of Tasmania. The infra red spectrum of the cationic active complex was compared with that of free glycine. A potassium bromide pellet of the complex was prepared and the spectrum accumulated over 100 scans. The resolution was 8 wavenumbers (Figure 6.4).

A multiple combination and overtones region common to both glycine and the complex was discernible from 2 000 to 3 000cm^{-1} with a strongly absorbing band from 3 300 - 3 600cm^{-1} in the complex being due to water. A prominent line at 2 129cm^{-1} in glycine, the asymmetrical $-NH_3^+$ and torsional oscillation, was absent in the spectrum of the complex. Another prominent line at 698.2cm^{-1}, likely to be a torsional $-NH_3^+$ oscillation was also found to be absent. If chromium was coordinated to glycine through the carboxylate ion, thus the $-NH_3^+$ was now NH_2, the loss of zwitterionic character would account for the change in the spectrum including the weakness of the 1 400cm^{-1} region compared with the 1 600cm^{-1} region in the complex.

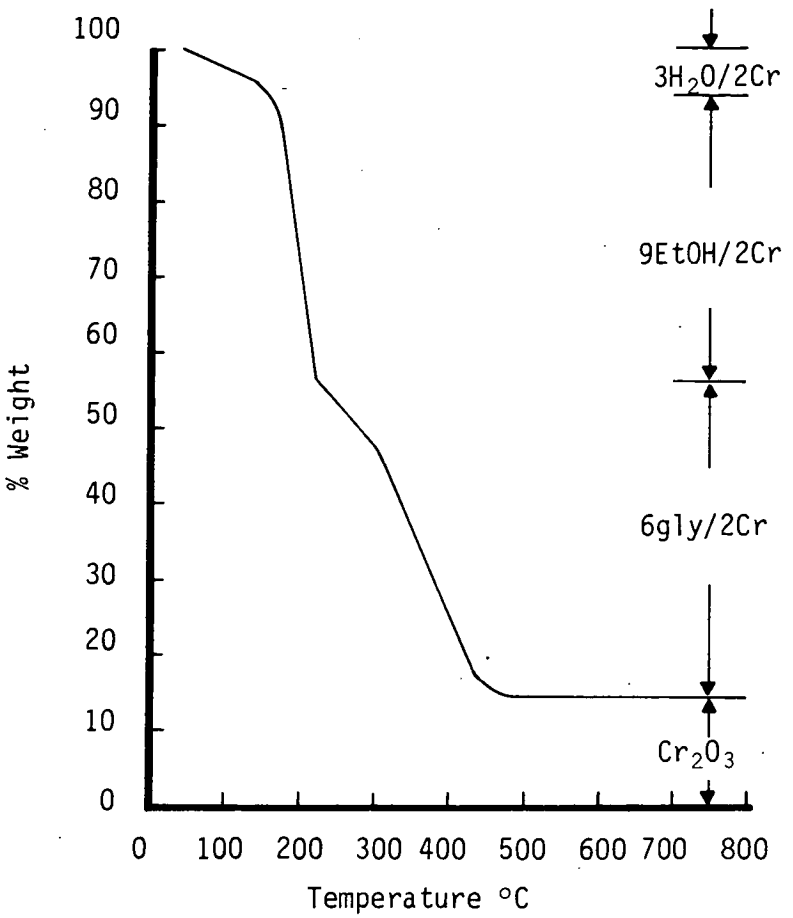


FIGURE 6.3 The thermal decomposition of the active chromium cationic complex and possible decomposition fragments. A molecular weight of 1 048 can be calculated from the final $\%Cr_2O_3$ if the starting product was a binuclear complex.

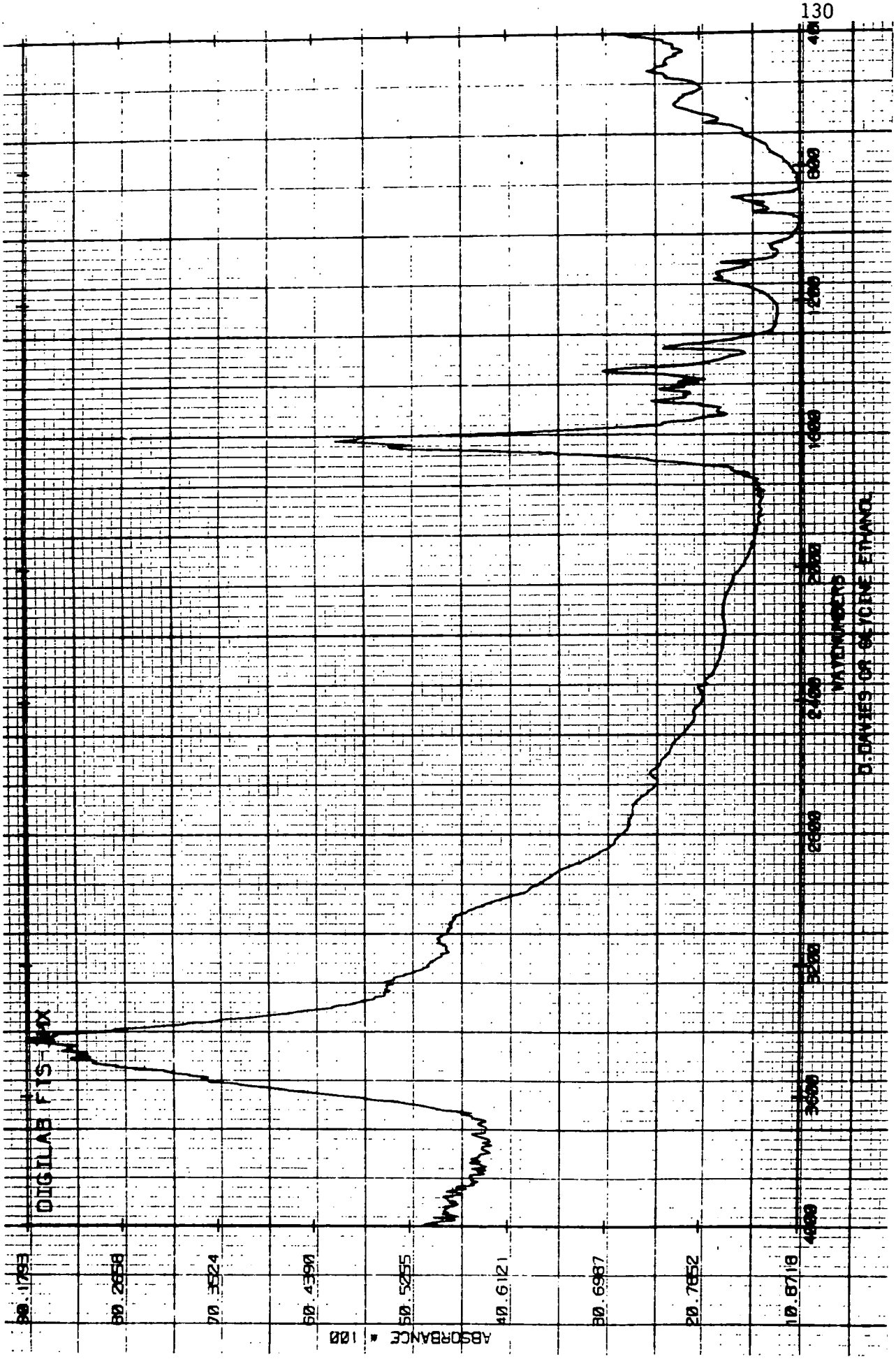


FIGURE 6.4 The IR spectrum of the biologically active chromium preparation. KBr pellet, 100 scans.

UV-visible solution spectra of an aqueous solution of fraction 3 (Table 6.4) showed visible region absorbance maxima of 535nm and 396nm similar to many defined octahedral solution species of chromium III.

6.4 DISCUSSION

Chromium acetylacetonate ($Cr(acac)_3$) was found to be inactive *in vitro* with both yeast and adipocyte assay systems in agreement with Mertz (1969) even though the compound was reported to be absorbed in the gut of rats to the extent of 40% (Anderson *et al* 1980). The problems associated with the use of chromium acetate, as used by Toepfer *et al* (1977) were highlighted in the preparation of the $Cr(acac)_3$ complex as olation products formed even in the presence of the strong acetylacetone ligand. As acetate ligands can act as bridges in the same way as OH^- in olation (Rollinson 1973), chromium nitrate was substituted resulting in no olation products precipitating in the preparations described (Section 6.2) and in contrast to the preparation of Toepfer *et al* (1977).

The reason for the addition of ethanol by Toepfer *et al* (1977) at an early stage of synthesis was not made apparent. The addition of alcohol at an early stage of the synthesis of a chromium glycine complex was found to increase olation problems by precipitating glycine and thus reducing the concentration of ligand available to compete with water. The addition of increasing concentrations of ethanol at a later stage of synthesis was found to be necessary for biological activity in the adipocyte assay, an aspect of the synthesis not previously reported. Biologically active material was also synthesized in the absence of nicotinic acid and although not outstandingly active in the yeast assay, was found to be active in the adipocyte assay.

Ammonia was used in all experiments to adjust the pH of the reaction mixture in accordance with the method of Toepfer *et al* (1977). As a small colour change was generally associated with the addition of ammonia and extra nitrogen was detected by the micro-analysis, the presence of ammonia in the active complex seems likely as glycine is not a completely effective chelating agent (Rollinson 1973). The active material could be described as a tris-glycine-chromium complex with associated ethanol, ammonia and water. The complex was most likely to be a binuclear complex and was, in solution, a positively charged complex ion. Tris-(amino acidato)chromium III complex reported by Oki *et al* (1976)dissociated in acid solution as did the active complex when attempting purification by paper chromatography in a butanol/ acetic acid solvent. The complex is not related to the simple tris-glycinato chromium III monohydrate reported by Bryan *et al* (1971), although the visible absorption maxima were very similar to those reported by Oki and Otsuka (1976) in strong acid solution (Table 6.4).

	Absorption Maximum	
	λ(nm)	λ(nm)
Oki *et al* (1976)	538	400
Reported complex	535	396

TABLE 6.4 The absorption maximum of the active chromium glycine preparation reported in this thesis compared with the absorption of comparable tris-chromium glycine complexes reported by Oki *et al* (1976).

The high solubility of the active complex contrasts with the limited solubility of the simple $[Cr(gly)_3]H_2O$ and $[Cr_2(gly)_4(OH)_2]H_2O$ (Bryan *et al* 1971).

The visible region solution spectrum shifts reported in this thesis were larger than observations made by Krishnamoorthy *et al* (1980) for the interaction of chromium III solution complex ion with nucleosides. Anghileri (1971) reports the preparation of aspartic and asparagine complexes with ^{51}chromium in aqueous solution for *in vivo* distribution studies. The reported cationic complexes were found to be products of polymerization with mono and binuclear compounds being formed. Thus the formation of discrete complexes in aqueous solution can be seen to be difficult and the general consensus of opinion is that, with increasingly dilute solution and time, weak ligands will eventually interact with water molecules resulting in the formation of polymers. The storage of active material after lypholization overcame the problems of precipitation and deactivation in aqueous solution reported by Nath *et al* (1979).

The preparation of Silio (1980) was found to be a cobalt III complex and strongly sorbed by cation exchange resin as was the chromium complex. The reported molecular weight of 1 500daltons compares closely with an estimated molecular weight of 1 048daltons for the active chromium complex and although the material was prepared with glutathione, no insulin mimicking activity was found, thus establishing GTF activity. The complexes were thus similar with respect to size and overall charge. The fact that a cobalt preparation with nicotinamide rather than nicotinic acid was active is completely contrary to the requirements of Toepfer *et al* (1977) of an axial nicotinic acid arrangement with a chromium nucleus and the requirement of Mertz (1969) that the complex should be a chromium coordination complex.

6.5 CONCLUSION

A chromium complex has been prepared without nicotinic acid or sulphydryl functional groups and found to be active. A cobalt complex prepared by Dr F. Silio has also been found to be active in the adipocyte assay.

CHAPTER SEVEN

THE STRUCTURE OF YEAST GTF

7.1 INTRODUCTION

The isolation of a biologically active, cationic fraction from whole yeast cell extract has been described in Chapter 5. The ninhydrin positive material produced a maximal stimulation of glucose oxidation with sub-optimal levels of insulin when tested in the adipocyte assay, and thus was considered to be GTF since no activity was found in the absence of exogenous insulin.

The characterization of the active material is described in this chapter. A wide range of instrumental facilities were used in order to elucidate the structure of the material which proved to be a difficult task because of its physical properties. Some indication of the polarity of the material and associated problems of sorption have already been discussed in Chapter 3.

7.2 RESULTS

7.2.1 Physical description

Approximately 15mg of active material in two degrees of purity (2^e, 2") were isolated from a 200ℓ yeast culture grown in synthetic medium. The lypholized solids were white and similar in appearance to albumin. The solids were extremely water soluble and hygroscopic to such an extent as to preclude grinding with "nujol" to form a mull for IR spectroscopy. The material was also found to be totally insoluble in dimethyl sulphoxide (DMSO) thus obtaining exchangeable proton NMR spectra

was not possible. The insolubility in DMSO highlights the high polarity of the material.

7.2.2 Biological activity

A dose response curve of % enhancement (increase over control, Chapter 4) of anaerobic glucose oxidation by yeast cells was prepared by accurate dilutions of the active material 2^e (Figure 7.1).

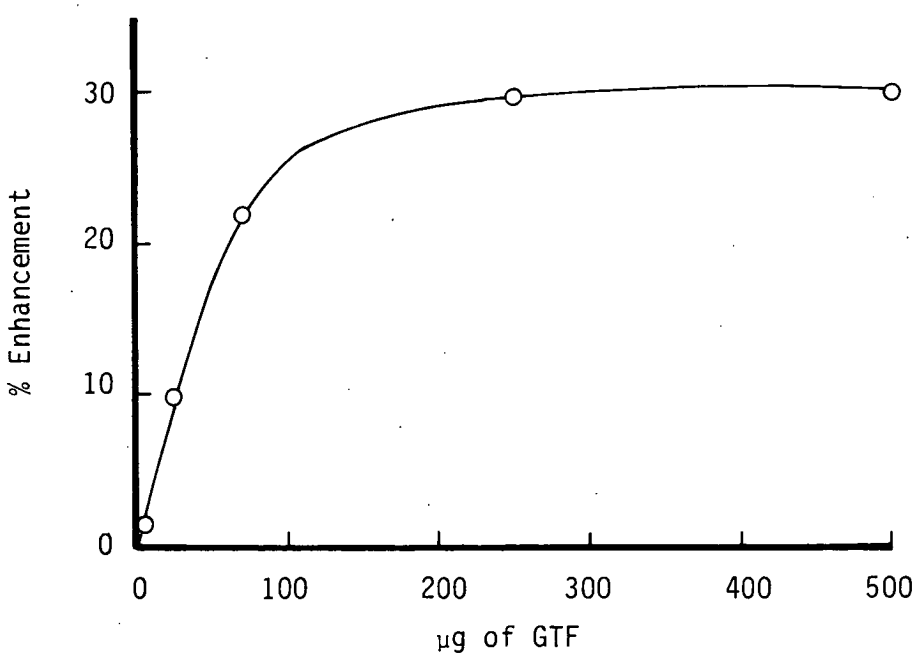


FIGURE 7.1 The dose response curve for the active fraction 2^e as tested by the yeast assay described in Chapter 4.

Although not outstanding in activity, there is a clear relationship between dose and % enhancement showing saturation at greater than 200µg/1ml test volume.

Fraction 2^e and 2" were tested for activity in the adipocyte assay by Ms J. Sherriff (MSc thesis, 1983) at a level of 50µg/1ml test

volume. As has been indicated, the effect of the fraction was to increase the oxidation of glucose by adipocytes in the presence of 10μU of insulin to equal the effect produced by 200μU of insulin. Duplicate experiments on the duplicate preparation confirmed the activity. No effect was found in the absence of insulin thus ruling out the presence of insulin mimicking substances.

7.2.3 Chemical analysis

The chromium content of the active material was determined by CRA-AAS using the method described in Chapter 2 for yeast extract fractions. The chromium content was found to be 8×10^{-4}% and was regarded as being insignificant.

Micro-chemical analysis of the material was performed by the Australian Micro-analytical Services (AMDL) who were supplied with 2mg of sample. The results were as follows:

% carbon	=	34.63
% hydrogen	=	13.86
% nitrogen	=	7.10
% oxygen by difference	=	44.41

The ratio of carbon to nitrogen can be calculated to be 6 carbon:1 nitrogen in comparison with 6 carbon:2 nitrogen for lysine.

7.2.4 Amino-acid analysis

0.2mg of active material was acid hydrolysed in a 0.5ml "Reactivial", a conical vial with a teflon cap, by addition of 5μl of

5N hydrochloric acid and heating for 18hrs at 110°C. Excess acid was removed by lypholization. The sample was diluted in the starting buffer (0.2N sodium citrate pH 6.28) for analysis by a programmed Dionex amino acid analyzer developed by J. Jordan of the Biochemistry Department. The column used was 4.6mm x 250mm packed with Aminex A-7 resin. The column temperature was 60°C and a ninhydrin detection system was used. Only one peak was observed eluting at the exact volume corresponding to lysine.

Remaining hydrolyzed material was spotted onto paper for chromatography in the iso-propanol/ammonia solvent system described in Chapter 3. Unhydrolyzed material, lysine, poly-L-lysine (MW 4 000) and partially hydrolyzed poly-L-lysine (18hr, 80°C, 1N hydrochloric acid) were also applied to the paper prior to development. The material on the paper was visualized by spraying with ninhydrin. All compounds developed without heating thus indicating the presence of an α-amino group.

	Rf
L-lysine hydrochloride	0.33
poly-L-lysine (MW 4 000)	0.01
Sample 2^e	0.31

TABLE 7.1 The Rf of poly-lysine and the biologically active material 2^e in iso-propanol/ ammonia solvent.

The mildly acid hydrolyzed material yielded a series of compounds with Rf values from 0 to 0.31. The difference in Rf between lysine and the active material was small whereas the difference between poly-L-lysine was considerable thus indicating the active material was of considerably lower molecular weight.

7.2.5 Nuclear magnetic resonance

Spectra were recorded using the 100M Hz Fourier Transform spectrometer at the National NMR Centre in Canberra. The instrument was operated by Dr Alan Jones and research assistant Ms B. Butler. Spectra were accumulated at 298°K unless otherwise specified.

7.2.5.1 Proton NMR (^1H)

(i) The effect of pD and temperature

The NMR proton spectrum of sample 2^e was found to pD dependent. The pD of the sample dissolved in D_2O was alkaline (~8). Spectra recorded in alkaline solution showed broad lines. Adjustment of pD using DCl so that the sample was mildly acidic, resulted in a spectrum with defined spin-spin splitting multiplets. The line widths, when compared with those of L-lysine hydrochloride, were broadened (Figure 7.2). The ε-protons were found to yield a quartet thus establishing the absence of hydroxy-lysine. A similar behaviour was observed in the spectrum of poly-L-lysine MW 50 000 (Sigma) in acid and alkaline solution.

As hydrogen bonding was suspected to be the mechanism causing broadening at alkaline pD, spectra were obtained with the sample dissolved in urea, a hydrogen bond breaking solvent. The pD was varied from 5.0 up to 12.5. Increasing broadening of line widths with increasing pD resulted as before, with the lines showing a decreasing chemical shift with increasing pD. If hydrogen bonding had been responsible for the broadening in alkaline solution, the presence of urea would have removed the effect. The conclusion drawn was that a strong zwitterionic structure was involved rather than hydrogen bonding.

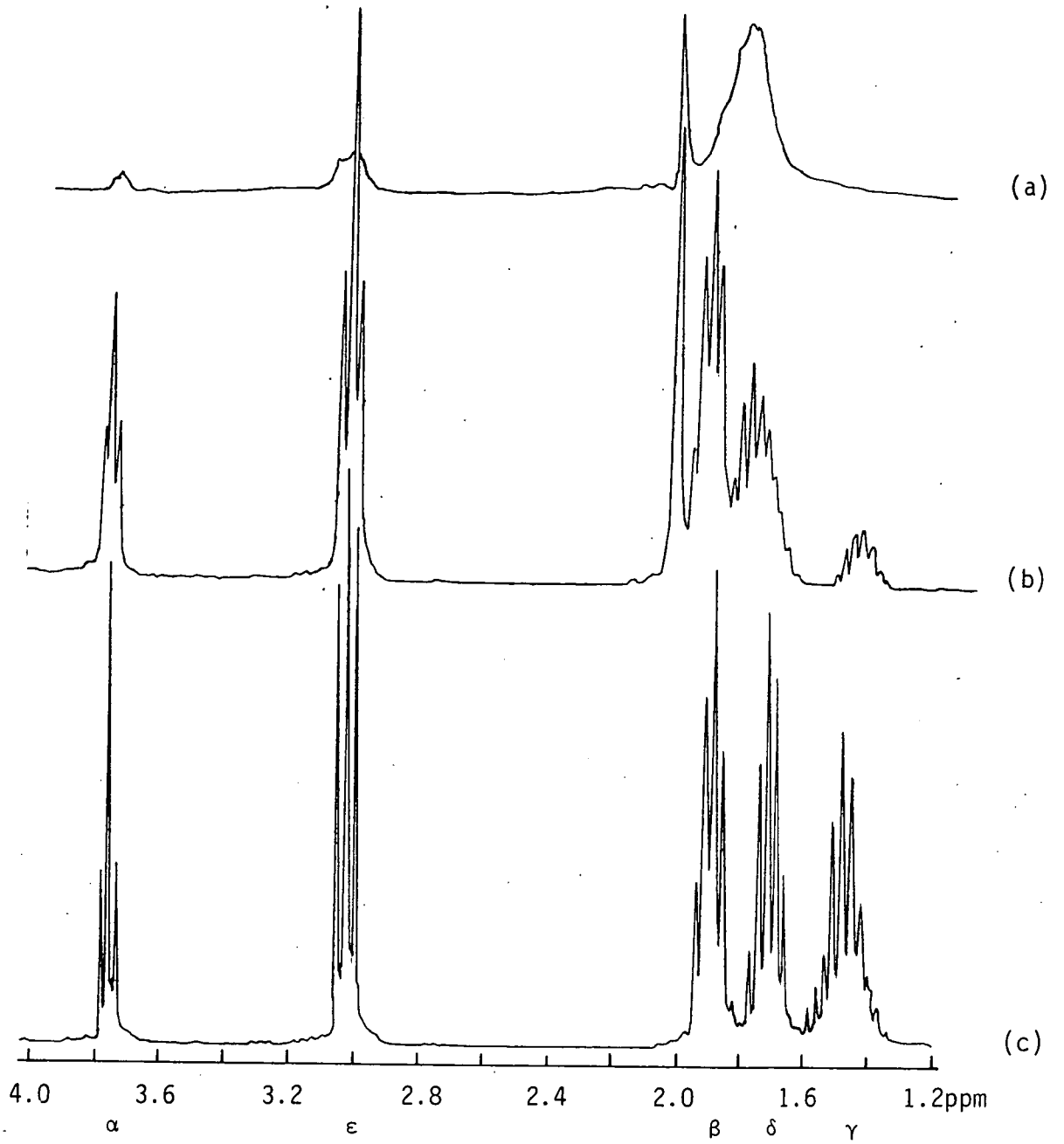


FIGURE 7.2 The ^1H spectrum of 5mg of 2^e in (a) alkaline solution (pD 8) (b) acid solution (pD 5) and the spectrum of L-lysine in acid solution (c).

FIGURE 7.3 The ^1H spectrum of 5mg of 2^e in acid D_2O solution at 348°K.

Further evidence that the effect of line broadening was not due to hydrogen bonding was found in the spectra obtained at increased temperature. The probe temperature was increased to 348°K and the spectrum recorded. Line broadening and chemical shifts to higher values resulted, returning to the original room temperature spectrum on cooling (Figure 7.3), thus the effect was reversible. As breaking of hydrogen bonds occurs at higher temperature, the contrary would have been expected if hydrogen bonding was significant.

(ii) Decoupling experiment

Before evaluation of the proton spectrum, it was necessary to establish that the protons observed were part of the same molecule to rule out the possibility of impurities.

The ^{1}H spectrum was assigned based on the similarity to lysine thus the protons have been labelled as if they were lysine protons.

Assignment	Chemical Shift	Spin-spin splitting	J (Hz)
α CH	3.69	doublet	6.2
ε CH_2	2.69	quartet	8.0
? CH_2	1.83	singlet	-
β CH_2	1.83	sextet	8.0
δ CH_2	1.74	octet	8.0
γ CH_2	1.42	octet	8.0

TABLE 7.2 The proton spectrum of the active material 2^{e}.

The spin-spin coupling constant (J) was similar for each multiplet indicating that all protons were of the same molecule.

Decoupling was performed by irradiation at the frequency of each group of protons. The effect was observed on the remaining protons.

(a) 998Hz (αCH) resulted in collapse of the βCH_2 region with no effect on the singlet at 1.83ppm.

(b) 810Hz (εCH_2) resulted in the collapse of the δCH_2 region.

(c) 495Hz (βCH_2) resulted in the collapse of the αCH and γ and δCH_2 region.

(d) 380Hz (γCH_2) resulted in changes in the βCH_2 and δCH_2 regions.

Thus it can be seen that all protons except the singlet with a chemical shift of 1.83ppm are part of the same molecule. The line at 1.83ppm was regarded as an impurity.

(iii) The analysis of the proton spectrum

As the spectral features of amino acids with ionizable side chains are pD dependent (Roberts and Jaradetzky 1970), comparative spectra of lysine, poly-lysine and the sample 2^e were obtained in acid conditions. As can be seen from Table 7.3, the chemical shifts compare more closely with lysine than poly-lysine with the proton integral also indicating lysine.

Proton	Chemical Shift (ppm)			2^e proton integral
	Poly-lysine	Lysine	Sample 2^e	
α CH	4.31	3.74	3.70	1
β CH	1.69	1.84	1.83	2
γ CH	1.45	1.47	1.40	2
δ CH		1.71	1.68	2
ε CH	3.01	3.01	2.97	2

TABLE 7.3 The comparison of H spectra of L-lysine hydrochloride, poly-L-lysine (MW 4 000) and the biologically active material 2^e in acidic D_2O at 298°K.

7.2.5.2 ^{13}C NMR

The effect of pD was found to be an important factor in obtaining a spectrum with a high signal to noise ratio. The sample (15mg) was dissolved in D_2O and the pD adjusted to ~5. The spectrum was accumulated over a 64hr period with a 10second time delay.

The chemical shifts of lysine, poly-L-lysine and the active material are compared in Table 7.4.

Assignment	Chemical Shift (ppm)		
	Lysine	Poly-lysine	Sample 2^e, 2"
C=O	174.7	174.6	175.7 175.2
α C	54.5	54.7	55.8 55.4
ε C	40.5	40.2	40.4 40.2
β C	27.5	31.6	31.1
δ C	31.8	27.3	28.6 27.6
γ C	23.1	23.2	24.0 22.7

TABLE 7.4 The ^{13}C spectrum of lysine, poly-DL-lysine (MW 5 000) and the active material in acidic D_2O.

The apparent discrepancy in assignment between lysine and poly-lysine was compared with literature (Wüthrich 1976, Smith *et al* 1973) but was found to be correct thus posing problems in assigning the line at 31.1ppm in 2^e - is it a β or δ carbon?

The assignment of the carbon atoms fits lysine better than other amino acids but when compared to poly-lysine and lysine, larger shifts are found for the carboxyl carbon, α carbon and γ carbon and

difficulty in assigning the β carbon. The "paired" carbon atoms were originally thought to be due to the presence of D and L forms of lysine but was found to be incorrect as Table 7.4 demonstrates that poly-DL-lysine did not show a similar spectrum to 2^e, Thus, it must be concluded that two slightly different lysine polymer molecules are present, causing difficulty in the ^{13}carbon integration.

If an almost insignificant band at 182.4ppm is regarded as being the terminal carbon atom of a polypeptide, the ratio of the two different peptide banded carbon could be calculated from the integral to be 1 terminal C:1 ^{13}C 175.7ppm:4 or 3 C 175.2ppm. If all were part of the same molecule, a very approximate molecular weight of 800 daltons is implied but the two sets of lines would not be predicted. If the band at 182.4ppm is disregarded it may be that two small polymers of different size are present in the ratio of 1:3.

7.2.6 Infra-red spectroscopy

Infra-red spectra were obtained by J. Bignall of the Central Science Laboratory, University of Tasmania, using a Digilab Fourier Transform FTS-20E spectrometer. Samples of poly-L-lysine, L-lysine hydrochloride and the active preparation, 2^e, were prepared as potassium bromide pellets. Spectra were accumulated from 100 scans with a resolution of 2 wavenumbers (cm^{-1}).

A strong band from 3 300 - 3 600cm^{-1} was observed for sample 2^e and poly-L-lysine. Both materials were hygroscopic so the band was attributed to water which absorbs in the 3 500cm^{-1} region. Poly-L-lysine (MW 4 000) was found to have a broad absorbance band below 800cm^{-1} in comparison with lysine, due to the much greater length of the poly-lysine. The sample 2^e showed detail at less than 800cm^{-1}

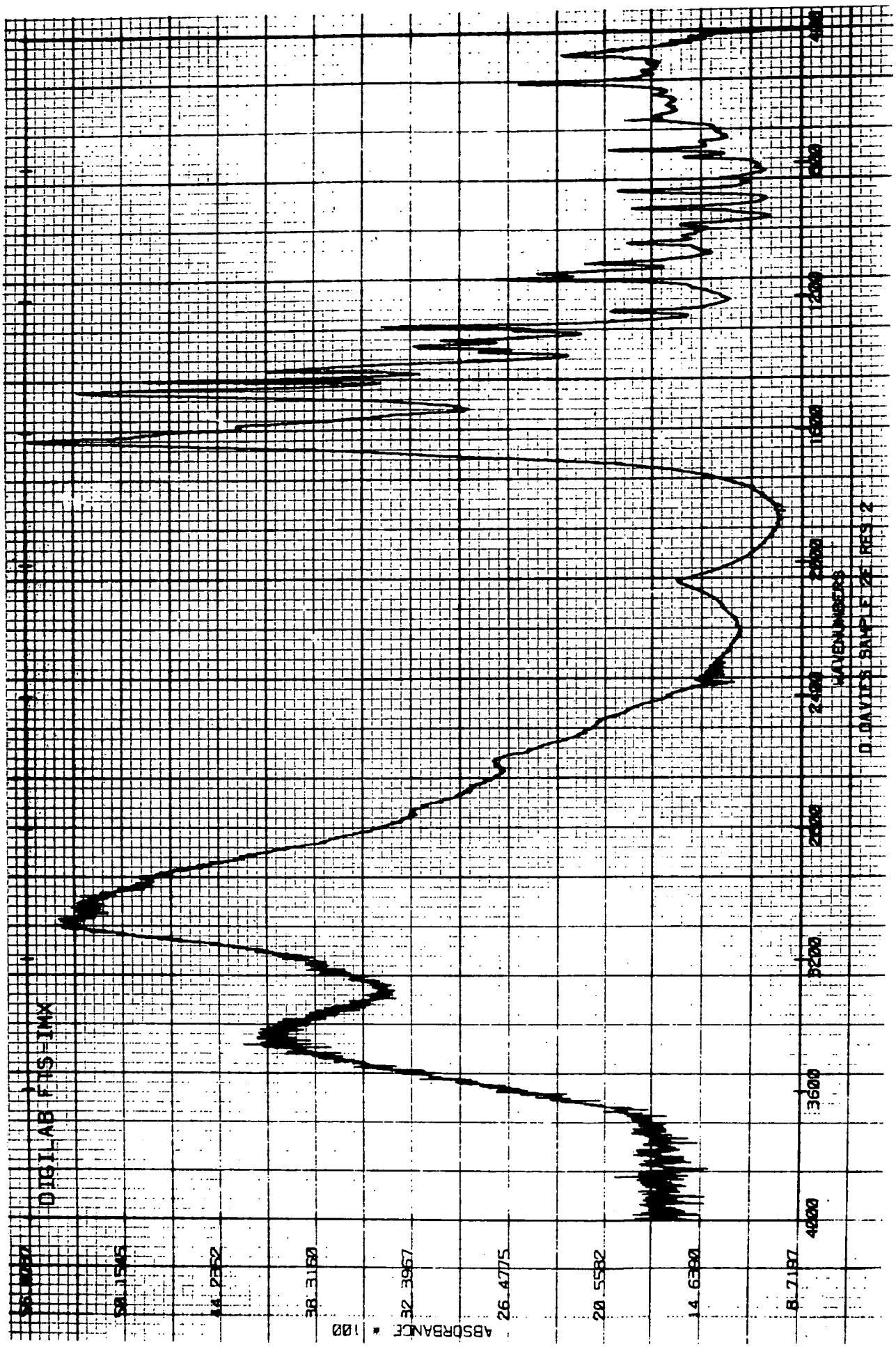


FIGURE 7.4 The IR spectrum of the biologically active material 2^e. KBr pellet, 100 scans.

similar to lysine. Most significant differences were found in the 1 650cm^{-1}(C=O) and N-H region which would involve peptide linkage oscillations.

	Assignment (cm^{-1})		
	C=O	N-H symmetrical	$-NH_3^+$ asymmetrical
lysine	1 585	1 504	2 120
poly-L-lysine	1 660	1 540	2 000
sample 2^e	1 630	1 480	2 060

TABLE 7.5 Comparison of the main absorbance bands of L-lysine hydrochloride, poly-L-lysine (MW 4 000) and the biologically active material 2^e.

The sample 2^e can be seen to be intermediate between the lysine monomer and the 26-lysine polymer with the exception of the symmetrical -NH bending band which is shifted further.

7.2.7 UV-solution spectrum

The solution spectrum was recorded using a Cary 219 spectrophotometer (Varian). An aqueous solution of 1.33mg/1ml of the active fraction was scanned from 300 to 200nm. A single symmetrical absorbance band at 260nm had an absorbance of 0.905 absorbance units. Strong absorbance was found at less than 230nm. If a molecular weight of 800daltons is assumed, the molar extinction coefficient can be calculated to be ε_{260} = 543 (1cm pathlength). No such band was observed for lysine or poly-L-lysine.

7.2.8 Mass spectrometry

Following lack of success in obtaining a parent ion in the expected region of 800 mass units by ammonia chemical ionization mass spectroscopy (Central Science Laboratory, University of Tasmania), a sample was submitted to Dr I. Lewis of UG Analytical Ltd., Manchester, for fast atom bombardment (FAB source) mass spectroscopy. Unfortunately, he was unable to obtain a spectrum despite several attempts. The difficulty was not made apparent but may have been due to insolubility in the organic solvent used.

7.2.9 The biological activity of poly-L-lysine

As has been shown in Chapter 4, poly-L-lysine (MW 4 000) and poly-DL-lysine (MW 5 000) were found to be inhibitory in the yeast assay. As a smaller lysine fragment was required, the poly-L-lysine (MW 4 000) was mildly acid hydrolyzed in 1N HCl for 18hrs at 80°C, similar to the hydrolysis of Tuman *et al* (1978). Six discrete compounds were found to be present by paper chromatography in the iso-propanol/ammonia solvent system.

100µg of the acid hydrolysate was found to be active in the yeast assay, with a % enhancement of 19%. L-lysine hydrochloride was found to be inactive. Higher levels of the hydrolysate were inhibitory and would seem to be due to unhydrolyzed material. The adipocyte assay was not available due to the exhaustion of Teklad food and thus discontinuation of the rat breeding programme.

7.3 DISCUSSION

The active fraction 2^e was found to have physical and spectral similarities with a low molecular weight poly-lysine. The Merck Index (1968) describes poly-lysine as a transparent, film-like polymer, readily soluble in water and practically insoluble in usual organic solvents as was the case with the active material. Both the active material and poly-lysine were found to be hygroscopic.

Acid hydrolysis of the sample 2^e yielded only lysine by amino acid analysis. As mass spectroscopy did not give a result and gel filtration chromatography was not possible due to strong sorption (Chapter 3), the NMR spectra were to provide the majority of structural information, including estimated chain length.

Initially, the extent of ^{13}C line broadening was thought to indicate a large molecular weight as has been demonstrated in the crosslinking of polystyrenes but this was discounted on the basis of paper chromatography. Poly-lysine, having a molecular weight of 4 000daltons, had a low Rf in the alkaline solvent system whereas 2^e had an Rf of 0.31. Wüthrich (1976) reports that cyclic peptides exhibit line broadening, thus the possibility of a cyclic peptide linkage deserves closer examination. Smith *et al* (1973) have studied the ^{13}C chemical shifts of many amino acids in cyclic peptide hormones and find that the carboxyl carbon of lysine has an average chemical shift of 175.5ppm in cyclic peptides which compares closely with 175.7 and 175.2ppm in sample 2^e. Poly-lysine and lysine carboxyl carbon shifts were 174.6 and 174.7ppm. Thus, the line broadening seen in both the proton and ^{13}C spectra may be attributed to cyclic peptide linkage as can the larger chemical shifts for the carboxyl carbon atoms.

It has been indicated in Section 7.2.5.2 that two slightly different molecules are present in the proportion of 1 to 3. The slight differences in ^{13}C shifts may arise from the presence of two ring sizes differing by 1 lysine unit. A possibility is the minimum of two lysine units, peptide linked to give a cyclic central unit, and a larger molecule of 4 lysine residues. The increased broadening with pD may result from the interaction of the protruding lysine chains as poly(lysine)$_{12}$ is reported to undergo a coil-helix transition at high pH (Smith *et al* 1973).

The IR spectra would also appear to favour a cyclic structure as the absorbance bands associated with peptide formation were shifted to a greater extent than those of a linear poly-lysine.

UV spectra indicated a weak absorbance band at 260nm which was difficult to account for as no aromatic structure was found in the NMR spectra which, if present, would have been obvious. Initially, it was thought that 6-amino-piperidine-2-carboxylic acid (a lysine like compound) may have absorbed in the 260nm region but, as L-pipecolinic acid was found to be transparent in that region, it seemed unlikely. A two lysine cyclic peptide may absorb in that region, however, it seems more likely that an impurity of a small amount of a strongly absorbing compound (e.g. nicotinamide) would have been responsible.

The presence of saccharopine (ε-N-L-glutar-2-yl-L-lysine), an intermediate in the biosynthesis of lysine by *Sacch. cerevisiae* (Jones *et al* 1965), was difficult to discount on the basis of spectral evidence, and if the micro-analytical result is considered, the ratio of 5 carbon atoms to 1 nitrogen correlates better than lysine. Saccharopine has been listed by the Merck Index (1968) as being sparingly soluble in water in

contrast with the ease of solution of the isolated active material. The micro-analysis would be expected to be in error in relation to oxygen and hydrogen analysis since a carefully desiccated sample was found to contain water by IR spectroscopy (Section 7.2.6). The micro-analysis carbon to nitrogen ratio still remain difficult to explain but, as the preparation had a higher degree of purity than commercially available poly-lysine, it may be that the analysis was in error due to the presence of water.

7.4 CONCLUSION

The NMR spectra strongly support the existence of a small cyclic lysine peptide. As partial hydrolysis fragments of poly-lysine were active in the yeast assay but to a lesser extent, it seems that lysine is the active principle of GTF. This finding contrasts with the historic structure of GTF, a chromium-nicotinamide-amino acid coordination complex described in Chapter 1.8.3.

CHAPTER EIGHT

DISCUSSION

The purification of GTF from brewers' yeast described in this thesis resulted in decreasing chromium levels with increasing activity in the adipocyte assay, with the final active product containing a negligible chromium concentration (8×10^{-4}%). The divergence of chromium content from biological activity was not unexpected since the concentration of chromium in reported yeast fractions with activity in the adipocyte assay was exceedingly low (Chapter 1), indicating some other factor may have been responsible for the biological activity. Toepfer *et al* (1977) had found that an increasing ratio of chromium to dry weight of yeast fraction resulting from purification procedures that did not result in the concentration of glucose tolerance factor activity as would be expected if chromium was important. The importance of nicotinic acid has also been questioned in reviewing the structure of GTF (Chapter 1) and it was not found in the final isolated product. The structure of GTF reported in Chapter 7 was that of a small, cyclic lysine peptide, a previously unreported structure in relation of glucose tolerance factor activity. The lysine peptide was found to stimulate the action of a sub-optimal level of insulin on glucose oxidation, to that of a maximum effect. As no stimulation of glucose oxidation was found in the absence of exogenous insulin, the biological activity of the lysine peptide was deemed to be a true GTF effect by the criteria of Anderson *et al* (1978, 1979) and Kienle *et al* (1979).

The active material was isolated from *Sacch. carlsbergensis* grown in a completely synthetic medium with no additions such as bacto-peptone which may have been a source of GTF. Active material was isolated from

yeast grown in a chromium free medium but a higher yield of material was obtained when chromium (2.0ppm) was added to the culture (Chapter 5). The growth of yeast was found to be inhibited by chromium and was thus toxic in agreement with Kumpulainen *et al* (1978) and contrary to the findings of Burkeholder and Mertz (1966), thus the yeast used can be regarded as being chromium sensitive.

Ono and Weng (1982) obtained ten mutant forms of *Sacch. cerevisiae* and genetically analyzed them finding all to be recessive. Among six mutations found in mutants with single mutations responsible for the chromium sensitivity, 6 loci were distinguished, one of them being identified as LYS 7, the gene previously known as the structure gene for homocitric dehydrase which was found to be involved in the detoxification of chromium. It is proposed that the production of the lysine peptide may be associated with a detoxification mechanism that results in the production of higher levels of lysine. It may be that a chromium lysine complex is formed with the chromium initially bound to the ε-NH_3^+ which has been reported by Brill (1977) to participate in metal binding and which has an intrinsic pK of 10.0. As most sample handling involved the use of ammonia (Toepfer *et al* 1977), it could well be that the chromium was lost to cation exchange resin while eluting with ammonia.

An amphoteric material was reported in Chapter 5 from which active material was derived after mild acid hydrolysis and which was more active in the yeast assay than the final product, the lysine peptide. It may be that the crude brown amphoteric material with associated chromium was the original GTF synthesized by the yeast, and that the active cationic material was a low molecular weight fragment of the original. In Chapter 5 (Table 5.2) it was demonstrated that crude anionic material from a synthetic medium culture was also active in the yeast assay and may have been equivalent to the chromium containing

anionic complexes of Kumpulainen *et al* (1978) and Votava *et al* (1973). No activity for such complexes was found in the adipocyte assay thus the different size and charge of the original compound would appear to have been responsible for the division of biological activity between the yeast and adipocyte assays.

As has been reported in Chapter 6 of this thesis, a cobalt-nicotinamide-glutathione preparation was also found to be active in the adipocyte assay but not in the yeast assay developed for screening of GTF containing fractions (Chapter 4). A chromium-glycine complex reported in Chapter 6 was found to be active in both assay systems but not to the extent of the cationic lysine peptide isolated from yeast. All three compounds were found to be basic and the cobalt complex was of similar molecular weight to the chromium complex. It may be that for adipocytes, the similarity of shape and charge of the complexes had resulted in a GTF effect. If the IR "fingerprint" spectrum of the chromium glycine preparation (Chapter 6) is compared with the lysine polymer (Chapter 7), many spectral similarities are seen in the 3 100cm^{-1} region, and the 1 630cm^{-1} to 800cm^{-1} region which would otherwise be unexpected for two very different compounds. Thus it seems that chromium, with its low rate of ligand exchange, has a purely structural function. As the cobalt complex was not found to be active in the yeast assay, there is also the possibility that the mode of action may be different in adipocytes when compared with the lysine peptide. The mode of action of the cobalt GTF was thought to be the reoxidization of NADH which was necessary for the action of insulin at the pyruvate dehydrogenase level in contrast to the chromium complexes of Toepfer *et al* (1977) which were thought to inhibit pyruvate carboxylase (Silio, personal communication 1982, Silio 1975, 1977). No inhibition of pyruvate carboxylase has been found in this laboratory in yeast (Appleby and Holdsworth, to be published).

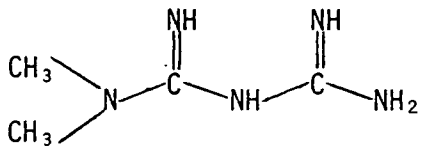
Three very different compounds were thus found to be active in the adipocyte assay. The adipocytes were isolated from rats which were not chromium deficient since the "low chromium" diet supplied by Teklad was found to contain 0.5 - 0.8μg/g Cr (Chapter 2) in comparison with the colony rat diet containing 0.8μg/g Cr. Both levels are in excess of the minimum dietary requirement for rats of 0.1ppm (Mertz and Anderson 1977), thus the rats were not chromium deficient yet GTF activity could only be demonstrated using tissue from the Teklad diet raised rats. It has been found that the rats fed the higher sugar Teklad diet had a lower growth rate than the colony rats and this has been attributed to the quality of available protein as the diet appeared to be adequate in all other respects including chromium (Sherriff, MSc thesis, 1983). The "low" chromium Teklad diet containing torula yeast as the only source of protein was similar to the diet first reported by Mertz and Schwarz (1955) in conjunction with the first indication of impaired glucose tolerance.

It may be that the continual use of a high sugar diet was responsible for the observed effect in the isolated tissue rather than low quality protein. Schwarz and Mertz (1957) have reported impaired glucose tolerance in rats raised on a similar diet but with casein as the source of protein. Before further discussion of the effect of GTF, it is necessary to consider the yeast assay in relation to the adipocyte assay.

An improved yeast assay is reported in Chapter 4 of this thesis. Yeasts have been used as the basis of a microbiological assay of vitamin B6 and its components (Toepfer and Polansky 1970). The possibility that a yeast vitamin may have promoted activity was ruled out by testing the complete vitamin mixture used to grow the yeast (Chapter 4).

The use of ^{14}C-glucose with substrate depleted yeast cells showed that GTF stimulates the anaerobic oxidation of glucose to yield stoichometric quantities of carbon dioxide and ethanol. The effect of GTF on adipocytes was also increased glycolysis but only in the presence of a small amount of insulin, thus a common effect would appear to exist in the different assay systems. Nicotinic acid (0.033mM) was reported to be active in the fat pad assay with the effect reduced considerably when isolated adipocytes were used (Anderson *et al* 1978). Nicotinic acid and also adenosine have been reported to potentiate insulin stimulated glucose oxidation in fat cells via a decrease in c-AMP accumulation (Wieder and Fain 1975, Fain *et al* 1979). N6-methyl adenosine (100μM) was also found to stimulate glucose oxidation by Souness *et al* (1981), thus a wide variety of substances can produce a GTF effect in the adipocyte assay. None of the above substances were found to produce the maximal effect demonstrated with the isolated lysine peptide.

The very basic material isolated can be likened to a group of hypoglycemic agents, the biguanides. They differ in the mechanism of action from the sulphonyl urea hypoglycemic agents which stimulate the secretion of insulin by the pancreas. *In vitro*, anaerobic glycolysis is increased by biguanides in the presence of small amounts of insulin resulting in an accumulation of lactate in mammalian cells which would correspond to an increase in ethanol production in yeast cells. *In vivo*, biguanides inhibit intestinal absorption of glucose. The biguanides do not act as readily in a normal patient compared with a diabetic, presumably because the increase in peripheral glucose utilization is compensated for by an increase in hepatic glucose output (Bowman 1980). Lower plasma levels in triglycerides and cholesterol have also resulted during treatment with biguanides.



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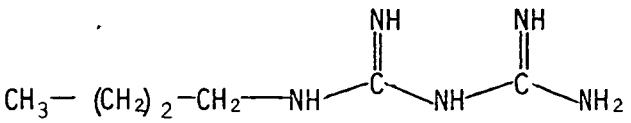

BUFORMIN

FIGURE 8.1 The structure of two biguanide hypoglycemic agents used in the treatment of abnormal glucose tolerance in obese patients.

If the action of pure cationic fractions isolated from brewers' yeast are likened to the effect of biguanides *in vivo*, the glucose tolerance of rats fed on the typical high sugar, "low" chromium diet would be expected to show blood glucose lowering on supplementation with yeast fractions. Thus a non-chromium containing glucose tolerance factor from yeast could be active in a glucose tolerance test. The glucose tolerance test is regarded as the ultimate specific test of glucose tolerance factor (Chapter 1) but one which is technically difficult to perform on rats (Sherriff, MSc thesis, 1983).

The testing of synthetic complexes *in vivo* appears to have been confined to the intestinal absorption of complexes and, as has been indicated in Chapter 1, a high intestinal absorption does not correlate with activity in the adipocyte assay. Chromium tris-acetylacetonate has been reported to have a high intestinal absorption (Anderson *et al* 1974) but no activity in the adipocyte assay (Mertz 1969), a finding that has been confirmed in this thesis. Tuman *et al* (1978) were the only group found who compared the *in vivo* effect of synthetic complexes with yeast complexes by injecting mice with both preparations but without a chromium III control. A small decrease in plasma glucose was found with synthetic preparations. A large number of diverse synthetic preparations have been prepared with structures varying from the pyridoxal nicotinic acid structure of Gonzalez-Vegera (1981) to a group of interesting porphyrin

chromium complexes prepared by Riley (1980) on the basis of their acid stability in the gut. Because of the lack of *in vitro* testing of the various compounds, it is not possible to draw any analogies between yeast GTF or the complex reported in this thesis. Once again, glucose tolerance tests would be the final measure of the value of such complexes as synthetic GTF. Using *in vitro* activity only may have shortcomings as the synthetic preparation of Toepfer *et al* (1977), found to be active in the fat pad assay, was not found to cross the placenta (Mertz and Anderson 1977), a specific requirement of Mertz (1969) for GTF activity.

If a division is made in recent controlled studies between the effect of chromium supplementation and supplementation with high chromium brewers' yeast, two different effects can be postulated. Riales and Albrink (1981) found a slight improvement in glucose tolerance on supplementation with inorganic chromium but a more significant effect on the lowering of low density lipo-protein cholesterol. As one site of cholesterol synthesis is the liver (Harper *et al* 1977), it may be that the liver is a site of action of absorbed chromium. Evidence of the role of liver in chromium metabolism was found by Hamamoto *et al* (1980) who injected rabbits with ^{51}chromium and then isolated a low molecular weight chromium complex from the livers by electrophoresis and by chromatography.

Offenbacher *et al* (1980) studied the beneficial effect of a high chromium brewers' yeast with that of a low chromium yeast on diabetic and non diabetic groups of older Americans. The group receiving the high chromium yeast showed a decrease in blood glucose levels despite lowered or unchanged insulin levels suggesting enhanced insulin sensitivity. The response described is the same as reported for the biguanide hypoglycemic agents. The hypothesis that a hypoglycemic agent can be extracted from yeast in higher quantities in response to toxic levels of chromium is reinforced by the findings of Offenbacher *et al* (1980) who

also suggested that some factor other than chromium may be involved. Gill *et al* (1981) have conducted a similar experiment that supported the findings of Offenbacher *et al* (1980) but used high chromium yeast based cookies as a supplement in comparison with low chromium torula yeast cookies. The conclusion was that the high chromium yeast based cookie decreased the relative amount of insulin required to deal with a given glucose load. Thus, two effects may be distinguished, one of GTF activity and the other, a beneficial effect of chromium as a possible protective agent in coronary heart disease (Riales, 1979).

As has already been indicated in Chapter 1, it has been stated by Anderson (1981) that the form of dietary chromium is not as important as has previously been postulated but that it is the total chromium intake. This casts some doubt on the worth of continuing research into the production of chromium complexes which may have high intestinal absorption, since satisfactory supplementation in humans has been shown with chromium chloride (Jeejeebhoy 1977). Chromium has traditionally been regarded as a toxic element and studies by Okada *et al* (1981) indicate that chromium III stimulates RNA synthesis which may relate to the role of chromium in newborn human babies whose chromium content has been reported to be high, and also the carcinogenicity of chromium. As no reliable measure of chromium deficiency is available (Chapter 1), the question of supplementation with inorganic chromium should be approached with caution.

An interesting area arising from this thesis could be the production of compounds with potential as hypoglycemic agents produced by yeast in presence of chromium.

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