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AN EXAMINATION OF THE PUTATIVE GLUCOSE TOLERANCE FACTOR ACTIVITY OF  
AMINO ACID AND PEPTIDE FRACTIONS ISOLATED FROM BREWER'S YEAST

A THESIS PRESENTED IN PARTIAL FULFILMENT OF THE  
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TIMOTHY GRAHAM JACKSON

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This thesis is dedicated to my aunt, Miss J. Meyer (Q.S.M.) and to my father, Dr G.L. Jackson, without whose support it would never have been completed.

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## Section 1

INTRODUCTION

The first report of the possible existence of a glucose tolerance factor (GTF) was made by Mertz and Schwarz (1955) who noticed that a dietary additive, termed factor 3, isolated from an enzymatic casein hydrolysate (Schwarz (1952)), maintained normal glucose removal rates in diabetic-like rats. These rats were the subject of a study on the development of dietary necrotic liver degeneration. The immediate cause of death, in these rats, could be demonstrated to be severe hypoglycaemia (Mertz and Schwarz (1955)) that initially manifested itself, during the latent period of degeneration, as impairment of excess blood glucose removal. The diet used to induce the development of necrotic liver degeneration was a semi-purified, vitamin E-free, ration of 30% Torula yeast which also represented the sole protein source. The vitamin E prevented the development of necrotic liver degeneration but did not affect the removal of excess blood glucose. In 1957, Schwarz and Mertz reported that the factor 3, in itself, was not responsible for the maintenance of normal glucose removal rates but rather that it contained an active fraction separable by fractionation procedures involving evaporation, in vacuo, of a NaCl-containing, factor 3 concentrate. The NaCl was removed by filtration and the GTF activity was found to be present in the separated salt fraction, from which it could be removed by treatment with 65% ethanol. A further claim was made that this separated substance, now termed the glucose tolerance factor (GTF), not only prevented but cured impairment of glucose removal when administered in the diet and that the initial glucose impairment observed was not a symptom of necrotic liver degeneration but a result of a dietary deficiency. GTF preparations were reported (Mertz and Schwarz (1959)) to be routinely obtained from brewer's yeast as well as acid hydrolysates of dried, defatted, pork kidney powder.

Identification of the active ingredient contained within the GTF preparations was then undertaken, with Schwarz and Mertz (1959) proposing that the active ingredient in the brewer's yeast extract was trivalent chromium. This proposal was initially based on the finding that wet-ashing of the GTF preparations did not remove the ability of the preparations to affect removal rates, indicating the presence of a

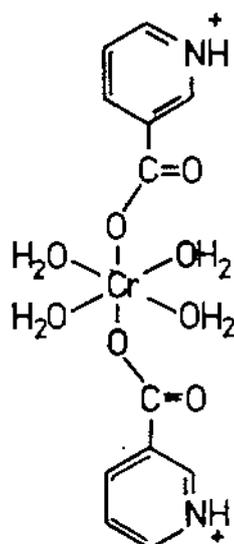
trace element. After a series of screening tests, on various combinations of trace elements components, it appeared that chromium was the common denominator in those combinations that exhibited a GTF-like response when administered as a dietary additive. Tests with various chromium compounds followed, resulting in the identification of Cr(III) compounds as those which, in general, showed effects similar to GTF preparations from brewers yeast (Schwarz and Mertz (1959)). Other methods were also used to attempt to isolate the active fraction from the brewer's yeast extract (Votava et al (1973), Burkenholder and Mertz (1967)). Mertz et al (1974), using their own separation procedure, claimed that their active fraction contained chromium, nicotinic acid, and amino acids and with no experimental evidence available relating to the structure of GTF, Mertz et al proposed that GTF was a cationic Cr(III) complex with 2-axial N-coordinated, nicotinic acid ligands and 4 amino acid ligands, possibly glycine, glutamic acid and cysteine, arranged in an undefined configuration.

Toepfer et al (1977) attempted to prepare a synthetic mixture with GTF-like activity by mixing together a chromium (III) salt with nicotinic acid, glycine, glutamic acid and cysteine, on the assumption that a Cr(III) complex, such as that proposed by Mertz et al (1974), would be formed. The resulting reaction mixture did show GTF-like activity, in their fat pad bioassay system, but nothing could be concluded about the nature of the complex or complexes responsible for the observed activity.

This work was then extended by Cooper et al (1984a,b) who synthesised defined Cr(III) complexes, firstly aquo-nicotinic acid complexes then various aquo-amino acid complexes. In each case the activity of the individual complexes were tested using a yeast bioassay system (Mirsky et al (1980), Haylock et al (1982)). The effect of N-coordination of the nicotinic acid ligands compared with O-coordination was studied along with a comparison of mono- and bidentate complexes of Cr(III) and nicotinic acid (Cooper et al (1984a)).

It was found that only the O-coordinated Cr(III) aquo-dinicotinic acid complex (fig. 1) showed any GTF-like activity.

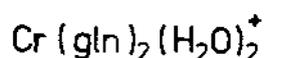
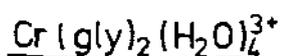
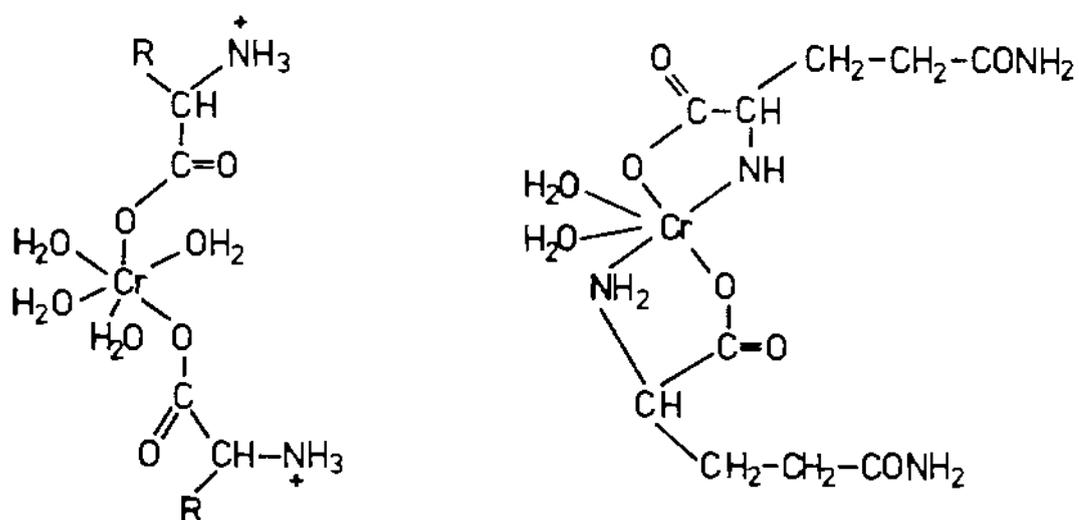
Fig.1.  $\text{Cr(III)(nic)}_2(\text{H}_2\text{O})_4^{3+}$   
 note trans arrangement  
 of ligands.



This was followed with a study on various amino acid complexes with the general structure  $\text{Cr}(\text{amino acid})_2(\text{H}_2\text{O})_2$  where the bidentate amino acid ligands were glycine, glutamic acid and glutamine. Also studied were, a solution of monodentate glycine aquo complexes and Cr-nicotinic acid-glycine and Cr-nicotinic acid-cysteine complexes of undetermined structure (Cooper et al (1984b)). The results showed that only  $\text{Cr}(\text{glutamine})_2(\text{H}_2\text{O})_2^+$ , Cr-nicotinic acid-glycine and the mixture of chromium glycine aquo complexes, showed any significant GTF-like activity in the yeast bioassay. From these results it was hypothesised that a trans arrangement, about the central metal ion, of the non-coordinated nitrogen atoms in the ligands (fig 2) resulted in a structure with the ability to mimic the structure of natural GTF and was responsible for the observed activity.

The actual structure of GTF however, remained undetermined.

Figure 2 Active Cr(III)-amino acid Complexes

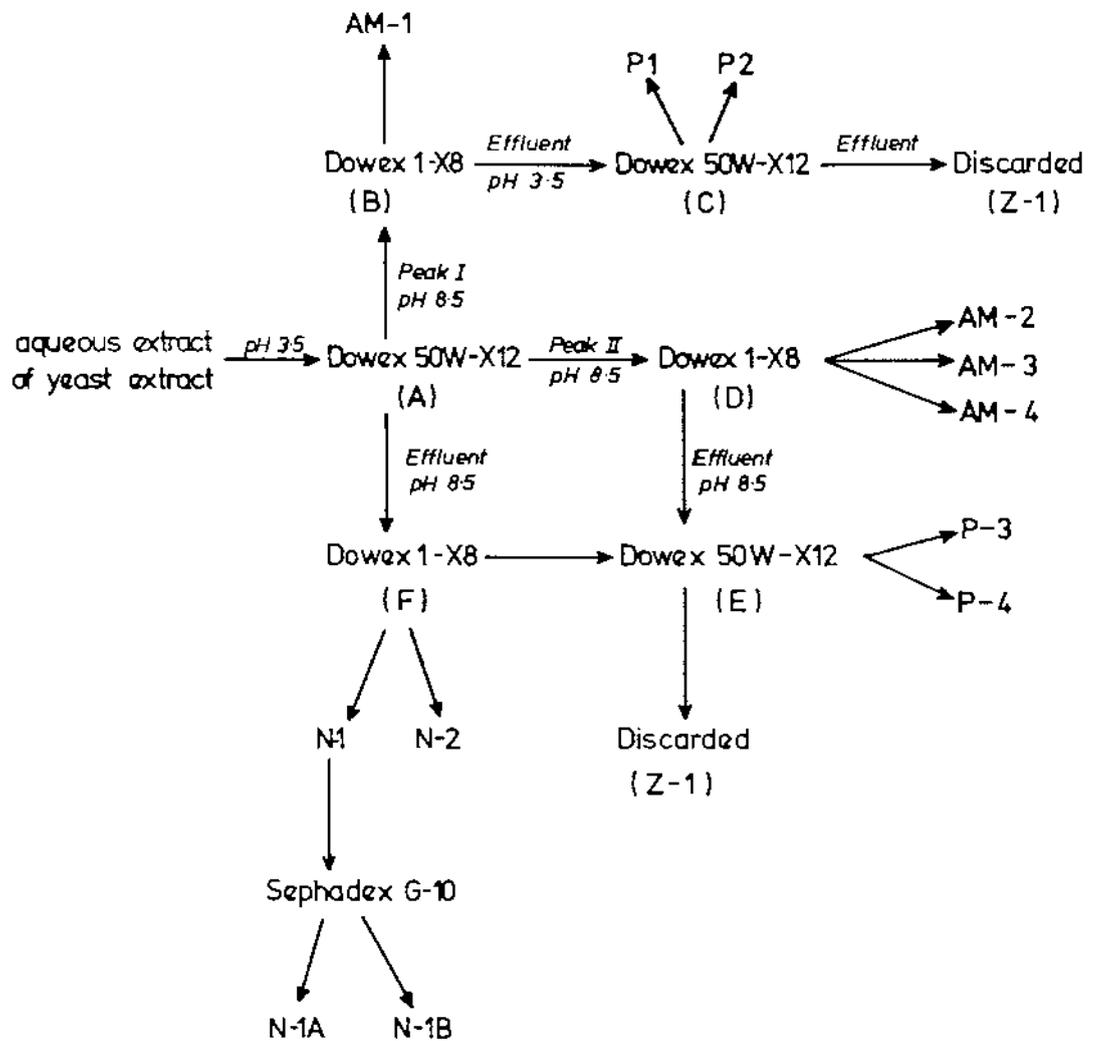


Note: arrangement of non-coordinated nitrogens compared with figure 1.

In an attempt to finally determine the structure of the GTF contained in brewer's yeast, Haylock et al [(1983a)-Diagram 1] subjected an autolysed, high chromium, commercial yeast extract (Merck) to ion-exchange columns, with varying pH gradients, resulting in the separation of 3 anionic, 4 cationic and 4 amphoteric fractions.

Of these separated fractions only the 4 cationic fractions showed any significant GTF-like activity in the yeast bioassay, and, of these biologically active fractions, only the fractions designated P<sub>3</sub> and P<sub>4</sub> (diagram 1) were not attributable to complexing between chromium and the constituents of the growth medium. This was determined (Haylock et al (1983b)) by a comparison between the chromium containing fractions separated from: (i) harvested brewer's yeast cells grown on a medium containing added chromium chloride, (ii) the liquid remainder from the yeast growth in the high chromium medium, (iii) a control solution identical in all respects to the medium used for yeast growth except

Diagram 1: Summary of separation procedure



- Z = Zero charged  
 AM = Amphoteric  
 P = Positive charged  
 N = Negative charged

(E. O'Donoghue, Honours Project (1983), Massey University)

that no yeast was added and (iv) the chromium-containing fractions separated previously from the high chromium commercial yeast extract (Merck).

As only the fractions P<sub>3</sub> and P<sub>4</sub> (Haylock et al (1983b)) were not able to be isolated from the control solution it was decided that these must be a component of the yeast cells and therefore would be the most likely fractions to contain GTF. The fractions P<sub>3</sub> and P<sub>4</sub> were selected to undergo further purification using ion-exchange and gel-filtration chromatography. Only a small percentage of the chromium-containing material and all of the biological activity were retained on the cation-exchange column, for both of the fractions P<sub>3</sub> and P<sub>4</sub>, indicating a separation of biological activity and the chromium-containing material. Structural analysis of the biologically-active fractions followed ultra-violet, and visible absorption and mass spectral analysis, which indicated the presence of tyramine in both of the separated fractions (ie. P<sub>3</sub> and P<sub>4</sub>). However, accurate analysis of the P<sub>3</sub> fraction was precluded as it was not found possible to separate the active material from the phosphate buffer used in the elution of this fraction from the cation-exchange column.

The activity of tyramine was determined by both the yeast bioassay system (Haylock et al (1983b)) and a rat adipocyte assay (E.M. Holdsworth, University of Tasmania, Personal Communication), however the tyramine was not found to be active in either assay system.

The separation of biological activity and the majority of the chromium present in the separated fractions indicated that the activity observed was not due to the presence of chromium but rather to some other factor (Haylock et al (1983b)).

Further work by J. Cooper (unpublished results), based on the procedure of Haylock et al [(1983a)-Diagram 1], resulted in the separation of the active fraction of the Merck yeast extract, using a series of gel-filtration and ion-exchange columns as follows:

1 kg of yeast extract (Merck) was dissolved in water and loaded onto a Dowex 1-X8 column which bound and removed the anionic fraction

(predetermined as being non-active (Haylock et al (1983a)). The filtrate was collected and loaded onto a Dowex 50W-X12 column and washed with water to remove the neutral portion (also predetermined as being inactive (Haylock et al (1983a)). The column, was then washed with a pH gradient generated in situ by the use of 0.6M  $\text{NaH}_2\text{PO}_4$  (1000  $\text{cm}^3$ ) versus 0.2M  $\text{Na}_2\text{HPO}_4$  (1000  $\text{cm}^3$ ) and finally eluted with 0.2M  $\text{Na}_2\text{HPO}_4$  (1000  $\text{cm}^3$ ) until a pH of 9.0 was attained and then with 0.05M  $\text{Na}_3\text{PO}_4$  (1000  $\text{cm}^3$ ) until the pH was 12.0. The biological activity of the resulting cationic samples was determined using a yeast bioassay system (Mirsky et al (1980); Haylock et al (1982)). The fraction showing activity was loaded onto a Dowex 50W-X2 column and eluted using volatile buffers (e.g.  $\text{NH}_4\text{HCO}_3/\text{NH}_4\text{OH}$ .) The biological activity of the fractions was again determined and the active fractions collected, freeze dried and run through a Sephadex G-15 column to remove the salt. The biological activity was again followed and the active portion collected and freeze-dried. The resultant, active, cationic fraction comprised 0.9g of yellowish solid, exhibited high biological activity and was essentially chromium-free. This fraction was then designated as 'yeast GTF' and will be referred to as such for the duration of this work.

The selection of the assay system for determining GTF-like activity, in the samples under study, was primarily based on the requirement for an assay which was simple enough for use in a routine manner to screen large numbers of samples.

The original assay for the presence of GTF was developed by Mertz and Schwarz (1959) and involved the induction of impaired glucose tolerance in rats by the administration of a controlled, vitamin E-free, diet of Torula yeast and sucrose. The test for the presence of GTF in a sample was to orally administer that sample to the rat and determine whether the rate of excess blood sugar removal returned to normal.

Mertz et al (1961) developed a similar assay based again on the induction of impaired glucose tolerance in rats. A piece of rat epididymal adipose tissue, obtained from the diabetic-like rats, was incubated in a carbonate or Krebs-Ringer phosphate buffer, for 2 hours, with insulin, sample and carbon-14 labelled glucose.

The  $^{14}\text{CO}_2$  produced was absorbed in base and measured radiometrically. The biological activity generated by the test sample was determined by comparing the amount of  $^{14}\text{CO}_2$  produced by the sample with that produced by insulin alone. This assay method was modified by Anderson et al (1978) to account for variations between individual pieces of adipose tissue. The epididymal adipose tissue was digested with collagenase and the adipocytes were separated by flotation. Incubation of the adipocytes required the presence of 2% albumin in the Krebs-Ringer phosphate buffer to disperse the washed fat cells.

The two assay methods outlined, while they are possibly the most specific methods available, require the growing and nurturing of the diabetic-like rats, which is an expensive and time consuming process. The rats must be kept on a controlled diet, to induce impaired glucose tolerance, and kept in stainless steel free cages to prevent chromium from being absorbed by either eating or gnawing at the metal. The time required to run the assay means it is difficult for the assay to be used in a routine manner to screen a large number of samples for GTF-like activity.

In this work, the assay system selected was a modification of a standard yeast bioassay system (Mirsky et al (1980)). For this assay, three strains of yeast cells were individually grown for use in the bioassay (i.e. *Saccharomyces carlsbergensis*, *Saccharomyces cerevisiae*, *Saccharomyces ellipsoideus*). The fermentation rates of the yeast cells, in the presence of various test samples, as measured by Warburg manometric techniques (Manometric Techniques 3rd Ed.), were used as the basis for detection of GTF-like activity in those samples. The fermentation rates observed, in the presence of added sample, were compared with the fermentation rates of a control solution which contained no added sample but was identical in all other respects to the test sample solutions (i.e. a sample blank). A modification of this system (Haylock et al (1982)) was a lowering of the cell count in the bioassay system from the original  $3 \times 10^8$  cells/cm<sup>3</sup> to  $1.5 \times 10^8$  cells/cm<sup>3</sup> in order to enhance the effects of any observed GTF-like activity in the test samples. The justification for the use of yeast cells to assay for the presence of a factor responsible for increased glucose removal from blood, was on the basis that as there appeared to be such a high concentration of GTF in yeast, there must be some physiological necessity for it in the cell.

Thus it was originally assumed that external administration of GTF to a GTF deficient cell (i.e. it is assumed that Cr-free means it is GTF-free) in the presence of glucose under anaerobic conditions, should have much the same effect as with blood and tissue cells in animals. If the sample supplied to the yeast cells did not facilitate glucose uptake, as seen by increased CO<sub>2</sub> production rates, then it was assumed that it was also unlikely to affect glucose removal rates from blood in mammals.

At the beginning of this study the hypothesis formulated to account for the results of both Haylock (1983 a,b) and Cooper (1984 a,b) was that the activity of the yeast GTF was due to the presence of a peptide, or possibly an amino acid, with a specific arrangement of nitrogen atoms similar to that observed by Cooper (1984 a,b) in chromium-amino acid and chromium-nicotinic acid complexes (figs 1 and 2). To account for the observed cationic character of the yeast GTF fractions (Haylock et al (1983 a,b)) it was presumed that amino acids with the greatest basic character were present, such as arginine and lysine, in the yeast GTF and also that such amino acids would be most likely to have an orientation of nitrogen atoms similar to that proposed by Cooper (1984b).

The objective of this study was to attempt to isolate a specific peptide or amino acid (or several related amino acids and/or peptides) from the yeast GTF which would account for the total observed activity of the sample.