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

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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 existance 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 maintainance 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

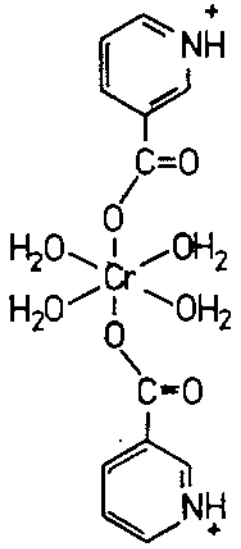
trace element. After a series of screening tests, on various combinations of trace elements components, it appeared that chromium was the common denominator in those combinations that exhibited a GTF-like response when administered as a dietary additive. Tests with various chromium compounds followed, 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.

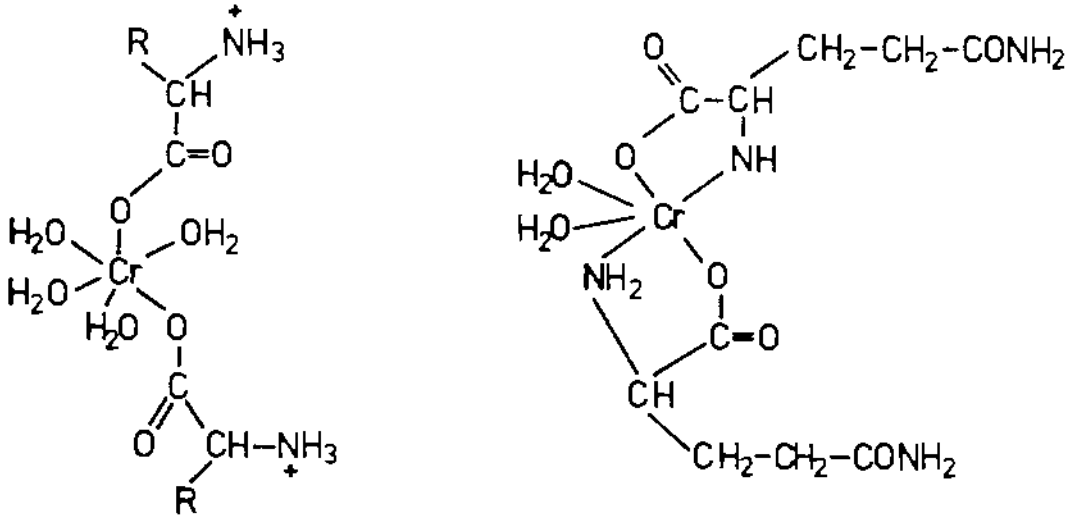
Fig.1. $Cr(III)(nic)_2(H_2O)_4^{3+}$
note trans arrangement
of ligands.



This was followed with a study on various amino acid complexes with the general structure Cr (amino acid)$_2$(H_2O)$_2$ 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 Cr(glutamine)$_2$(H_2O)$_2^+$, 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 acutal structure of GTF however, remained undetermined.

Figure 2 Active Cr(III)-amino acid Complexes



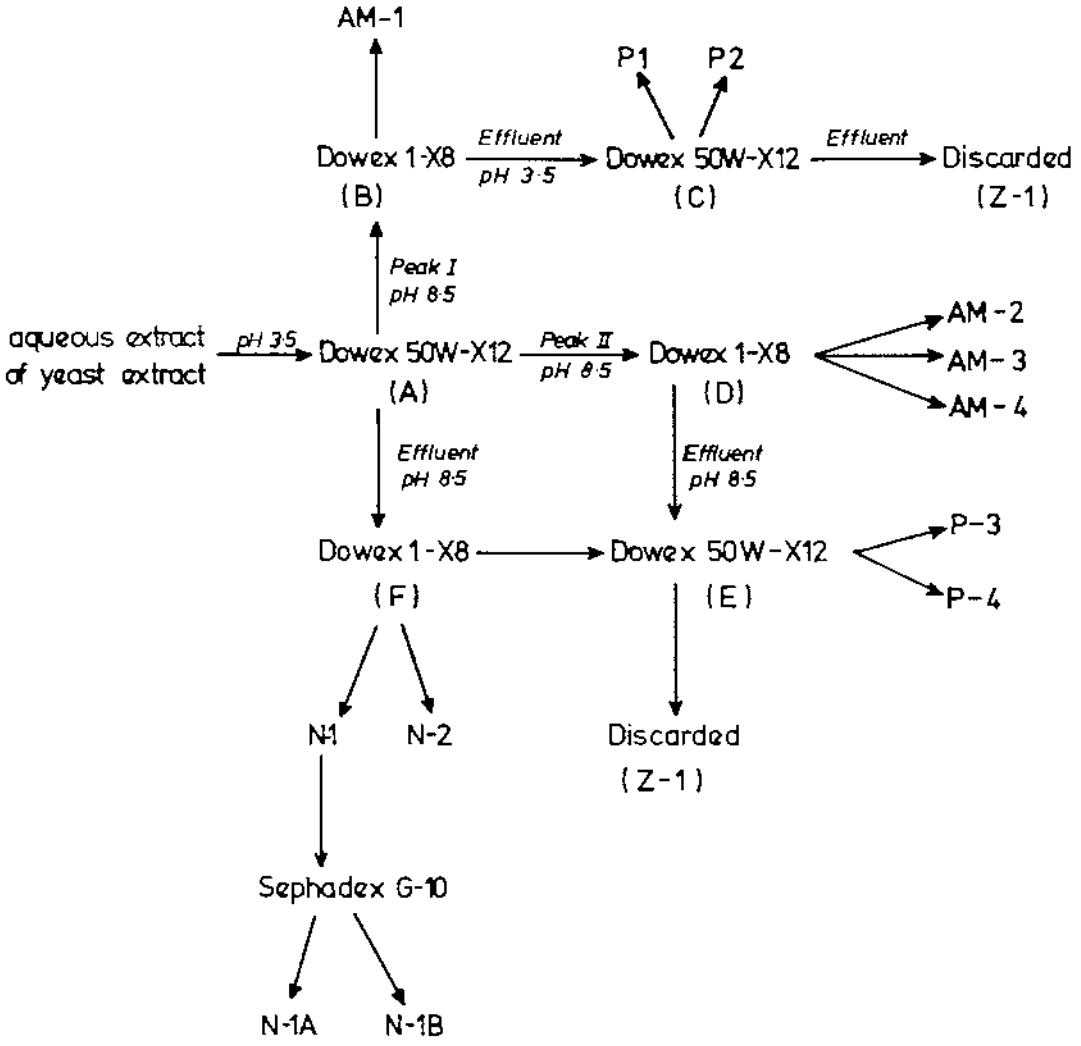
$Cr(gly)_2(H_2O)_4^{3+}$ $Cr(gln)_2(H_2O)_2^{+}$

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_3 and P_4 (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_3 and P_4 (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_3 and P_4 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_3 and P_4, 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_3 and P_4). However, accurate analysis of the P_3 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 NaH_2PO_4 (1000 cm^3) versus 0.2M Na_2HPO_4 (1000 cm^3) and finally eluted with 0.2M Na_2HPO_4 (1000 cm^3) until a pH of 9.0 was attained and then with 0.05M Na_3PO_4 (1000 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. NH_4HCO_3/NH_4OH.) 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 $^{14}CO_2$ produced was absorbed in base and measured radiometrically. The biological activity generated by the test sample was determined by comparing the amount of $^{14}CO_2$ 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 orginal $3x10^8$ cells/cm^3 to $1.5x10^8$ cells/cm^3 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_2 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.

SECTION 2: PURIFICATION, SEPARATION AND IDENTIFICATION OF FRACTIONS FROM THE YEAST GTF SAMPLE

2.1 INTRODUCTION

The methods described in this section are those which were employed in

(i) the purification of the yeast GTF sample and the separation of the peptide and amino acid fractions from the purified yeast GTF and
(ii) the identification of the peptides and amino acids contained in those separated fractions.

These procedures were necessary to supply fractions containing single amino acids and peptides, in order to be able to assay the GTF like peptide and amino acid constituents of the yeast individually for GTF activity.

2.2 PURIFICATION AND SEPARATION PROCEDURES APPLIED TO THE YEAST GTF SAMPLE

2.2.1 Electrophoresis

The yeast GTF sample was initially dissolved in doubly distilled deionised water and streaked in a narrow band, with a pipette, across a sheet of Whatman chromatography paper, with a thickness grading of either 1 MM or 3 MM depending on the amount loaded. The sample was loaded approximately 250 mm from the bottom of the sheet and 50 mm in from each edge. Two standard chromatography markers, 'R' and 'T', containing the free amino acids asp, glu, thr, ser, ile, ala, his, gly, and tyr, phe, met, leu, val, arg, lys respectively, were placed as two individual spots in each of the 50 mm margins. Placed at 50 mm intervals along the length of the sample band was a fluorescent marker, 'F', containing dansyl-arg, dansyl-arg-arg and dansyl-OH. The standard chromatographic markers 'R' and 'T' were applied to allow the movement of the sample bands, on application of current, to be compared with the movement of the free amino acids contained in those markers. The

fluorescent marker 'F', applied along the sample band, moved with the solvent front, thus when the paper was finally placed under ultra-violet light, the general position of the solvent front could be seen from the positions of the fluorescent markers, in this way the general shape of the sample bands could also be determined.

Using a 10 cm^3 pipette, electrophoresis buffer, containing 10% pyridine, 0.4% acetic acid v/v in water at pH 6.5, was applied to the Whatman sheet in such a way that the buffer fronts generated focused onto the sample band, thus saturating the entire sheet while, at the same time, narrowing the sample band further without moving it from the origin. The complete electrophoretogram was then suspended in an electrophoresis tank so that the top and bottom ends of the electrophoretogram were in separate electrolyte solutions, while the centre was contained in a solution of the same electrophoresis buffer used to saturate the Whatman sheet. Also contained in the separate electrolyte solutions were electrodes to enable the applied current to pass over the whole electrophoretogram. As a consequence of the known basic character of the yeast GTF sample, as determined by S. Haylock (1983a), the electrophoretogram was arranged, in the tank, to allow maximum movement of any peptides in the cathodic direction.

The whole electrophoresis system was then enclosed in the tank and a potential of 3 kv applied between the electrodes with an initial current of 100 mA, for approximately 40 minutes. The electrophoretogram was then removed from the tank and allowed to dry. Determination of the band positions was accomplished by cutting off the 50 mm margins plus about 2 mm of the sample containing sheet and staining this with a ninhydrin dip [add 15 cm^3 of (15 g cadmium acetate plus 300 cm^3 HOAc, plus 600 cm^3 H_2O) to 85 cm^3 of (1% ninhydrin in acetone)]. By comparing the positions of the corresponding bands, as shown by the ninhydrin stain, and by using the shape of the solvent front, as shown by the fluorescent 'F' marker, the separate bands could be cut from the Whatman sheet. The mobilities of the sample bands (R_f values) were calculated by comparing the distances run by the sample bands with a basal line as shown by the neutral components of the standard 'R' and 'T' markers. The results could then be expressed as a dimensionless number characteristic for the sample under the electrophoresis conditions employed.

2.2.2 Paper Chromatography - One Dimensional

The selected strips of Whatman chromatography paper removed from the electrophoretogram (section 2.2.1) were first treated with M/50 ammonia so as to focus the contained sample into a narrow strip. This was achieved by applying the M/50 ammonia with a small pipette and allowing the solvent fronts to focus along a predetermined central line. The narrow strip was then cut to a uniform width, approximately 1 cm across and then sewn onto a fresh sheet of Whatman chromatography paper, of identical thickness grading to the sheet used as the initial electrophoretogram, (i.e. 1 MM or 3 MM), approximately 100 mm from the bottom and 50 mm in from each side. The strip of blank Whatman paper behind the sewn sample strip was carefully removed using a razor-blade, leaving a complete chromatogram of uniform thickness. The standard markers 'R' and 'T' and the fluorescent marker 'F', were then applied in the same manner as described previously (section 2.2.1).

A solvent mixture, of butanol, acetic acid and water 5:1:4 (BAW) or of ammonia, isopropanol and water 4:14:1, was used in the descending phase (i.e. using gravity and capillary action to separate the constituents). The chromatogram was firstly allowed to equilibrate for 24 hours in the aqueous phase, in the sealed chromatography tank, to build up the moisture content of the chromatography paper to enhance the degree of separation in the second stage, where the chromatogram was developed with the organic solvent mixture for 24 hours. When the chromatogram was dry the 50 mm margin plus approximately 2 mm of the sample containing sheet were cut off and stained with ninhydrin dip (section 2.2.1) to determine the positions of the bands separated. By using this information and from the position of the solvent front, as determined by the fluorescent 'F' marker, the positions and shapes of the separated sample bands could be determined. Mobilities (R_f values) could then be determined by using the method described previously (section 2.2.1).

2.2.3 Paper Chromatography - Two Dimensional

Chromatography in the 2nd dimension proceded following electrophoresis of the yeast GTF sample (section 2.2.1) using a 5 cm sample band length,

from which three sections were arbitarily selected and subjected, individually, to chromatography in the first dimension, using the BAW solvent (section 2.2.2). The resultant sample bands were completely removed from the chromatogram, divided into grid form, and sewn as origins onto separate sheets of fresh Whatman chromatography paper, (Diagram 3), resulting in chromatograms of uniform thickness.

These chromatograms were equilibrated for 24 hours in the aqueous phase of a 28% phenol/water solvent, to build up the whatman sheets moisture content. This enhanced separation in the second stage where the chromatograms were developed with the organic phase of the solvent mixture for 20 hours, in the descending phase, then allowed to dry.

To align the separated peptides over a comparative background the chromatogram was divided into a grid pattern using the origin as the basal horizontal line (Diagram 3). On application of the ninhydrin stain a chromatographic map was revealed in which the principle peptide spots appeared in their separate positions (Diagram 3, section 3).

2.2.4 High Performance Liquid Chromatography (HPLC)

The top strips from five separate electrophoretograms (section 2.2.1) were cut from the sheets and subjected independantly to one-dimensional paper chromatography (section 2.2.2) in the BAW solvent system. The resultant bands were cut from the separate chromatograms and the peptides eluted off the paper with M/50 ammonia, bulked together and freeze-dried. HPLC was carried out on each of the separated samples using an R.C 18 column. Each sample was dissolved in 500 ul of H_2O, centrifuged for 5 minutes to remove any solids present and then half of the sample (250 ul) was loaded onto the column. A linear (1-100%) solvent gradient was used, starting with solvent A (0.1M ammonium bicarbonate) changing to solvent B (0.1M ammonium bicarbonate/isopropanol/acetonitrite (1:1:1)), over a 2 hour period at a flow rate of 1 cm^3/minute. The various fractions were collected from each sample on the basis of their absorbance at 230 nm, and then were frozen until needed for further analysis.

2.2.5 Ion-Exchange Separation

Ion-exchange separation was carried out using a sulphonated polystyrene Dowex-50 ion-exchange column (0.9 cm x 50 cm, 22 micron spheres) similar to the resin used in an amino acid analyser. 100 mg of the yeast GTF was loaded onto the column in 1 cm^3 acetic acid (pH 2.0) and washed into the column also with acetic acid ((pH 2.0); 2 x 0.5 cm^3) using N_2 at 5 psi. The eluting solvent mixture was applied as a linear gradient and consisted of: 350 cm^3 pyridine acetate at pH 3.1 (16.13 cm^3 (0.2M) pyridine plus 279 cm^3 acetic acid per litre) and 350 cm^3 pyridine acetate at pH 5.4 (80 cm^3 (2M) pyridine plus 71.63 cm^3 acetic acid per litre). The temperature used was 50^oC and the flow rate of the solvent (20 cm^3/hour) was controlled by a Beckman accuflow pump. The fractions which separated were collected at 20 minute intervals.

Column resolution was assessed by the use of electrophoretic mapping. Aliquots (200 ul) were obtained from each fraction, dried under vacuum over P_2O_5, and taken up in 10 ul of M/50 ammonia. Each sample was then streaked onto a 1 cm section of the origin of an electrophoretogram using 1 MM Whatman chromatography paper (section 2.2.1). Electrophoresis was carried out via the method described previously (section 2.2.1) and, on application of ninhydrin stain, an electrophoretic map was obtained showing which of the separated fractions contained peptides and how well they had separated.

The remainder of the fractions obtained were placed in a dessicator and dried under vacuum over P_2O_5 and NaOH pellets. The dried fractions were then taken up in H_2O (2 x 100 ul).

On the basis of the electrophoretic mapping results, selected fractions were removed from their tubes and streaked across a 15 cm portion of the origin of an electrophoretogram using 2 MM Whatman chromatography paper. Therefore on each electrophoretogram 2 selected fractions were placed, as well as the electrophoresis markers ('R', 'T' and 'F') and electrophoresis buffer (section 2.2.1). The separate bands obtained were cut from the electrophoretograms and the contained peptides eluted off with M/50 ammonia, freeze-dried to remove any pyridine remaining from the

2.3 IDENTIFICATION PROCEDURES FOR THE FRACTIONS SEPARATED FROM THE YEAST GTF SAMPLE

2.3.1 Amino Acid Analysis

The amino acid analyser used was a Beckman 119 BL Amino Acid Analyser operated by Mr J. Reid. The samples for analysis (10 ul) were placed in special hydrolysis tubes (which could be easily sealed under vacuum) and dried over P_2O_5 under vacuum in a dessicator, to remove the original M/50 ammonia solvent (sections 2.2.4, 2.2.5). A solution of HCl (50 ul; 6M) and phenol (0.05 cm^3 of a 0.1% solution) was added and the tubes sealed under vacuum prior to heating for 16 hours at 100°C. The acid present cleaved the peptide bonds while the phenol protected liberated tyrosine from any oxidising agents present (e.g. Cl_2) by preferentially reacting with them. The tube contents were then dried, under vacuum in a dessicator over P_2O_5, and loaded onto a Beckman W-2 cation-exchange column in a sodium citrate loading buffer (pH 3.25). The ion-exchange column separated the mixture of amino acids and then the effluent from the column was mixed with ninhydrin reagent [1 l of 4M NaOAc buffer plus 3 l methyl cellosolve, add 80 g ninhydrin, filter, and add $SnCl_2$].

The colour that developed in this mixture was detected colorimetrically at 570 nm and 440 nm. The results obtained were recorded graphically on an automatic recorder and the amino acids present identified by their characteristic positions on the chart paper. To obtain quantitative information about the constituent amino acids, the area under the characteristic curve for each amino acid was calculated (height x width at half height) and this figure divided by the standard conversion factor 'C'. This standard conversion factor was calculated from an analysis of a free amino acid solution of known concentration. [The conversion factor was found by calculating the ratio of the area under the standard curve, for the amino acids, to the nanomoles of the amino acids present.] Thus the concentration in nanomoles of the sample amino acids could be calculated.

2.3.2 N-terminal Analysis

N-terminal analysis was carried out using the fluorescent marker dansyl-chloride which specifically reacts with, and attaches to, the N-terminal end of a peptide chain. 2M $NaHCO_3$ (10 ul) and a solution of 2.5 mg/cm^3 dansyl chloride (10 ul) were placed in a 5 mm diameter test tube along with the test sample (1 ul) and heated for 30 minutes at 45^{o}C. During this period the N-terminal amino acid was tagged with the fluorescent marker. The tube contents were then dried, taken up with 6M HCl (50 ul), sealed under vacuum and placed in a 100^{o}C oven for 16 hours. This served to sever the dansylated N-terminal amino acid from the peptide chain. The sample was again dried and then taken up with glass distilled 95% ethanol.

This sample solution was then applied to both sides of a polyamide plate 2 cm in from one corner. On only one side of this plate a mixture of dansylated amino acids (1 ul) was applied on the same spot as the sample solution. These dansylated amino acids acted as spatial markers for comparison with the dansylated ex-N-terminal amino acid(s) present in the sample solution. The plate was then run in two dimensions in a series of solvents to gain the maximum separation possible of the dansylated ex-N-terminal amino acid(s) and spatial markers. The solvents used were:

Solvent 1 (1.5% v/v) formic acid (12 minutes)
2 (9:1 v/v) toluene/acetic acid (11 minutes)
3 (20:1:1 by vol.) ethyl acetate/methanol/acetic acid (13 minutes)

After running the sample in these solvents, separation was checked via ultra-violet light. If it was found that arginine, lysine and/or histidine were present as the dansylated ex-N-terminal amino acid(s) then solvents 4 and 5 were used to gain separation between these.

Solvent 4 (3:1 v/v) pyridine acetate/ethanol
5 (1:1) 1M ammonia/95% ethanol

After the sample had been run in the solvent series, the solution of dansylated amino acids, acting as spatial markers, moved to positions on the polyamide plate characteristic of each amino acid and were detectable by ultra-violet light. The cleaved dansylated ex-N-terminal amino acid(s) could then be compared with the spatial markers and be identified.

Section 3 RESULTS AND DISCUSSION

Electrophoresis was used as a method of obtaining some separation of the charged constituents of the yeast GTF mixture. Electrophoresis is defined as the migration of particles under the influence of an electric field where they separate on the basis of charge and, to a lesser extent, molecular size. The charge determines the direction of movement of the particles, either cathodically or anodically, and the size of the particles determines the distance they will move. The peptides present in the yeast GTF were found to have moved exclusively in the cathodic direction which indicated that the overall charge of the material, at the pH 6.5 used in the elctrophoresis procedure (section 2.2.1), was positive and confirmed the basic character of the sample (S. Haylock Ph.D. Thesis (1981), Massey University).

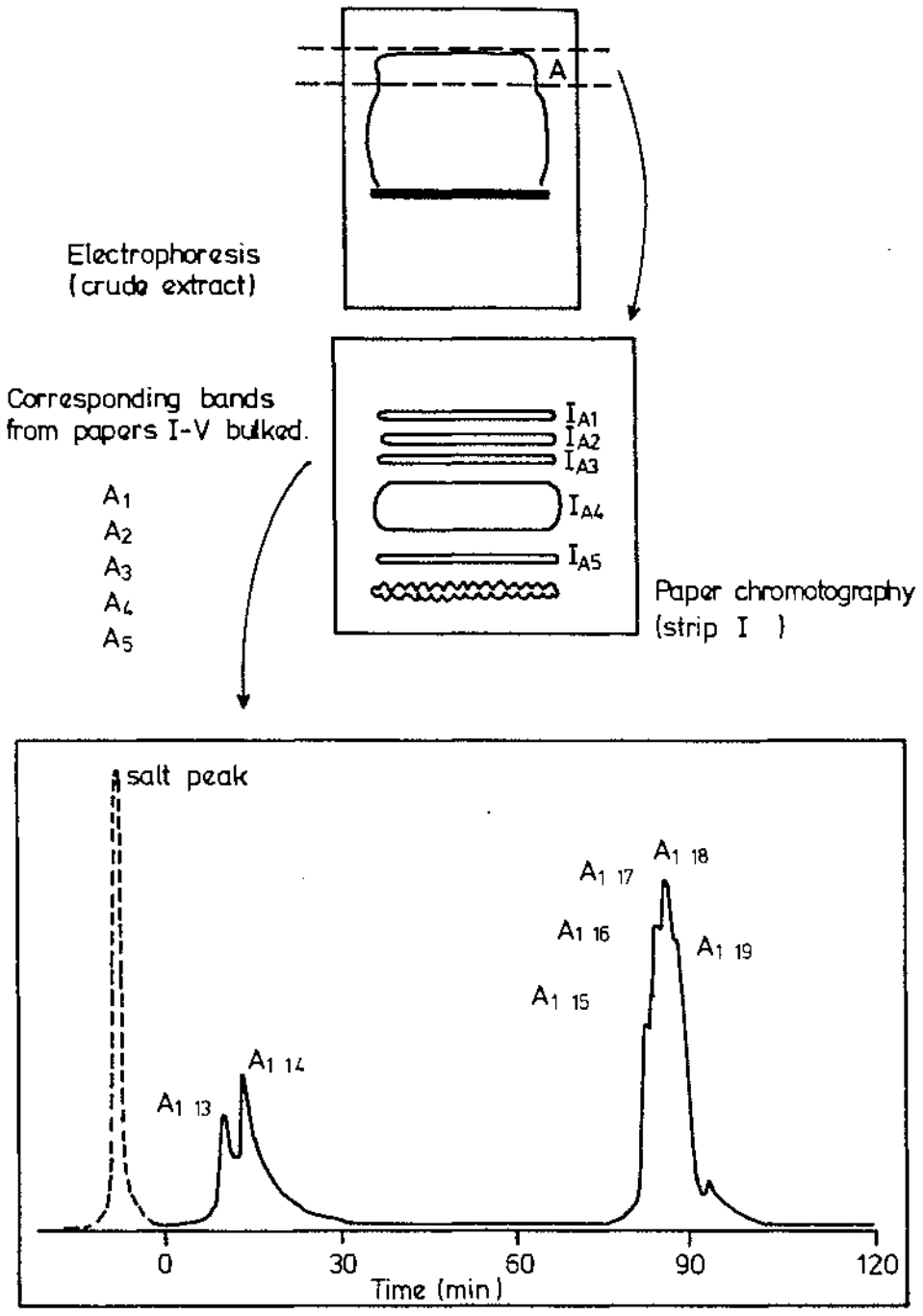
Five electrophoretograms were run on the yeast GTF sample (50 mg/0.2 cm^3 H_2O per run) and the corresponding top strips were cut from the separate electrophoretograms and labelled 'A' with the superscripts I-V denoting from which of the five electrophoretograms the strips originated.

This was followed by one-dimensional paper chromatography (section 2.2.2) to separate the constituents of the yeast GTF contained in he top strips ('A') of the electrophoretograms. The solvent system used to separate the constituents was the BAW solvent, as it was found that the ammonia/isopropanol/water (4:14:1) solvent did not move the yeast GTF from the baseline.

Each of the electrophoretic strips ('A') were found, after paper chromatography, to have separated into five clearly defined bands. These bands were labelled, as subscripts, from 1-5, with the top band denoted as 1 and the band closest to the origin as 5. (Diagram 2).

The R_f mobility values of each of the bands are shown in table 1.

Diagram 2 Flow Chart of Electrophoresis and HPLC Separation Procedures



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

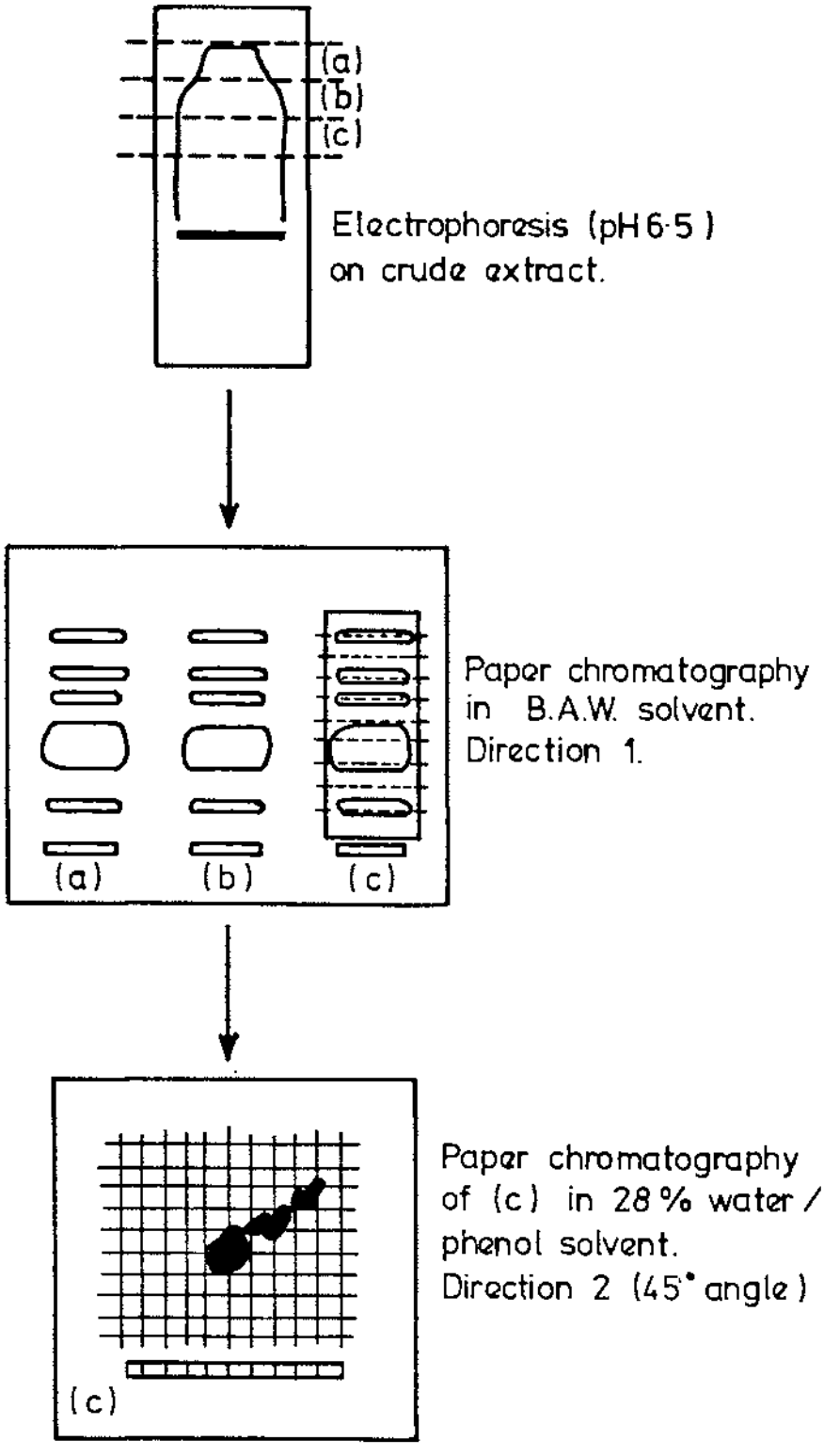
TABLE I

R_f values for initial Electrophoresis Separation

Separated bands	R_f	Separated bands	R_f
$^{I}A_1$	0.415	$^{II}A_1$	0.551
$^{I}A_2$	0.351	$^{II}A_2$	0.432
$^{I}A_3$	0.248	$^{II}A_3$	0.329
$^{I}A_4$	0.198	$^{II}A_4$	0.25
$^{I}A_5$	0.081	$^{II}A_5$	0.082
$^{III}A_1$	0.523	$^{IV}A_1$	0.479
$^{III}A_2$	0.432	$^{IV}A_2$	0.338
$^{III}A_3$	0.332	$^{IV}A_3$	0.306
$^{III}A_4$	0.249	$^{IV}A_4$	0.242
$^{III}A_5$	0.082	$^{IV}A_5$	0.084
$^{V}A_1$	0.429		
$^{V}A_2$	0.337		
$^{V}A_3$	0.255		
$^{V}A_4$	0.195		
$^{V}A_5$	0.084		

As the separated sample bands were clearly defined it was decided to bulk the corresponding bands together, following elution from the paper with M/50 ammonia. This procedure resulted in five samples ready for further separation by HPLC. The samples were designated $^{I\text{-}V}A_1$, $^{I\text{-}V}A_2$, $^{I\text{-}V}A_3$, $^{I\text{-}V}A_4$, $^{I\text{-}V}A_5$. In an attempt to achieve further separation via chromatography, prior to HPLC, two dimensional paper chromatography was used (section 2.2.3). Chromatography in the first dimension, of three

Diagram 3 Flow Chart of One Dimensional and Two Dimensional Paper Chromatography Separation Procedures



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

electrophoretic strips resulted in the same five bands being separated as were obtained previously. On running the strips in the second dimension, in 28% phenol/water solvent, it was found that in all cases, after application of ninhydrin stain, the peptide spots had aligned themselves on a 45^{o} diagonal across the grid pattern (Diagram 3). This result indicated that a greater degree of separation had not been achieved with the second solvent system.

The bulked samples eluted from the one-dimensional chromatogram were then loaded onto the HPLC column (section 2.2.4). The resultant separated fractions were labelled 'A', the superscripts being dropped, with the first subscript denoting the band number from the chromatogram and the second denoting the number of the tube used to collect the fraction from the HPLC column (Diagram 2).

The selection of the fractions to undergo amino acid analysis depended on the resolution of the peak obtained, on the HPLC traces. The position of elution along the solvent gradient was used to select the most basic fractions as, at this time, it was thought that GTF was most probably a peptide of basic character (section 1).

The selected separated fractions and the amino acids present in them are shown in table II. The amino acid analysis (section 2.3.1) indicated numerous amino acids present, up to six in fractions $A_{2\ 8}$ and $A_{2\ 9}$, and all fractions showed a high degree of impurity as shown by the presence of trace amounts of other amino acids.

To test whether the HPLC had separated an individual peptide in any of the selected fractions, N-terminal analysis of the fractions was carried out (section 2.3.2). Analysis of the selected HPLC fractions indicated a mixture of N-terminal amino acids to be present (table III) with five being present in one fraction ($A_{2\ 9}$) along with contamination by other, less abundant, amino acids.

TABLE II

Amino Acids present in HPLC separated Fractions

Fraction	Amino acids present
A_1 14	lys ile leu
A_1 13	lys arg val ile
A_2 8	lys arg gly val leu ile
A_2 9	lys arg pro gly val ile
A_4 26	lys arg gly pro val

TABLE III

Major N-terminals present in HPLC Fractions

Fraction	Major N-terminals
A_1 14	lys leu
A_1 13	lys leu
A_2 8	lys ile
A_2 9	lys val gly pro ile leu
A_4 26	lys val gly

This information, coupled with that obtained from the amino acid analysis of these fractions (table II), indicated that a mixture of peptides was present in each fraction as, at best, there were 2 N-terminals present in a fraction containing 2 amino acids ($A_{1\ 14}$; $A_{1\ 13}$). From this evidence it was apparent that the HPLC had not achieved sufficient separation to enable further peptide identification to be carried out.

Due to the failure of the HPLC to separate the constituent peptides of the yeast GTF satisfactorily, another method was required to obtain fractions containing peptides pure enough for further analysis. It was decided to try ion-exchange separation (section 2.2.5) as a method to obtain fractions containing single peptides and, in all, 99 separate fractions were collected from the ion-exchange column. In assessing the resolution of the column via electrophoretic mapping it was found that each electrophoretogram could accommodate approximately 30 separate, 1 cm, samples. Thus 4 electrophoretograms were needed to map all 99 separate fractions. Electrophoresis was carried out under the usual conditions (section 2.2.1) and the resultant electrophoretic map indicated high column resolution with, at most, 3 separate, distinguishable, spots per fraction being observed.

From this result it was decided to use large scale electrophoresis to separate the fractions obtained from the ion-exchange column into their peptide constituents.

Selection of the fractions to undergo further purification and analysis was made on the basis of the electrophoretic map as this indicated which of the fractions contained peptides, all those that did not could be discounted and were freeze-dried and stored at $0^{o}C$.

All of the remaining fractions were analysed for their constituent amino acids (section 2.3.1), their R_f values recorded and from the amino acid analysis trace, the micromoles, and hence the number of grams of peptide in each fraction was calculated (table IV). Peptides obtained from the same fraction, from the ion-exchange column, and separated by electrophoresis were denoted by 'a', 'b' and 'c', where 'a' denoted the peptide farthest from the line of origin and 'c' the peptide closest to

TABLE IV

Ion Exchange Separated Fractions Selected for Bioassay Analysis

Fraction	R_f	ug/200 ul	Major Amino Acids
36a	0.89	187.4	lys
49a	0.84	383	(3-4)lys,pro,gly
49b	0.72	218	lys,pro
50b	0.58	323	2lys,thr
52a	0.85	1254.6	arg
52b	0.68	702	arg
63a	0.65	614.2	lys,leu
63b	0.43	446	2lys,glu,ile
66a	0.64	384.2	2arg,asp
67a	0.54	586.8	2arg,thr
67b	0.44	707.8	2arg,asp
68	0.53	1163.2	2arg,thr
69a	0.55	147.3	arg,ser
69b	0.43	173	lys,arg,asp,glu,val
70a	0.75	233.3	arg,gln
70b	0.54	183.8	lys,asn
71a	0.73	122.5	arg,gly
71b	0.62	128.5	2arg,ser
72a	0.55	483	arg,thr
73a	0.58	398.5	arg,ser
74a	0.54	147.8	ser (some arg)
75	0.48	52	2lys,glu
76/77	0.64	284.5	arg,pro
83	0.69	9.15	lys
84	0.48	123.4	2lys,glu
85a	0.67	117.5	arg,gly
85b	0.61	138.8	arg,val
85c	0.48	101.8	2lys,glu
86b	0.77	152.3	arg,val

the line of origin. The numbers denote the number of the collection tube the fractions were obtained from.

Certain of the fractions analysed by amino acid analysis (table IV) were selected to undergo sequencing, to determine the amino acid sequence of the contained peptide, by the Dansyl-Edman method (E.M. O'Donoghue, Honours Project (1983), Massey University). The fractions selected, and the sequences obtained, are shown in table V. The selection of these fractions was on the basis of amino acid content and the amount of sample present in the fraction, as it was necessary to retain enough sample for further analysis should this be required.

The fractions shown in tables V and VI were those selected to undergo further analysis with the yeast bioassay system. This selection was made on the basis of the fractions basicity (i.e. fractions containing lysine or arginine peptides) as at this time it was envisaged that if the GTF was an amino acid or a peptide it would most likely be basic due to the basic character of the yeast GTF sample and to the availability of nitrogen to arrange in a manner similar to that observed by Cooper [1984b - (figs 1 and 2)].

The sequencing of the fractions (table V), the mole ratio data (table IV) and the electrophoretic mobilities (R_f - table IV) allowed peptide molecular weights to be calculated (E.M. O'Donoghue - Honours Project (1983), Massey University) using an Offord mobility versus Molecular Weight graph (Offord 1966). In this procedure probable charge on the peptide was estimated and the di- or tri- peptide character of the sample confirmed. From the charge on the peptide it was possible to distinguish between glutamic acid and glutamine and between aspartic acid and aspargine.

TABLE V

Peptide Sequences of Selected Ion-Exchange Separated Fractions

Fraction	Sequence
72a	thr-arg
73a	ser-arg
76/77	arg-pro/ala-arg
84	lys-glu-lys
85c	lys-glu-lys
85b	arg-val/val-arg
85a	arg-gly
86b	arg-val/val-arg

TABLE VI

Ion-Exchange Separated Fractions Selected for Bioassay Analysis

Fraction	Amino Acid Content
36a	lys
49a	(3-4)lys,pro,gly
49b	lys,pro
50b	2lys,thr
52a	arg
63a	lys,leu
70a	arg,gln
70b	lys,asn

[Note: the fractions shown in both table V and table VI were assayed in the yeast bioassay system.]

Section 4 SUMMARY

It has been shown (section 3) that it was not possible to obtain sufficient separation of the peptides contained in the yeast GTF by HPLC. It was possible to identify their constituent amino acids but their order and distribution were unobtainable due to the impurity of the fraction and the absence of any well defined amino acid ratios.

With the ion-exchange separation scheme it was found to be possible to identify a limited number of peptides per fraction and, in some cases, the structures of these peptides were also confirmed. It was therefore decided that the fractions separated by ion-exchange chromatography would be subjected to further analysis by the yeast bioassay system in an attempt to determine if any of those fractions contained GTF-like activity.

Section 5 YEAST BIOASSAYS

5.1 INTRODUCTION

The determination of which of the peptide fractions separated from the yeast GTF sample (section 3) actually contained GTF-like activity was achieved via the use of a standard yeast bioassay system (Mirsky et al (1980), Haylock et al (1982)).

The yeast bioassay system theoretically determines GTF-like activity via the detection of increased glucose utilisation rates as seen by increased CO_2 production by a standard number of contained yeast cells. In this way it was possible to determine which of the peptide fractions would be most likely to contribute to the maintainance of normal glucose removal rates as observed by Schwarz and Mertz (1957).

5.2 METHODS

5.2.1 Yeast Growth and Harvesting

The yeast strain, S.ellipsoideus (Dr Zvi Dori, Technion Institute, Haifa, Israel), used in the bioassay system was grown in three successive, chromium free, growth media to ensure chromium deficiency (J. Cooper, MSc Thesis (1982), Massey University). The media used was prepared by the addition of Yeast Nitrogen base (2.0 g) without amino acids (Difco) and glucose monohydrate (6.0 g) to 300 cm^3 of doubly distilled deionised water. The solution was then placed in three separate conical flasks, numbered 1 to 3, with 100 cm^3 in each, and sterilised in a pressure cooker for 10 minutes at 15 lbs/in^2.

Once sterilised, the solutions were allowed to stand and cool to room temperature prior to innoculation. Innoculation was via the transferral of a single cell colony from an agar storage plate to growth medium number 1, with an innoculating loop using aseptic transferral techniques. This yeast containing medium was then placed in a temperature controlled environment (30°C) for approximately 24 hours. 0.1 cm^3 of this

solution was then transferred into growth medium number 2 using a sterile pipette (from a stock of sterile pipettes kept in an oven at 100°C), and aseptic transferral techniques. This solution was then placed in the temperature controlled environment (20°C) for 24 hours and the process was repeated for the third growth of yeast culture. The final growth of the yeast colony was in a defined medium consisting of 10 cm^3 of essential elements (table VII) placed in 90 cm^3 of doubly distilled deionised water with 2.0 g glucose monohydrate. This solution was then sterilized for 10 minutes in a pressure cooker at 15 lbs/in^2. The defined medium was then allowed to cool to room temperature prior to the addition of 0.1 cm^3 of essential vitamins (table VII) and 0.1 cm^3 of the yeast growth medium number 3, again using sterile pipettes and aseptic transferral techniques. This defined medium was then placed in the 30°C temperature controlled environment for approximately 18 hours.

TABLE VII

Essential Elements and Vitamins for Yeast Cell Growth

Essential elements:	$CaCl_2.6H_2O$ (0.14 g l^{-1}), $(NH_4)_2SO_4$ (5.0 g l^{-1}), $MgSO_4.7H_2O$ (0.5 g l^{-1}), NaCl (0.1 g l^{-1}), KH_2PO_4 (0.875 g l^{-1}) and K_2HPO_4 (0.125 g l^{-1}) (in doubly distilled deionised water, sterilized at 15 lbs/in^2 for 10 minutes)
Essential vitamins:	thiamine hydrochloride (1.25 mg l^{-1}), vitamin B_{12} (1.25 mg l^{-1}), calcium panthotherate (1.25 mg l^{-1}), nicotinic acid (1.25 mg l^{-1}), pyridoxal phosphate (0.25 mg l^{-1}), para-aminobenzoic acid (0.25 mg l^{-1}), inostol (0.25 mg l^{-1}), folic acid (0.025 mg l^{-1}) and biotin (0.20 mg l^{-1}).

(J. Cooper, MSc Thesis (1982), Massey University)

The yeast colony was harvested in its logarithmic growth phase to avoid a buildup of dead cells that could adversely affect the bioassay system.

Harvesting of the yeast cells was achieved via the use of a Sorval RC-5B centrifuge at 4000 rpm for 3 periods of 6 minutes each. After each centrifugation the supernatant was discarded and the yeast cells were washed with doubly distilled deionised water to remove any of the growth medium adhering to the yeast cells. The resulting yeast pellet was taken up in 50 cm^3 of phosphate buffer (pH 5.75) and stored in a fridge. The composition of the phosphate buffer used was: 1.7 g KH_2PO_4 in 200 cm^3 H_2O and 1.08 g K_2HPO_4 in 100 cm^3 H_2O, with the K_2HPO_4 solution being added to the 200 cm^3 KH_2PO_4 until the pH reached 5.75 (0.062 M).

The cell count was determined by measuring the turbidity of the solution by absorbance measurements at 540 nm on a SP500 spectrophotometer. A standard curve of absorbance versus cell count (J. Cooper MSc Thesis (1982) Massey University) was used to calculate the number of cells per cm^3 in the solution. The solution was diluted with the phosphate buffer (pH 5.75) to give a cell count of 1.5×10^8 yeast cells per cm^3 for use in the bioassay.

5.2.2 Calculations

The determination of the increase in the rate of CO_2 production via a fermentation process, as an indication of the presence, or absence, of GTF-like activity was achieved in the following manner:

The volume of CO_2 produced by the samples was measured, at 30°C, using calibrated manometers. This was then converted into micromoles CO_2 produced via the standard Warburg manometric equation (Manometric Techniques 3rd Ed.)

$$\text{moles } CO_2 = H \times \frac{[[(V_f+V_m)-V_s] \times \frac{273}{303} + (V_s \times S_{CO_2})]}{22.4}$$

where H = corrected manometer height $((h_t-h_o) - (T_t-T_o))$
V_f = volume of Warburg flask (cm^3)
V_m = volume of manometer (cm^3)
V_s = volume of test solution (2.5 cm^3)
S_{CO_2} = solubility of CO_2 at 30°C (0.665 cm^3)

The rates of CO_2 production were calculated from the slope of a plot of micromoles CO_2 produced versus time (in minutes) taken over the last 140 minutes of a total bioassay time of 300 minutes. The activity of a sample is reported as the percentage enhancement of CO_2 production, by the sample to be assayed in the yeast bioassay, compared to a sample blank rate (i.e. CO_2 production rate by an equivalent number of yeast cells in the presence of only glucose monohydrate). The percentage enhancement of the test samples compared with the blank rate is calculated thus:

$$\% \text{ Enhancement} = \frac{\text{umoles } CO_2/\text{min(sample)} - \text{umoles } CO_2/\text{min(blank)}}{\text{u moles } CO_2/\text{min(blank)}} \times \frac{100}{1}$$

5.2.3 The Yeast Bioassay

The standard bioassay technique involves the detection of variations in the rate of CO_2 production by a set number of chromium deficient yeast cells, anaerobically, at 30°C on the addition of test samples as compared with a blank rate of CO_2 production (i.e. no sample present). The variations were measured using Warburg manometric techniques.

To the base of each Warburg flask was added 2 cm^3 of yeast solution (1.5 x 10^8 cells/cm^3) in pH 5.75 phosphate buffer (section 5.2.1). Placed in the Warburg flask sidearm, separate from the yeast solution, was 0.4 cm^3 of a 2% glucose monohydrate solution and 0.1 cm^3 of the test sample (in doubly distilled deionised water). All additions were made using sterile pipettes. The blank solution consisted of 0.1 cm^3 of phosphate buffer (pH 5.75) being added to the Warburg flask sidearm, instead of

test solution. The thermobar, which compensates for variations in temperature and atmospheric pressure, contained 2.5 cm^3 of water only.

In preparation for the assay, the sidearm stopcock was greased, with lanolin, and gently ground into the glass joint leaving the passage through to the flask open (this could be checked by sucking air through the stopcock). The manometer joint was then greased, also with lanolin, the flask was attached, ground into place, and secured with a rubber band leaving the top of the manometer open. The flask, secured to the manometer, was then placed in a 30°C Braun agitating waterbath, to allow the lanolin seal to adapt to the waterbath conditions (the lanolin would thin out, due to viscosity changes and should seal more efficiently). This was done individually for each Warburg flask so that as the last flask was placed in the waterbath the first had already adapted to the water bath conditions.

The flask was then removed from the waterbath, ground into the manometer joint again, to ensure an airtight seal, and oxygen-free dry nitrogen was flushed through the system to purge it of any oxygen, thus creating an anaerobic environment when the system is closed. The anaerobic conditions ensured that any CO_2 produced was due to yeast cell utilisation of the glucose provided. The nitrogen gas purge was continued for approximately 3 minutes per manometer then the nitrogen flow was turned off, the sidearm stopcock closed and finally the top of the manometer closed. If no leaks were present in the system a slight change in manometer fluid levels should be observed (right hand side down, left hand side up) due to the slightly elevated pressure inside the Warburg flask compared with the external pressure. The flask was then placed back into the 30°C waterbath and the resultant internal pressure buildup, due to the temperature increase, is exhibited in a sudden change in manometer fluid levels. The pressure must be immediately released by opening the top of manometer then quickly shutting it again before oxygen, from the atmosphere, was allowed back into the system. The system was then allowed to thermally equilibrate in the waterbath, for 15 minutes.

All manometer levels were then adjusted so that the right hand side read 15. The left hand side should read between 0 and 10. This was achieved

by raising the level of the manometer fluid until the right hand side read 30. With the top of the manometer closed, the levels of the fluid were then lowered until the right hand side read 15.

Prior to commencement of the bioassay, the agitator was started and run for 5 minutes. The right hand side fluid level was then readjusted to 15 and the left hand side's fluid level was recorded. This was repeated until the readings were constant (this should take 3 or 4 readings) and then the bioassay proper could be carried out. If the manometer readings did not become constant then there was probably a leak in one of the seals and all of that manometer's seals should be checked.

To start the bioassay the contents of the sidearms were tipped into the yeast solution in the base of the Warburg flask (the thumb was placed over the opening at the top of the left hand side of the manometer and the whole system tipped gently). The flask was then placed back in the waterbath, the right hand side fluid level adjusted to 15 and the left hand side's fluid level recorded, giving the reading for time = zero (i.e. t_0 in the CO_2 production calculation (section 5.2.2)). Readings were taken every 20 minutes by adjusting the right hand side fluid level to 15 and recording the fluid level of the left hand side. If at any stage the fluid level of the left hand side should be about to exceed 30 (the maximum on the manometer scale) the current fluid level should be recorded (at the 20 minute mark) and the pressure released through the top of the manometer. The right hand side's fluid level should be raised to 30 and then adjusted to read 15. The left hand side fluid level is then recorded and the bioassay restarted.

5.3 Bioassays of the Identified Peptide Fractions

The results of the bioassays run on the peptide fractions selected from the yeast GTF (section 4) are shown in table VIII. Also shown are the moles and milligrams of peptide present, of each sample, in the bioassay system. (i.e. per 0.1 cm^3).

The amounts of each peptide used in the bioassay were selected on the basis of the amount of peptide present in the fraction. 200 ul of

Table VIII

Bioassay Results of Selected Ion-Exchange Separated Fractions

Sample	Amino acid content	moles/0.1 cm^3	mg/0.1 cm^3	% Activity
36a	lys	6.09×10^{-7}	0.09	0
49a	(3-4)lys,pro,gly	2.47×10^{-7}	0.2	74
49b	lys,pro	3.96×10^{-7}	0.1	32
50b	2lys,thr	3.72×10^{-7}	0.15	48
52a	arg	3.42×10^{-6}	0.59	517
63a	lys,leu	1.05×10^{-6}	0.29	63
63b	2lys,glu,ile	3.72×10^{-7}	0.21	118
66a	2arg,asp	3.8×10^{-7}	0.18	189
67a	2arg,thr	5.95×10^{-7}	0.28	180
68	2arg,thr	1.19×10^{-6}	0.56	185
+70a	arg,gln	6.7×10^{-7}	0.22	75
+70b	lys,asn	6.1×10^{-7}	0.17	39
*72a	thr-arg	1.5×10^{-6}	0.45	92
*73a	Ser-arg	1.32×10^{-6}	0.37	75
*76/77	arg-pro/ala-arg	9.52×10^{-7}	0.26	171
*84	lys-glu-lys	2.5×10^{-7}	0.11	-34
*85c	lys-glu-lys	2×10^{-7}	0.09	6
*85a	arg-gly	4.3×10^{-7}	0.11	113
*86b	arg-val/val-arg	4.6×10^{-7}	0.12	118

* Bioassays and sequencing by Miss E.M. O'Donoghue, Honours Project (1983), Massey University

\+ Ratios established only

doubly distilled deionised water was added to the solid obtained from freeze-drying each fraction and 10 ul of this solution was removed for amino acid analysis.

From the amino acid analysis trace, the concentration of peptide per 200 ul could be calculated (section 2.3.1). A 0.1 cm^3 aliquot of each fraction was then removed for use in the bioassay (table VIII, mg/0.1 cm^3), in order to have as much peptide as possible present in the bioassay to ensure maximum response to the peptide added, while leaving enough peptide for use in further analyses if they become necessary (i.e. N-terminal analysis, bioassay repetition).

5.4 DISCUSSION

On studying the results for the bioassays of the ion-exchange separated fractions (table VIII) it was apparent that the fractions containing only arginine (No. 52a) and only lysine (No. 36a) occupied opposite ends of the activity scale, with the ariginine fraction exhibiting 517% activity and the lysine fraction exhibiting 0% activity.

The general trend that arose from the bioassay results, regarding the lysine- and arginine-containing fractions, indicated that those peptides containing lysine (49a, 49b, 50b, 63a, 63b, 70b, 84, 85c) exhibited lower activities, in general, than those peptides containing arginine (66a, 67a, 68, 70a, 72, 73, 76/77, 85a, 86b). This was shown in the results with the activity exhibited by the most active lysine-containing fraction being at 118% (fraction 63b) while the lowest activity exhibited by the arginine containing fractions was 75% (fractions 70a, 73a). This trend however, may be slightly biased as the concentration of the arginine peptides, in the bioassay, tend to be slightly higher than those of the lysine peptides. With the fraction containing only arginine exhibiting over double the activity shown in any other fraction, and the arginine-containing peptides also showing high levels of activity, it appeared that the simple presence of arginine in a fraction resulted in increased activity for that fraction. The lowering in activity, seen in arginine-containing peptides compared with the

fraction containing only arginine, would therefore presumably be due to the lower concentration of arginine present in those fractions.

In order to test this theory it was decided to carry out a series of bioassays on individual, synthetic, amino acids at varying concentrations and to obtain activity ranges for these amino acids. In this way it was hoped that an insight would be gained into their effects on the bioassay system.

Section 6 YEAST BIOASSAYS WITH INDIVIDUAL AMINO ACIDS

6.1 INTRODUCTION

Based on the results of the previous section (section 5) it was decided to measure the activities of various individual amino acids, over a range of concentrations, to determine their effects on the yeast bioassay system. The effects of individual amino acids on the yeast bioassay have been studied previously (J. Cooper, MSc Thesis (1982), Massey University), however, the concentrations of the amino acids used in the bioassay were much lower than those present in the separated fractions obtained by ion-exchange chromatography, as the concentrations used were based on amounts present when the active chromium-amino acid complexes were assayed.

Of particular interest was the behaviour of arginine as this amino acid, present in the separated fraction 52a (table IV), exhibited the greatest activity, 517% (table VIII). It was necessary therefore to determine the effects of the amino acids at the concentrations present in assays using the ion-exchange separated fractions (table VIII), in order that any additional stimulation caused by possible specific peptides may be clearly recognised.

In order to determine whether the response of the bioassay system to the peptide fractions separated from the yeast GTF sample by ion-exchange chromatography (table VIII), was a response by the yeast cells in the bioassay to the individual amino acids present in the peptides, a bioassay was run using a synthetic arginine-containing peptide, gly-arg-val, (provided by Dr D. Harding), and also on an equimolar mixture of the amino acids glycine, arginine and valine at the same concentrations as for the tripeptide. Also the individual constituent amino acids, at the same molar concentrations were assayed.

A range of concentrations of the gly-arg-val tripeptide was assayed along with a concentration range of the glycine, arginine and valine mixture for an activity comparison with the concentration ranges of the other individual amino acids.

6.2 METHOD

The methods used in the bioassays of the various samples in this section were the same as those described previously (section 5.2) with all samples dissolved in doubly distilled deionized water at concentrations ranging between $6x10^{-5}$ mmoles/0.1 cm^3 and $5x10^{-2}$ mmoles/0.1 cm^3. All amino acids used are of the L-form unless otherwise indicated.

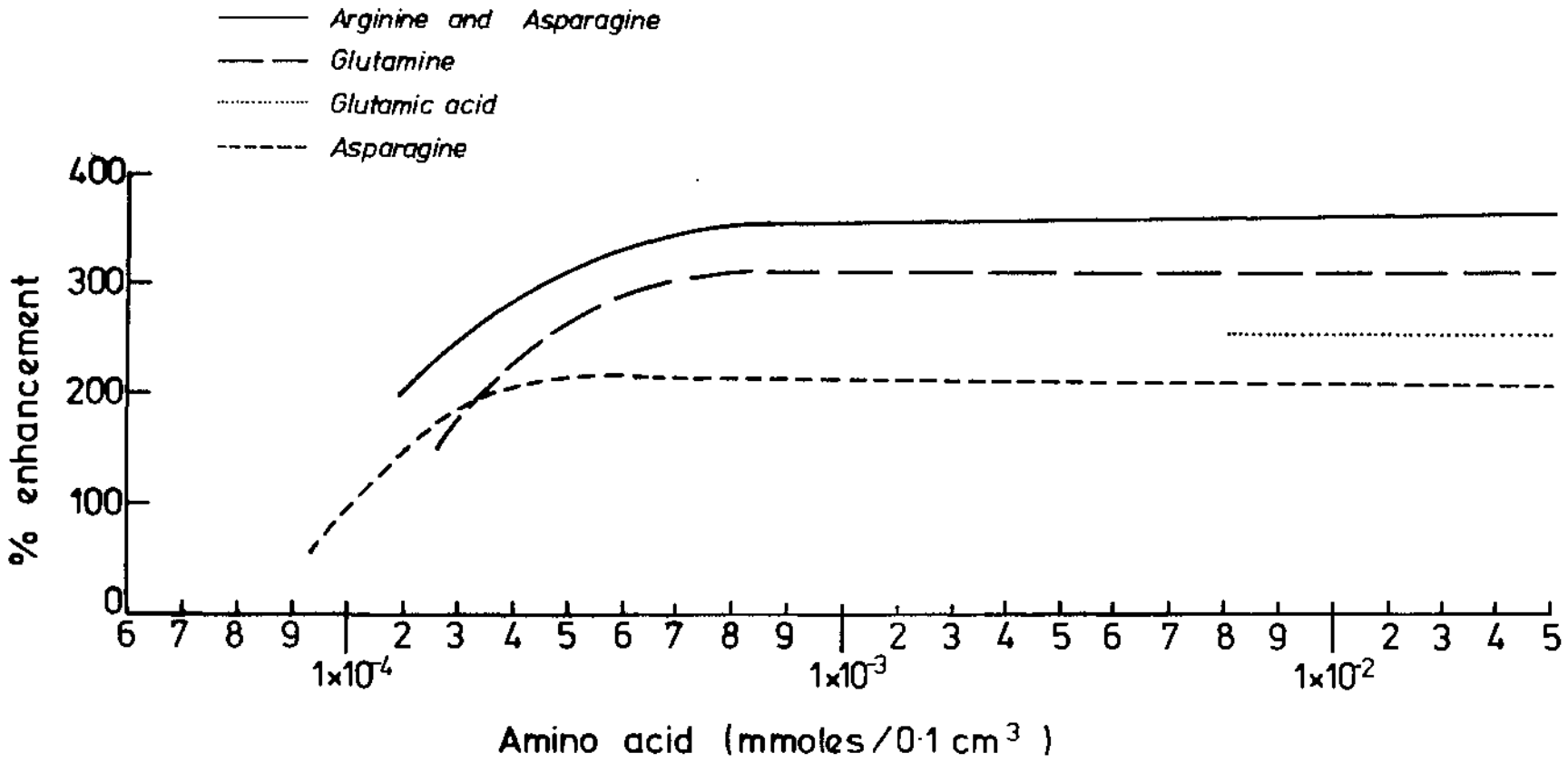
The concentration of the gly-arg-val tripeptide ($1.2x10^{-3}$ mmoles/ 0.1 cm^3) was chosen so that the amount of arginine present in the bioassay would be on the plateau of maximum activity for free arginine (fig. 3).

6.3 RESULTS

All concentrations of amino acids used in the bioassays are given in millimoles per the 0.1 cm^3 added to start the assay (i.e. millimoles of amino acid actually present in the bioassay system). Figure 3 shows the concentration dependance of those amino acids which exhibited the greatest activity, namely arginine, asparagine, glutamine and glutamic acid. Aspartic acid was also expected to be amongst this group (based on the activity level observed for glutamic acid) but due to its relative insolubility in water it was not possible to attain a concentration range at comparable levels to the other amino acids. Of these very active amino acids, glutamine, asparagine and arginine all could possibly conform to the initial hypothesis that certain amino acids and peptides may mimic the action of GTF via the orientation of their contained nitrogen groups (section 1). Glutamic acid, however, does not completely conform to this hypothesis on a structural basis, due to the presence of its side chain carboxylic acid group.

It was found that all of the amino acids assayed for an activity rise response exhibited approximately the same type of activity response curves, with the plateau levels being reached at approximately the same concentration of amino acid in each case (i.e. approximately $3x10^{-4}$ mmole/0.1 cm^3). The only major variant between the amino acid response curves was the level of the activity plateau (Fig. 3, Tables X and XI). While pure arginine does not exhibit the highest level of activity at saturating concentrations, in the data shown in Figure 3, but is

Figure 3 Amino Acids Exhibiting Activities of 200%+



approximately equal to that of asparagine, other results (table XI) indicated that it does exhibit the highest activity levels of the individual amino acids tested.

TABLE IX

Interassay Arginine Activity Variations

arginine/0.1 cm^3	% activity
0.2	448
0.2	387
0.2	326
0.2	500
0.2	582*
0.05	348*
0.025	328*

* Bioassays run on the same day.

The variation in results between bioassays (i.e. interassay variation) is shown by the results obtained for asparagine, in figure 3, and arginine, in table IX. As can be seen in figure 3, asparagine, under the same bioassay conditions but assayed on different days, exhibits differing activity plateau levels. The variation in arginine activity can be seen in table IX as arginine, under the same bioassay conditions but, again, run on different days, exhibits varying activities using identical concentrations. The standard deviation ($\pm$99%) for these varying activities, gives an idea of the variations between other, corresponding, amino acid bioassays. Intraassay comparisons however appeared to result in consistent responses regarding activity levels for different amino acids in the same bioassay run.

TABLE X

Amino Acids Exhibiting 100%-200% Activity

Valine		Leucine		Proline		Serine		Ornithine	
mmoles	% Activity	mmoles	% Activity	mmoles	% Activity	mmoles	% Activity	mmoles	% Activity
8.5×10^{-4}	129	7.6×10^{-4}	197	4.3×10^{-3}	128	4.7×10^{-3}	193	7.5×10^{-5}	70
4.26×10^{-3}	122	3.8×10^{-4}	174	2.1×10^{-3}	175	2.4×10^{-3}	191	2.0×10^{-4}	121
8.5×10^{-3}	105	7.6×10^{-3}	189	1.0×10^{-3}	135	1.2×10^{-3}	148	7.5×10^{-4}	101
1.2×10^{-2}	129	1.1×10^{-2}	197					2.3×10^{-3}	127
1.9×10^{-2}	128	1.5×10^{-2}	123					5.3×10^{-3}	127
2.7×10^{-2}	186							1.0×10^{-2}	172
3.0×10^{-2}	186							1.7×10^{-2}	106
3.5×10^{-2}	150								

TABLE XI

Amino Acids Exhibiting less than 100% Activity

Glycine		Tryptophan		Threonine		Isoleucine		Lysine	
mmoles	% Activity	mmoles	% Activity	mmoles	% Activity	mmoles	% Activity	mmoles	% Activity
7.4×10^{-4}	23	1.2×10^{-4}	50	4.2×10^{-3}	30	3.8×10^{-3}	33	2.05×10^{-4}	-22
1.5×10^{-3}	43	2.4×10^{-4}	47	2.1×10^{-3}	0	1.9×10^{-3}	2.5	3.4×10^{-4}	-30
4.1×10^{-3}	47	4.9×10^{-4}	28	1.0×10^{-3}	60	9.5×10^{-4}	22	1.4×10^{-3}	-40
8.1×10^{-3}	19.7	1.2×10^{-3}	25					3.2×10^{-3}	-45
1.4×10^{-2}	22	2.4×10^{-3}						7.0×10^{-3}	-35
2.5×10^{-2}	36							1.2×10^{-2}	-39
2.7×10^{-2}	70							1.6×10^{-2}	-61

Those amino acids exhibiting percentage enhancements of CO_2 production rates compared with blank rates (section 5), of between 100% and 200% are shown in table X. Of these amino acids ornithine and valine were the only amino acids tested for a rise in activity response. The others, leucine, serine and proline, were tested only for activity plateau levels as it had been determined that activity rise responses were approximately constant in all cases (fig. 3).

Table XI contains the data for those amino acids, tested in the bioassay system, which exhibited less than 100% activity (as defined in section 5.2.2) at their saturation plateau levels. As can be seen in table XI the amino acids tested, namely glycine, tryptophan, threonine and isoleucine, all exhibited limited enhancement responses in the bioassay system, while lysine appeared to actively inhibit CO_2 production with a saturation activity plateau level of approximately -35%. Again only the plateau levels were tested.

As a test of the effect that dextro forms of the amino acids had on the bioassay system, D-ornithine and L-ornithine were assayed simultaneously at identical concentrations. The results are shown in table XII, with the D-amino acid giving no stimulation of the rate of CO_2 production by the yeast. Such specificity indicates that proteins and/or enzyme systems could be involved in the observed effects. The result was not unexpected as in general the D-forms of amino acids are not utilised aS nitrogen sources (H. Suomalainen and E. Oura (1971)).

TABLE XII

Optical Isomer Activity Comparison

Sample	$mg/0.1cm^3$	% Activity
L-ornithine	0.2	128%
D-ornithine	0.2	0%

The activities generated by the synthetic tripeptide gly-arg-val, the individual component amino acids, at equimolar levels to those in the tripeptide, and the equimolar mixture of amino acids contained in the tripeptide are shown in table XIII. Concentrations are given at 1.2x 10^{-3} millimoles/0.1 cm^3. It can be seen (table XIII) that arginine gives a significantly greater activity than either the model mixture or the tripeptide, with the model mixture exhibiting a greater activity level than the tripeptide.

TABLE XIII

Bioassays on the Tripeptide Gly-Arg-Val

Sample	% Activity
gly-arg-val	106
gly-arg-val	106
glycine	20
glycine	3
arginine	413
valine	89
mixture	266

The results of a comparison of the concentration ranges used for the tripeptide and its model amino acid mixture are shown in table XIV. As can be seen the activity ranges support the results of table XIII, in that the amino acid mixture exhibited a greater activity plateau level than that observed for the tripeptide.

TABLE XIV

Bioassay Composition of the Tripeptide Gly-Arg-Val and its Model Amino Acid Mixture over a Range of Concentrations

Tripeptide		Model Mixture	
mmoles/0.1 cm^3	Activity	mmoles/0.10 cm^3	Activity
$1.5x10^{-3}$	87.5%	$1.5x10^{-3}$	368%
$1.2x10^{-3}$	94%	$1.2x10^{-3}$	340%
$7.5x10^{-4}$	94%	$7.5x10^{-3}$	354%
$3.8x10^{-4}$	94%		
$1.9x10^{-4}$	118%		
$9.5x10^{-5}$	129%		

6.4 DISCUSSION

The response of the bioassay system to the concentration ranges of the various amino acids was typical of saturation effects in biological systems, with the activity increasing to a plateau level. This saturation was obtained in all of the initial bioassays run (arginine, asparagine, glutamine and ornithine), but in later cases only the plateau levels were measured so that activity comparisons were always in terms of the maximum effect for any given amino acid. As can be seen (fig. 3 tables X, XI) the response of the bioassay system to the various amino acids could be divided into three general groupings: (a) those with activities above 200% (fig. 3), (b) those with activities between 100% and 200% (table X) and (c) those with activities below 100% (table XI). The wide range of activities of these groupings is due to the variations between different bioassays. This is demonstrated in table IX, where variation in the activity of arginine between different bioassays shows the reproducability of the data. Over 5 separate bioassays the mean plateau value for arginine obtained at concentrations of

0.2 mg/0.1 cm^3 ($1.15x10^{-6}$ moles/0.1 cm^3) was 448.6%, and the standard deviation for the data of 99% meant that only differences greater than 100% could be considered significant, when studying interassay variations. Relative intraassay comparisons however, show much more reproducable results and, as such, are not affected by the large standard deviation of interassay comparisons.

In general therefore, with the main aim of the individual amino acid bioassays not being a comparison between the samples tested but rather to test whether the activities of the ion-exchange separated fractions could be accounted for by the simple presence of constituent amino acids, the high standard deviation of interassay comparisons did not affect conclusions reached.

The comparisons of the results of the bioassays for the fractions separated by ion-exchange chromatography, from the Merck yeast extract (section 5), and the individual amino acids determined in this section, indicated that there could be a cummulative effect occurring, with each amino acid present in the separated fractions contributing in some way to the activity observed for that fraction. For example, while free arginine (fraction 52a) exhibited the greatest activity of the separated fractions, 517% (table VIII), fractions containing arginine plus another, less active, amino acid (e.g. arginine, serine (fraction 73a)), exhibited a considerably reduced activity (i.e. for fraction 73a, 75%). This can also be demonstrated with the lysine-containing fractions. Fraction 36a, containing only lysine, exhibited 0% activity, however when lysine was combined with another more active amino acid (i.e. lysine, leucine (fraction 63a)) the activity observed was considerably enhanced (for fraction 63a, to 63%). It can also be said that in general (table VIII) those separated fractions containing arginine exhibited greater activities than those fractions containing lysine, with the lowest activity generated by an arginine-containing fration being 75% (fractions 73a, 70a; table VIII) and the greatest activity generated by a lysine-containing peptide being 118% (fraction 63b, table VIII).

The only result that does not conform to this pattern is the activity for fraction 70a (i.e. arginine, glutamine) where two very active amino acids, as determined in section 6.3 (fig 3), are combined in one

fraction. It would be expected, therefore, that the observed activity would be greatly enhanced but, as seen in table VIII, this does not occur, with fraction 70a exhibiting just 75% activity, indicating the possibility of some form of competition occurring between active amino acids resulting in lower CO_2 production rates by the yeast cells.

The general order of activity for individual amino acids in the yeast bioassay can be compared to yeast cellular growth doubling times when single amino acids are utilised as the sole sources of nitrogen (table XV), with the ability of the amino acid to produce cellular growth paralleling its ability to stimulate CO_2 production in the yeast bioassay system.

The growth of the yeast cells, prior to use in the bioassay system, utilized a yeast nitrogen base without amino acids (Difco) as the nitrogen source to initiate yeast growth. The growth medium was then washed from the cells (section 5.2.1) with doubly distilled deionised water after centrifugation, to separate the cells from the growth medium, and this was repeated three times. The yeast pellet was then taken up in phosphate buffer (pH 5.75) and stored, in a fridge, for at least 24 hours prior to use in the bioassay system.

This procedure was such that no amino acids were supplied to the yeast cells, although the cells were not starved of nitrogen. As a result of this treatment, when the yeast cells were placed in the bioassay system, in phosphate buffer with glucose supplied (section 5.2.3), the amino acid sample added was the only available nitrogen source for the yeast cells, also the 30^{o}C environment (i.e. waterbath temperature) was conducive to cell growth. Thus a comparison made between the two sets of data appears valid.

To support this claim, turbidity measurements were taken to determine whether an increase in cell number had in fact occurred over the duration of the bioassay and whether this could be related to the observed activity. The results of this were inconclusive but did indicate that cell growth had occurred although no direct relationship could be determined between activity and the increase in cell numbers.

TABLE XV

Doubling times (min) for growth on various amino acids

	Strains				
Amino acid	M25	M25-12b	S288C	1278b	M970
Ammonia	149	140	144	148	130
Glutamine	136	155	132	144	120
Asparagine	147	162	134	173	113
Arginine	153	181	169	202	138
Glutamate	169	161	149	136	180
Serine	175	175	153	160	153
Alanine	187	194	180	163	212
Aspartate	197	170	175	171	136
Phenylalanine	193	217	224	153	164
Leucine	244	270	287	271	211
Tyrosine	278	291	326	164	265
Valine	289	205	204	212	216
Isoleucine	318	373	222	288	197
Tryptophan	319	349	260	241	322
Proline	322	284	269	268	158
Methionine	363	308	329	-	397
Histidine	531	n.d.	n.d.	423	470
Threonine	549	n.d.	312	154	176
Glycine	635a	n.d.	n.d.	580a	n.d.
Lysine	n.d.	n.d.	n.d.	n.d.	n.d.
Cysteine	n.d.	n.d.	n.d.	n.d.	n.d.
Allantoin	181	156	190	222	293
Allantoate	388	172	315	424	362
Urea	148	153	164	158	166
Citrulline	188	264	208	186	192
Ornithine	190	187	193	206	238

Whickersham's medium was used in this experiment, with 0.6% glucose as carbon source.

n.d. indicates insignificant growth in which the cells failed to double one time.

a Cells did not continue to grow.

[From, Molecular Biology of the Yeast Saccharomyces - Metabolism and Genetic Expression, J.N. Strathern, E.W. Jones and J.R. Broach ed. (1982)]

In studying table XV, notice must be taken of the time period required for cell doubling to occur using the amino acids indicated as the sole nitrogen sources. The time period between 160 minutes and 300 minutes is the period used to calculate the rate of CO_2 production by the cells in the bioassay system (section 5.2.2). From table XV it can be seen that the amino acids capable of inducing cell doubling at approximately 160 minutes are also those that exhibit the most enhanced activities in the bioassay (fig. 3). This observation links the activity of these amino acids not only to their abilities to stimulate the rate of CO_2 production but also to their abilities to act as nitrogen sources and to their speed of uptake and utilisation by the yeast cells, as increasing the metabolic rates, by supplying the cells with a good nitrogen source in a favourable environment, will increase CO_2 production and thus will be observed as an activity increase in the bioassay system. This increase in activity will also be enhanced by the fact that based on the yeast cell doubling times (table XV) for the more active amino acids, there may be approximately twice as many cells present in the bioassay at the time that the rate calculations of CO_2 production, for that sample, are commenced (section 5.2.2).

Those amino acids with cell doubling times closer to the end limit of the bioassay time would therefore exhibit a lower level of activity due to a lower cell count in the bioassay system. This can be seen with the results of the bioassays for the amino acids in tables X and XI corresponding to their effects on cell doubling times (table XV), when compared with the more active amino acids of figure 3. The same reasoning can be applied to those amino acids in table X, that exhibit activities below 100%. Lysine has been reported (Bourgeois (1969)) to be an amino acid not utilised by yeast cells as a nitrogen source and this is reflected both in the results of this section (table X) and also in the results of section 5, where it was isolated in fraction 36a (table VIII), free of any other amino acid, and exhibited 0% activity.

Amino acids, supplied to a yeast culture, are not in general incorporated in yeast protein intact (A.A. Eddy (1980) but are rather involved in a series of transamination reactions. Strains of Saccharomyces cerevisiae utilise wide varieties of amino acids as nitrogen sources, for example arginine is readily absorbed and degraded by arginase to produce ornithine and urea. Ornithine is degraded to

glutamate (Bossinger and Cooper (1977)) and urea is carboxylated to allophanate which in turn yields CO_2 and NH_3 (Waheed and Castric (1977)). Thus, from this example, it is apparent that CO_2 production could be enhanced by the presence of certain amino acids over and above that enhancement that could be accounted for simply by cell doubling in the bioassay.

The lower activities observed with the peptide fractions isolated from the yeast GTF compared to the most active of the individual, constituent, amino acids can also be explained in this manner. Uptake of peptides into the yeast cell is accomplished via a separate transport system to that utilised for individual amino acids (Becker and Naider (1980)). The peptide is transported into the cell as a whole unit and, once inside the cell, is hydrolysed by peptidases into its component amino acids, making them available for use in metabolic processes. Thus it is conceivable that the lowered activity response of the separated peptide fractions (table VIII), compared to their active amino acid consitutents, is due to the time required for this hydrolysis step.

The response of the synthetic tripeptide, gly-arg-val, also supports the hypothesis of a general amino acid/peptide effect on the yeast bioassay. It is obvious that (table XIII) the activity generated by the gly-arg-val tripeptide, and that of the model equimolar amino acid mixture, are both lower than that of the most active constituent amino acid (arginine) when each is assayed at the same molar concentrations in the same bioassay run. The activities shown for the levels of individual amino acids glycine, arginine and valine (table XIII) are consistant with earlier bioassay results (fig. 3, tables X, XI).

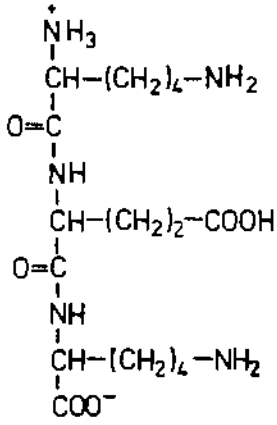
The activity shown by the model amino acid mixture tends rather to support the proposal that the activity generated by the amino acid and peptide fractions, isolated from the yeast GTF, and perhaps the yeast GTF itself, is a general, non-specific, effect of those amino acids and peptides acting as nitrogen sources. The lower activity of the model mixture, compared to equimolar amounts of arginine (table XIII) could be seen as a competition effect where the amino acids present in the mixture are competing for available amino acid transport sites into the yeast cell.

The increased activity of the model mixture of amino acids, glycine, arginine and valine, with respect to the gly-arg-val tripeptide (tables XIII, XIV) may also be due to the time required for peptidase hydrolysis in the yeast cell prior to the amino acids of the tripeptide becoming available for metabolic processes. As discussed previously, if this time is longer than the bioassay time then little or no activity would be seen in the bioassay. With this explanation of the data it is necessary to propose that some hydrolysis of the gly-arg-val tripeptides had in fact taken place during the bioassay to account for the observed activity of 106% (table XIII). This level of activity can in fact be nicely accounted for on the basis of a doubling in the cell number during the bioassay.

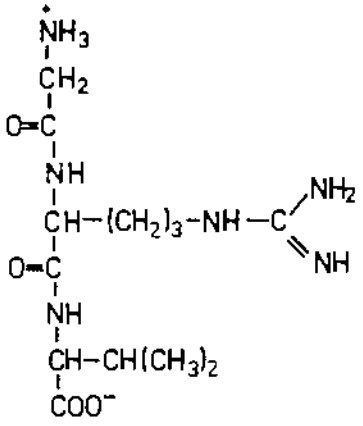
The results of this section and those of the previous section, on the activities of the peptides, separated from the yeast GTF do not however, completely discount the initial hypothesis (section 1) of a specific GTF effect of certain peptides and possibly amino acids. As mentioned previously (section 6.3) of those amino acids that exhibited the greatest activity (fig. 3) only glutamic acid did not contain nitrogen groups in the trans arrangement similar to that observed for the nicotinic acid ligands of the active $Cr(nic)_2(H_2O)_4^{3+}$ complex (Cooper, MSc Thesis (1982), Fig. 4). The structures of the other amino acids which exhibited the greatest activity levels (i.e. arginine, glutamine and asparagine) are shown in fig. 4 with a comparison to the structures of the active chromium complexes synthesised by J. Cooper (MSc Thesis (1982)), and the structure of the gly-arg-val tripeptide. The gly-arg-val tripeptide, which does show significant activity in the bioassay, can adapt the trans 'geometry', involving the N-terminal nitrogen atom and the nitrogens of the arginine side-chain (fig. 4) and seems to support the Cooper suggestion.

However, as mentioned previously, the suggestion of Cooper (section 1), regarding the specific trans arrangement of contained nitrogen groups, is not completely supported by the results of this section, with the activity of the tripeptide gly-arg-val, being considerably less than that of a mixture of the three individual amino acids glycine, arginine and valine (table XIII) which is not what would be expected if a specific GTF mimicing action was responsible for the activity. Also the presence of glutamic acid amongst the most active amino acids arginine,

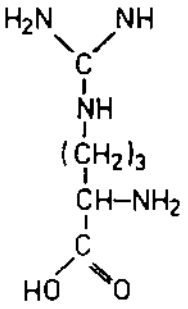
Figure 4 Structural Comparisons of Various Active and Inactive Samples



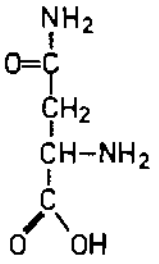
Lys-Glu-Lys



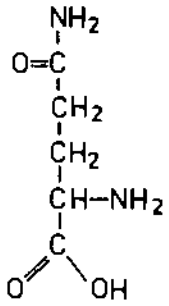
Gly-Arg-Val



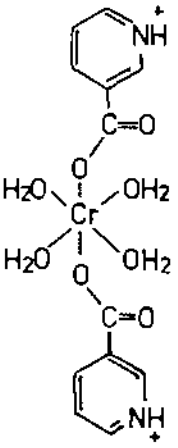
Arginine



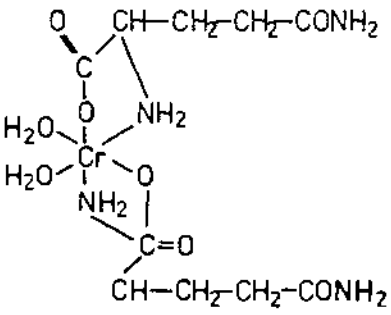
Asparagine



Glutamine



$[Cr(III)(nic)_2(H_2O)_4]^{3+}$



$Cr(gln)_2(H_2O)_2^+$

asparagine and glutamine (fig. 3), all of which could be seen to possibly conform to the GTF-mimicing hypothesis (Cooper, MSc Thesis (1982)), the similarity between the results of this section on the individual amino acids effect on the yeast bioassay system (fig. 3, tables X, XI) the data on yeast doubling times using amino acids as the sole nitrogen sources (table XV), and, the low response of the yeast bioassay to peptides such as lys-glu-lys tripeptides (85c and 84, table VIII) separated from the yeast GTF sample, which were initially expected to exhibit enhanced activities based on the suggestion of Cooper (MSc Thesis (1982), fig 3), all do not support the idea of a specific nitrogen arrangement being responsible for enhanced activities. All of the examples mentioned above tend rather to support the idea of a general non-specific amino acid/peptide effect on the yeast bioassay system.

The hypothesis of a specific GTF-mimicing structure being able to be formed by certain amino acids and peptides therefore appeares doubtful.

The results of this section indicate that the activity observed in the peptide fractions separated from the yeast GTF (table VIII) and in the pure amino acid samples (fig. 3, tables X, XI) can best be explained in terms of a general non-specific amino acid/peptide effect on the yeast bioassay system. The relatively high levels of amino acids required to give activity in the bioassay system, when compared to the levels used by Cooper et al (1984b) in the chromium-amino acid complexes found to be active, does not appear to support the hypothesis of a specific amino acid or peptide being responsible for the activity observed in the GTF preparations as, on the basis of Cooper et al's results, the concentration required should be significantly lower. Thus it may be that another factor is responsible for the activities observed.

Section 7 BIOASSAYS WITH YEAST GTF

7.1 INTRODUCTION

In order to compare the results of the bioassays with the peptide fractions separated from the yeast GTF (section 5.3), the individual amino acids (section 6.3) and with the synthetic tripeptide gly-arg-val (section 6.3), a detailed study on the behaviour of the original yeast GTF sample in the yeast bioassay was required. To compare the results of the previous sections with the bioassays of the yeast GTF, the concentrations used in the bioassays, reported in previous sections needed to be recorded on a weight basis, rather than a molar basis, since there was no known molar mass for the yeast GTF sample. Initially a comparison was made between the effects of the yeast GTF on the bioassay system over a concentration range (in mg/0.1 cm^3) and the concentration ranges of activities generated by the individual active amino acids, (in mg/0.1 cm^3). This was done in an attempt to test the theory that the activity generated by the yeast GTF, as a whole, could be accounted for simply by the effect of its peptide's individual constituent amino acids on yeast cell growth and metabolism processes under the bioassay conditions, after hydrolysis in the yeast cell itself. The yeast GTF fraction was also hydrolysed in vivo, so that only single amino acids would be present and the resulting hydrolysate was bioassayed over a range of concentrations. A model amino acid mixture was also prepared with a composition based on that determined by amino acid analysis of the yeast GTF hydrolysate. This would be expected to give a solution equivalent in amino acid content to the yeast GTF hydrolysate and should reveal what proportion of the activity observed, in the hydrolysate, was due to the presence of the individual amino acids.

Amino acid analysis was also carried out on the unhydrolysed yeast GTF sample to determine which single amino acids were present in the yeast GTF sample and hence to what extent any activity observed in the yeast GTF could be accounted for by their presence.

7.2 METHODS

The methods for assaying the yeast GTF, and associated samples, in this section are the same as described previously (section 5.2). The yeast GTF sample was hydrolysed by the method described in section 2.3.1 (i.e. prior to amino acid analysis). The amino acid analysis method was also the same as described in section 2.3.1, with 7.8 mg of yeast GTF being hydrolysed and taken up in 500 ul of citrate loading buffer (pH 3.25). A 10 ul aliquot was then loaded onto the amino acid analyser, thus the total amount of hydrolysed yeast GTF analysed was 0.156 mg. A further 7.6 mg of yeast GTF was then hydrolysed to supply the yeast GTF hydrolysate for use in the bioassay system as a comparison with the yeast GTF sample. The model amino acid mixture was based on the amino acid analysis trace, obtained from the 0.156 mg of yeast GTF hydrolysate mentioned previously. All amino acids used in this mixture were of the L-form unless otherwise stated.

7.3 RESULTS

The bioassay of the yeast GTF, over a concentration range, resulted in an activity saturation curve similar to those of the individual amino acids (Fig. 3).

The plateau in the activity versus concentration curve observed at saturating levels of the yeast GTF (Fig. 5) was however seen to be much higher than that observed for arginine and asparagine. The activity plateau was approximately 200% higher than that of arginine and asparagine but was reached at a higher concentration in the bioassay (i.e. yeast GTF, 0.5 mg/0.1 cm^3; arg, asn, 0.2 mg/0.1 cm^3). This higher concentration level is probably not surprising since the effective molar mass is greater for the yeast GTF sample (perhaps on a molar basis the plateau level would occur at a similar concentration). When the bioassay of the yeast GTF hydrolysate was compared with that of the yeast GTF itself, at comparable concentration levels in the same bioassay run, it was found that the activity plateau for the hydrolysate was approximately 150% higher than that of the yeast GTF sample (fig. 6),

Figure 5 Bioassay Comparison of the Yeast GTF sample with Active Free Amino Acids

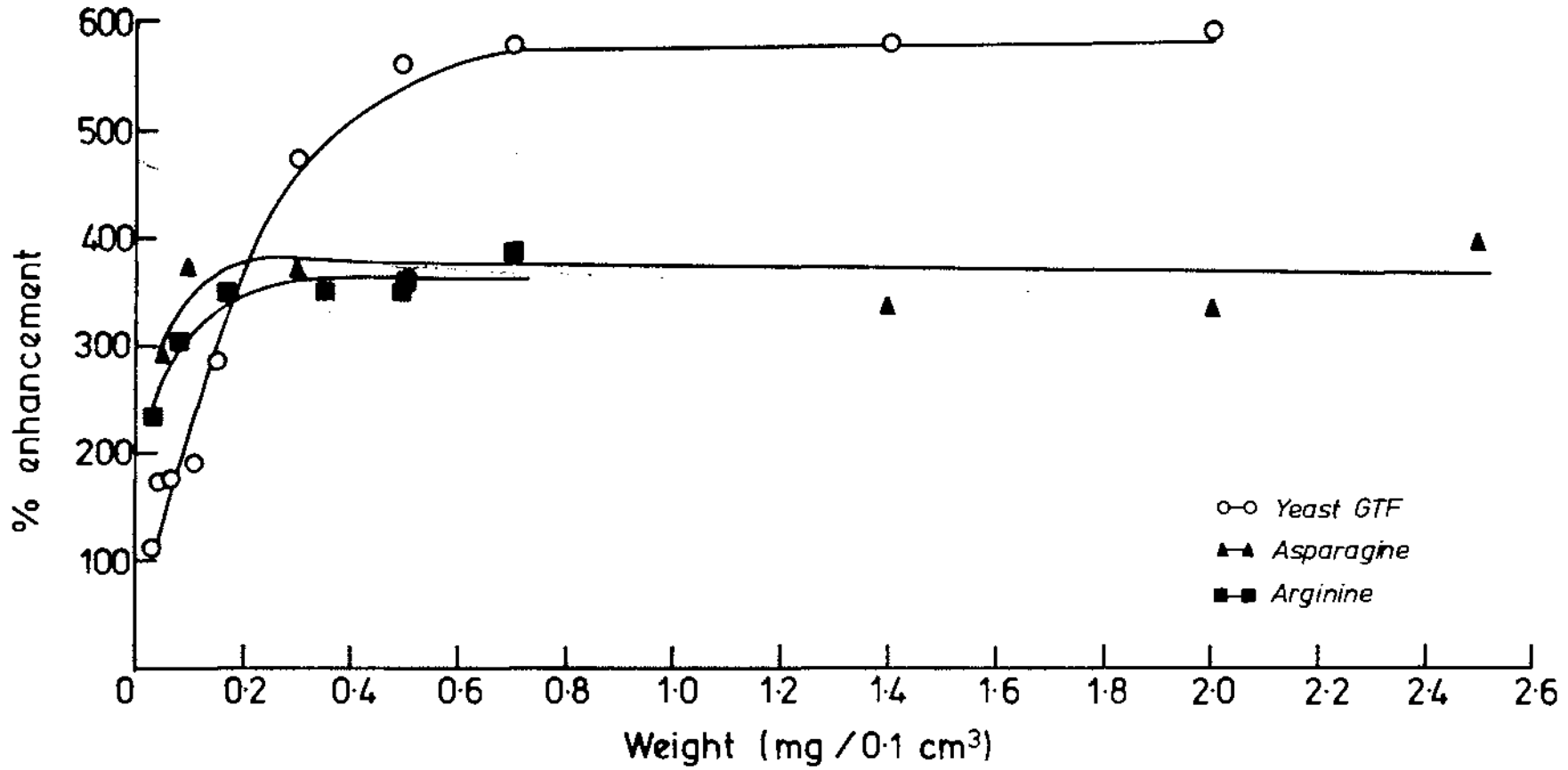
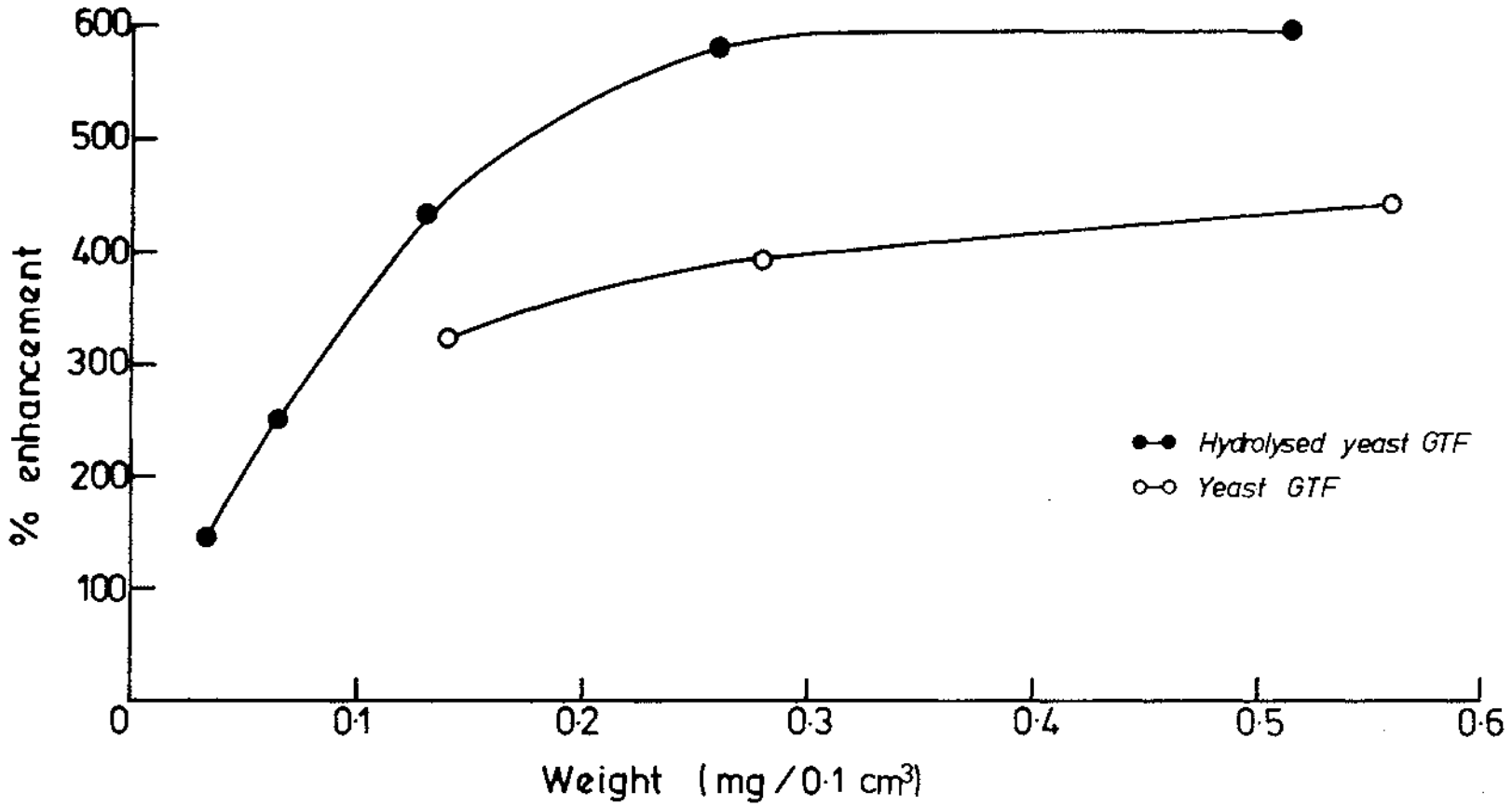


Figure 6 Bioassay Comparison of the Yeast GTF Sample and its Hydrolysate



with the plateaus being reached at approximately the same concentration levels (i.e. approximately 0.25 mg/0.1 cm^3).

Amino acid analysis of the yeast GTF hydrolysate also resulted in the identification of all the individual amino acids present (table XVI) and thus a model amino acid mixture of the yeast GTF hydrolysate could be prepared.

The approximate total number of moles of the required amino acids, determined from the amino acid analysis of the yeast GTF hydrolysate, was calculated assuming an average amino acid molar mass of 150 g l^{-1}. Six grams per litre total was required to ensure a position on the activity plateau for the yeast GTF hydrolysate (i.e. 0.6 mg/0.1 cm^3) which corresponds to an approximate total molar requirement of 0.04 moles per litre. To calculate the number of moles of each amino acid required in the model mixture, the mole percentage, determined from the amino acid analysis, was multiplied by the total number of moles required (i.e. 0.04 moles l^{-1}).

The model mixture of amino acids (table XVI) was dissolved in 1 litre of doubly distilled deionised water. Different combinations of the constituent amino acids were also assayed to test their effect on the bioassay. These variations included, the model amino acid mixture without lysine or arginine (-arg-lys), without lysine (-lys+arg), without arginine (+lys-arg), the complete mixture (+arg+lys) and finally the complete mixture including an amount of ammonia, estimated from the ammonia peak on the amino acid analysis trace, (+lys+arg+NH_3) to account for NH_3 released via hydrolysis of peptides contained in the yeast GTF (Table XVII).

As can be seen, in table XVII, the total amino acid mixture and variations exhibited a significantly lower activity than an equivalent amount of the yeast GTF hydrolysate. There was a possibility of a certain amount of phenol being present in the yeast GTF hydrolysate, as a result of the hydrolysis procedure, (where phenol is added as a protective agent for liberated tyrosine (section 2.3.1)), therefore an approximately equivalent amount of phenol was added to the model amino acid mixture to test its effect on the yeast bioassay system (table XVIII), and also half of this estimated amount to test for any concentration effect. An

TABLE XVI

Results of Amino Acid Analysis of the Yeast GTF Hydrolysate

Amino acid	Nanomoles/ 10 ul	Mole % of total	Moles needed/l	Wts used (g)/l
Lysine	227.85	24.8	9.92×10^{-3}	1.45
Histidine	9.72	1.06	4.24×10^{-4}	0.066
Arginine	169.49	18.4	7.36×10^{-3}	1.28
Asparatic acid	37.27	4.05	1.62×10^{-3}	0.22
(D-L) Threonine	38.61	4.2	1.68×10^{-3}	0.2
(D-L) Serine	31.02	3.37	1.35×10^{-3}	0.14
Glutamic acid	38.17	4.15	2.66×10^{-3}	0.39
Proline	53.9	5.86	2.34×10^{-3}	0.27
Glycine	61.5	6.69	2.68×10^{-3}	0.2
Alanine	33.2	3.61	1.44×10^{-3}	0.13
(D-L) Valine	63.56	6.91	2.76×10^{-3}	0.32
Methionine	2.9	0.36	1.44×10^{-4}	0.02
Isoleucine	74.63	8.12	3.25×10^{-4}	0.43
Leucine	64.1	6.97	2.79×10^{-3}	0.37
Tryptophan	7.9	0.86	3.44×10^{-4}	0.06
Phenylalanine	5.73	0.62	2.48×10^{-4}	0.04
TOTALS	919.51	10D%	0.D4101	5.586 g

assay was also run on the model amino acid mixture plus approximately 0.5 mg/0.1 cm^3 of nicotinic acid, as nicotinic acid had been reported to be present in the active fraction of the yeast GTF isolated by Mertz et al (1974) (table XVIII). Also run was a check assay on the effect of added ammonia on the behaviour of the model amino acid mixture in the bioassay system.

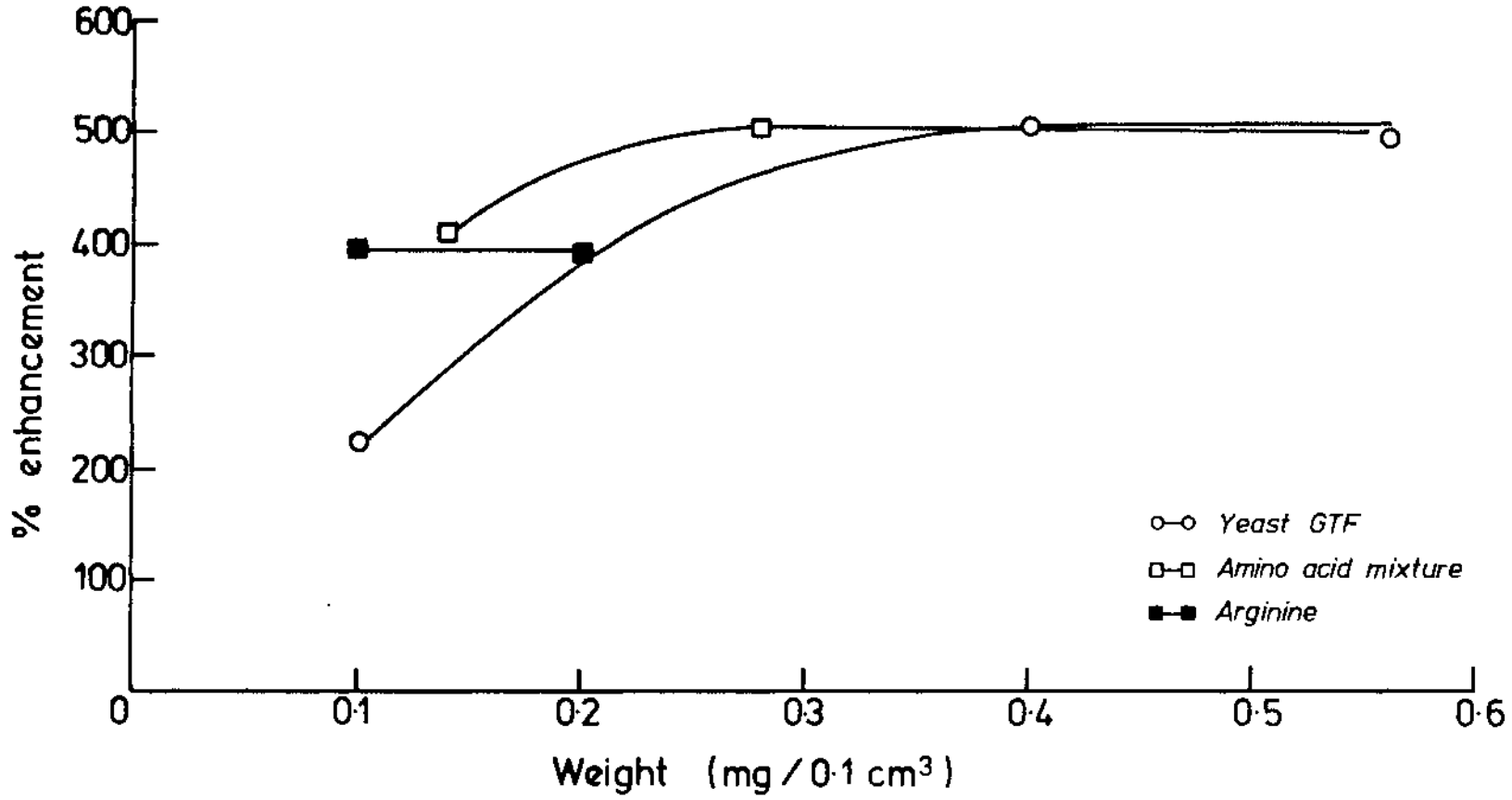
Since the relationship between the activity of the yeast GTF and its hydrolysate is known (Fig. 6) and also the difference between the activity of the yeast GTF hydrolysate and the model amino acid mixture (table XVII), a comparison between the activity of the yeast GTF sample and the model amino acid mixture was required to obtain an overall view of the activity relationships between the samples (fig. 7).

As can be seen in the figure the yeast GTF sample and the synthetic mixture of amino acids showed the same dependance on sample concentration and the same maximum activity, within experimental error. The assay was not as sensitive to the concentration of the yeast GTF and synthetic mixture samples as it was to arginine but the plateau in activity was approximately 100% higher.

Calculations based on the traces from the amino acid analysis of the yeast GTF hydrolysate showed that of the 0.156 mg of sample loaded onto the analyser, about 0.127 mg was amino acids. Therefore approximately 20% of the weight of the yeast GTF hydrolysate was not attributable to amino acids.

Amino acid analysis of the unhydrolysed yeast GTF sample (carried out in order to determine what free amino acids were present in the sample) resulted in the identification of the two amino acids arginine and lysine. Unfortunately it was not possible to determine how much lysine was present due to interference from other substances present but, of the 1.7 mg of yeast GTF loaded, 0.0374 mg was free arginine which corresponds approximately to 2% of the yeast GTF weight. Therefore it is unlikely that the activity observed for the yeast GTF sample (fig. 5) could be attributed solely to contained free amino acids.

Figure 7 Bioassay Comparison of the Yeast GTF sample, its Hydrolysate's Model Amino Acid Mixture and Arginine



7.4 DISCUSSION

The yeast bioassay results (figs. 5, 6) show the effects of a range of concentrations, expressed in mg/0.1 cm^3, on the yeast bioassay of the yeast GTF sample and the yeast GTF hydrolysate and allows a comparison with the results obtained for the individual amino acids.

Even when the experimental variations in the activities for different bioassay runs are taken into account, the general trend is clear and reproducible, in that the activity plateau, at saturating levels for the yeast GTF sample, is significantly higher than for the most active individual amino acid, arginine, at a lower concentration in the yeast GTF sample due to its incorporation in peptides (i.e. 2% of added yeast GTF is free arginine (section 7.3)).

On the basis of the results from section 6, with the tripeptide gly-arg-val, it would be expected that for a sample such as the yeast GTF, which contains mainly a range of small peptides, the activity of the most active constituent amino acid, arginine, should be greater than that observed for the whole yeast GTF sample, as the individual arginine should be more readily available for metabolic processes (section 6.4). As this is not experimentally observed (figs. 5, 7) it indicates that either there is a synergistic effect occurring between the constituent amino acids and peptides in the yeast GTF sample that results in enhanced activity in the yeast bioassay system, or, that there is some other factor present in the yeast GTF that accounts for the enhanced activity over and above that expected on the basis of the amino acids and peptides contained.

To distinguish between these two possibilities the yeast GTF sample was hydrolysed to break down the peptides present into their constituent amino acids. This would not only allow a test of the possibility of synergism, as breaking down the peptides contained in the yeast GTF sample would alter its composition but also of the original hypothesis that the active fraction of the yeast GTF could be a small peptide (section 1). If such a peptide existed and had a specific effect on the uptake and utilisation of glucose, removal of all the peptides present should then destroy any observed activity. The results show that the hydrolysis of the yeast GTF (fig. 6) in fact increased the activity

observed for the sample, from approximately 400% to 600%, thus effectively excluding both of the possibilities outlined. However, the results did support the general amino acid/peptide response hypothesis (section 6.4) as the process of hydrolysis would release more individual amino acids into the bioassay system therefore, based on the results of the bioassays on the gly-arg-val peptide and its model amino acid mixture (section 6.3), the increase in activity would be expected, although we would still expect it to be less than arginine alone since dilution with other amino acids should reduce its effect.

The model amino acid mixture was then prepared based on the amino acid analysis of the yeast GTF hydrolysate (table XVI). Results of a bioassay comparing the amino acid mixture, to the yeast GTF hydrolysate were expected to show similar activities due to the similar amino acid content of the two samples, however it was shown (table XVII) that the hydrolysate generated a significantly higher activity than any of the variations, on the model amino acid mixture which were tried. This supported the possibility of an additional factor being present in the yeast GTF hydrolysate and the yeast GTF itself. The results of the amino acid analysis of the yeast GTF hydrolysate was consistant with this possibility since it showed (section 7.3) that approximately 20%, by weight, of the yeast GTF hydrolysate was not due to amino acids. The possibility of either the phenol, added prior to hydrolysis to prevent oxidation of any liberated tyrosine, the nicotinic acid, reported present in the active fraction of GTF preparations (Mertz et al (1974)), or the ammonia, liberated from the peptides contained in the yeast GTF sample via the hydrolysis process, enhancing the activity observed in the bioassays, could be discounted as the bioassay results (table XVIII) showed no significant increase in its activity.

Finally a comparison was made between the yeast GTF sample, the model amino acid mixture and free arginine over comparable concentration ranges run on the same day. As seen in figure 7, the yeast GTF and the model amino acid mixture exhibited approximately identical activity plateaus while free arginine gave a significantly lower result. This shows that the model amino acid mixture exhibits a greater activity enhancement than the most active individual amino acid (i.e. arginine) at a comparable concentration level. This is in contrast to the results shown in section 6.3 where arginine exhibited a greater activity than

TABLE XVII

Bioassay Comparison of the Yeast GTF Hydrolysate and its Model Amino Acid Mixture

Sample	% Activity
Yeast GTF hydrolysate	909
Amino acid mixture (-lys-arg)	491
(+lys-arg)	453
(-lys+arg)	670
(+lys+arg)	757
(+lys+arg+NH_3)	649

TABLE XVIII

Bioassays of Variations on the Model Amino Mixture of the Yeast GTF Hydrolysate

Sample	% Activity
Amino acid mixture (complete)	464
(+phenol)	427
(+ ½phenol)	463
(+NH_3)	427
(+Nicotinic acid)	395

that shown by the amino acid mixture of the tripeptide gly-arg-val or the tripeptide itself. This increase in activity of the model amino acid mixture compared to free arginine may be a result of supplying the yeast cells with a variety of amino acids thus allowing direct stimulation of various metabolic pathways but the explanation of this result is still unclear.

The similarity between the yeast GTF and its model amino acid mixture, prepared on the basis of the composition of the yeast GTF hydrolysate, supports the hypothesis of an additional factor being present that enhances both the activity of the yeast GTF and its hydrolysate. This factor is not, therefore, affected by hydrolysis. The hypothesis of an additional factor is based on the idea that if the activity exhibited by the yeast GTF sample was due solely to the effect of the amino acids and peptides contained in it, then the increased activity observed with the yeast GTF hydrolysate is explainable as there are more free amino acids directly available to the yeast cells than with an equal amount of the original yeast GTF sample. However, if this amino acid/peptide effect was the only factor operating then the model amino acid mixture should show a comparable activity to the yeast GTF hydrolysate which is contrary to the experimental results obtained. In fact the amino acid mixture shows activity similar to that of the original yeast GTF (fig. 7). Therefore it seems that there must be an additional factor present which augments the amino acid and peptide effects on the rate of CO_2 production. It is this factor which increases the activity of the yeast GTF sample to the level expected for the model amino acid mixture and also increases the activity of the yeast GTF hydrolysate above that expected, (i.e. that of the model amino acid mixture (table XVII)).

CONCLUSION

The earlier work of Cooper (1984 a,b) and Haylock (1983 a,b) led to the separation of peptide fractions (O'Donoghue Honours Project, section 3) from a brewer's yeast extract by ion-exchange chromatography as part of a search for a small peptide, or amino acid, with the ability to mimic the action of the glucose tolerance factor. The separation of peptide fractions, from the yeast GTF sample, was carried out using electrophoresis, as the initial purification step, followed by 1-Dimensional paper chromatography on selected bands (section 3). HPLC was then used in an attempt to separate identifiable peptide fractions from the selected bands. Amino acid and N-terminal analyses, however, indicated that insufficient separation of the peptides (section 3) had been achieved by this method. Ion-exchange chromatography was then used to obtain single peptide fractions from the yeast GTF sample and subsequent N-terminal and amino acid analyses indicated that adequate peptide separation had been achieved by this method (section 3).

Of the peptide fractions which were bioassayed the arginine-containing peptides showed high activity levels, with the fraction containing only free arginine exhibiting the highest level of all. Therefore bioassays on pure arginine, and other constituent amino acids of the separated peptide fractions, were run to determine if the effects on the bioassay of these separated peptides were due to the individual amino acids contained in them or to some other factor. (i.e. a specific effect attributable to their overall three-dimensional structure.)

A comparison of the effects of various amino acids on the yeast bioassay system with the results of a yeast growth study using individual amino acids as the sole nitrogen sources (table XV section 6) indicated that the activity observed in the separated fractions could be explained on the basis of a non-specific amino acid/peptide effect. This amino acid/peptide effect was envisaged as being the result of their role as nitrogen sources. To check this and to determine the effect the separated fractions should have if utilised as nitrogen sources on the yeast bioassay system, it was decided to assay the synthetic, arginine-containing, tripeptide gly-arg-val (section 6). This work indicated that the results obtained for the separated peptide fractions could be

accounted for by the action of the contained peptide, as a nitrogen source, on the yeast bioassay system. The bioassay run on an equimolar amino acid mixture, with the same overall composition as the tripeptide, showed a greater level of activity than that exhibited by the tripeptide itself (section 6 tables XIII, XIV). The difference in effect of a mixture of individual amino acids compared with a tripeptide, on the bioassay system, presumably arises because the individual amino acids can be utilised directly while the peptide requires a hydrolysis step prior to utilisation by the yeast cells (section 6.4).

In order to be able to compare more quantitatively the results of the bioassays run on the separated peptide fractions, the individual amino acids, and the tripeptide with those for the yeast GTF it was necessary to assay the yeast GTF sample over a wide range of concentrations (section 7). This comparison showed that the maximum activity, observed at high concentrations of the yeast GTF, was greater than that for the most active of the samples assayed previously (fig. 5). The results obtained with the model amino acid mixture, based on the gly-arg-val tripeptide (section 6) led to the formulation of a model yeast GTF hydrolysate mixture. In order to prepare this mixture, the yeast GTF was hydrolysed, the amino acid content determined and the model mixture of amino acids calculated from the amino acid analysis trace obtained (section 7, table XVI). The synthetic tripeptide was less active, in the bioassay, than the equimolar mixture of its constituent amino acids thus if this result was general, the hydrolysis of the yeast GTF should increase the activity observed for the sample. On the other hand, if the activity, in the bioassay, of the yeast GTF was due to the presence of a small amount of a very active peptide then the activity should be seen to drop after hydrolysis. However, in either case, the activity of the yeast GTF hydrolysate would be expected to be similar to that of the corresponding model amino acid mixture.

The results of the bioassay run as a comparison between the yeast GTF and its hydrolysate showed that hydrolysis did increase the observed activity significantly (fig. 6) thus effectively discounting the possibility of a peptide with any specific properties able to affect the bioassay. However the results of the comparison between the yeast GTF hydrolysate and its model amino acid mixture showed that the hydrolysate still exhibited a significantly higher activity than that of its model

mixture (table XVII). This indicated the possibile existence of some other factor in the yeast GTF hydrolysate that contributed to the observed activity. Finally it was necessary to compare the activities of the yeast GTF sample and the model amino acid mixture of the yeast GTF hydrolysate. If the activity observed in the yeast GTF was due solely to a general amino acid/peptide effect the model amino acid mixture should exhibit a greater activity than that of the yeast GTF due to the presence of a variety of peptides in the yeast GTF sample (fig. 7). The results obtained showed that the activity levels generated, by both the yeast GTF and the model amino acid mixture of the hydrolysate, were approximately the same. This result supported the hypothesis of the presence of some other factor, present in both the yeast GTF and its hydrolysate, that was responsible for the enhanced activities observed.

The conclusion of Toepfer et al (1977) that the active fraction separated from brewer's yeast could be a chromium complex containing nicotinic acid, and possibly glutamic acid, glycine and cysteine appears, in view of the limited amount of separation and purification that their active fraction underwent, to be a little premature. With the high activity generated by the yeast GTF sample, obtained from the brewer's yeast extract by Cooper (unpublished results), and tested in this study, it is reasonable to assume that the contents of this yeast GTF sample would also be present in the yeast extract sample reported by Toepfer et al (1977).

Enhancement of activity in the active fraction of Toepfer et al, could also have occurred as a result of the hydrolysis of the sample carried out by refluxing it with 5N HCl which would be expected to convert any peptides into a mixture of their component amino acids resulting in an increased activity. This effect was in fact seen with a similar hydrolysis of the yeast GTF carried out in section 7 of this work. Also, the use of NH_4OH as the eluting solvent, as used by Toepfer et al (1977), has been shown (Cooper MSc Thesis (1982)) to produce false activity levels for samples tested in the yeast bioassay system. The activity observed by Toepfer et al (1977), using the adipocyte assay, could therefore be due to a similar non-specific amino acid effect perhaps enhanced by the use of NH_4OH as the elution solvent. However, this cannot be stated categorically as the effects of amino acids, small peptides and ammonia on the adipocyte assay system are still largely unknown.

In support of this suggestion however, it has been shown (E.M. Holdsworth, University of Tasmania, personal communication) that ornithine does exhibit significant activity in the adipocyte assay system thus perhaps the amino acid effect can account for some of the activity observed in adipocyte bioassay systems as well.

Further work must be done on the effect of amino acids and peptides in the other GTF assaying systems. It may be that there is a glucose tolerance factor present in the yeast extract samples that have been prepared, but its effect may be masked by the presence of amino acids and peptides, in those samples, on the assay systems used to detect it.

This work has shown that the initial hypothesis, of a small peptide, or amino acid, present in the GTF preparations with the ability to mimic the action of the GTF (section 1) is unlikely to be correct at least for an autolysed yeast sample such as the Merck yeast extracts. It has also been demonstrated that there is a significant amount of activity generated by a non-specific amino acid/peptide effect in the yeast bioassay system. There is still the possibility however, of a factor, contained in the yeast GTF sample, and unaffected by hydrolysis, that either enhances the amino acid/peptide effect on CO_2 production or directly affects glucose uptake of the yeast cells and consequently the CO_2 production rates in some unknown manner (section 7). In order to make the yeast bioassay system more quantitatively correct it may therefore be necessary to convert the bioassay parameters to percentage enhancement relative to the live cell count, thus negating the effect of the amino acids and peptides as cell growth factors.

While more work needs to be done on the amino acid/peptide effects a study also should be undertaken on the differences between Torula yeast, used to induce impaired glucose tolerance in rats (section 1), and the brewer's yeast used to prepare the GTF samples. It could be that the differences in the composition of the yeasts, either amino acid or otherwise, will give a further insight into the identity of the GTF if it exists. The results of Cooper (Thesis (1982); Cooper *et al* (1984b)) showing the biological activity of various synthetic chromium-amino acid complexes however cannot be explained simply by an amino acid effect. With the activity of the most active complex, $Cr(gln)_2(H_2O)_2$, being

322%, at a chromium concentration of 6 ug/cm^3, the glutamine contained in the complex has a concentration, in the bioassay, of 2.29×10^{-8} moles/0.1 cm^3. This glutamine concentration is well below the concentrations necessary for the generation of activity by pure glutamine (fig. 3). Thus it appears that the complexing of glutamine by chromium, did have some additional effect on the yeast bioassay system and more work will need to be done with this, and other active chromium complexes. Since neither the activity generated by the $Cr(gln)_2(H_2O)_2$ complex nor the activity of the yeast GTF and its hydrolysate can be completely explained as being merely due to the sum of the constituent amino acids and peptides. Whether amino acids affect the excess blood glucose removal rates in diabetic-like rats is also an interesting question which should be answered.

In conclusion, the results of this work, while pointing out the existance of a general effect of yeast extracts (unrelated to a single unique glucose tolerance factor) on the yeast bioassay system, has not been able to show conclusively that all of the activity generated by the yeast GTF sample could simply be accounted for by the amino acids and peptides contained in that sample. Thus it appears that the presence of glucose tolerance factor material in the yeast GTF sample cannot yet be discounted.

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