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STUDIES ON CHROMIUM-CONTAINING FRACTIONS
IN YEAST AND THEIR RELATION TO THE PROPOSED
GLUCOSE TOLERANCE FACTOR

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ABSTRACT

Chromium-containing fractions were isolated both from whole yeast cells and yeast extracts in attempts to isolate the glucose tolerance factor (GTF).

Techniques for the isolation of chromium-containing fractions from whole yeast cells were investigated by first identifying the chromium fractions by means of chromium-51 labelling. An anionic complex was isolated from yeast cells which had been supplemented in a medium with high levels of chromium chloride. This complex was identified as a chromium-glucose complex but did not show any of the properties expected for the GTF.

An isolation procedure based on ion-exchange chromatography achieved the isolation of eleven apparently discrete chromium-containing fractions from yeast extract. The activity determination of these fractions, by use of a yeast-fermentation assay, resulted in generally only the cationic fractions showing high activity levels.

The origin of the isolated fractions was investigated by determining which fractions resulted from reaction with media components and those which probably resulted from association with yeast. These investigations showed that the majority of complexes formed were not synthesized by the yeast cells but were a result of reaction with media components. After these studies only two cationic fractions remained as possible candidates for the glucose tolerance factor.

The cationic chromium-containing fractions from black peppercorns, sage, pork kidney powder, wheat bran and molasses were investigated. The cationic fractions obtained from these samples were identified as all having different ion-exchange elution positions and all having widely varying activities. Hence there did not appear to be a single discrete chromium-containing fraction which was capable of being identified with the glucose tolerance factor.

Preincubation of chromium-deficient yeast in media containing chromium was undertaken in an attempt to demonstrate some requirement of the yeast for chromium. No such requirement could be demonstrated.

On further purification of the active-cationic fractions the activity was shown to separate from the chromium but the factor causing the activity could not be identified.

Collectively these results support the existence of a non-chromium containing factor, capable of increasing the rate of fermentation of yeast and hence a factor, which apart from the lack of a chromium atom, shows close similarity to the glucose tolerance factor as reported by Schwarz and Mertz.

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SECTION I

INTRODUCTION

Since 1957 when Schwarz and Mertz (1957) first proposed the existence of a Glucose Tolerance Factor (GTF) which was necessary to maintain a normal blood glucose removal rate in rats, much research has been carried out to try and determine the mechanism of its action and to ascertain its chemical composition. In 1959 Schwarz and Mertz (1959) suggested that chromium was in fact the active ingredient in the glucose tolerance factor and since then various systems both in vivo and in vitro have been used to assess the biological effects of chromium and crude GTF preparations.

Attempts have been made to isolate GTF from various sources but the most commonly used are brewer's yeast (or yeast extract) and pork kidney powder. However so far only fairly impure samples have been isolated by the published procedures and conclusions drawn about the type of structure giving the observed biological effects using such impure samples must be open to doubt, and may be totally misleading.

Various groups (Schwarz and Mertz (1959), Mertz (1969), Votava et al (1973), Toepfer et al (1977), Kumpulainen et al (1978) and Mirsky et al (1980)) have reported isolation and partial purifications of chromium-containing fractions obtained from various sources. The methods used and the results achieved are critically discussed in section 4.4 of this thesis.

Despite some of the more obvious shortcomings of the reported preparations, some very interesting effects have been observed when the samples are tested in various assay systems. The fundamental effect which has been attributed to GTF is its ability to maintain normal glucose tolerance in the rat. However, since glucose metabolism is a common feature of most cells, several related cellular systems have been used to investigate the effects of GTF and chromium complexes.

The main function of GTF was demonstrated by inducing an apparently chromium-deficient state in the rat by feeding on a controlled diet (Schwarz and Mertz(1957)). This resulted

in the removal rate of excess blood glucose dropping from 4.0% per minute to 2.0% per minute. The impaired glucose removal rate was reversed by doses of crude GTF preparations obtained from yeast extract or acid hydrolysates of pork kidney powder.

Various in vivo experiments have been carried out to detect possible interactions or associations between chromium, glucose and insulin in a variety of different animals raised on normal and chromium-deficient diets. Using rats in much of the earlier work, Schroeder (1966) proposed a link between chromium deficiency and a diabetes like syndrome. He took great care to produce an apparently chromium-deficient rat strain from which he examined the serum glucose levels and the growth rates. Schroeder claimed to have shown that the rats receiving the chromium-deficient diets were more likely to be associated with hyperglycemia, with the hyperglycemic rats having an average blood serum glucose level of 137.2 ± 6.8 mg/100 cm³ and those receiving chromium supplementation having an average level of 116.6 ± 1.7 mg/100 cm³. The chromium-deficient rats had a lower body weight than those receiving a diet containing adequate chromium supplied as chromium acetate and glycosuria was detected in a significantly greater number of the chromium-deficient rats. Schroeder did not attempt to show that the symptoms which developed as a result of inducing chromium deficiency could be reversed by chromium supplementation but the symptoms developed by the rats receiving the low-chromium diet were characteristic of diabetes. It is of course possible that the specialised diet used by Schroeder was deficient in factors other than chromium, capable of causing diabetic symptoms.

With the possible association between a chromium-deficient state and diabetes having been shown by Schroeder, Roginski and Mertz (1969) attempted to demonstrate the existence of a chromium-insulin interaction. Various in vitro experiments are described later but their in vivo work involved the demonstration of a greater sensitivity to injected insulin (measured by a lower blood glucose level) for rats receiving a chromium-chloride supplementation in

their drinking water compared with those rats receiving no chromium supplementation and hence being classified as chromium-deficient. A curious difference in the results for fasting blood glucose levels for the chromium-deficient rats compared with the supplemented rats appears when the results of Roginski and Mertz (confirmed by Wooliscroft and Barbosa (1977)) are compared with the results reported by Schroeder (1966) for the blood glucose levels of similar groups of rats. Schroeder had observed a difference in the blood serum glucose levels for the two groups of rats whereas the other workers found similar levels for rats in both chromium states. Roginski and Mertz found that the blood glucose level for "low chromium rats" was $58 \text{ mg}/100 \text{ cm}^3$ while the chromium-supplemented rats had a similar level of $59 \text{ mg}/100 \text{ cm}^3$. Wooliscroft and Barbosa found the average blood plasma glucose level for chromium deficient rats to be $105 \pm 9 \text{ mg}/100 \text{ cm}^3$ while that for the rats receiving chromium supplementation was $116 \pm 4 \text{ mg}/100 \text{ cm}^3$.

Wooliscroft and Barbosa also reported that they found little difference in the glucose removal rates for both groups of rats when using the accepted method of calculating the glucose removal rate. They found the low-chromium rats had an average glucose removal rate of $2.06 \pm 0.13\%/ \text{min}$ while the chromium-supplemented rats had a removal rate of $2.29 \pm 0.14\%/ \text{min}$. They also inferred that the results obtained from the method of calculation of the glucose removal rate employed by Schwarz and Mertz (1959) (i.e. using the excess blood glucose level) may have been in fact a methodological artifact. By using two different glucose loads ($50 \text{ mg}/100 \text{ g}$ and $125 \text{ mg}/100 \text{ g}$), Wooliscroft and Barbosa showed that on comparison of the glucose removal rates for the two groups of rats, that both had similar removal rates for the low-glucose load and again similar, but this time higher glucose removal rates for the higher glucose load. These results were consistent with normal β cell function in the chromium-deficient rats and hence presumably a non-diabetic state.

Several other experiments have been carried out where the effects of chromium supplementation on the glucose removal rates of chromium-deficient rats have been examined

(Mertz et al (1960), Mertz et al (1965)). In each case the non-accepted method of calculating the glucose removal rates has been used and hence the significance of the results must be in doubt.

In an attempt to determine the intracellular localisation of chromium Mertz et al (1965a) and Onkelinx (1977) injected chromium-51 intravenously in the rat. Both groups proposed the existence of compartmentalized chromium pools within the rat, as well as various excretion pathways on the basis of the results obtained. Mertz et al showed that the decay curve obtained by whole body counting was similar for both chromium-supplemented and chromium-deficient rats. If a chromium-deficient state had been achieved in the rat, the injection of the chromium would be expected to result in altered decay curve for the chromium-deficient rats to allow for the build up of a depleted chromium compartment. Similar chromium-51 tracer experiments were carried out on diabetic strains of rats by Mathur and Doisy (1972) and more recently by Kraszeski et al (1979). No clearcut answer was obtained from the investigation since in many cases contradictory results were obtained by the two groups of investigators and thus it is not clear whether there is any significant difference in chromium uptake by diabetic (and presumably chromium-deficient) rats and normal rats. One major point of agreement however was the observation of increased chromium-51 levels in the serum of the diabetic rats which perhaps suggests loss of chromium in the diabetic state. In the more recent study, Kraszeski et al extended the work to involve the effect of insulin on the diabetic rats. From their published data they concluded that the tissue sources from diabetic rats treated with insulin have higher chromium levels showing that insulin may be able to protect against the loss of chromium.

Recent results reported by Ghafghazi et al (1979) have tended to cast doubt on some of the earlier work. Although he did not use chromium-deficient rats, Ghafghazi has reported effects in rats receiving administration of chromium chloride quite different to those found by Mertz et al (1965) and Schroeder (1966). Ghafghazi has claimed

that after administration of either 5.1 mg/kg or 50 mg/kg of chromium daily for up to 23 days there was no difference in the blood glucose levels of rats given the chromium administration over controls receiving saline injections. He has also shown that rats when injected with chromium at levels between 100 mg/kg to 300 mg/kg, develop hyperglycemia in the resting blood glucose levels. Although experimental conditions were different since the rats were raised on diets unlikely to cause chromium deficiency and a higher level of chromium supplementation was used, Ghafghazi has produced evidence which does not support the idea that chromium is involved as the active ingredient of GTF in its ability to maintain normal glucose tolerance in rats.

While many researchers have attempted to demonstrate an association in rats between chromium and blood glucose levels, and the response of these to insulin administration no unequivocal conclusion can be reached from the conflicting results that have been reported. It appears in many cases that researchers have started with preconceived ideas as to the effects they expected to observe and consequently they have tried to fit the data to these ideas.

No clear conclusion can be drawn from the results obtained when other animals are tested for their response to chromium supplementation. Davidson and Blackwell (1968) while investigating the effects of chromium chloride and chromium acetate supplementation in the diet of squirrel monkeys which exhibited abnormal glucose tolerance tests reported an improved glucose tolerance. On the other hand Preston et al (1976) when experimenting with chromium-deficient guinea pigs found that chromium chloride supplementation in the diet did not effect the glucose tolerance of the animals. Tuman and Doisy (1978) showed during comparative experiments on normal and genetically diabetic mice that a single intraperitoneal injection of GTF concentrated from brewer's yeast was sufficient to reduce the average non-fasting plasma glucose level in the diabetic mice by up to 29%. However the normal mice also showed a reduction in their plasma glucose of 36% with a similar injection.

A number of studies have also been carried out to try and demonstrate a link between chromium deficiency and abnormal glucose metabolism in humans. Schroeder (1968 review) reported a statistical analysis of the amounts of chromium in various tissues which was conducted to determine whether there was any variation in the level for different populations and also whether this varied with age. He discovered a gradual decline in the chromium levels in the human tissues investigated (aorta, liver, heart, kidney, spleen) with increasing age. This decline was most rapid in the first 10 years of life and thereafter a slower decline was observed reaching background levels at approximately 70-80 years. On the basis of this data Schroeder proposed the existence of a dietary chromium deficiency which results in the gradual depletion of chromium in human tissues. A comparison of similar tissue types from different geographical origins was conducted by Tipton and Cook (1963) and by Tipton et al (1965) who found lower chromium levels in tissues from American subjects than in African, Near Eastern or Far Eastern subjects. This prompted Schroeder et al (1970) to propose that the widespread refinement of foods was a major factor in causing chromium deficiency in American diets. In this same study by Schroeder et al (1970), exhaustive analysis was carried out on a wide variety of different human tissue and organ sources. Although higher chromium levels were found in the skin, lungs and ovary, no significantly high chromium levels were encountered in any of the other major tissue or organ sources. Thus if GTF was present in humans, then its action was not confined to any particular organ.

Analysis of plasma chromium levels after glucose administration has led to widely conflicting results. Glinsmann et al (1966) and Levine et al (1968) have all found that on administration of glucose (in a glucose tolerance test) to normal non-diabetic subjects there was a rise in plasma chromium. Using apparently similar experimental techniques, again on healthy non-diabetic subjects, both Davidson and Burt (1973) and Pekarek et al (1975) reported the complete reverse, in that the plasma chromium levels showed a fall after the glucose administration. From the

contradictory nature of these results it seems apparent that a further unknown factor (or factors) is present in the experimental procedures used and until this is resolved, further detection and comparison of insulin, chromium and glucose levels in blood serum will not be very helpful. One of these factors is probably the wide variety in analytical results from chromium analysis on biological samples. This has been demonstrated in a WHO study (Parr (1977)) in which the same sample gave widely varying results in different laboratories where a wide range of different analytical methods such as flame and flameless atomic absorption, colorimetry, activation analysis and emission spectroscopy were used.

Urinary excretion has been proposed as the pathway for the removal of chromium from the body. Schroeder (1968) has demonstrated a rise in urinary chromium after a glucose load in normal subjects. This finding was partially confirmed by Gursan and Saner (1978) when in a similar experiment 8 out of 10 normal subjects showed higher urine chromium levels after glucose administration. When diabetic subjects were examined the number of subjects showing the increased chromium levels after glucose administration dropped to 3 out of 8. Again the magnitude of the changes in these experiments were of doubtful significance.

The most recent and extensive survey published on the chromium levels in the various body fluids and tissues of normal and diabetic subjects has been carried out by Rabinowitz (1980). In the tissues and fluids measured (hair, red blood cells, plasma and urine) wide variations in chromium levels were observed. These levels however overlapped and thus there was no direct evidence to suggest that the diabetic subjects had lower chromium reserves than the normal subjects. This information was in contrast with the earlier results of Scheoeder (1965 review) where he based some of his argument for the low chromium-diabetic state association on the lower chromium levels in older tissue sources.

Several researchers have attempted cures or at least improvements in diseases resulting from disorders in glucose metabolism by means of both short and long term chromium supplementation. The early chromium supplementation work was

carried out by Levine et al (1968) and Glinsmann and Mertz (1966). By using daily chromium supplementation of 150 μg on elderly subjects with impaired glucose removal rates, Levine et al were able to label 4 of the 10 subjects as "chromium responders" but a further 6 did not respond to the chromium supplementation. In the case of the chromium responders the effect was shown not to be a result of increased insulin release. Glinsmann and Mertz conducted similar experiments using subjects suffering from maturity-onset diabetes. These subjects received a range of chromium supplementation doses up to 3 mg/day. Results reported here were again inconclusive as only 3 out of 6 subjects showed any improvement in glucose tolerance. Gurson and Saner (1971) investigated the effect of a single 250 μg chromium chloride supplementation on patients suffering from marasmus (a disease caused by a deficiency in total calories in children which is often associated with the diabetic symptom of a low glucose removal rate). Although the data had large standard deviations they did establish a trend towards higher glucose removal rates in 9 out of 14 cases. Perhaps the most striking effect in which short term administration of chromium has been used is that reported by Hopkins et al. In treating malnourished infants from Jordan and Nigeria who had impaired glucose tolerance curves they observed that a single 250 μg dose of chromium as chromium chloride was sufficient to raise the excess blood glucose removal rate from 0.6%/min (Jordan) and 1.2%/min (Nigeria) to 2.9%/min (both locations). Hopkins et al have explained the initially low glucose removal rate in the case of the Jordanian infants by the very low chromium level found in their drinking water. Successive glucose tolerance tests carried out on malnourished infants who did not receive a chromium supplement showed large variations in the glucose removal rate which must consequently decrease the overall value of the results reported. The superior diet given to the infants in the hospitals where these tests were undertaken could also have had a large and immediate effect on the glucose tolerance of the infants.

In a recent experiment, Liu and Morris (1978) have also noted the effect of chromium supplementation but in this

case the supplementation was taken in the form of brewer's yeast. They found that the hyperglycemic people in their group after receiving 4 μg of chromium in brewer's yeast daily for a 3 month period did show a reduced fasting blood glucose level compared to their pre-supplementation blood glucose levels. The observed difference however was not greater than the standard deviations for either blood glucose level.

Human diets normally contain a wide variety of foods, often with widely varying chromium concentrations. Consequently it has been difficult to obtain results from an apparently chromium-deficient human. Recently though, two cases have been reported where patients receiving long term total parenteral nutrition (TPN) have shown interesting effects on chromium supplementation. Jeejeebhoy et al (1977) have reported the case of a patient receiving TPN for 5 years. This patient after receiving TPN for 3 of the 5 years developed weight loss and glucose intolerance even with a constant caloric intake. These symptoms were associated with low levels of chromium in the blood and hair. The above symptoms were reported to be corrected by 250 μg chromium supplementations as chromium chloride daily for 2 weeks. In a similar case Freund et al (1979) have described a patient receiving TPN for 5 months who also developed symptoms of severe glucose intolerance and weight loss. Again with a daily supplementation of 150 μg of chromium (III), the symptoms were reversed. Both of these isolated cases may be taken as evidence of a chromium-deficient state being created, which in turn results in symptoms of glucose intolerance and weight loss, not unlike the symptoms seen in chromium-deficient rats. These two cases do not represent a statistical sample of patients receiving long term TPN and hence the question arises as to why similar symptoms do not arise in other cases. In both of these reported cases, there is also the possibility that alternative treatments such as increased insulin levels and altered calorie intake, given on development of the symptoms, may have in fact brought about the cure.

As with much of the other chromium-GTF research, work by other groups has also revealed conflicting results where

chromium supplementation has been used. Sherman et al (1968), using both normal and diabetic subjects receiving either a 50 μg chromium supplement or a placebo orally 3 times daily for a period of 16 weeks, found no improvement in the hyperglycemia in the diabetic patients receiving chromium compared with those receiving the placebo. The glucose tolerance of the normal subjects was unchanged. Carter et al (1968) while examining children suffering from Kwashiokor, a symptom of which is an impaired glucose tolerance, again found no improvement in glucose tolerance with a single dose of 250 μg chromium (III). In some cases subjects receiving lower levels of chromium (8 μg) fed with milk, showed a greater improvement than subjects receiving higher dose levels (750 μg Cr). The patients receiving a high-protein, high caloric diet, irrespective of chromium levels showed the greatest improvement in glucose tolerance. More recently Wise (1978) reported no change in glucose tolerance on chromium supplementation of subjects with abnormal fasting glucose levels.

Clearly then much controversy exists over the effect of chromium supplementation in humans. For such a wide range of results to have been reported other non-controlled experimental conditions must be important. Fluctuations in day to day glucose tolerance tests may lead to erroneous conclusions as may the variation of diet received by the subjects. The nature of the diseases tested may also mean that the causes of the impaired glucose tolerance may not be the same for all the various types (diabetes, kwashiorkor, marasmus or for the TPN-diet condition). Certainly there is the need for more careful experimentation with far greater control placed on subjects diet and the cause of the impaired glucose tolerance. Also the type of supplementation given must be considered. Most work has been carried out using chromium chloride supplementations but it may be necessary to use preparations of GTF from yeast extract which have been purified to the highest possible state. In the absence of such purification it is impossible to distinguish between effects brought about by chromium and effects due to unspecified impurities in the sample.

Ghafghazi et al (1980) have recently reported a further effect of chromium on the insulin release from isolated islets of langerhans. They have reported that chromium chloride inhibits insulin release from the islets of langerhans, a finding which seems contradictory to the idea that chromium plays a role in glucose metabolism. However it must be pointed out that since the islets of langerhans are insulin secreting cells, the effect of chromium on these may be different to its effect on other cells in which glucose is metabolised via the intermediacy of insulin. These findings reported by Ghafghazi et al have been countered by Shapcott (1980) who pointed out that the chromium levels used by Ghafghazi were 3 orders of magnitude greater than those normally found in man. Despite this challenge by Shapcott supplementation of the diets of animals and humans with chromium as chromium chloride to produce restored glucose removal rates would seem to be an unwise procedure.

As well as the in vivo work, experiments have also been performed on the in vitro effects of chromium and GTF preparations. Several of these have been developed into assays used to detect the presence of GTF-like activity. Those used to date for assay purposes are reviewed in section 3.1.

Attempts have been made to demonstrate interactions between insulin and, GTF, synthetic chromium complexes and solutions of chromium salts since insulin is a necessary requirement in determination of GTF activity using the epididymal rat fat pad assay. Christian et al (1963), Campbell and Mertz (1963) and Belusko (1977 thesis) all using different analytical methods have demonstrated an interaction between chromium and insulin. Evans et al (1972) using GTF fractions prepared from brewer's yeast as well as a synthetic, biologically-active chromium fractions, have shown that binding of these samples to insulin occurs. Anderson and Brantner (1977) found that the insulin bound only to the biologically active chromium fraction and not to any of the other chromium fractions produced during the synthesis. Here there appears to be some uncertainty in not knowing exactly which forms of chromium will bind or interact with insulin and consequently

the interactions observed may not be the result of a chromium-insulin combination having a specific function but may in fact be due merely to random interactions which may be expected for proteins and most charged species in solution.

The present understanding of chromium and GTF in glucose metabolism is confused and beset by conflicting results. However in view of the possibility that GTF (or chromium complexes) may be useful in the treatment of some forms of diabetes it is important to either obtain a supply of the pure, biologically-active GTF or to demonstrate that no such single active factor exists.

It was the aim of the work reported in this thesis to achieve one of these goals. If in fact a pure GTF fraction was isolated, attempts would be made to chemically characterise and to try to determine unequivocally its biological efficacy.

SECTION 2

ISOLATION OF CHROMIUM COMPLEXES FROM WHOLE YEAST CELLS

2.1 INTRODUCTION

Although the published procedures (Votava et al (1973), Toepfer et al (1977) and Kumpulainen et al (1978)) for the isolation and purification of chromium complexes from yeast are markedly different, they all involve the use of whole yeast cells as the basic starting material. Brewer's yeast has been reported to be the best source of the glucose tolerance factor (Mertz (1969)). As the glucose tolerance factor has been reported to contain chromium it seemed likely that chromium-containing fractions from brewer's yeast would have a high biological activity as measured by assays for the glucose tolerance factor. An assay to determine biologically active chromium was not initially available so it was decided to isolate the chromium-containing fractions from extracts of whole yeast cells, monitoring the chromium levels by atomic absorption spectroscopy. If the chromium levels were too low for accurate detection in this way then the yeast would be grown on a medium containing chromium-51. The chromium-51-labelled complexes extracted from the yeast cells could be monitored throughout the isolation procedure by counting the gamma radiation emitted by the radioactive chromium-51 label.

After development of an isolation procedure, attempts could then be made to accumulate significant quantities of the major chromium complexes by supplementing brewer's yeast in a growth medium containing very high levels of chromium. If such accumulation experiments were successful it would then be possible to chemically characterise the major complex isolated, a step that is necessary if an understanding of the role of chromium in yeast and hopefully of the glucose tolerance factor, is to be obtained.

The work described in this section involves the attempts to isolate chromium complexes from (a) the commercially available brewer's yeasts, (b) the brewer's yeast grown on a medium containing radioactively labelled chromium-51 as a constituent and (c) from brewer's yeast supplemented

with chromium as chromium chloride at levels much higher than those found in naturally occurring media.

2.2 METHODS AND MATERIALS

2.2.1 Yeast sources.

Throughout the isolation attempted from the whole yeast, three different types of yeast source were used. These were the commercially available yeasts, brewer's yeast grown on a medium with radioactively labelled chromium-51 as a constituent and brewer's yeast supplemented with chromium chloride at chromium levels much higher than those found in naturally occurring media. These yeasts were obtained as follows:

(a) Commercially available yeast.

Two sources were used for bulk extractions, a true brewer's yeast (ex-Brewery) and a Saccharomyces cerevisiae strain used primarily for baking. Chromium analyses were performed on different batches of these.

(b) Yeast grown on media containing chromium-51.

The yeast source for the isolation of chromium-51 labelled fractions was a strain of Saccharomyces cerevisiae (employed primarily for brewing) grown on a complete mineral medium containing radioactively labelled chromium-51. This effected the incorporation of chromium-51 into the yeast cells and hence made it available for complex formation. The complete mineral medium was constituted as follows:

Sugar and Major Elements: Glucose monohydrate (100 g/l), NH_4Cl (1 g/l), $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (0.7 g/l), KCl (0.6 g/l), Na_2SO_4 (0.3 g/l), $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ (0.4 g/l), $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ (1.8 g/l) and citric acid (1.8 g/l).

Minor Elements: H_3BO_3 (0.50 mg/l), $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ (0.47 mg/l), $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ (0.20 mg/l), $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ (0.20 mg/l), ZnCl_2 (0.19 mg/l), KI (0.10 mg/l) and $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (0.027 mg/l).

Vitamins: Inositol (2.0 mg/l), thiamine-HCl (0.40 mg/l), pyridoxine-HCl (0.40 mg/l), biotin (0.002 mg/l), calcium-pantothenate (0.20 mg/l), folic acid (0.04 mg/l) and vitamin B_{12} (0.10 mg/l).

Radioactive chromium-51 was added to a level of ca 5 mCi/l. The vitamins were sterilized by micro-filtration while the remainder of the medium components were sterilized by autoclaving at 15 lb/in² for 5 minutes.

Glucose additions to the medium have been reported by Burkeholder and Mertz (1967) to stimulate the uptake of chromium-51 by the yeast and this phenomenon was considered to be desirable here in order to increase the yield of chromium-51 containing complexes, thus for the medium used here, additions of glucose (100 g/l) were made at day 0 and day 8 of a 25 day incorporation period. Samples were removed every second day for analysis. Chromium-51 was determined by radioactive counting and dry weights were determined by centrifugation of the yeast pellet with subsequent drying overnight in an oven at 80°C.

(c) Chromium chloride supplemented yeast .

Chromium chloride supplemented yeast was prepared by adding fifty grams of commercially available Saccharomyces cerevisiae to 1 litre of medium with $\text{CrCl}_3 \cdot 6\text{H}_2\text{O}$ (1 g) and the same quantities of sugar and major elements, minor elements and vitamins as described in the above medium. This yeast medium solution was shaken aerobically for 14 days before the cells were harvested and washed in distilled water to remove adhering medium solution.

2.2.2 Chromium determination .

(a) Solutions .

Aqueous solutions were analysed by flame atomic absorption spectrophotometry using an acetylene enriched flame. A slit width of about 0.2 nm was used along with the chromium band of 357.8 nm. Chromium standards were prepared from chromic nitrate standard solution for atomic absorption spectroscopy and were acidified to 2M with hydrochloric acid to minimize oxidation (Rollinson et al (1967)).

Flameless atomic absorption spectroscopy was sometimes used and was generally found to have a higher sensitivity. However solutions containing high concentrations of extracted yeast were unsuitable for this method as high background readings resulted. Routine analyses were therefore always carried out using flame atomic absorption spectroscopy.

(b) Solids .

The following wet ashing procedure was used to determine the chromium content of solid material. Between five and ten grams of solid sample were weighed into a 250 cm³

pyrex beaker and analar nitric acid (30 cm^3) was added. After stirring, the mixture was allowed to stand until the yeast had dissolved (about 1/2 hour) and the beaker was then placed on a hot plate and the nitric acid was gently evaporated off. The solid residue remaining was charred at a greater heat. This sample was then placed in a muffle furnace at 600°C overnight for ashing. For most samples this ashing procedure did not produce a uniformly white ash, therefore a further aliquot of nitric acid (5 cm^3) was added to the ash and gently evaporated. Re-ashing in the muffle furnace for 2 hours at 600°C produced a clean white ash which was suitable for analysis.

This ash was then taken up in between 4 and 7 cm^3 of 2.0 M HCl (depending on the weight of yeast sample taken), from which the chromium concentration was determined by atomic absorption spectroscopy.

(c) Chromium-51 detection.

Chromium-51 levels were determined in both solids and solutions by gamma counting using either a Packard Tri Carb or a Packard Model 5285 Auto Gamma Scintillation Spectrometer. Both machines were set to maximise counting efficiency which was 3.6% and 7.0% respectively.

2.2.3 Cell breakage techniques .

(a) Ball Mill .

Both aqueous yeast slurries and dried yeast samples were used in cell breakages attempted by means of the ball mill. Cell breakage was achieved by continuous revolution of a cylindrical mill containing the yeast sample along with a variety of marble balls (diameter range 0.5 cm - 1.7 cm) for periods of up to 48 hours.

(b) Polytron Homogeniser.

This method involved taking aqueous slurries of yeast and processing them for up to 5 minutes. The method relied on achieving the cell breakage by mechanical and high frequency processing.

(c) The French Press.

Aqueous slurries of yeast cells were processed by the French Press at pressures of ca 7600 psi. To obtain

an adequate cell breakage, most yeast slurries were processed by passing through the French Press twice. The French Press achieves the cell breakage by means of forcing the yeast slurry through a small aperture under very high pressure.

2.2.4 Ion-exchange and gel filtration resins.

Both ion-exchange and gel filtration resins were used during the course of the isolation procedures. Cellulose ion-exchangers (Whatman DE 23 and Whatman CM 23) were prepared according to the manufacturers instructions.

For gel filtrations Sephadex G10 and G25 were used. These were also prepared according to the manufacturers instructions.

2.2.5 Isoelectric focusing.

Isoelectric focusing was undertaken in a tube gel system by preparation of a 7.31% acrylamide gel by mixing an aqueous solution of 0.065% v/v N,N,N,N' tetramethylethylenediamine, 0.195% w/v N, N' methylene bisacrylamide and 7.31% w/v acrylamide, 0.96% v/v ampholyte solution, 0.22% w/v of the sample to be tested and finally 0.06% w/v potassium persulphate. After pouring into the cylindrical gel tubes the gels were allowed to stand until completely rigid. The gels were then placed in a holder so that one end was protruding into an aqueous 0.2% v/v sulphuric acid solution while the other end protruded into an aqueous 0.4% v/v ethanolamine solution. These solutions were connected to a power supply so that the sulphuric acid solution was at the anode and the ethanolamine solution was at the cathode. The sample was focused by means of applying a current of ca 2.0 mA for a period of about 2 hours during which time the monitored voltage rose to ca 350 volts.

Various commercial ranges of ampholyte solutions were available with broader ranges encompassing pH 3.5 - 10.0 while the narrower ranges best suited for this work were those covering the range from pH 2.5 - 4.0 and pH 3.0 - 6.0.

Analysis was carried out by slicing duplicate gels into 0.5 cm³ sections for pH and chromium measurements. Measurements of pH were made by washing the ampholyte from the gel with 2.0 cm³ of distilled water over a 1 hour period,

while the duplicate gel was used to determine the chromium position by standing the gel segment in 1.0 cm³ of 2.0 M HCl overnight and analysing the solution by atomic absorption spectrophotometry. Chromium concentrations were then plotted against the pH gradient.

2.2.6 Gel electrophoresis.

Gel electrophoresis was undertaken in a tube gel system by preparation of acrylamide gels composed of various percentages of acrylamide. The method used was essentially that described by Orstein (1964).

2.2.7 Paper electrophoresis.

Paper electrophoresis was carried out at pHs 6.5 and 2.1. A standard marker solution containing the amino acids tryosine, phenylalanine, methionine, leucine, valine, arginine and lysine was run and the direction of flow and velocity of the chromium sample was compared to these. The electrophoretograms were developed in ninhydrin to enable determination of the markers as well as any amino acid content in the loaded samples. Chromium determination was undertaken by cutting the paper electrophoretogram into segments and washing the chromium from these with 1.0 M HCl and determining the chromium concentration by atomic absorption spectroscopy.

2.2.8 Butanol-acetic acid-water paper chromatography.

The mobility of the anionic chromium complex on paper was determined in a butanol-acetic acid-water solvent system made up in the ratio of 5 : 1 : 6. The chromatogram was developed in ninhydrin and then cut and eluted with 1M HCl for chromium determination by atomic absorption spectroscopy.

2.2.9 Acid and base hydrolysis for amino acid analysis.

Acid hydrolysis was performed with 6 M hydrochloric acid for 24 hours at 110^o C in sealed tubes under vacuum. Water and HCl were removed from the sample under vacuum in a dessicator over phosphorous pentoxide and sodium hydroxide pellets. The sample was then taken up in water and neutralised with 0.5 M sodium hydroxide. Base hydrolysis was performed with 0.4 M barium hydroxide for 24 hours at 110^o C under vacuum. Carbon dioxide was bubbled through the

solution until barium carbonate precipitation was complete. The aqueous hydrolysate was then collected by centrifugation. Both samples were used for amino acid analysis by means of a Beckman 119 BL amino acid auto analyser.

2.2.10 Infra-red, ultra-violet and visible spectral analyses.

Infra-red spectra were determined on a Pye Unicam SP3 - 300 infra-red spectrophotometer while the ultra-violet visible spectra were determined on a Shimadzu MPS 500 spectrophotometer.

2.2.11 Micro analysis.

Micro analysis was kindly carried out by the Department of Chemistry at Otago University.

2.2.12 Carbohydrate tests.

(a) Molisch test.

Molisch reagent (a 1% solution of α -naphthol in ethanol) was used as a qualitative test for the presence of carbohydrate. The tests involved adding 0.2 cm^3 of the Molisch reagent to a 3 cm^3 aqueous solution of the sample to be tested and adding to this (by pouring down the side of the test tube) 3 cm^3 of concentrated sulphuric acid. A positive test is shown by a purple or violet colouration at the interface. This is caused by the dehydration of the carbohydrate by the concentrated sulphuric acid to form a hydroxymethyl furfural and the reaction of this with the α -naphthol to produce the coloured condensation product.

(b) Phenol-sulphuric acid test.

The quantitative analysis of carbohydrates was determined by the phenol-sulphuric acid test as described by Hirs (1967). Standard samples were prepared with glucose concentrations of $0.12 \times 10^{-4} \text{ M}$, $0.60 \times 10^{-4} \text{ M}$, $1.84 \times 10^{-4} \text{ M}$ and $3.68 \times 10^{-4} \text{ M}$ and samples were examined at the maximum wavelength for hexoses of 489 nm.

2.2.13 Nuclear magnetic resonance spectroscopy.

Where practical both proton and carbon-13 spectra were determined for samples. Both types of spectra were determined on a JEOL FX60 Pulse Fourier Transform Nuclear Magnetic Resonance Spectrometer using a magnetic field of 1.4T with frequency transmissions of 60 MHz for ^1H and 15 MHz for ^{13}C .

2.2.14 Absorbance, conductivity and pH measurement.

Absorbance measurements were made using a Unicam SP 500 spectrophotometer, pH measurements on a Radiometer model 28 and conductivity on a Radiometer model CDM 2e.

2.3 RESULTS

The isolation and purification of chromium complexes from whole yeast was attempted by three different approaches:

(1) Bulk extraction and purification from brewer's yeast sources shown to be high in chromium.

(2) Extraction and isolation of radioactive fractions from yeast grown in media containing chromium-51.

(3) Extraction and isolation of chromium complexes from brewer's yeast where the chromium levels had been artificially increased by supplementation of the growth medium with inorganic chromium (as $\text{CrCl}_3 \cdot 6\text{H}_2\text{O}$).

2.3.1 Bulk extraction and purification from brewer's yeast.

The yeast obtained from the Brewery and the Saccharomyces cerevisiae strain were analysed on receipt of each new batch. The chromium concentrations were determined by the wet ashing method (Section 2.2.2b) and the results are shown in Table 2.1.

TABLE 2.1

CHROMIUM LEVELS IN BREWER'S YEAST USED IN EXTRACTIONS

Yeast sample	Average chromium concentration	Range
	$\mu\text{g/g}$	$\mu\text{g/g}$
Brewery yeast	0.38	0.20 - 0.72
Saccharomyces cerevisiae	0.32	0.13 - 0.64

Of the three cell breakage methods described the French Press gave the best results. While the other methods described (Polytron Homogeniser and Ball Mill) did have advantages in terms of ease of operation, the highest cell breakage was found, by microscopic observation, to be returned by passage of the yeast slurry twice through the French Press. Since the working volume of the press was only 100 cm^3 , disruption of larger volumes of yeast slurry was time consuming but was still completed more rapidly and with higher cell

breakage than by any other method.

Separate aqueous extractions on the broken yeast cell homogenate were carried out, in conjunction with n-butanol and chloroform, to determine their ability to act as precipitants of extraneous matter and also solvents of extraneous matter. The n-butanol was found to be successful in solubilizing organic material but the total yield of chromium in the aqueous layer was never better than 30%, with the remaining chromium being associated with the large cell debris or unbroken cells. Within the limits of detection (0.04 ppm) there was no chromium in the butanol. Chloroform was ineffective as both a precipitating and solubilizing reagent. Aqueous extracts of broken yeast cells always produced higher chromium yields than similar extracts on unbroken cells but it should be noted that unbroken yeast cells when extracted with water did yield trace amounts of chromium.

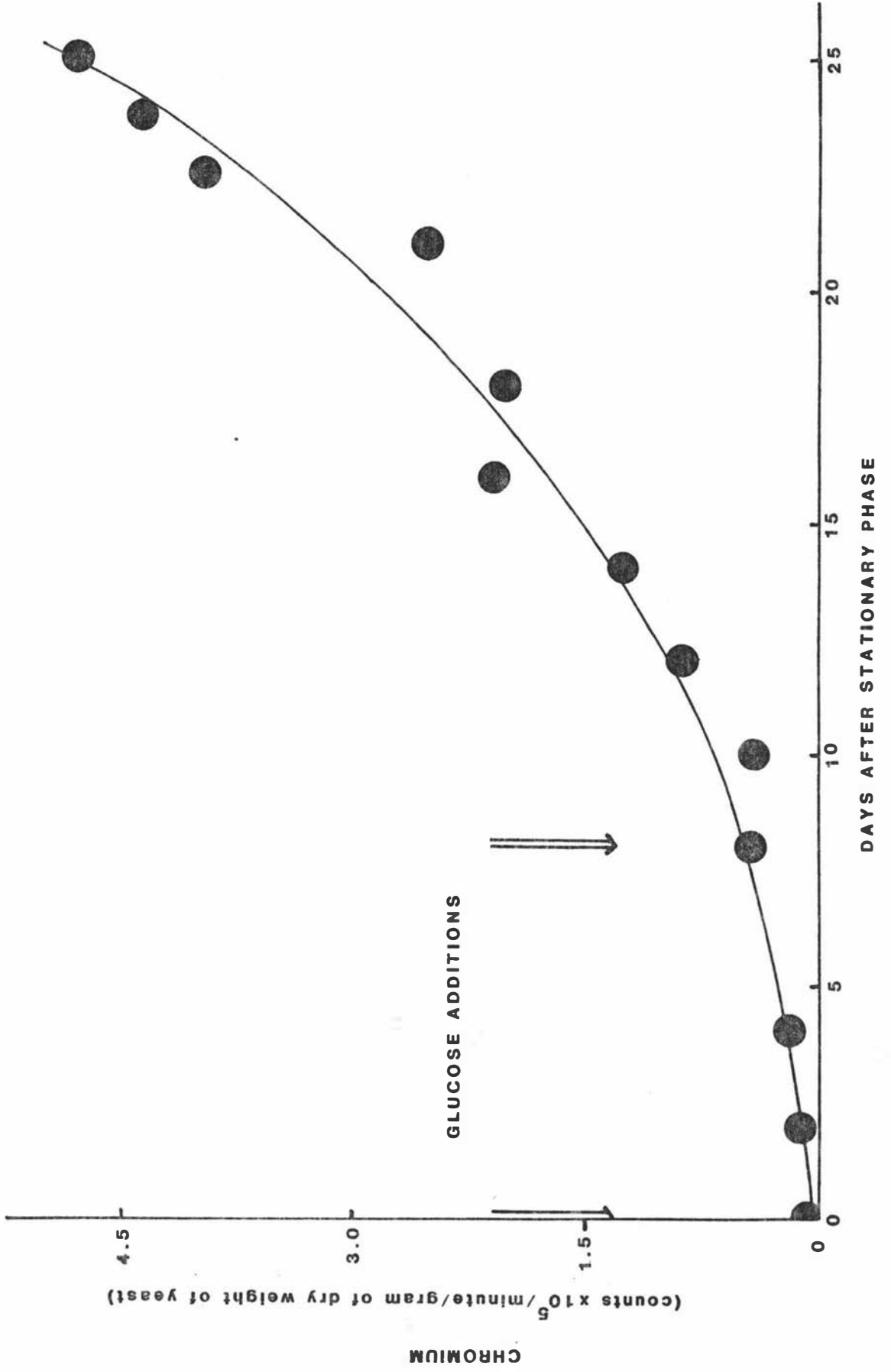
Two types of chromatography were used in an attempt to isolate and purify the chromium fractions in the aqueous extract. DE 23 cellulose resin and activated charcoal were used but the low levels of chromium present in the aqueous extract (15 μg Cr from 100 g of yeast) meant that analysis of fractions on elution from these columns could never unambiguously locate the various chromium peaks. Thus it was necessary to change to radioactive counting of chromium-51 in order to have any chance of success in the attempt to develop an isolation procedure for the chromium complexes.

2.3.2 Yeast growth in chromium-51-containing media. Isolation of chromium-51-containing complexes.

The incorporation of chromium-51 into the yeast sample over a 25 day period is shown in Figure 2.1. On day 25 the suspended yeast cells were centrifuged and the yeast pellet so obtained was washed twice in distilled water to remove adhering medium solution from the yeast.

Cell cleavage was achieved using the French Press and the broken yeast cell slurry was extracted in a 1:1 water-butanol mixture. By this procedure fifty-five percent of the total chromium-51 was extracted into the aqueous layer. Polyethylene glycol and ammonium sulphate were tried as precipitants in an attempt to separate the chromium-51-

FIGURE 2.1



containing fraction from high molecular weight material such as proteins, but without success.

Thus in order to separate the lower molecular weight chromium-51-containing fraction from the extraneous material gel filtration columns were employed. The aqueous extract was concentrated by freeze drying and then applied separately to both Sephadex G10 and Sephadex G25 columns. These were run using both water and pH 7.7 Tris-HCl buffer (0.01 M). However none of these treatments were able to separate the chromium from the extraneous material.

Both anion and cation-exchange chromatography were undertaken on the chromium-51-containing aqueous extract using the cellulose resins DE 23 and CM 23. The aqueous extract was first loaded onto a DE 23 column at pH 7.7 and the bound material was eluted with a pH 7.7 Tris-HCl buffer gradient (0.01 M - 1.0 M). Figure 2.2a shows the separation achieved where a bound anionic fraction and a non-binding fraction are apparent. (The void elution volume was ca 100 cm³).

The optical density at 280 nm and the conductivity measurements are also shown. The fraction not binding to the DE 23 column, labelled as Peak A was then titrated to pH 5.2 and loaded onto a CM 23 column. This column was eluted with a pH 5.2 sodium acetate-acetic acid buffer gradient (0.01 M - 1.0 M). Figure 2.2b shows the separation achieved with the larger fraction not binding to this column but a smaller chromium fraction did bind and was eluted with the sodium acetate-acetic acid buffer gradient. Application of Peak B to a CM 23 column resulted in no binding of chromium containing material and hence confirmed its anionic nature.

Examination of the amounts of chromium-51 under the peaks B, C and D in Figures 2.2a and 2.2b, and the pH conditions used in the separation gave an indication of the relative amounts of cationic, anionic and neutral chromium present. These peak areas indicated that the relative amounts were : anionic 80% (Peak B), cationic 1% (Peak D) and neutral 19% (Peak C). It must be noted that the elution conditions used in this separation may not have been sufficient to elute all of the bound chromium containing fractions and

FIGURE 2.2a

Elution profile of the DE 23 column (4.6 cm x 15 cm) after separation of the chromium-51 labelled aqueous extracted yeast sample. The tube volume was 10 cm^3 and the eluent reagents are described in the results. The chromium peaks (————) are designated as A and B. Conductivity (-----) and optical density at 280 nm (-----).

FIGURE 2.2b

Elution profile of the CM 23 column (4.6 cm x 15 cm) after separation of Peak A into peaks C and D. The tube volume was 10 cm^3 and the eluent reagents are described in the results. Chromium (————), conductivity (-----) and optical density at 280 nm (-----).

FIGURE 2.2a

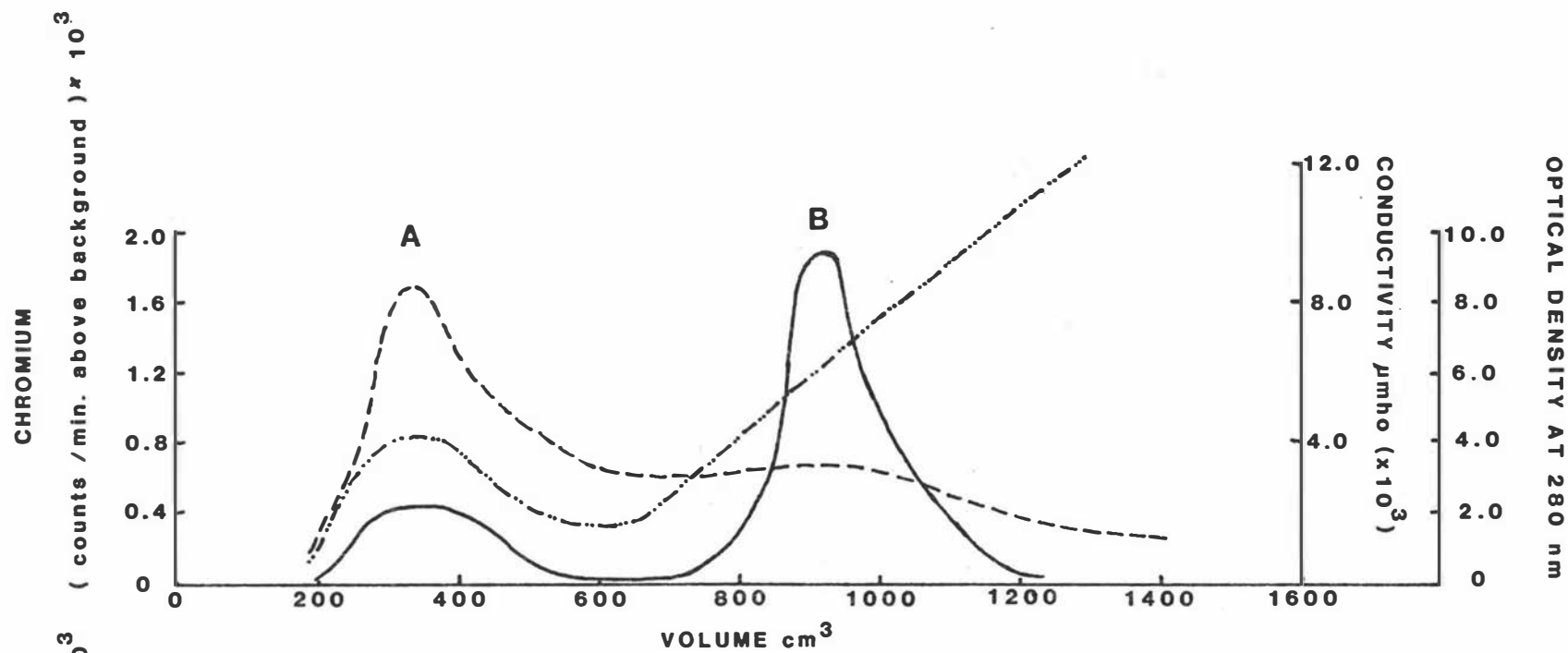
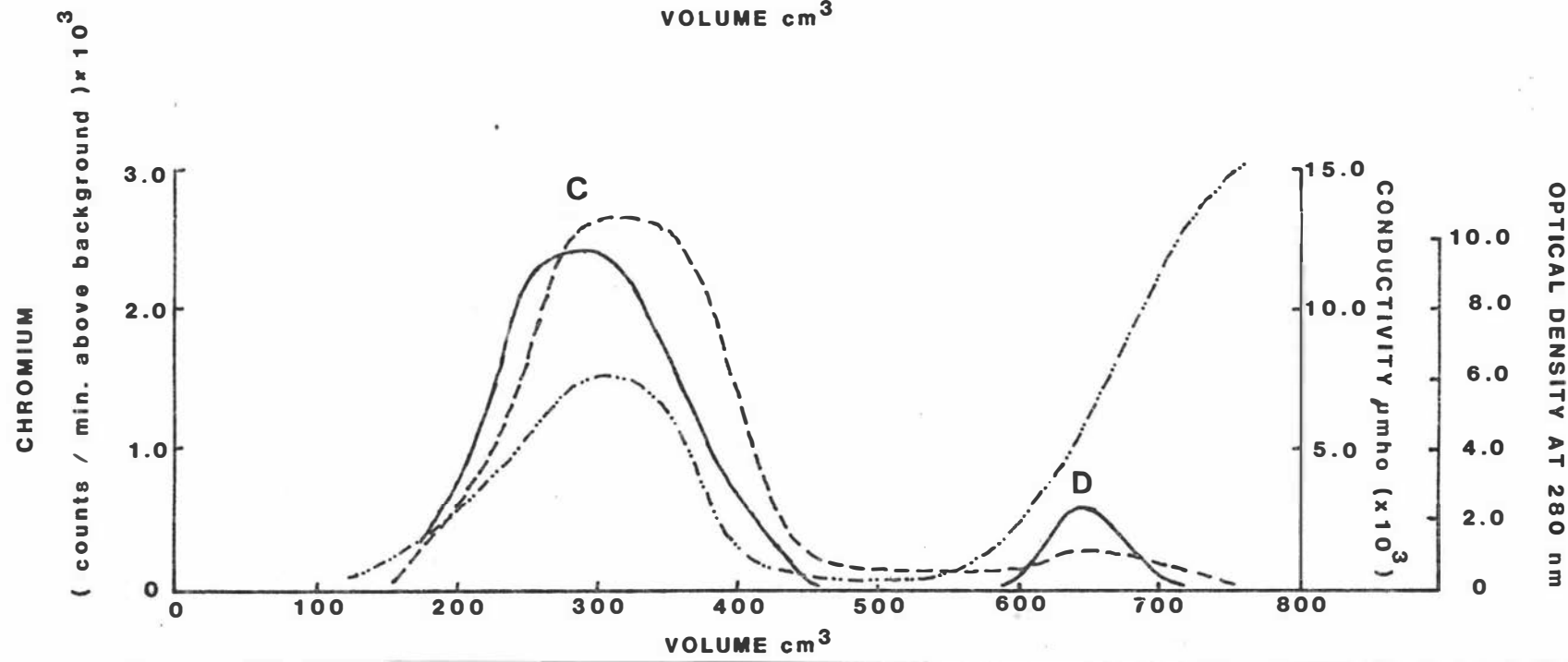


FIGURE 2.2b



may not have separated possible different bound chromium fractions from each other. (For a discussion of other gradients used see section 4). This work clearly showed however the major aqueous extracted chromium fraction to be anionic but also revealed the presence of at least two other types of chromium containing fractions which were cationic and neutral (or charged chromium complexes incapable of binding to cellulose resin). Since chemical characterisation of the very small samples obtained from the radioactive isolation was clearly impracticable attempts were then made to artificially increase the amount of chromium in the yeast cell by fermenting the yeast on a medium supplemented with chromium chloride.

2.3.3 Extraction from chromium chloride supplemented yeast.

A similar isolation and extraction procedure to that used for the yeast grown on chromium-51 containing medium was adopted. Cell breakage was carried out using the French Press, followed by aqueous extraction in a 1:1 butanol-water mixture and chromatography on a DE 23 anion-exchange column at pH 6.5. Figure 2.3a shows the separation achieved with elution by a single step pH 6.5 Tris-HCl buffer (0.4M). This shows the elution of a bound fraction containing 8500 μg Cr representing 85% of the total chromium loaded and also a small chromium-containing fraction not binding to the column which contained 800 μg Cr representing 8% of the total chromium. These amounts represented a 500 fold increase in the aqueous extractable chromium over the levels obtained from natural brewer's yeast sources. The large peak which bound to the DE 23 column was concentrated by freeze drying and applied to Sephadex G25 gel filtration column. Figure 2.3b shows the separation of the chromium into two fractions (these were designated as the high molecular weight anionic fraction and the low molecular weight anionic fraction). The larger of the two peaks, the high molecular weight anionic fraction was selected for further investigation. Both fractions as aqueous solutions were green and on freeze drying of the high molecular weight sample a green solid was obtained.

FIGURE 2.3a

Elution profile of the aqueous extract from the chromium chloride-supplemented yeast separated on a DE 23 anion-exchange column (4.6 cm x 15 cm). The tube volume was 10 cm³ and the eluent reagents are described in the results. Chromium (—————), optical density at 280 nm (-----).

FIGURE 2.3b

Elution profile of the bound fraction from Figure 2.3a on a Sephadex G 25 column (2.8 cm x 81 cm). The tube volume was 10 cm³ and the column was run using water as the eluent. Chromium (—————) and optical density at 280 (-----).

OPTICAL DENSITY AT 280 nm

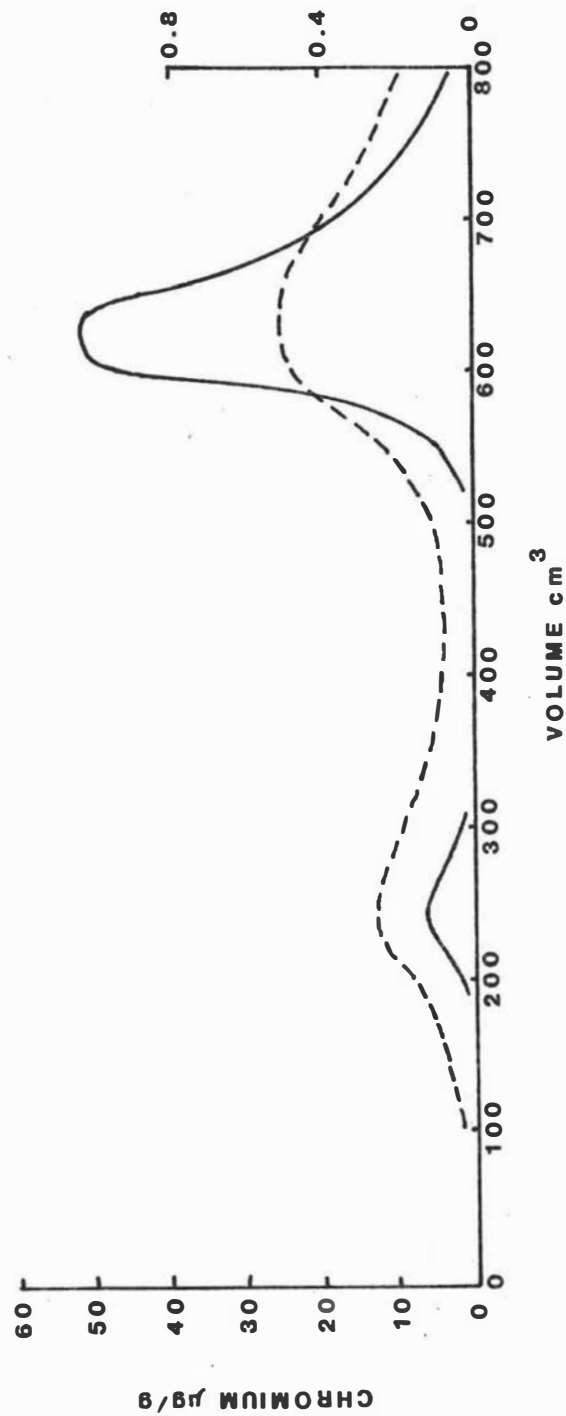


FIGURE 2.3a

OPTICAL DENSITY AT 280 nm

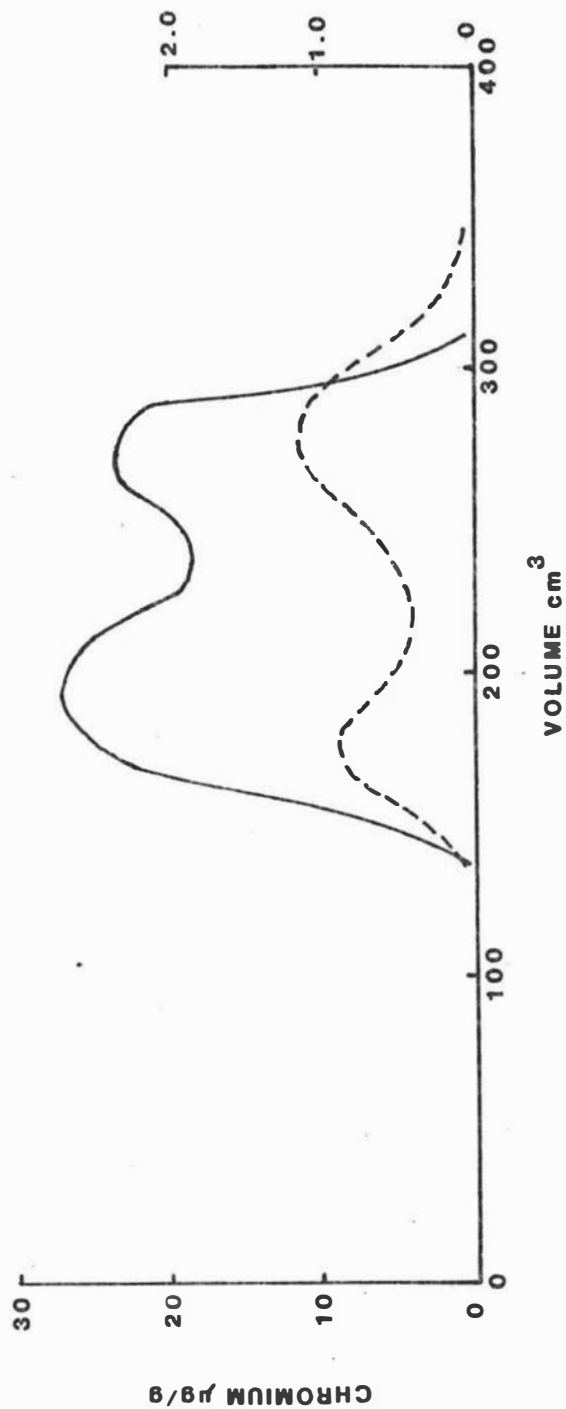


FIGURE 2.3b

2.3.4 Ultra-violet, visible and infra-red spectra.

The ultra-violet and visible absorption spectra of the high molecular weight anionic fraction are shown in Fig. 2.4. In the visible spectrum two characteristic chromium peaks are apparent at 415 nm and 595 nm. The 595 nm band has an extinction coefficient of ca 30 if the complex is assumed to be pure and to have a molecular weight of about 1000. This data compares favourably with typical six co-ordinate chromium (III) complexes such as the green $\text{Cr}(\text{Cl})_2(\text{H}_2\text{O})_4^+$ which has an extinction coefficient of 16 and peaks at 440 nm and 630 nm.

In the ultra-violet spectrum a peak was observed at 262 nm which on the basis of the above information would have had an extinction coefficient of 1090.

It was not easy to obtain an infra-red spectrum as the sample was difficult to mull and even a spectrum determined as a KBr pellet was poor. The only readily distinguishable characteristic was a broad peak characteristic of H_2O or $-\text{OH}$ in the region of 4000 cm^{-1} .

2.3.5 Isoelectric focusing.

Results from a variety of ampholine solutions showed apparent focusing of the high molecular weight anionic fraction as a single peak positioned well towards the anode. Figure 2.5a shows a typical example in which the chromium peak has almost run off the lower pH end of the gel. In this gel the pH range used was from 3.5 to 6.0. All the isoelectric focusing results were consistent with the complex being highly anionic.

2.3.6 Gel electrophoresis.

Gel electrophoresis results were generally unrewarding in that the high molecular weight anionic fraction appeared to run as a single peak. The speed at which the sample ran necessitated increasing the acrylamide concentration to 24%. A typical example is shown in Fig. 2.5b where the speed of the complex through acrylamide gel is evident by its faster movement compared with the bromophenol blue marker band, possibly suggesting a highly charged low molecular mass species (or mixture of species).

FIGURE 2.4

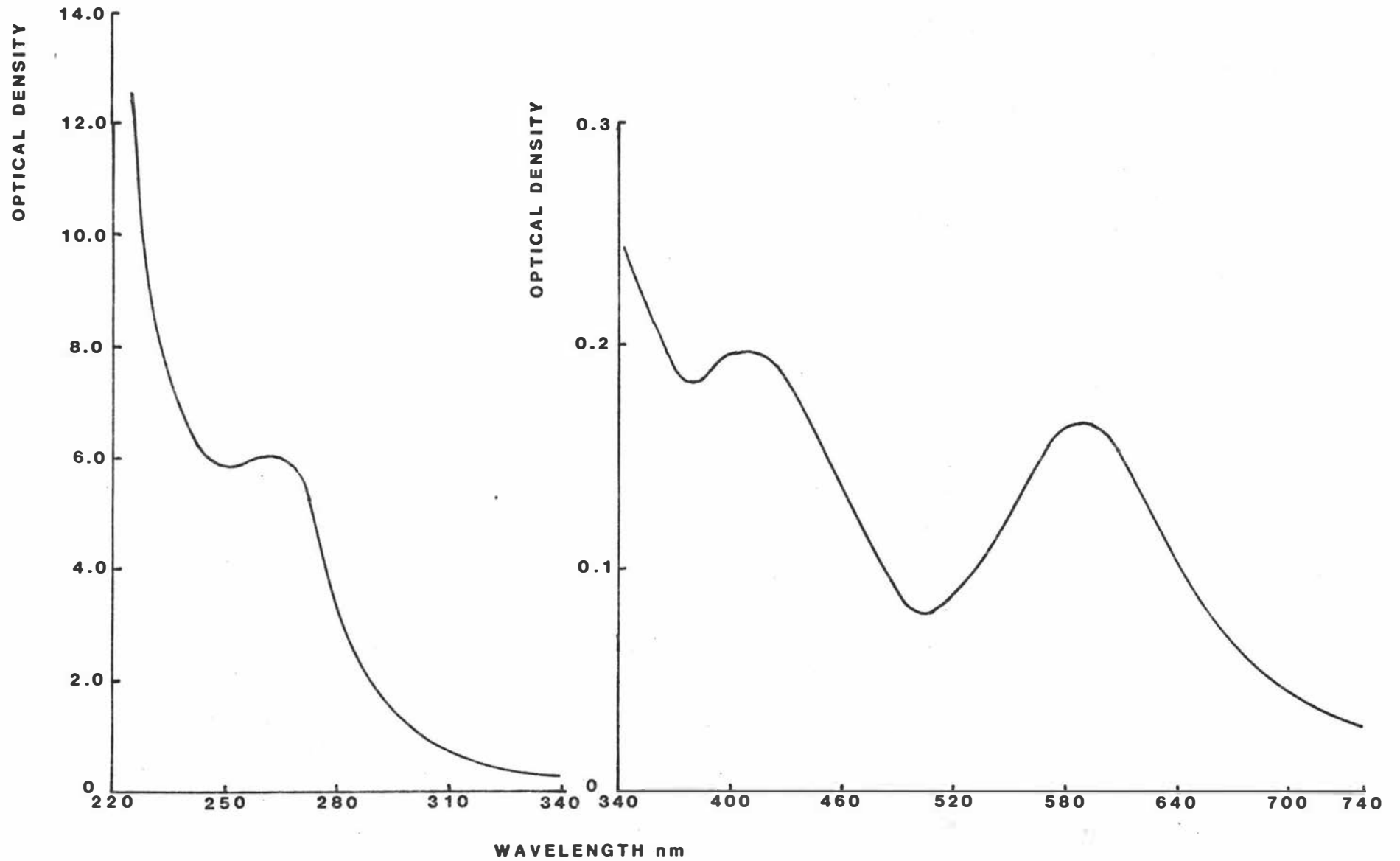


FIGURE 2.5a

PH (○.....○) and chromium (●————●) profiles from an isoelectric focusing gel using an ampholine pH range of 3.5 to 6.0 for the high molecular weight anionic fraction.

FIGURE 2.5b

Profile of the high molecular weight anionic fraction on gel electrophoresis where chromium is shown by (●————●) as determined by atomic absorption spectrophotometry. The visual appearance is shown by the green chromium band (||) and the bromophenol blue marker band (|||).

FIGURE 2.5a

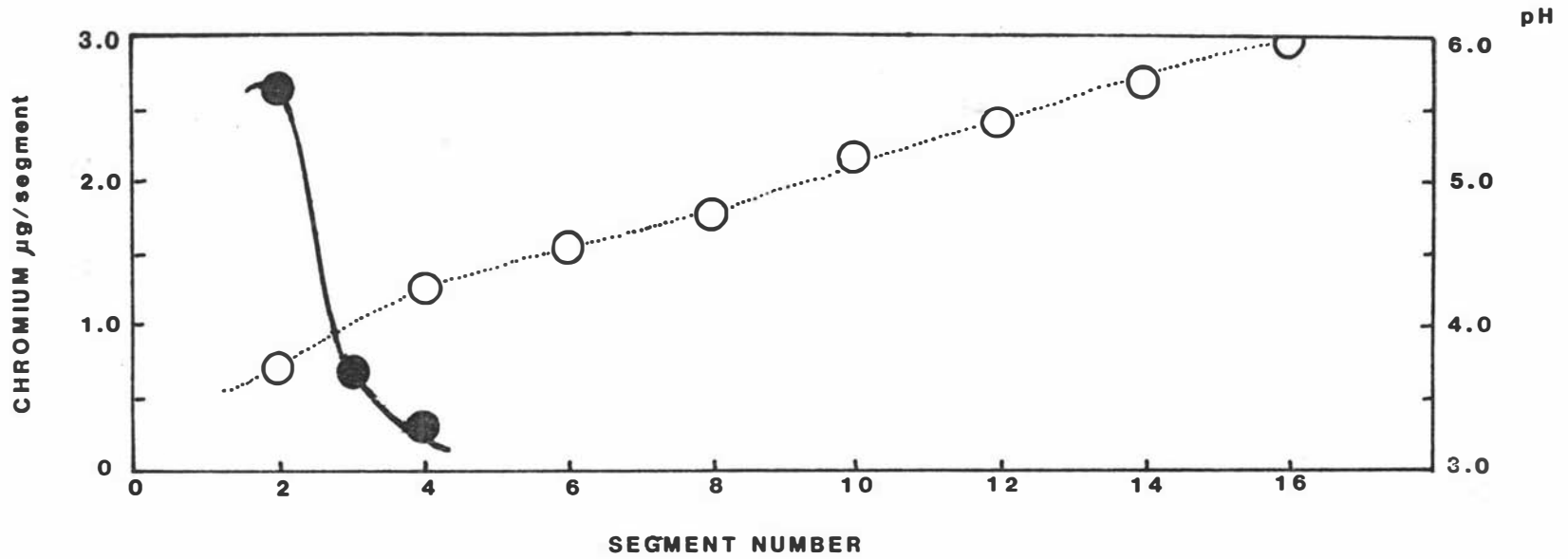
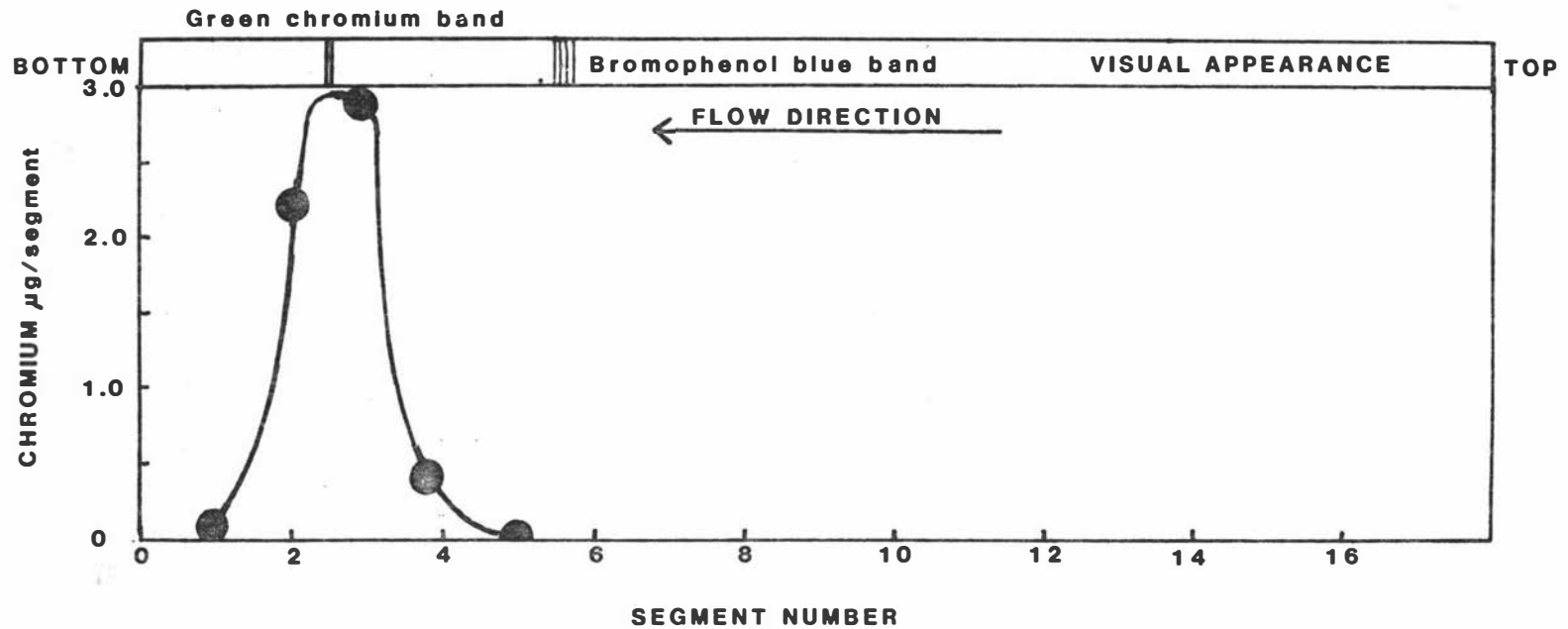


FIGURE 2.5b



2.3.7 Paper electrophoresis.

Paper electrophoresis was run at the pHs of 6.5 and 2.1. The results after developing in ninhydrin and analysing for chromium are shown in Figures 2.6a and b. It is apparent in both cases that the high molecular weight anionic fraction was moving toward the anode so that even at pH 2.1 it was still anionic. At the chromium peak on both electrophoretograms there was no associated ninhydrin stain indicating that the complex was not associated with protein or amino acids.

2.3.8 Butanol-acetic acid-water paper chromatography.

After ninhydrin development and chromium analysis it was apparent that virtually no movement of the chromium complex from the origin had occurred in this solvent system. Again the ninhydrin development suggested that the high molecular weight anionic fraction was not associated with amino acids.

2.3.9 Amino acid analysis.

Although preliminary work suggested that amino acids were not present in the high molecular weight anionic fractions it was considered worthwhile to carry out an amino acid analysis in case the chromium was effectively blocking the reaction of ninhydrin with any bound amino acids. However results from the auto analyser did not reveal any amino acids but did reveal some highly basic but undefined species.

2.3.10 Micro analysis.

Micro analysis results on the high molecular weight anionic fraction received from Otago University were as reported in Table 2.2. The chromium value was determined by atomic absorption spectroscopy and dry weight determination.

TABLE 2.2

ELEMENTAL PERCENTAGE COMPOSITION OF THE HIGH MOLECULAR WEIGHT ANIONIC COMPLEX BY WEIGHT.

<u>Element</u>	<u>Percentage of total sample</u>
C	25.40
H	5.34
N	3.87
P	4.09
Cr	4.0

FIGURE 2.6a





Paper electrophoretogram of the high molecular weight anionic fraction at pH 6.5 showing the amino acid markers () as described in methods and the chromium band peak ().

FIGURE 2.6b

Paper electrophoretogram of the high molecular weight anionic fraction at pH 2.1 showing the amino acid markers () as described in methods and the chromium band peak ().

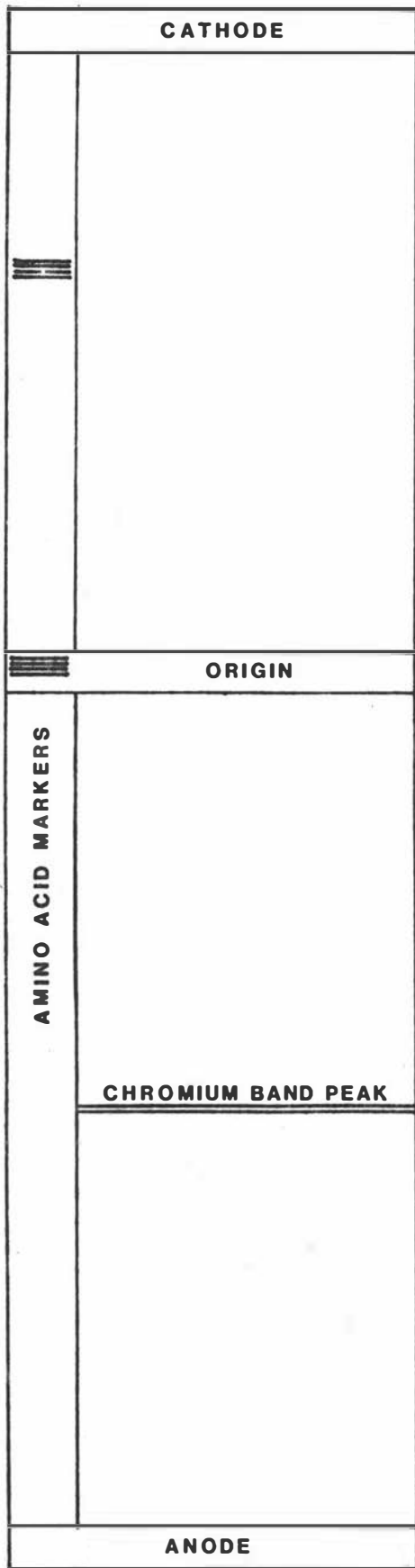


FIGURE 2.6a pH 6.5

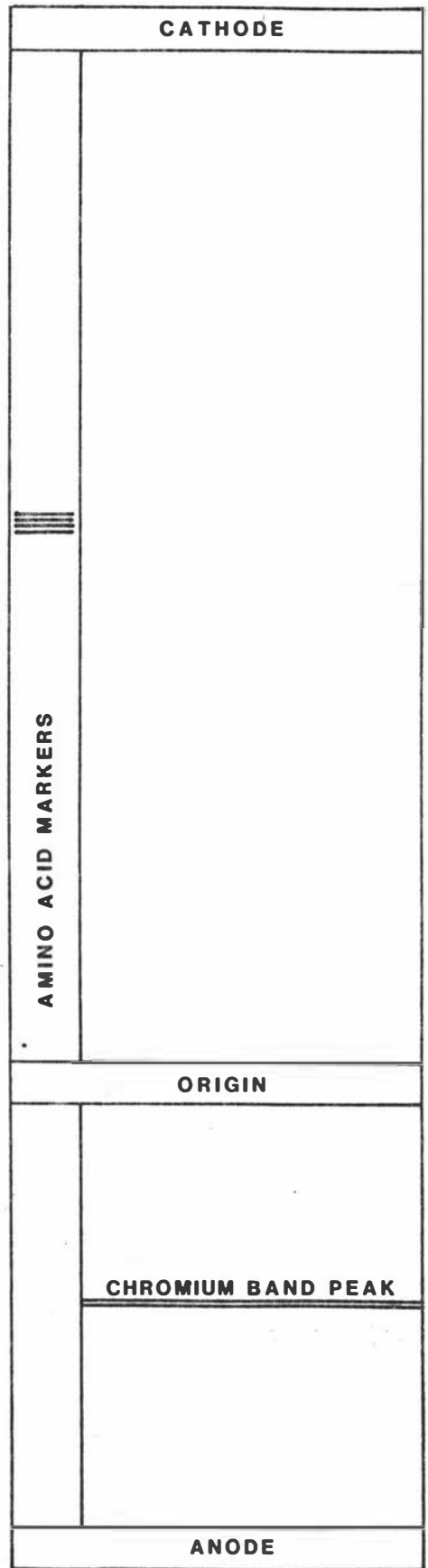


FIGURE 2.6b pH 2.1

Unfortunately oxygen was not determined but presumably must have made up a large part if not all of the remaining 58%.

2.3.11 Carbohydrate tests..

(a) Molisch test.

On analysis of the high molecular weight anionic fraction a faint purple colouration was observed at the interface between the aqueous layer and the sulphuric acid. This was indicative of a positive test and the presence of carbohydrate.

(b) Phenol-sulphuric acid test.

A standard curve prepared from the glucose concentrations described in methods is shown in Figure 2.7. A solution of the green complex analysed by this test showed that 7.5×10^{-5} grams of sample contained 3.05×10^{-7} moles of carbohydrate, or in terms of glucose 5.49×10^{-5} grams which would represent a glucose percentage by weight of 73% of the total sample.

It must be noted that neither of the carbohydrate tests used were specifically designed for carbohydrate bound to metal ions and because of this differences may have occurred particularly in the quantitative analysis by the phenol-sulphuric acid test described above.

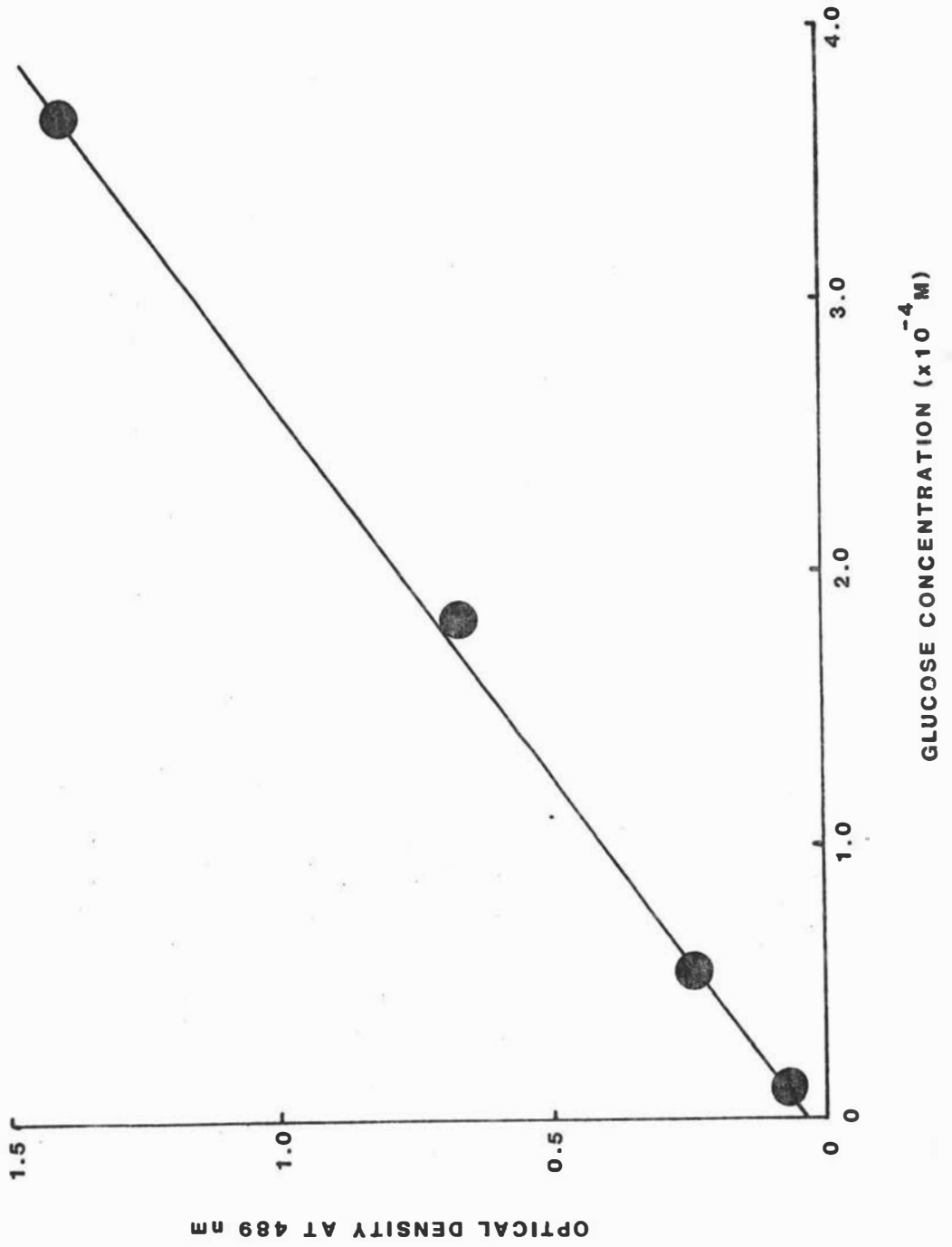
2.3.12 Nuclear magnetic resonance spectroscopy.

Figures 2.8a and b show the ^1H and ^{13}C spectra of the high molecular weight anionic fraction in D_2O . Figure 2.8c shows for comparison the ^{13}C spectrum for glucose also dissolved in D_2O . If as seems likely, the high molecular weight anionic fraction contains chromium in the +3 state then the configuration of the chromium will have three unpaired electrons. This would cause paramagnetic line broadening in the nmr spectrum particularly in bands representing atoms in close proximity to the chromium atom and also may be expected to cause some paramagnetic contact shifts. A single peak 4.17 ppm from the reference T.P.S. (3-(Trimethylsilyl) - propanesulfonic acid sodium salt) was observed. (Figure 2.8a). This peak is most likely due to the presence of H_2O either bound to the chromium or associated with the sample in some other way. No C-H peaks were observable in the spectrum. There are six distinct peaks in Figure 2.8b

FIGURE 2.7

Standard curve used in the phenol-sulphuric acid test for the determination of carbohydrate concentration. Glucose concentrations for the standards were between 0 and 4.0×10^{-4} M while the optical density was measured at 489 nm.

FIGURE 2.7



with shifts from the T.P.S. reference of 104.05, 80.19, 75.16, 72.07, 68.66 and 62.82 ppm. These can be compared with the glucose spectrum shown in Figure 2.8c with seven peaks at 97.23, 93.34, 77.10, 74.02, 72.72, 70.94 and 61.85 ppm. There is a striking resemblance between the two spectra. Without a considerable amount of further work a detailed assignment of the ^{13}C bands can not be given.

2.3.13 Mass spectrometry.

No sensible spectrum was obtained for the high molecular weight anionic fraction as the material did not readily volatilize.

FIGURE 2.8a

^1H nmr spectrum of the high molecular weight anionic fraction in D_2O .

FIGURE 2.8b

^{13}C nmr spectrum of the high molecular weight anionic fraction in D_2O .

FIGURE 2.8c

^{13}C nmr spectrum of glucose D_2O .

FIGURE 2.8a

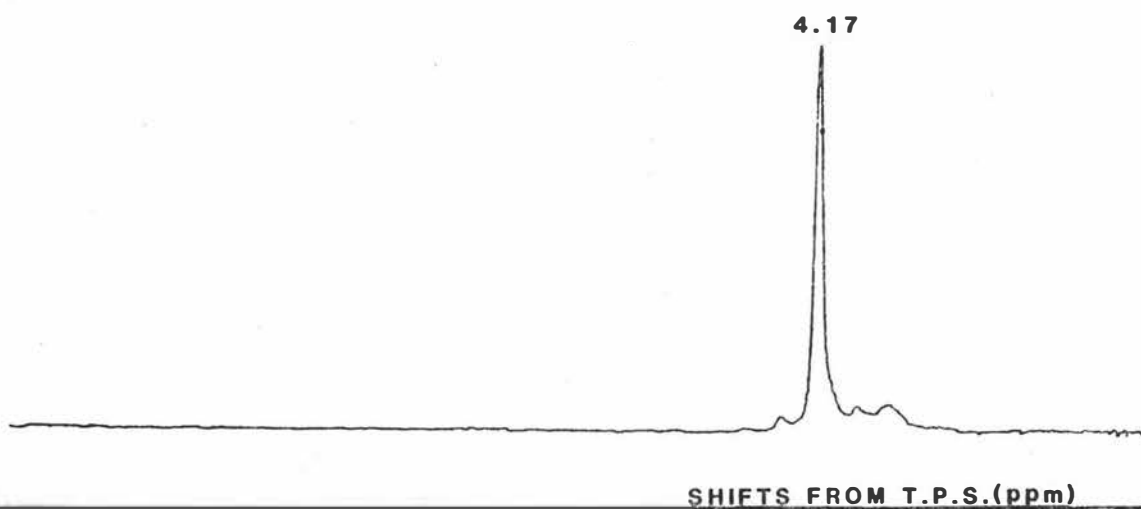


FIGURE 2.8b

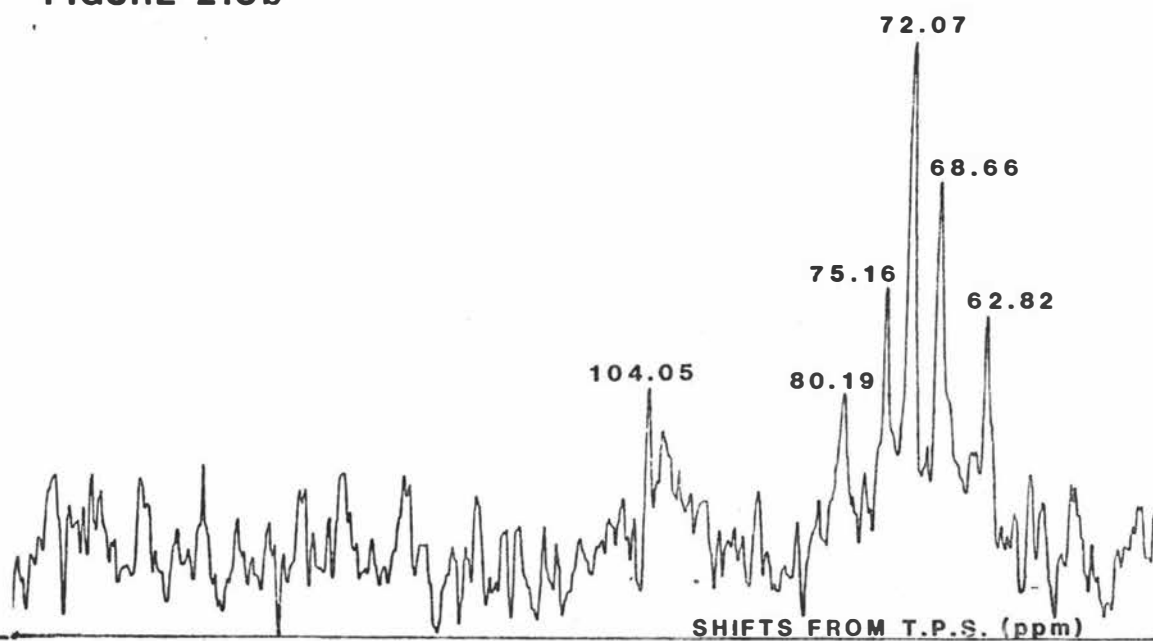
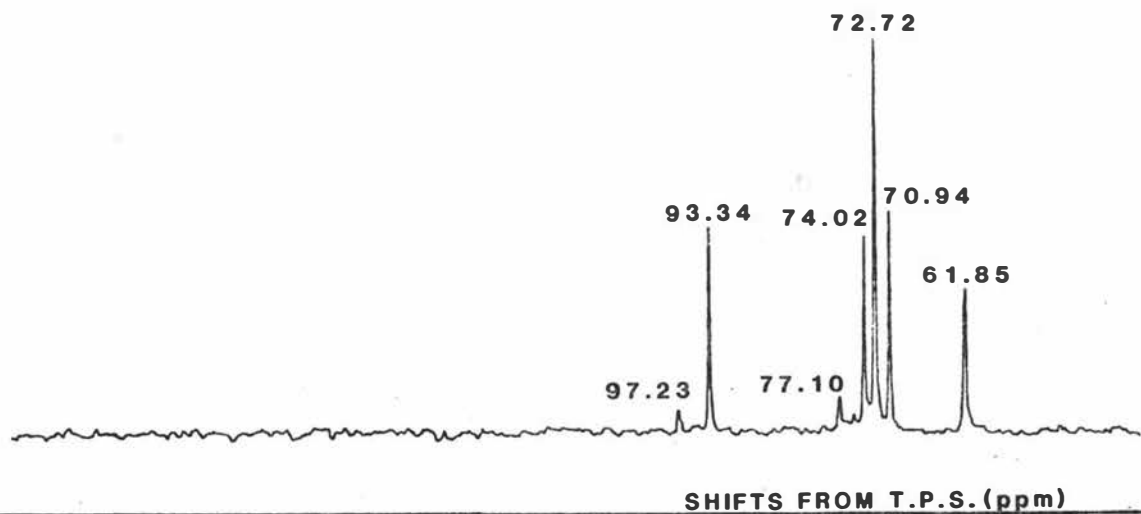


FIGURE 2.8c



2.4 DISCUSSION

The two commercially available yeasts had an outwardly similar appearance, with perhaps the Saccharomyces cerevisiae type having a cleaner appearance. The chromium levels varied widely for different batches of these two yeasts although as shown in Table 2.1 the average chromium levels were very similar. The S. cerevisiae type had a shorter growth period, or a shorter period in contact with the growth medium, than the ex-brewery yeast but whether this caused any metabolic changes which could effect the chromium levels is not known.

Using whole yeast cells as a basis for any extraction and purification procedure requires that some method of cell cleavage is used as a first step. Best results were achieved using the French Press whereby an aqueous extraction of chromium from yeast cells yielded about 30% recovery on average. Taken at face value this percentage does not seem too prohibitive but when it is realised that the chromium in the yeast does not exist as a unique complex then the amount of each chromium fraction becomes an important factor.

Methods reported in the earlier papers by Votava et al (1973) and Toepfer et al (1977) involved alcohol-water extraction without any cell cleavage, with Votava using n-butanol and Toepfer using ethanol. Results determined here indicate that some chromium can in fact be extracted from the yeast cell without cell cleavage but the percentage obtained is markedly increased by using a cell cleavage technique. If the yeast cell wall is not ruptured there is a danger that only simple chromium salts may be extracted leaving the larger and perhaps more interesting chromium complexes retained by the yeast. In a recent paper Kumpulainen et al (1978) have used a cold press for cell disintegration coupled with ethanol-water extraction. Values obtained by Kumpulainen for the amount of chromium extracted, range from 30%-50% which is in agreement with the data obtained in the present work.

In the preliminary search for chromium complexes in yeast, the problems associated with isolating a complex from natural brewer's yeast in amounts large enough for character-

isation experiments become apparent. Assuming a 100% extraction of the chromium in the yeast and only one chromium complex (of molecular weight about 500), then it would be necessary to take about three kilograms of brewer's yeast to obtain 10 mg of the complex. In practice however the best extraction achieved was only ca 50% and there was a loss of sample of about 10% at each of the purification steps. Also the ion-exchange chromatography undertaken on the aqueous extract obtained from yeast grown on the chromium-51 containing medium clearly showed the presence of at least three different types of chromium; anionic, cationic and neutral (Figures 2.2a and 2.2b). It would therefore have been necessary to take many kilograms of yeast in order to get 10 mg of any given chromium fraction.

For this reason the strategy of growing brewer's yeast in a medium containing chromium-51 was adopted. Figure 2.1 shows the increase in chromium-51 incorporation into the yeast over a 25 day incubation period. This data indicates that yeast once it has reached its stationary growth phase does assimilate chromium to quite a considerable extent. The enhanced uptake of chromium-51 brought about by glucose additions seems consistent with the claims of Burkeholder and Mertz (1967) although a control experiment in which no glucose additions were made was not undertaken here. In the light of later work (see section 4) the resin and elution systems used here were not ideal for the isolation of relatively small molecular weight cationic molecules. A more detailed examination of the chromium complexes isolated from yeast grown on media containing chromium-51 is made in section 5. At this stage however the ability to bind and elute chromium-containing fractions from the yeast extract served as encouragement to try and increase the absolute levels of the various chromium fractions by allowing yeast to metabolise in a medium with chromium chloride as a constituent.

The method of chromium supplementation described (see section 2.2.1c) in fact resulted in a 500 fold increase in the amount of aqueous-extractable chromium over the levels obtained from a similar weight of natural brewer's yeast. Figure 2.3a shows the predominance of anionic chromium

material within the chromium extracted, with 85% of the chromium binding to the DE 23 anion-exchange column. (The fraction which did not bind to the anion-exchange column, containing 8% of the chromium was not further investigated.) In this respect the behaviour was similar to that observed for yeast grown on chromium-51 where eighty percent of the material extracted was anionic. This was a result which could not have been predicted since the vastly different molar amounts of chromium in the two systems could have resulted in quite different relative amounts of the anionic, cationic and neutral chromium fractions. The anionic peak shown in Fig 2.2a when chromatographed on gel filtration columns split into two fractions (Figure 2.2b). As the high molecular weight fraction was now the major fraction isolated from the yeast, it was subjected to a range of chemical characterisation tests. It was considered worthwhile to try and determine its chemical composition since this would give an indication of the origin of the material.

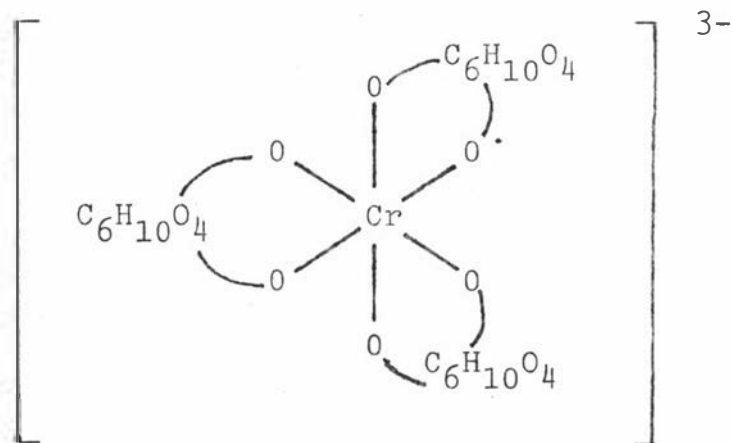
The peak position and the calculated extinction coefficient shown in the visible absorption spectrum (Figure 2.4) indicated that the green sample was a typical six coordinate chromium (III) complex. The peak at 262nm found here, has also been found in other chromium-containing fractions isolated from yeast extract (Toepfer et al (1977)). This has often been attributed to nicotinic acid, ($\epsilon = 2630$) but if the nicotinic acid was present in the same molar amounts as the chromium (as determined by atomic absorption spectroscopy) then the apparent extinction coefficient at 262 nm would be 1090. Clearly the nicotinic acid is not present as a ligand bound in a 1:1 mole ratio with the chromium and may be present only as an impurity. Chromatography and gel electrophoresis (Figures 2.5a, 2.5b, 2.6a and 2.6b) indicated that the chromium complex was anionic down to at least pH 2.1 and probably a single complex or at least a mixture of very similar complexes. The ninhydrin development of the paper chromatograms indicated a very low level of amino acid or protein material. These results were confirmed by acid and base hydrolysis followed by amino acid analysis, which showed that no amino acids were present.

Total chemical characterisation of the complex proved difficult. No reliable results were obtained from the mass spectrometric data because of the involatile nature of the species.

The phenol-sulphuric acid test clearly showed the presence of large amounts of carbohydrate in the sample which must have been glucose since this was the only carbohydrate to which the chromium had been exposed in the supplementation medium. When the ^{13}C nmr spectrum of the sample is compared with a ^{13}C nmr spectrum of glucose the similarity is obvious. Both spectra show the presence of six carbon atoms although there is considerable line broadening of the peaks in the complex and some shifts in the peak positions, presumably as a result of proximity to a paramagnetic chromium ion. Unfortunately the ^1H nmr spectrum was not helpful since the only observable peak was a broad H_2O peak at 4.17 ppm which was so intense that any C-H peaks were obscured.

The nmr spectra (Figure 2.8a,b and c) and the carbohydrate tests strongly suggest that the chromium complex had glucose as its predominant ligand type.

A possible structure for the chromium glucose complex is shown below;



To date no similar glucose complex has been reported in the literature, however Angyal (1971) has shown that sugars with the correct sequence of hydroxy groups will form complexes with some metal ions (Ca^{2+} , Ba^{2+} , Mg^{2+} , Na^+ and K^+). Co-ordination through two oxygen atoms for each of the three glucose molecules with subsequent loss of 2 protons per ligand would

result in a complex with three negative charges and hence considerable anionic character. As the protons on the glucose molecules are not very acidic, formation of such a complex would have to occur by binding of the -OH groups to the chromium through the oxygen followed by the expulsion of a proton for each bound oxygen atom.

The percentage of carbohydrate in this proposed anion can be calculated as 91%, which is larger than the value of 73% found by the phenol-sulphuric acid test. It must be remembered however that this proposed anion when in a solid form must be accompanied by a counter ion and thus the percentage carbohydrate would be reduced depending on the size of the counter ion. Also any impurities associated with the green material would result in a decrease in the value determined for the carbohydrate percentage. Impurities must be present in the form of nitrogen and phosphorous-containing compounds to account for their presence in the micro-analytical results. Since the identity of the counterion and the nature of the possible impurities is not known calculations of the elemental percentage composition of the proposed structure can not be compared quantitatively with the experimental values. (Table 2.2) The data are not however inconsistent with the proposed structure.

The biological activity of the isolated anionic complex is reported in section 4. It should be noted that such a complex could be formed by reaction of the chromium with the glucose in the medium and need not result from any metabolic action of the yeast. It would seem then that the fractions of biological interest may be the minor fractions which are neutral or cationic.

SECTION 3THE CARBON DIOXIDE PRODUCTION FROM YEAST ACTIVITY ASSAY;
ESTABLISHMENT AND THE ANALYSIS OF PARAMETERS.3.1 INTRODUCTION

During the development of any isolation procedure the need for a reliable and relatively specific assay system which can be utilized on a routine basis with large numbers of samples, soon becomes apparent. In the previous section successful isolations were assessed in terms of the amount of chromium detected by atomic absorption spectroscopy and not glucose tolerance factor activity. Glucose tolerance factor (GTF) has been defined by Schwarz and Mertz (1957) as the factor necessary to prevent the impairment of the rate of glucose removal in rats. This definition was proposed after Mertz and Schwarz (1955) found that rats fed on a diet in which the sole protein source used was American Torula yeast, developed an impaired rate of removal of excess blood glucose. The impairment detected took the form of a decrease from the normal rate of removal of 4.1% of the excess blood glucose/minute of the lower rate of 2.8% of excess blood glucose/minute. Schwarz and Mertz (1959) claimed that hydrolysates of brewer's yeast or pork kidney powder when fed orally in the diet, or by a stomach tubed dose, were capable of restoring the excess blood glucose removal rate to normal. Chromium was later claimed to be the active ingredient contained in both hydrolysates and in fact it was claimed that wet ashing in $\text{HNO}_3\text{-H}_2\text{SO}_4$ did not destroy the activity. Various chromium salts were also shown to be active in this respect. It is on the basis of this assay that the concept of a glucose tolerance factor was originally proposed.

A similar assay system has been published more recently by Tuman et al (1978) in which a diabetic strain of mice was used to monitor the effect of glucose tolerance factor (prepared as an extract from yeast) on the blood glucose levels. Similar results to those of Mertz and Schwarz were obtained with an interperitoneal injection of this GTF producing lower blood plasma glucose levels.

In order to locate the GTF containing fractions multiple activity assay determinations from various chromatographic

methods are required and clearly the above assay systems would prove to be far too time consuming for routine use in large numbers. For this reason various other assay systems for GTF have been investigated and those used to date are:

(a) adaptation of the standard epididymal rat fat pad assay monitoring glucose uptake or carbon dioxide production.

(b) modification of the epididymal rat fat pad assay by using isolated adipocytes as the tissue source.

(c) measurement of the rate of CO_2 production during yeast fermentation under anaerobic conditions.

Mertz et al (1961) using epididymal fat tissue obtained from rats which had been raised on a chromium-deficient diet and was therefore supposedly GTF deficient, monitored the glucose uptake and the effect on this of additions of chromium and insulin. It was found that the addition of chromalum ($\text{Cr}_2(\text{SO}_4)_3 \cdot \text{K}_2\text{SO}_4 \cdot 24\text{H}_2\text{O}$) increased the rate of glucose uptake from 32 μg of glucose/100 mg of epididymal fat/hour (for the control) to 62 μg /100 mg/hr when an insulin level of 0.1 mU/flask was used. This represented an increase of 94%. In a similar experiment Mertz and Roginski (1971) monitored CO_2 production from the epididymal fat tissue and determined the effect of glucose tolerance factor on this. Their data showed that in the presence of insulin (1 mU per flask), the rate of CO_2 production was increased by addition of GTF from 60 nmoles CO_2 /100 mg epididymal fat to 115 nmoles/100 mg- an increase of 92%. Unfortunately the chromium level of the GTF used in this study was not indicated. This system then became the standard assay for many of the earlier GTF activity determinations carried out in various laboratories.

A modified assay using isolated adipocytes in place of whole pieces of epididymal fat pad tissue, has been investigated by Anderson et al (1978) and (1979). Using adipocytes prepared from epididymal fat pads from rats which were raised on low chromium diets enabled a homogeneous tissue source to be used for each sample to be assayed. This overcame the problem of variability in different pieces of tissue which is inherent in the fat pad assay. In the modified assay addition of insulin to the adipocytes was again necessary so

that the activity could be determined by measuring the rate of CO_2 production for assayed samples as compared with controls which had only the insulin added. This assay showed no activity towards simple chromium compounds such as CrCl_3 but again was not specific for a unique chromium complex since various synthetic chromium complexes (Cr-nicotinic acid - glutathione, and Cr-nicotinic acid - glycine) along with the GTF prepared from yeast extract all showed similar activities. In all cases the rate of $^{14}\text{CO}_2$ production showed approximately 300% enhancement as compared to the controls. A difference between the specificities of the two fat cell associated assays is apparent. The activity shown by chromalum in the whole fat pad assay seems inconsistent with the fact that no activity was produced by simple salts in the isolated adipocyte assay. No data has been published for the activity of chromalum in the modified assay. A disturbing feature of the assay using isolated adipocytes is its requirement for the assay medium to be Krebs Ringer Phosphate buffer. If Krebs Ringer Bicarbonate buffer is used the rate of $^{14}\text{CO}_2$ production is similar for controls and GTF samples. Only when the Krebs Ringer Phosphate buffer is used does the distinction between the controls and the GTF sample become apparent.

The third assay system used does not obviously involve the supposed site of GTF action as used in the other two types but uses brewer's yeast. As brewer's yeast is claimed to be one of the richest sources of GTF, it would seem reasonable to assume that the GTF must have some biological function to perform in the yeast. Thus a comparison of the basal fermentation rate of a chromium-deficient yeast with that observed when an addition of GTF has been made should permit the detection of GTF samples. Burkeholder and Mertz (1967) first explored this idea when they discovered that GTF fractions prepared from yeast increased the rate of CO_2 production during fermentation by sixteen hour old, chromium-depleted yeast cultures by up to 45% when compared with controls. In marked contrast when so called inorganic chromium (III) solutions at concentrations up to 10 ng Cr/cm^3 were added to the fermentation mixture the stimulation in the rate of CO_2

production was only 5% compared with the controls. At concentrations of chromium (III) greater than this, retardation of the rate of CO_2 evolution occurs.

More recently Mirsky et al have used a chromium-depleted yeast fermentation assay to monitor the biological activity of chromium-containing fractions obtained from commercial yeast extracts. They were able to show an enhancement in the rate of CO_2 production for their active fraction (designated as GTF) in several yeast strains grown on chromium-depleted media. Under optimum conditions their GTF fraction increased the rate of CO_2 production by 230% over control rates and the amount of CO_2 produced was directly related to the amount of glucose used by the cells.

Control experiments were carried out which it has been claimed showed that the GTF fraction contained no fermentable substrates or stimulatory vitamins. Mirsky et al hypothesised that the effect of GTF was on the amount of glucose entering the cell and not on its metabolism since GTF did not enhance the rate of CO_2 production by cell-free extracts (which still undergo glycolysis).

As would be expected, the enhancement of CO_2 production depended on the amount of GTF added to the reaction mixture (in terms of chromium concentration) in a more or less hyperbolic fashion, the maximum effect being reached at 6 ng Cr/cm^3 . This result indicates a saturation effect.

Inorganic chromium salts and some well characterised chromium complexes ($6\text{-}150 \text{ ng Cr}^{3+}/\text{cm}^3$) were found to give no stimulation of the rate of CO_2 production even after prolonged contact with the yeast cells showing that the assay was specific for the GTF fraction.

Thus it appears that the yeast fermentation assay represents a fast and reasonably specific assay for the biological activity of GTF fractions. To the authors knowledge no inorganic chromium complex so far reported has significant activity in the yeast assay with the exception of the synthetic mixture reported by Toepfer et al (1977).

In summary, of the three assay systems described none can claim to be measuring the defined activity of the glucose tolerance factor and all have shown some degree of non-specifi-

city toward chromium compounds of various types. However each can be used as a method of quickly screening a large number of samples. While each system has its own particular merits and failings, the assay system adopted here was that of monitoring the rate of CO_2 production of yeast during fermentation.

Dr Zvi Dori (Technion Institute, Haifa, Israel.) has kindly provided a sample of his yeast culture used in his fermentation assay which has been used to establish a similar assay. This assay can be used as a screen for the various chromium containing fractions isolated in the course of this work so that potentially interesting fractions can be singled out for further study in an attempt to identify the glucose tolerance factor. The results reported in this section relate to the establishment of the activity assay.

3.2 METHODS AND MATERIALS

3.2.1 Yeast.

A strain of the yeast Saccharomyces cerevisiae was used in all assay determinations. This was kindly supplied by Dr Z. Dori (Technion Institute, Haifa, Israel) and was found by him to give a highly sensitive assay. (Mirsky et al (1980))

3.2.2 Pre-assay growth.

The yeast was made chromium deficient by three successive growths on a liquid medium (100 cm^3) consisting of yeast nitrogen base without amino acids (6.7 g/l) and glucose monohydrate (20.0 g/l). Stationary aerobic growth conditions were used with the successive inoculations occurring as near to the beginning of the previous stationary growth phase as possible. Chromium was undetectable in a wet ashing procedure carried out on yeast grown by this method. The detection limit was ca $0.04 \mu\text{g Cr/g}$ of sample.

3.2.3 Assay growth.

A 0.1 cm^3 sample from the final pre-assay growth was used to inoculate the defined growth medium. This medium was constituted as follows: Sugar and Major Elements: $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (0.1 g/l), $(\text{NH}_4)_2\text{SO}_4$ (5.0 g/l), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.5 g/l), NaCl (0.1 g/l), KH_2PO_4 (0.085 g/l), K_2HPO_4 (0.0125 g/l) and glucose monohydrate (20.0 g/l).

Vitamins; thiamine hydrochloride (1.25 mg/l), vitamin B₁₂ (1.25 mg/l), calcium pantothenate (1.25 mg/l), nicotinic acid (1.25 mg/l), pyridoxal phosphate (0.25 mg/l), para-amino-benzoic acid (0.25 mg/l) inositol (0.25 mg/l), folic acid (0.025 mg/l) and biotin (0.20 mg/l).

Growth conditions were again stationary aerobic. The normal growth time was 18-20 hours depending on the yeast concentration of the liquid inoculum. This growth time restricted the yeast to the logarithmic phase.

3.2.4 Harvesting and cell concentration determination.

Cell harvesting was performed by centrifugation at 4000 g for 5 minutes. Two water washes were carried out to eliminate any remaining growth medium constituents before taking the yeast cells up in pH 5.75 phosphate buffer (0.063 M) for cell number and assay measurement.

A requirement of the assay was a constant yeast cell concentration and to facilitate the determination of this, a standard curve of optical density against cell concentration was prepared (Figure 3.1). This was established using standard cell counting techniques on a hemacytometer.

3.2.5 Standard assay.

The Warburg manometric technique was used to monitor CO₂ production from yeast under the following standard conditions.

FLASK: 2.0 cm³ of chromium-deficient yeast (1.5 x 10⁸ cells/cm³) in pH 5.75 phosphate buffer (0.063 M).

SIDE ARM: 0.4 cm³ of glucose solution (0.126 M or 2.5 w/v), 0.1 cm³ of the sample to be assayed.

WATER BATH TEMPERATURE: 30°C.

After loading, the Warburg flask was purged with nitrogen (oxygen free) for three minutes to ensure that only anaerobic growth conditions prevailed throughout the assay.

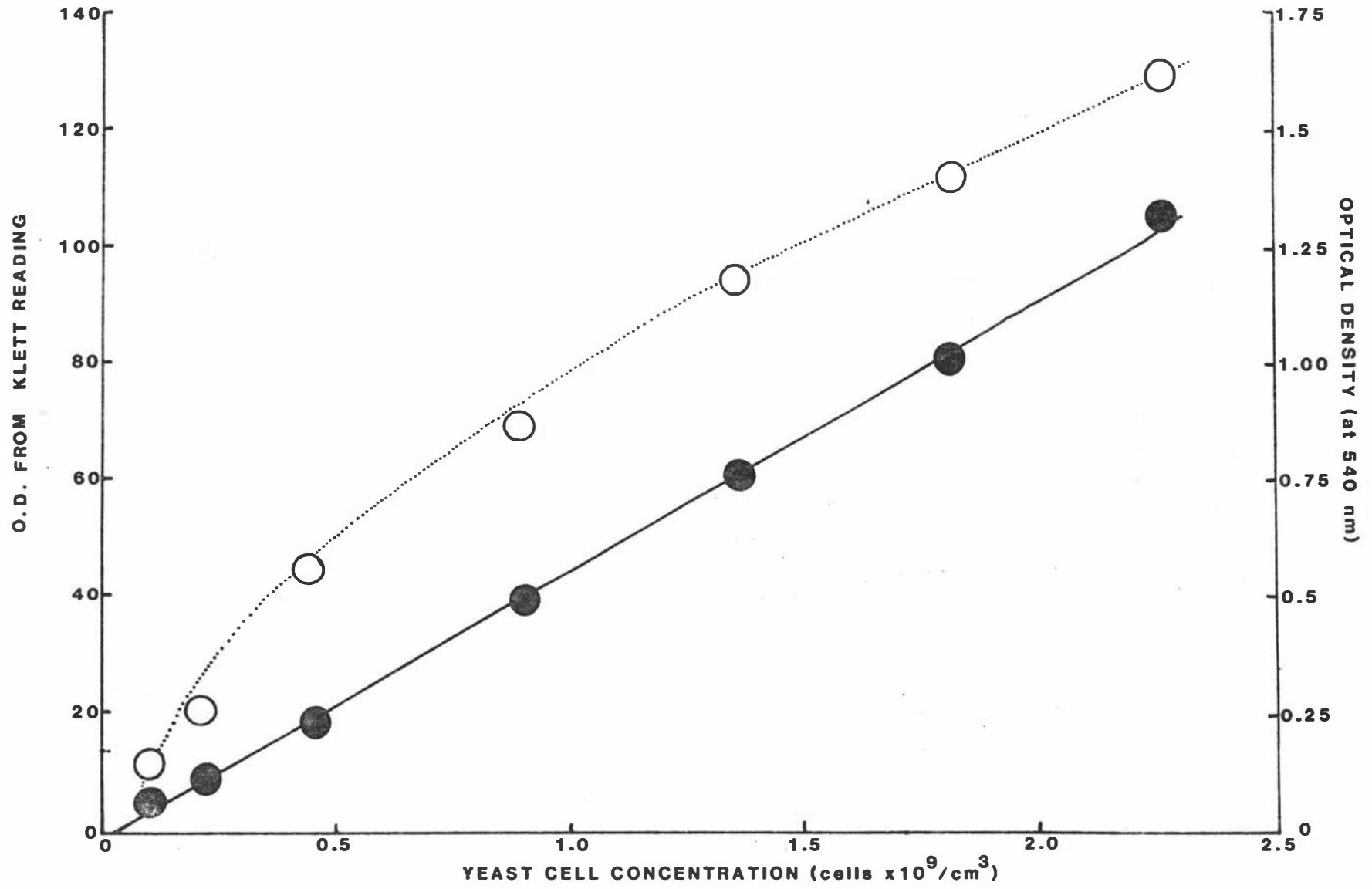
3.2.6 Water purity.

Redistilled, distilled-deionised water with a conductivity of ca 0.6 µmho was used for harvesting of the cells and in all yeast growth and assay media.

FIGURE 3.1

Standard curves used in the routine determination of yeast cell concentration after the growth in the defined medium and prior to assay. Optical density as determined from the Klett reading is shown (●—●) along with the optical density determined on an Unicam SP500 at 540 nm (○·····
·····○).

FIGURE 3.1



3.2.7 Calculation and interpretation of data.

Manometer height readings, after adjustment for atmospheric pressure variations recorded on the thermobar, were converted into nanomoles of CO₂ produced by means of the following formula.

$$\text{nmoles of CO}_2 = \left[\frac{(V_F + V_M) \frac{273}{T'} + V_S X}{22.4} \right] 1000 h'$$

where V_F = Flask volume (cm³)
 V_M = Manometer volume (cm³)
 V_S = Volume of assay mix (normally 2.5 cm³)
 X = Particular solubility gas constant (normally 0.665 for CO₂ at 303 K)
 h' = Manometer height (cm³)
 T = Waterbath temperature in Kelvin (normally 303K)

These values are plotted against time. After a lag phase of up to 60 minutes active samples showed a greater CO₂ production rate than did a buffer control. Carbon dioxide production rates were calculated from the near linear portion of the curve seen in the last 90 minutes of the 320 minutes for which the assay was run.

The percentage enhancement of the sample was calculated as:

$$\frac{\text{nmoles CO}_2/\text{min of sample} - \text{nmoles CO}_2/\text{min of phosphate buffer control}}{\text{nmoles of CO}_2/\text{min of phosphate buffer control}} \times \frac{100}{1}$$

Where comparison between different concentrations of chromium was required the assay was quantified by expressing the activity as a specific activity, calculated as percentage enhancement/100 pmoles of chromium.

3.2.8 Preparation of an active synthetic complex.

Extractions carried out by Toepfer et al (1977) from brewer's yeast revealed trace quantities of nicotinic acid, glycine, glutamic acid and cysteine along with chromium. Toepfer et al have published a procedure for the preparation of synthetic chromium complexes which are formed by reactants in the molar ratio of chromium:nicotinic acid:glycine:glutamic

acid:cysteine of 1:2:2:1:1. They claim that this procedure yields a solution whose properties mimic those of GTF isolated from yeast extracts in terms of biological activity in a rat fat pad bioassay and in spectroscopic properties. The procedure described here is based on that of Toepfer et al with the modifications described. Fifty grams of $\text{Cr}_2(\text{SO}_4)_3$ were dissolved in 80% ethanol (750 cm^3) containing acetic acid (2 cm^3), neutralized to pH 7.0 with NH_4OH . Nicotinic acid (4.0 g) was added to the flask and the mixture was refluxed for 1.5 hours. Solubilization was presumably incomplete since a grey-green precipitate was apparent at the base of the reflux flask at the end of this time. Glycine (2.4g), L-glutamic acid (2.4 g) and L-cysteine-HCl were then added and the mixture stirred under reflux for 18 hours. The solution was then filtered producing a grey-green precipitate and a bright purple filtrate. On standing for a further 48 hours a white material precipitated out of the purple solution and was removed by filtration. Chromium analyses were carried out on saturated solutions of the two precipitates as well as on the purple solution but of these solutions only those containing chromium were assayed for activity.

3.2.9 Chromium analysis; optical density, conductivity and pH measurements.

These were carried out as described in section 2.

3.3 RESULTS

3.3.1 Standard assay.

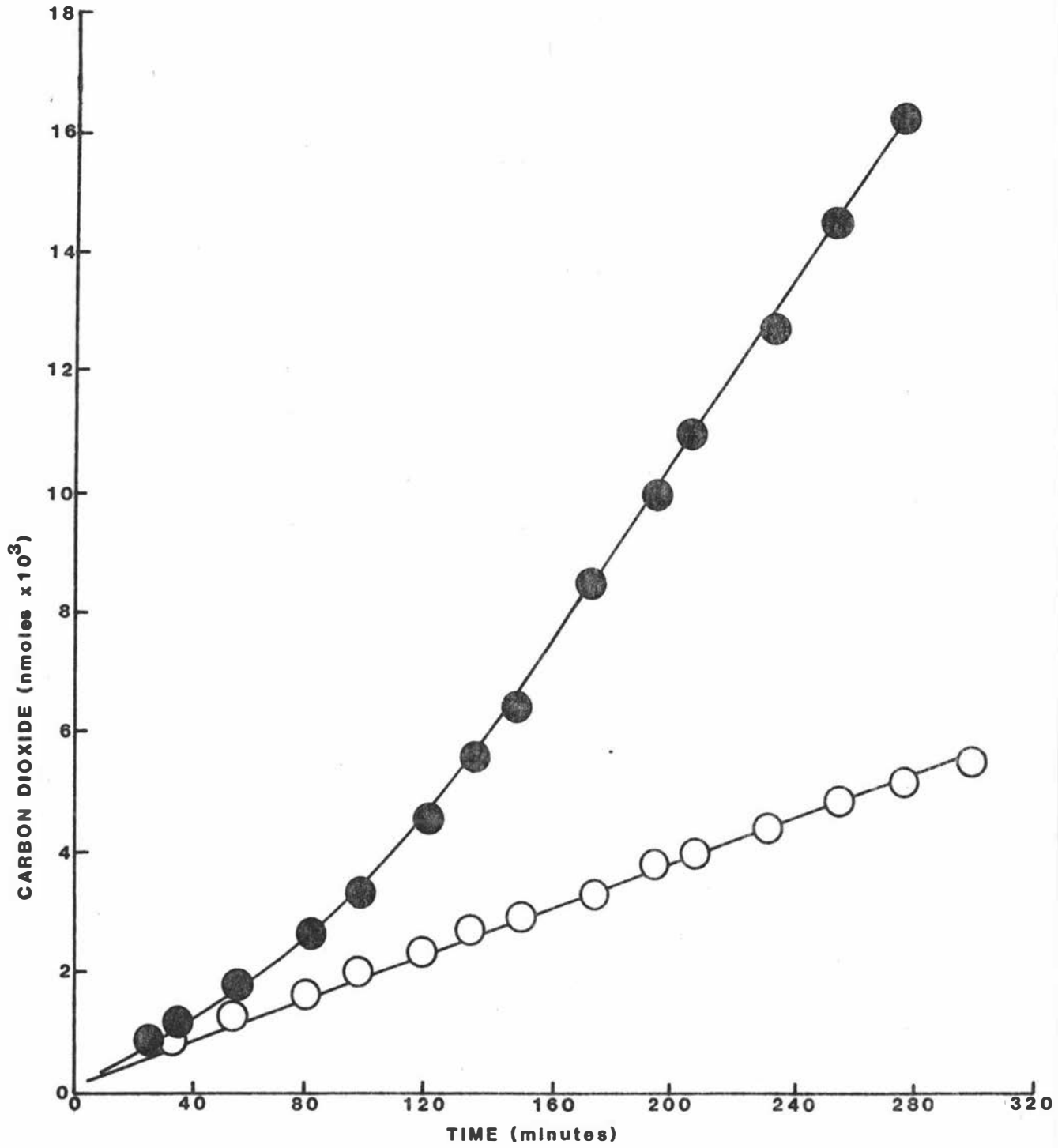
Data collected for a routine standard assay are shown in Figure 3.2. This shows the control rate derived from a phosphate buffer blank along with the rate of CO_2 production for an active sample. Calculations based on this typical example show a percentage enhancement of 269% and since the chromium concentration of the sample was $0.1 \mu\text{g/g}$ (192 pmoles in a 0.1 cm^3 sample) the specific activity was 140.

Assay results pertaining to isolation and purification procedures will be reported in later sections. The different parameters in the assay (yeast cell concentration, glucose concentration, pH and conductivity) were varied primarily to ensure that optimum assay conditions were used and also with the hope of gaining some insight into the mode of action of

FIGURE 3.2

Standard yeast assay showing the carbon dioxide production of an active sample P-3 (●—●) and a phosphate buffer blank (○—○) with respect to time. The rates of carbon dioxide production and the percentage enhancement and specific activity are described in results.

FIGURE 3.2



an active sample in causing the observed enhancement of CO₂ production.

3.3.2 Variation of yeast cell concentration in the standard assay.

Yeast cell concentration was varied between 0.5×10^8 cells/cm³ and 10.0×10^8 cells/cm³ in the original 2 cm³ standard assay volume. Over this range of yeast cell concentrations a phosphate buffer blank assay and two assays with different concentrations of an active sample derived from yeast extract (with chromium levels of 0.1 µg/g and 0.2 µg/g) were carried out and the results are shown in Figure 3.3.

There was difficulty in collecting data for highly active samples due to the very rapid CO₂ evolution rates which under these conditions resulted in very high errors.

3.3.3 Variation of glucose concentrations in the standard assay.

Glucose concentrations were varied from 1.26×10^{-2} M (0.25% w/v) to 1.26 M (25% w/v) in the standard assay for determination of both the blank rate using phosphate buffer and for an active sample. The data are shown in Figure 3.4.

3.3.4 Variation of pH in the standard assay.

The pH value of the buffer used in the standard assay was varied between pHs 2.9 and 12.2. The buffer was kept at a constant phosphate concentration of 0.063 M and varied by means of HCl or NaOH addition at the pH extremities. Blank rate activities are shown in Figure 3.5.

3.3.5 Variation of conductivity in the standard assay.

The phosphate buffer concentration was varied to give a conductivity range of 2600 µmho to 32500 µmho while maintaining the pH at 5.75. The activities from blank assays carried out with these buffers are shown in Figure 3.6.

3.3.6 Activity of the synthetic preparation and its components.

The results of chromium analyses performed on the three fractions obtained from the synthetic preparation are shown in Table 3.1.

FIGURE 3.3

Variation in the rate of carbon dioxide production over a range of yeast cell concentrations for a phosphate buffer blank and two concentrations of an active P-3 sample. Phosphate buffer blank (●—●), chromium concentration of P-3 of 0.1 $\mu\text{g/g}$ (○—○) and chromium concentration of P-3 of 0.2 $\mu\text{g/g}$ (⊗—⊗).

FIGURE 3.3

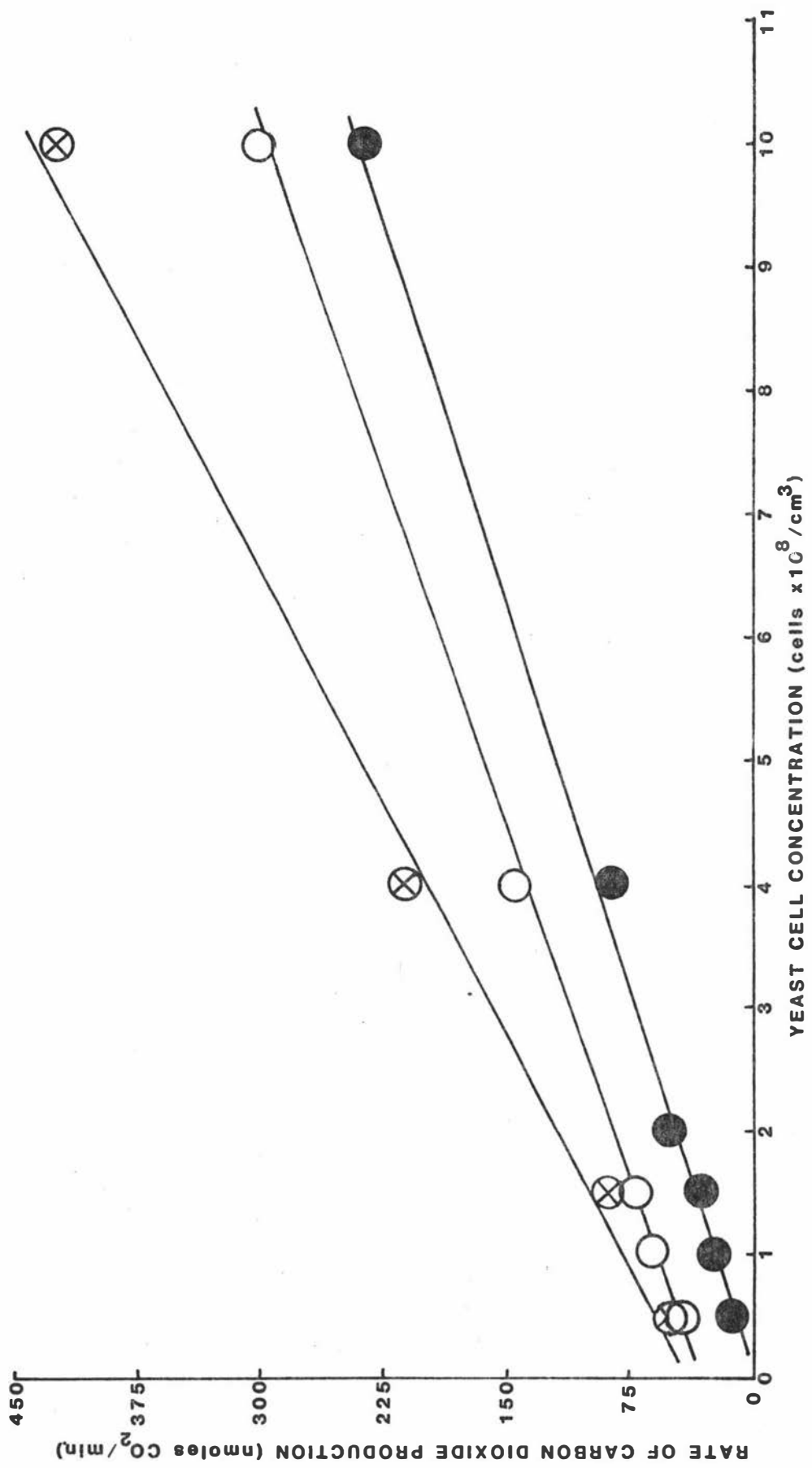


FIGURE 3.4

Variation in the rate of carbon dioxide production for a range of glucose concentrations for a phosphate buffer blank (●—●) and a moderately active sample isolated from yeast extract (○—○).

FIGURE 3.4

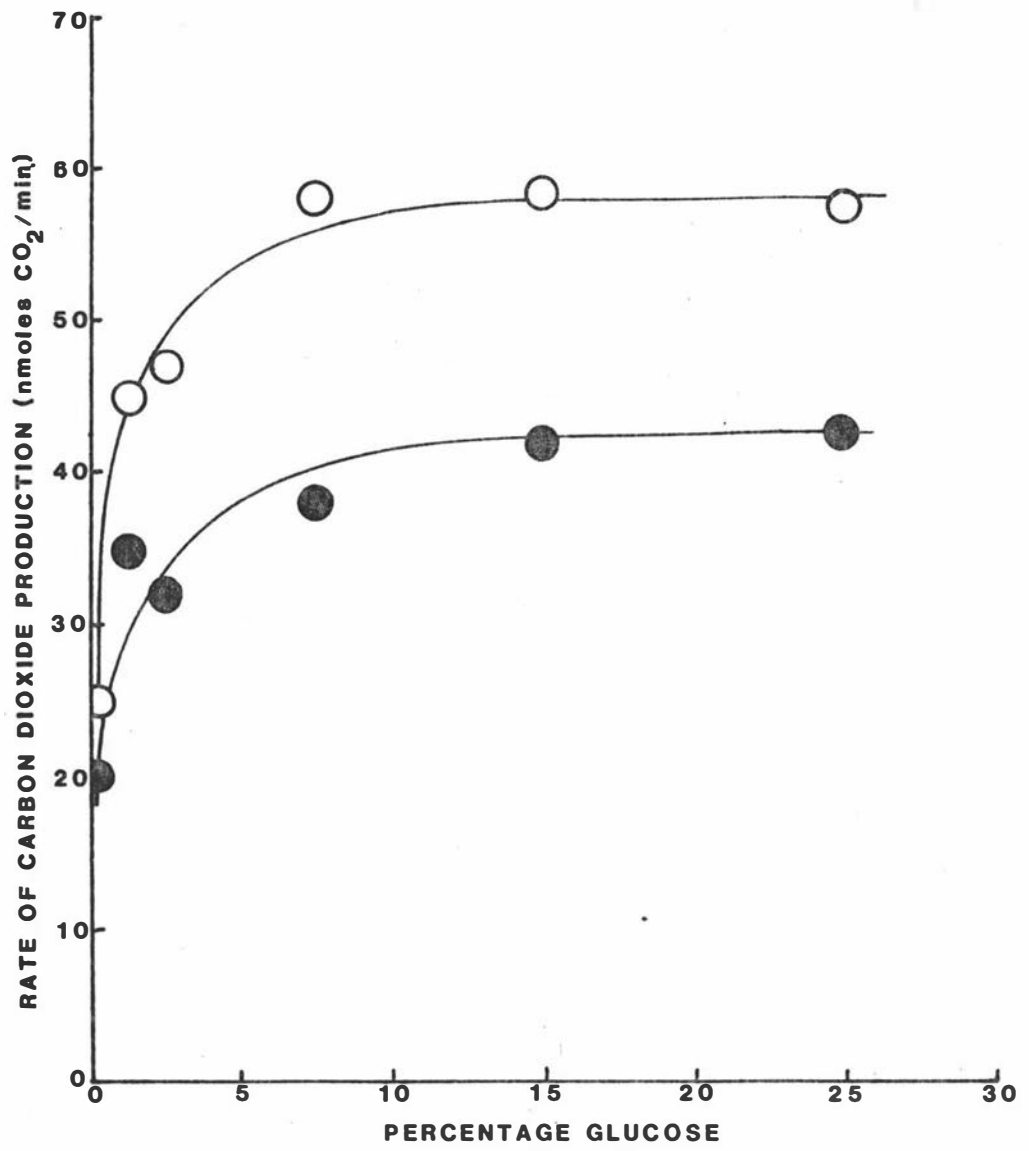


FIGURE 3.5

Variation in the rate of carbon dioxide production over a range in pH for the phosphate buffer blank in the standard assay.

FIGURE 3.5

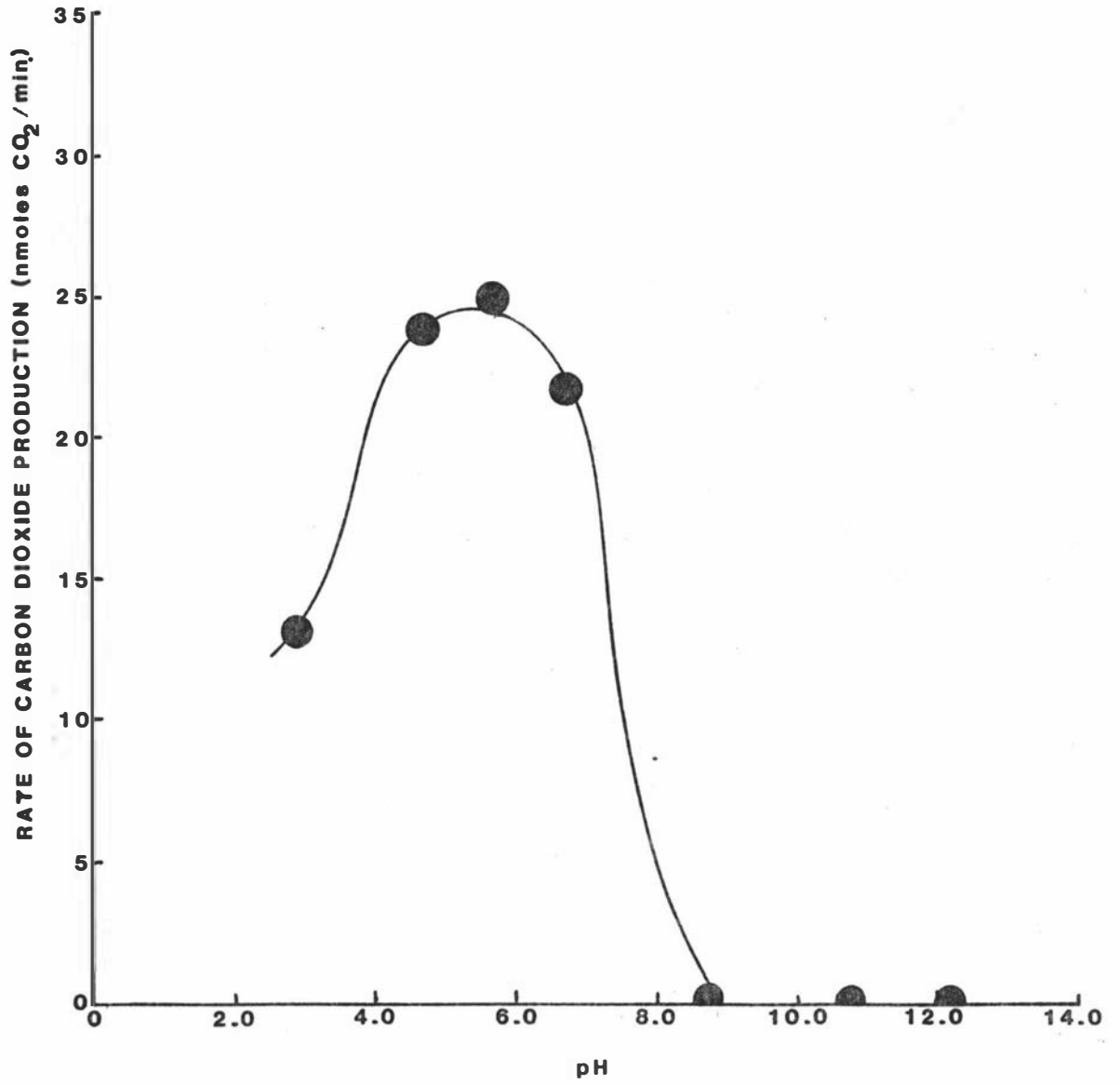


FIGURE 3.6

Variation in the rate of carbon dioxide production over a range in conductivity for the phosphate buffer blank in the standard assay.

FIGURE 3.6

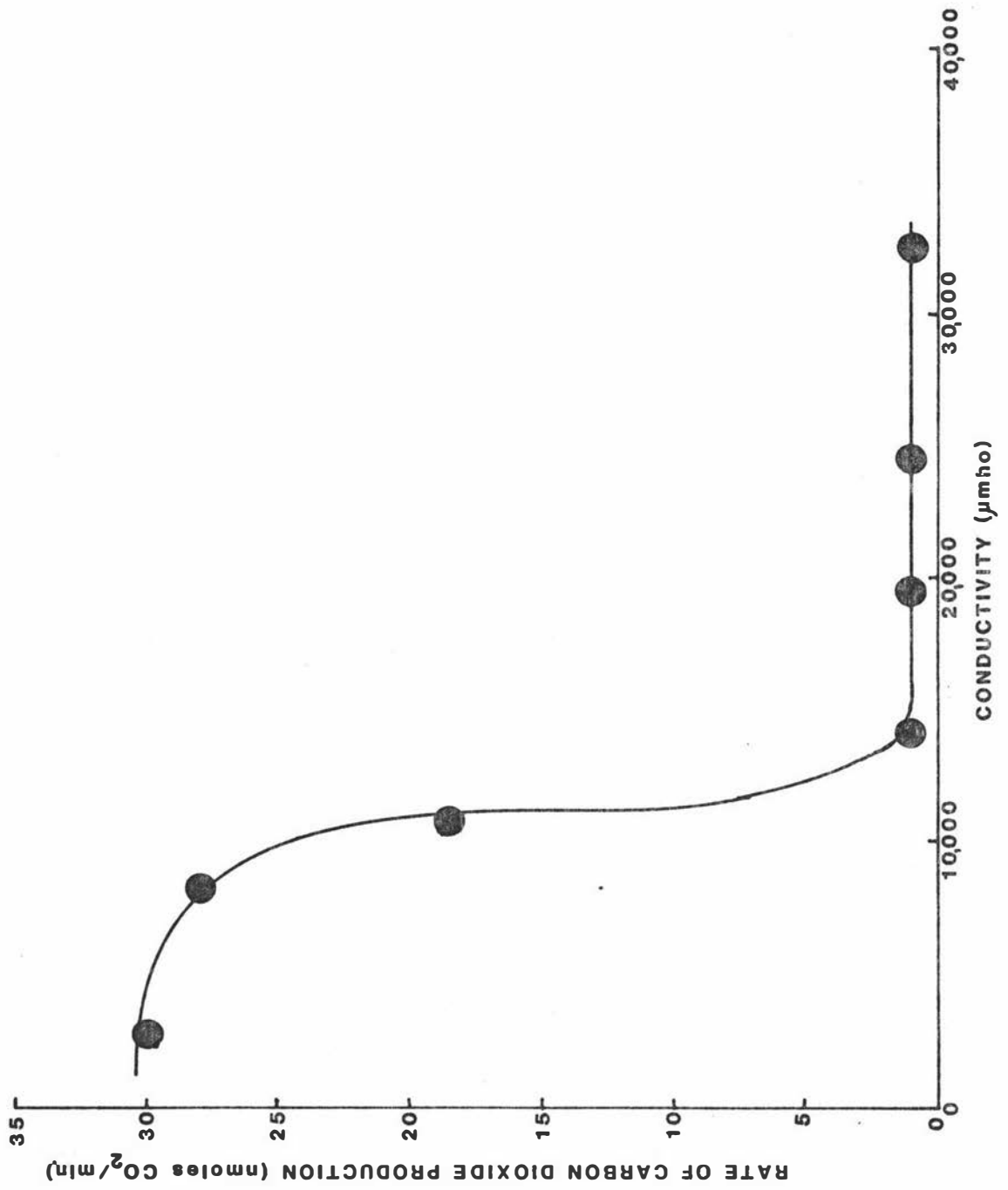


TABLE 3.1 CHROMIUM CONTENT OF FRACTIONS OBTAINED FROM THE SYNTHETIC PREPARATION

<u>Sample</u>	<u>Chromium ($\mu\text{g/g}$)</u>
Saturated aqueous solution prepared from the grey-green precipitate.	9.6
Saturated aqueous solution prepared from the white precipitate.	0.0
Purple solution.	835.8

The white precipitate which was free of chromium was presumably uncomplexed amino acids or nicotinic acid. Activity assays were determined for various chromium concentrations of the purple solution and of a solution made from the grey-green precipitate. The activities determined are shown in Table 3.2 along with the activities determined for aqueous saturated solutions of the component amino acids and nicotinic acid in order to show their contribution to the total activity determined for the purple solution.

TABLE 3.2 ACTIVITY OF REACTANTS AND PRODUCTS OF THE SYNTHETIC PREPARATION.

<u>Sample #</u>	<u>Rate</u> nmoles CO_2/min	<u>Percentage</u> <u>Enhancement</u>
Phosphate buffer blank	37.5	0
Purple solution (0.84 $\mu\text{g/g}$)	37.5	0
Purple solution (8.36 $\mu\text{g/g}$)	69.0	+84
Purple solution (83.6 $\mu\text{g/g}$)	126.0	+236
Purple solution (536 $\mu\text{g/g}$)	115.0	+206
Solution of the grey-green ppt (0.96 $\mu\text{g/g}$)	37.5	0
Solution of the grey-green ppt (9.55 $\mu\text{g/g}$)	55.0	47
L-glutamic acid	62.5	66
L-cysteine	23.7	-36.8
Nicotinic acid	33.7	-10.1
Glycine	42.5	+13
Combination *	48.8	+30

Where concentrations are specified these refer to chromium. All other solutions are saturated aqueous solutions.

* The combination involved a saturated aqueous solution of L-glutamic acid, L-cysteine, nicotinic acid and glycine.

3.3.7 Activity of inorganic chromium salts.

To determine whether inorganic chromium complexes were active in the yeast assay a range of different chromium salts was assayed. (Table 3.3). The percentage enhancements for a concentration range of $\text{CrCl}_3 \cdot 6\text{H}_2\text{O}$ ($2.0 \times 10^{-3} \mu\text{g/g}$ - $2.0 \times 10^4 \mu\text{g/g}$) were also determined. (Figure 3.7)

TABLE 3.3 ACTIVITY OF VARIOUS CHROMIUM SALTS

<u>Sample</u>	<u>Chromium Concentration</u> $\mu\text{g/g}$	<u>Rate</u> nmoles CO_2/min	<u>Percentage Enhancement</u>
CrCl_3	0.1	31.0	+33
CrCl_3	25.0	28.0	-66
$\text{Cr}_2(\text{SO}_4)_3$	0.1	24.0	-20
$\text{Cr}_2(\text{SO}_4)_3$	25.0	35.8	+19
$\text{K}_2\text{Cr}_2\text{O}_7$	0.1	17.5	-41.6
$\text{K}_2\text{Cr}_2\text{O}_7$	25.0	14.1	-45.9
$\text{Cr}(\text{NO}_3)_3$	10.3	34.0	- 2.8*

* N.B. Value recorded on a different day with a correspondingly different blank rate from the other values.

3.3.8 Activity due to extraneous material.

Yeast extract is a complex mixture of compounds and it is likely that compounds other than the hoped for chromium-containing GTF fraction will be active in the assay. For example a saturated solution of glutamic acid has already been shown to have a percentage enhancement of +66. The activity of a range of yeast extracts with optical densities at 280 nm ranging from 0.18 to 18.0 was measured and the results are shown in Table 3.4.

FIGURE 3.7

Variation in the rate of carbon dioxide production for a range of chromium chloride concentrations in the standard assay.

FIGURE 3.7

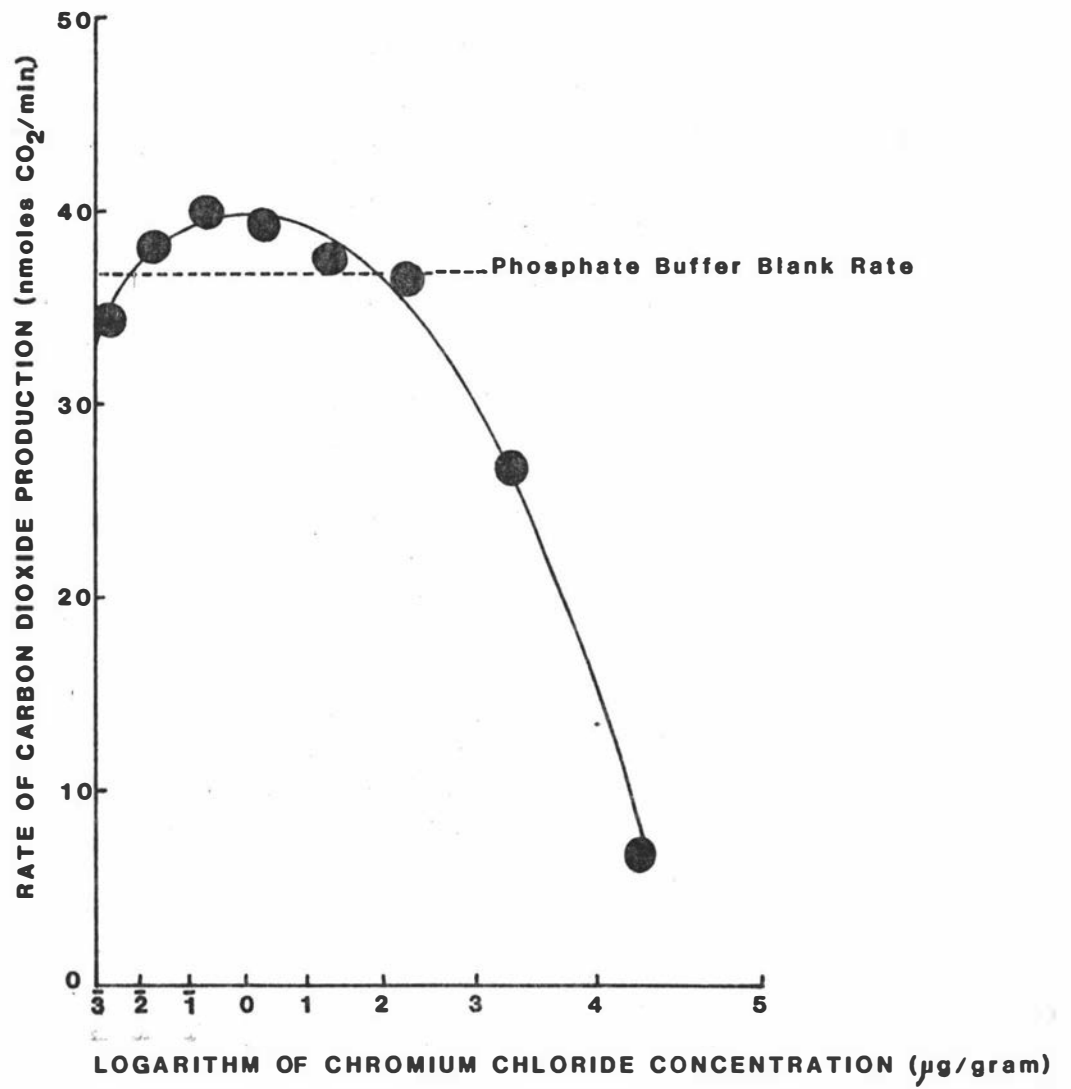


TABLE 3.4 ACTIVITY DUE TO DIFFERENT LEVELS OF YEAST EXTRACT

Percentage yeast extract in aqueous solution	Optical density at 280 nm	Chromium concentration ($\mu\text{g/g}$)	Rate nmoles CO_2/min	Percentage enhancement
0	0	0	28	-
0.01%	0.18	0.0001	33	17.8
0.02%	0.36	0.0002	29	3.5
0.1%	1.80	0.001	51	82
1.0%	18.0	0.01	127	353
2.0%	36.0	0.02	175	525
10.0%	180	0.10	156	457

3.4 DISCUSSION

The assay system described in this section enabled the determination of biological activity in terms of enhancement of the basal rate of carbon dioxide production during anaerobic yeast fermentation to be carried out on a routine basis. The standard conditions chosen for the assay on the basis of this work involved the use of 2.5% glucose solution and a concentration of yeast cells of 1.5×10^8 cells/cm³ at a pH of 5.75 in 0.063 M phosphate buffer.

Duplicate assays performed either on the same day or on different days gave good agreement (better than ± 10 percentage enhancement). Variations in blank rates did occur from day to day but were always between the range 20 nmoles CO_2/min to 37 nmoles CO_2/min . This variation seemed to depend on the rate of yeast growth on the defined medium.

Figure 3.2 shows the clearcut difference between an active sample and the blank rate. Not surprisingly this difference is very similar to that found by Mirsky *et al* (1980), although the absolute values for the moles of CO_2 produced are a little higher in their case.

Examination of the rates of CO_2 production for a range of yeast cell concentrations for both blank and active samples showed that there is little effect on the percentage enhancement for the lower activity sample (0) since the two lines

are nearly parallel (Figure 3.3). Thus it would not matter at which yeast cell concentration the percentage enhancement was determined for this sample. However the data for the higher activity sample (Q) showed that the percentage enhancement depended on the yeast cell concentration and this may account for the differences in enhancement between those reported by Mirsky et al (1980) and the results reported here. It is clearly important to maintain a constant yeast cell number in comparative assays. The concentration chosen was 1.5×10^8 cells/cm³ since this concentration ensured optimum rates of evolution for both the blank and test assays and was low enough to avoid any errors due to the decrease in the glucose concentration during the course of the assay. Cell concentrations much lower than this would lead to an unacceptably slow rate of CO₂ production while numbers much greater than this would result in rates which were too fast for precise measurement.

The rate of CO₂ production shows a hyperbolic relationship with glucose concentration for both the blank and active chromium containing samples, saturation being reached at about 15% glucose concentration. The maximum stimulation of CO₂ production was only 50% for the sample assayed in Figure 3.4 but in general the results are in agreement with those of Mirsky et al (1980) since the effect of the active sample was mainly on the maximum rate for the system.

The variations shown in the blank rate assay for pH and conductivity (Figures 3.5 and 3.6) show the importance of controlling the pH and conductivity range in the assay mixture. This necessitated, prior to assay, the titration of extremely acidic or alkaline samples to around pH 6.0 and the desalting of samples with conductivities high enough to cause inhibition.

A solution containing biologically active chromium complexes as reported by Toepfer et al (1977), was prepared according to the published instructions and used to test the response of the yeast assay towards a preparation which had been previously shown to possess biological activity in a bioassay using chromium-deficient rat epididymal adipose tissue (Kienle et al (1979) and Toepfer et al (1977)).

So far no researchers have managed to isolate a pure characterised complex from this solution but Toepfer et al (1977) have claimed that the general spectroscopic and chromatographic behaviour of the solution is similar to that exhibited by their 'GTF' fraction. In agreement with their findings are the results shown in Table 3.2. The purple solution, which undoubtedly contains a mixture of compounds including unreacted amino acids and nicotinic acid, clearly shows a stimulation of the rate of CO_2 evolution, reaching a maximum value of +236% at a level of 83.5 $\mu\text{g/g}$ chromium. Although the maximum contribution to this activity from unreacted starting material is +66% for a saturated solution of L-glutamic acid, a mixture of all reactants minus the chromium gave a percentage enhancement of only +30%. The assay is clearly responsive to the presence of some active species in the solution in the same way as is the more laborious fat-pad assay technique. In support of this contention are the data in Table 3.3 and Figure 3.7 which show that simple chromium (III) salts do not result in any significant enhancement of the basal rate of CO_2 production but rather result in an inhibition at levels significantly above 200 $\mu\text{g/g}$.

Thus the yeast assay detects 'biologically-active' chromium fractions in a similar fashion to other assay systems described in the literature (Mertz et al (1961), Tuman et al (1978), Anderson et al (1978), Burkeholder and Mertz (1967) and Mirsky et al (1980)). However during isolation of chromium containing fractions from yeast extracts there is always the possibility of a stimulatory effect due to extraneous material such as vitamins or fermentable substrates. An indication of this effect in the assay for different levels of added yeast extract was measured. The parameter used as a measure of extraneous material was the absorbance at 280 nm. The results (Table 3.4) show that once the absorbance (A_{280}) rises above 1.8 there is significant increase of the basal rate of CO_2 production. Obviously some of this increase may be a contribution from any biologically-active chromium fractions present in the yeast although for A_{280} values of less than 1.8 the chromium present in these samples would be unlikely to make a significant contribution to the total because of

the very low concentration ($>0.001 \mu\text{g/g}$). It is recognised that the A_{280} value is only a crude estimate of the extraneous material and thus once the material is fractionated the various fractions may respond quite differently in the assay for a given A_{280} value. Thus the increase in percentage enhancement from +3.5 to +82 as the A_{280} value increases from 0.36 to 1.8 is only an indication of the non-specific stimulatory effect due to the extraneous material. For crude samples however, if the A_{280} value is 1.8 or greater, enhancements of less than +82% cannot be used as indicative of the GTF activity of the chromium-containing fractions.

This observation is apparently in contradiction with results found by Mirsky et al (1980). They have stated that the observed activity in their case is not due to fermentable substrates or stimulation from possible vitamins. They have tested both of these possibilities by assaying various levels of glucose and vitamins. Without knowing the exact composition of their GTF sample the possibility of stimulation of the fermentation rate by factors other than a chromium complex cannot be ruled out.

Although the yeast assay superficially bears no obvious relationship to the insulin dependent systems commonly used to determine GTF activity, it does respond in a way which suggests that it is capable of measuring an effect on the glucose transport system since it gives the same results with the synthetic mixture prepared by Toepfer et al (1977) as do the insulin dependent assay systems and can be used with confidence as a convenient and rapid screening test for the various chromium fractions isolated from yeast and other biological samples.

SECTION 4ISOLATION AND ACTIVITY DETERMINATION OF CHROMIUM-CONTAINING FRACTIONS FROM YEAST EXTRACT.4.1 INTRODUCTION

With the availability of a high chromium yeast extract, and with the ability to determine GTF activity by the standard yeast assay (described in section 3), it became possible to attempt a systematic isolation and activity assay of the chromium-containing fractions in yeast.

With average chromium levels of 11.2 $\mu\text{g/g}$ the high chromium yeast extract that was available contained a far greater natural level of chromium than did whole yeast (ca 0.35 $\mu\text{g/g}$). Not only were the chromium levels some thirty fold higher but unlike the whole yeast samples, the yeast extract was readily soluble in water. Thus although only 50% of the chromium compounds present in the whole yeast cells were recovered after cell cleavage and butanol-water extraction, there was total solubilization of the chromium complexes from the yeast extract. One disadvantage of the yeast extract was the concomitant increase in the amount of soluble impurities.

A major assumption made in this work was that the autolysis process used in preparation of the yeast extract did not alter the natural chromium complexes present in yeast in any way. Yeast extract is used primarily as a growth medium and for this reason the process used to produce the yeast extract is designed to be as gentle as possible so that the maximum nutritive value is retained by the yeast extract. During autolysis large protein molecules should be broken down but smaller molecules such as the chromium complexes should remain largely unaffected.

The yeast assay enabled chromium-containing fractions isolated from the yeast extract to be classified as either active or inactive. This was important since further efforts to develop an isolation procedure could then be based on the information relating to the relative activities of the chromium-containing fractions.

Both of these factors have been utilized by Mirsky et al (1980) in a recent publication. Unfortunately they did not systematically isolate all the chromium-containing fractions from the yeast extract.

The systematic isolation attempted here was considered necessary in order to determine the number of chromium-containing fractions in yeast and their relative abundances and activities. Only from this information could the fractions most closely resembling GTF be singled out for further purification.

4.2 METHODS AND MATERIALS

4.2.1 Yeast extract sources.

The need for a commercially available source of yeast with high levels of chromium in order to facilitate the isolation of the various possible chromium complexes is apparent. Several brands of commercial yeast extract were therefore analysed for their chromium content by flame atomic absorption spectroscopy on aqueous solutions of the yeast extract and from wet-ashed samples taken up in 2 M HCl.

4.2.2 Cation-exchange chromatography.

Dowex 50 W-X 12 (hydrogen form, 100-200 mesh) was used in all cation-exchange columns for which results are described in this section. This was prepared by equilibration in 2 volumes of 2.5 M HCl for 30 minutes then in 2 volumes of distilled water (twice). This cycle was repeated and the column was packed and washed with distilled water until the column washings were neutral (pH 7.0). For optimum binding the columns were loaded at pH 3.5 at a flow rate of 3-5 cm³/minute, with the conductivity always less than 3000 μ mho. Column size depended on the sample size but was usually either 7.0 cm (diameter) x 16 cm or 3.4 cm x 11 cm. A mild pH gradient was found to be the most successful eluent and this was achieved by elution with salts of progressively weaker acids so that the gradient was set up in situ using the intrinsic acidity of the loaded columns. In a typical experiment the column was washed with 0.05 M NaCl, after loading was complete, until the absorbance at 280 nm returned to base line levels. This had the effect of reducing the pH of the

effluent of ca 3.0. The pH gradient was achieved by eluting with 0.05 M Na_2HPO_4 until the pH of the column effluent was 8.0. Finally 0.2 M Na_3PO_4 was passed through the column until the pH of the column effluent reached pH 12-12.5.

4.2.3 Anion-exchange chromatography.

Dowex 1-X8 (chloride form, 100-200 mesh) was used for all anion-exchange columns. The resin was prepared by standing in 2 volumes of 2.5 M HCl for 30 minutes, then 2 volumes of distilled water (twice). This process was repeated before the column was packed and distilled water was passed through the column until the pH of the column effluent was near neutral. Column size again varied with the size of the sample loaded, but was usually 3.4 cm x 11 cm. The samples were loaded at pH 8.5 with a conductivity of less than 2200 μmho . The flow rate for loading and elution was always between 2-4 cm^3 minute.

The conductivity gradient was formed by mixing equal volumes of a 0.19 M Tris-HCl pH 8.5 buffer (2000 μmho) with a 0.19 M Tris-HCl plus 0.16 M NaCl pH 8.5 buffer (10,000 μmho) in a gradient mixer. The pH gradients were then applied by first mixing equal volumes of the pH 8.5 Tris-HCl plus NaCl buffer and 0.35 M acetic acid plus 0.19 M NaCl buffer (10,000 μmho) until the pH was reduced to 4.0 in the effluent from the column. The second pH gradient applied consisted of equal volumes of the 0.35 M acetic acid plus 0.19 M NaCl solution (10,000 μmho) with 0.07 M HCl (100,000 μmho) which reduced the pH to about 2.0. Finally the column was eluted with 1.33 M HCl.

4.2.4 Gel filtration chromatography.

Gel filtration columns were run in water using either Sephadex G10 or Biogel P2 resin. The resins were prepared according to the manufacturers instructions.

4.2.5 Molecular weight determinations.

Approximate molecular weights were determined from a plot of the logarithm of molecular weight versus elution volume taken between the salt peak (indicated by the high conductivity at the lower molecular weight end) and the excluded volume peak (shown by an absorbance peak at 280 nm at the higher molecular weight end).

4.2.6 Chromium analyses; absorbance, pH and conductivity measurements.

These were undertaken as described in section 2.

4.3 RESULTS

4.3.1 Yeast extract sources.

The results of chromium determinations on the various commercially available yeast extracts are shown in Table 4.1. Little difference in the chromium levels was found by the two analysis methods described in section 2.2. Where multiple analyses were conducted a range of chromium concentrations is quoted.

TABLE 4.1 CHROMIUM CONTENT OF COMMERCIAL YEAST EXTRACTS

<u>Brand of Yeast Extract</u>	<u>Average chromium Concentration</u>		<u>Number of Samples</u>
	<u>µg/g</u>	<u>Range µg/g</u>	
Oxoid	1.48	-	1
Marmite	0.78	-	1
Difco	1.02	-	1
B.B.L.	1.70	-	1
Merck (high chromium yeast extract)	10.60	8.70-12.0	3
Merck (low chromium yeast extract)	0.91	0.88-0.96	3

As can be seen from the above table, values recorded for Merck yeast extract can be grouped into two classes, high chromium yeast extract and low chromium yeast extract. There was no outward difference in appearance in the two products although samples analysed as high chromium yeast extract were consistently found in batch numbers below 8535027.

4.3.2 Extraction procedure.

Merck yeast extract (200 g, 11.2 µg Cr/g) was mixed with a 1:1 butan-1-ol-H₂O mixture (1600 cm³) and stirred for 20 hours. At the end of this time the mixture was allowed to stand (30 min) and the lower aqueous layer separated from the butan-1-ol layer and precipitated material, either by standing in a separating funnel or by centrifugation at 4000 g. Chromium analysis gave a total of 2140 µg in the aqueous layer, representing 96% of the original chromium. The conductivity

was 1180 μmho and the solution pH was 6.45. In later preparations this aqueous extraction from butan-1-ol- H_2O was omitted as the purification achieved by this step was found to be small for commercial yeast extracts. The aqueous extract was adjusted to pH 3.5 and passed through a Dowex 50W-X12 column as described in methods. This procedure bound the cationic and amphoteric material, allowing material which was anionic or neutral to pass through the column.

4.3.3 Separation of complexes by ion-exchange chromatography.

When material bound to the cation-exchange column was eluted with a pH gradient (as described in methods), two main fractions were observed (Figure 4.1). A broad peak centred at pH 4.5 with a shoulder at pH 6-7 and a sharp peak centred at pH 10-11. The first peak (I) contained 250 μg of chromium and the second peak (II) contained 210 μg together constituting 21.5% of the chromium originally obtained from the Merck yeast extract in the aqueous extraction. Both peaks showed a high absorbance at 280 nm. The tubes containing the chromium peaks always appeared distinctly yellow and this remained associated with the chromium fractions during all subsequent separation procedures described in this section.

Attempts at elution of chromium complexes from the cation-exchange column with conductivity gradients were found to be largely unsuccessful as would also appear to be the case for similar elutions carried out by Votava et al (1973). Other workers have commonly used NH_4OH as a means of elution (Toepfer et al (1977), Kumpulainen et al (1978), Mirsky et al (1980)) for similar cation-exchange columns and their results are similar to those found in the present work. Ammonium hydroxide will elute chromium complexes from the cation-exchange resin but always as a single unresolved peak.

In order to separate any amphoteric material peaks I and II were separately applied to Dowex 1-X8 anion-exchange columns at pH 8.5. Peak I was loaded onto a 250 cm^3 (4.5 cm x 16 cm) column which on elution with a NaCl - Tris buffer, followed by successive pH gradients, as described in methods gave a single chromium-containing fraction (72 μg) designated AM-1 (Figure 4.2.). Similar treatment of Peak II after loading onto a 100 cm^3 (3.4 cm x 11 cm) column gave three chromium-

FIGURE 4.1

Elution profile for aqueous yeast extract after absorption on a Dowex 50W-X12 cation-exchange column (7.0 cm x 16 cm) at pH 3.5. The tube volume was 20 cm³ and the pH gradients (.....) were generated as described in methods. The chromium peaks (————) are designated as I and II and generally follow the A₂₈₀ profile (-----). This elution pattern was essentially the same for 10 separate column runs.

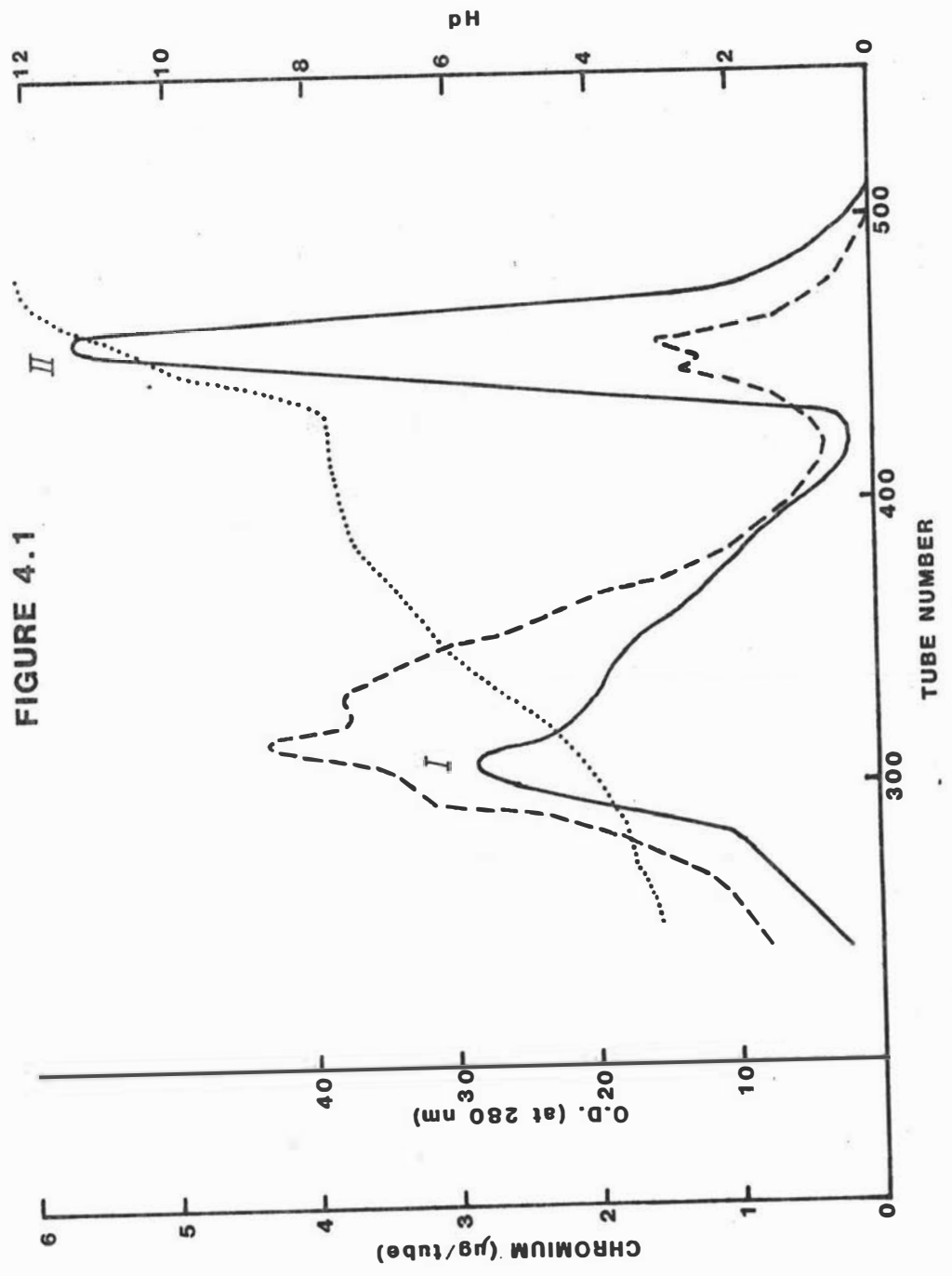
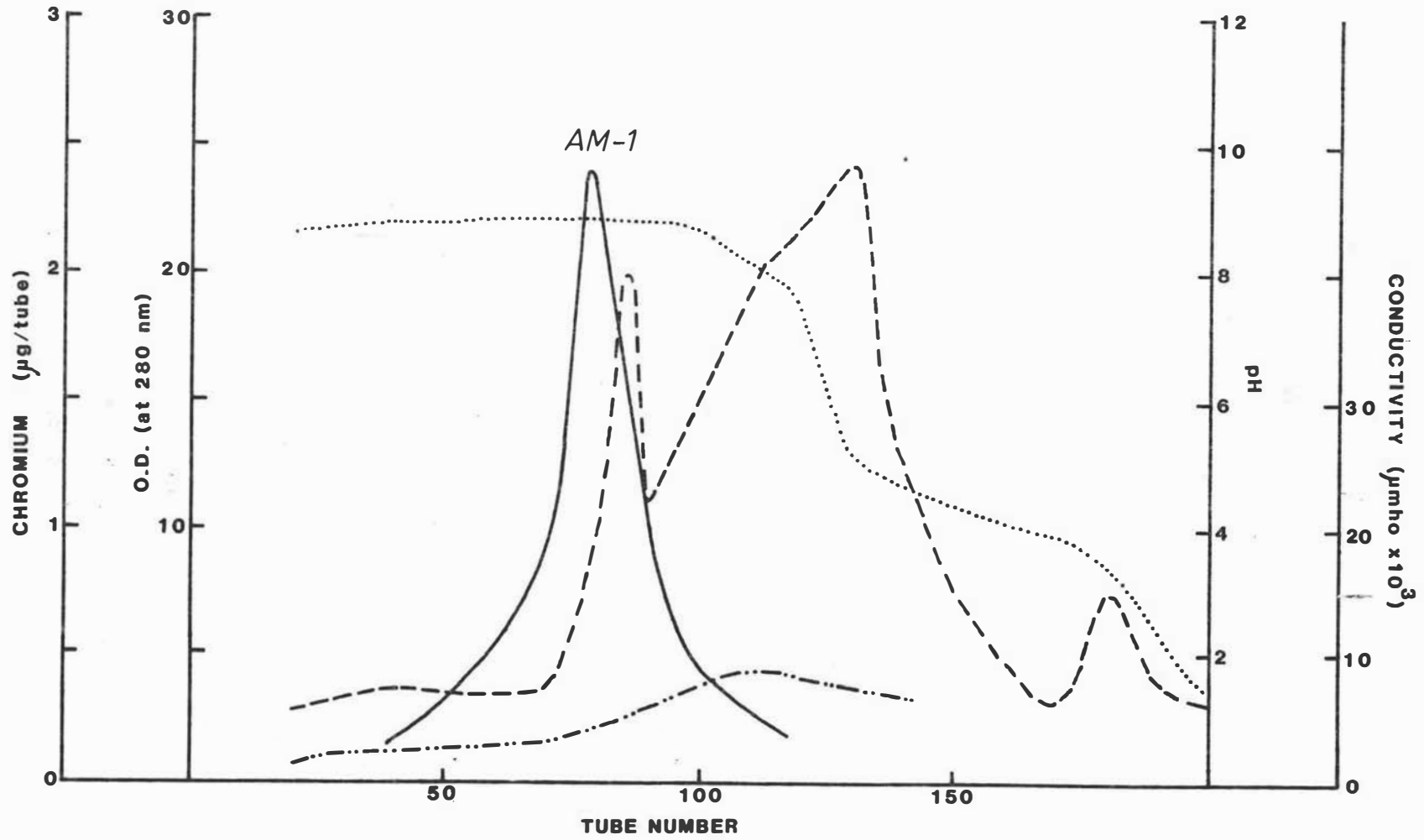


FIGURE 4.1

FIGURE 4.2

Elution profile for AM-1 from a Dowex 1-X8 anion-exchange column. The column (4.5 cm x 16 cm) was loaded at pH 8.5 and eluted as described in methods. Chromium (—————), pH (.....), conductivity (-----) and A_{280} (-----). The volume of each tube was 20 cm³.

FIGURE 4.2



containing fractions designated as AM-2 (21 μg), AM-3 (12 μg) and AM-4 (19 μg) respectively (Figure 4.3).

To date Dowex 1-X8 has not been used as an anion-exchange resin but Mirsky et al (1980) and Kumpulainen et al (1978) have used alternative anion-exchange resins. In both cases the resins have been eluted with a conductivity gradient formed by an ammonium salt, either chloride or carbonate. Both methods have been successful to some extent in separating chromium complexes but better results were obtained in this work by employing a conductivity gradient followed by a pH gradient.

The chromium material from peak I which was washed through the Dowex 1-X8 column without binding was collected, adjusted to pH 3.5 and reloaded onto a cation-exchange column (Dowex 50W-X12). Elution of the cationic material off the column (100 cm^3 , 3.4 cm x 11 cm) by using pH gradients generated in situ (as described in methods) resulted in two chromium peaks, P-1 and P-2 (Figure 4.4) P-1 (66 μg) was centred on pH 4.5 and presumably corresponds to the peak observed on the original Dowex 50W-X12 column (compare Figures 4.1 and 4.4) whereas P-2 (64 μg) was centred at pH 10 and did not obviously correspond with one of the original peaks, possibly a result of different column behaviour after removal of a large amount of extraneous material. The behaviour was however reproducible.

Similar treatment of the effluent obtained from the loading of peak II on a Dowex 1-X8 column also resulted in two peaks, P-3 (41 μg) and P-4 (31 μg). P-3 was eluted from the column at pH values around 7 while P-4 was eluted off the column at pH 10 (Figure 4.5).

The column effluent obtained after loading the original Dowex 50W-X12 column was titrated to pH 8.5, diluted to an appropriate conductivity and loaded onto a Dowex 1-X8 anion-exchange column. Elution of this column resulted in two large chromium peaks, N-1 (730 μg) and N-2 (205 μg), as shown in Figure 4.6. Chromatography of the anionic green complex isolated in Section 2, showed a similar elution profile to that of the N-1 peak isolated here.

FIGURE 4.3

Elution of fractions AM-2, AM-3 and AM-4 from a Dowex 1-X8 anion-exchange column. The column (3.4 cm x 11 cm) was loaded at pH 8.5 and eluted as described in methods. The volume of each tube was 18.5 cm³. Chromium (—————), pH (.....), conductivity (-----) and A₂₈₀ (-----).
-----).

FIGURE 4.3

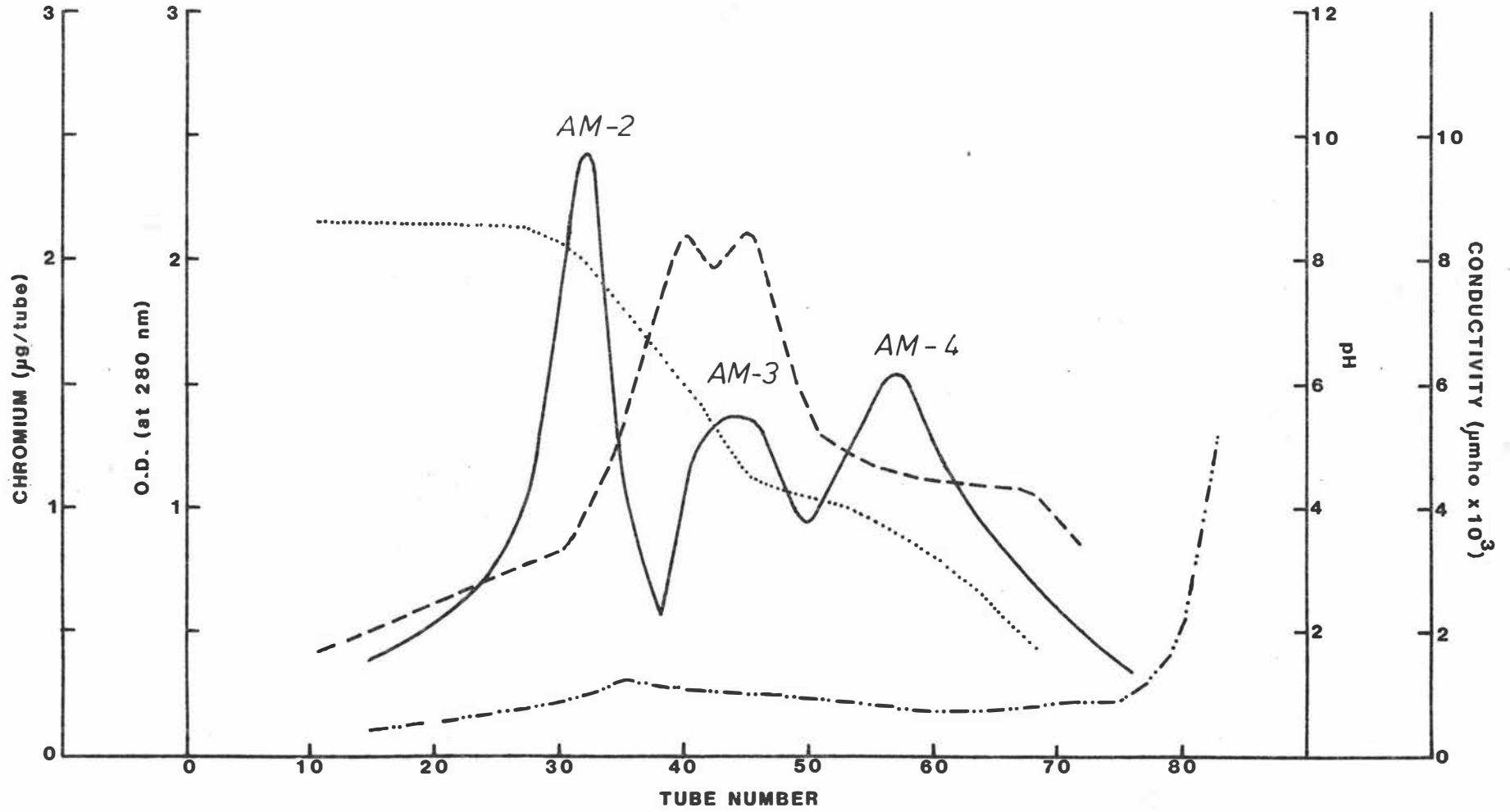


FIGURE 4.4

Elution of biologically-active P-1 and P-2 from a Dowex 50W-X12 cation-exchange column. The column (3.4 cm x 11 cm) was loaded at pH 3.5 with unbound material from Figure 4.2 and eluted as described in methods. The volume of each tube was 18.0 cm³. Chromium (—————), pH (.....), conductivity (-----) and A₂₈₀ (-----). This elution pattern was also obtained on three repeat columns.

FIGURE 4.4

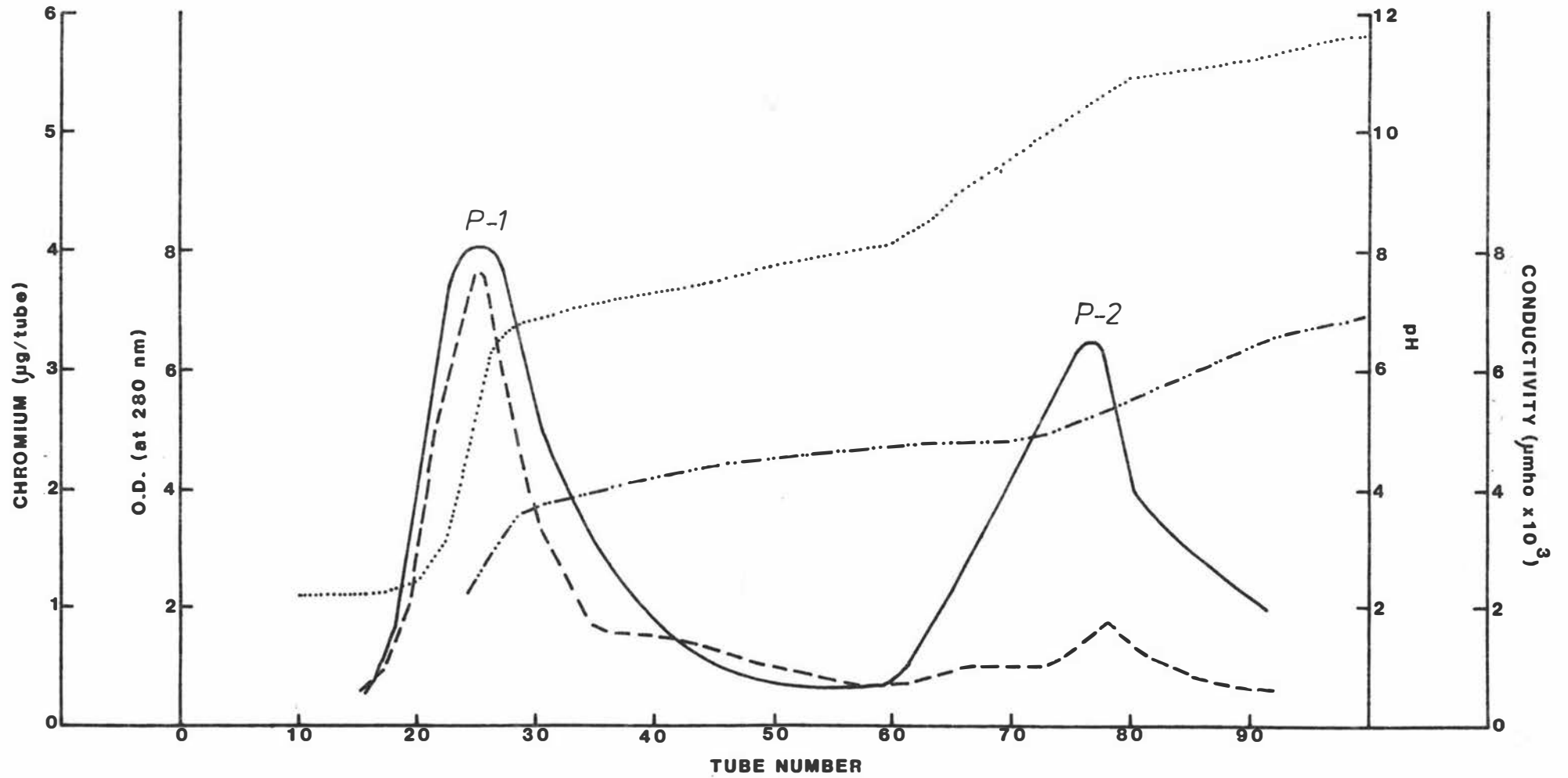


FIGURE 4.5

Elution of biologically-active fractions P-3 and P-4 from a Dowex 50W-X12 cation-exchange column. The column (2.7 cm x 9 cm) was loaded at pH 3.5 with unbound material from Figure 4.3 and eluted as described in methods. The volume of each tube was 20.0 cm³. Chromium (—————), pH (.....), conductivity (-----) and A₂₈₀ (-----). Four similar columns were run.

FIGURE 4.5

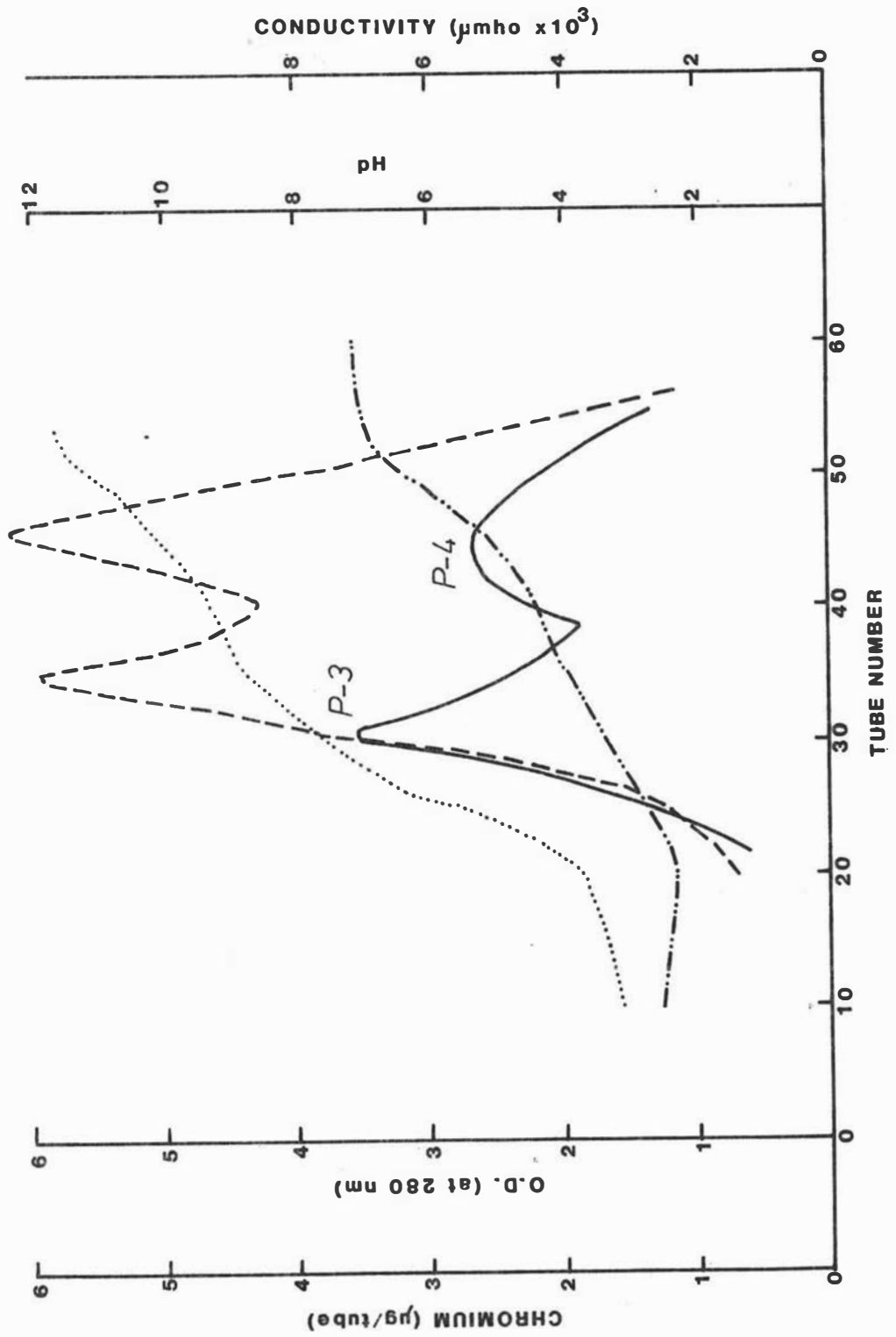
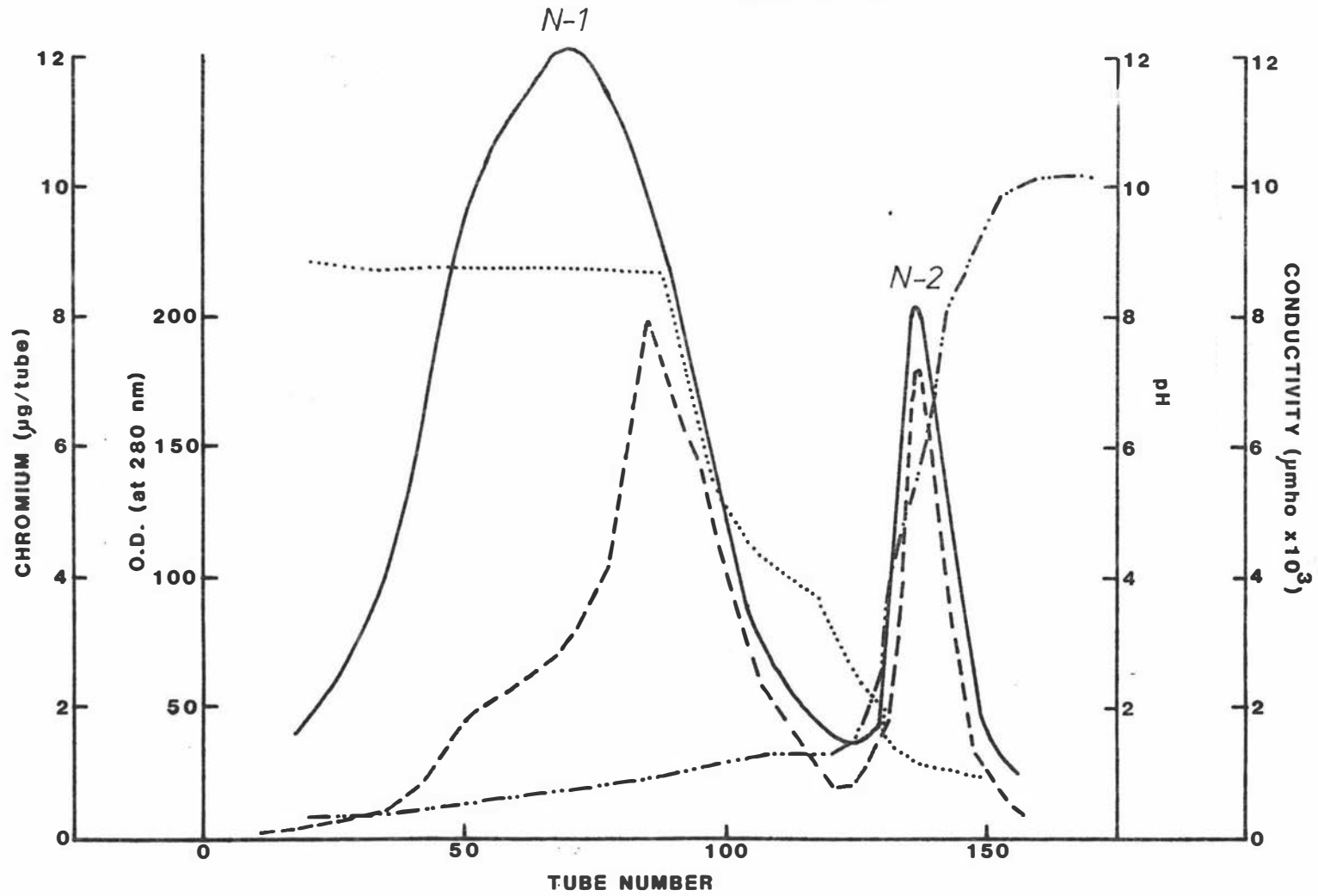


FIGURE 4.6

Elution of anionic-material N-1 and N-2 from a Dowex 1-X8 anion-exchange column. This column (4.5 cm x 18 cm) was loaded at pH 8.5, with the effluent (containing unbound material) from Figure 4.1, and eluted as described in methods. The tube volume was 20.0 cm³. Chromium (—————), pH (.....), conductivity (-----) and A₂₈₀ (-----). Three similar columns were run.

FIGURE 4.6



The fraction designated Z-1 comprises all chromium which did not bind to any column, possibly because it was neutral or, perhaps due to column over-loading phenomena. This fraction was the largest, comprising 877 μg of chromium as determined by means of an overall chromium balance.

4.3.4 Filtration columns.

The peak fractions from ion-exchange columns were bulked and freeze dried. The freeze-dried extracts were taken up in the minimum volume of distilled water and applied either to an 800 cm^3 (5.2 cm x 37 cm) Sephadex G-10 (40-120 μ) desalting column or to a Biogel P2 (50-100 mesh) column with a bed volume of 310 cm^3 (2.7 cm x 54 cm). The fractions collected were analysed for chromium and conductivity and in the cases where the separation of the chromium peak and conductivity peak was sufficiently large, the chromium peak was again bulked, concentrated by freeze drying and rechromatographed on the Biogel P2 column. In those cases where analysis showed little or no separation of the chromium and conductivity peaks, no further chromatography was carried out. Despite several literature reports about the loss of volatile chromium during ashing prior to atomic absorption spectroscopy (Tuman et al (1977), Shapcott et al (1977)), there was no apparent loss of chromium on freeze drying.

Of all the fractions collected from the ion-exchange columns only N-1 showed further fractionation on gel filtration. N-1, the major anionic fraction, split into two peaks N-1A and N-1B (Figure 4.7).

Estimates of molecular size were made from the elution volumes and these are shown in Table 4.2. It must be stressed that the values reported were only approximate as the method used did not take into account any non-ideal elution behaviour that may have occurred from sample association with the filtration resin bead. Effects of this type would have led to an apparent molecular weight which was less than the true value.

FIGURE 4.7

Gel filtration of peak N-1 on Sephadex G-10. The column (5.2 cm x 37 cm) was eluted with distilled water. Chromium (————) and conductivity (-----). The tube volume was 8.0 cm³. A similar result was obtained on a duplicate column.

FIGURE 4.7

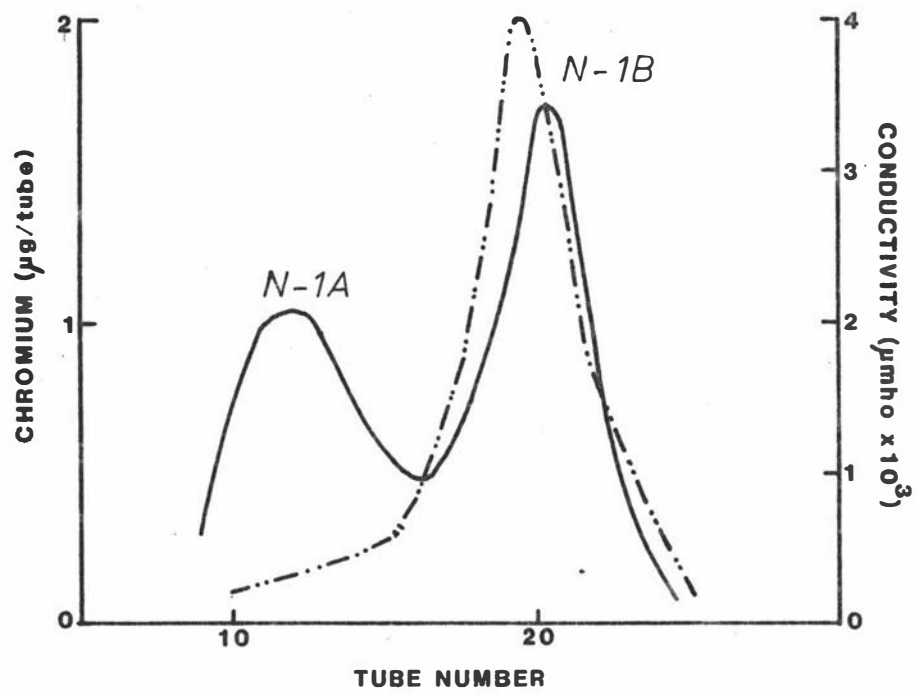


TABLE 4.2 MOLECULAR WEIGHTS OF CHROMIUM-CONTAINING FRACTIONS OBTAINED FROM YEAST EXTRACT

	FRACTIONS											
	AMPHOTERIC				CATIONIC				ANIONIC			
	AM-1	AM-2	AM-3	AM-4	P-1	P-2	P-3	P-4	N-1A	N-1B	N-2	
Molecular weight (Daltons)	295	501	700	389	355	110	324	138	543	347	700	
Percentage yield	3.4	1.0	0.6	1.0	3.1	3.0	1.9	1.4	34.2	9.6		

The total separation scheme used in the isolation of chromium peaks is summarised in Scheme 4.1.

4.3.5 Biological activity.

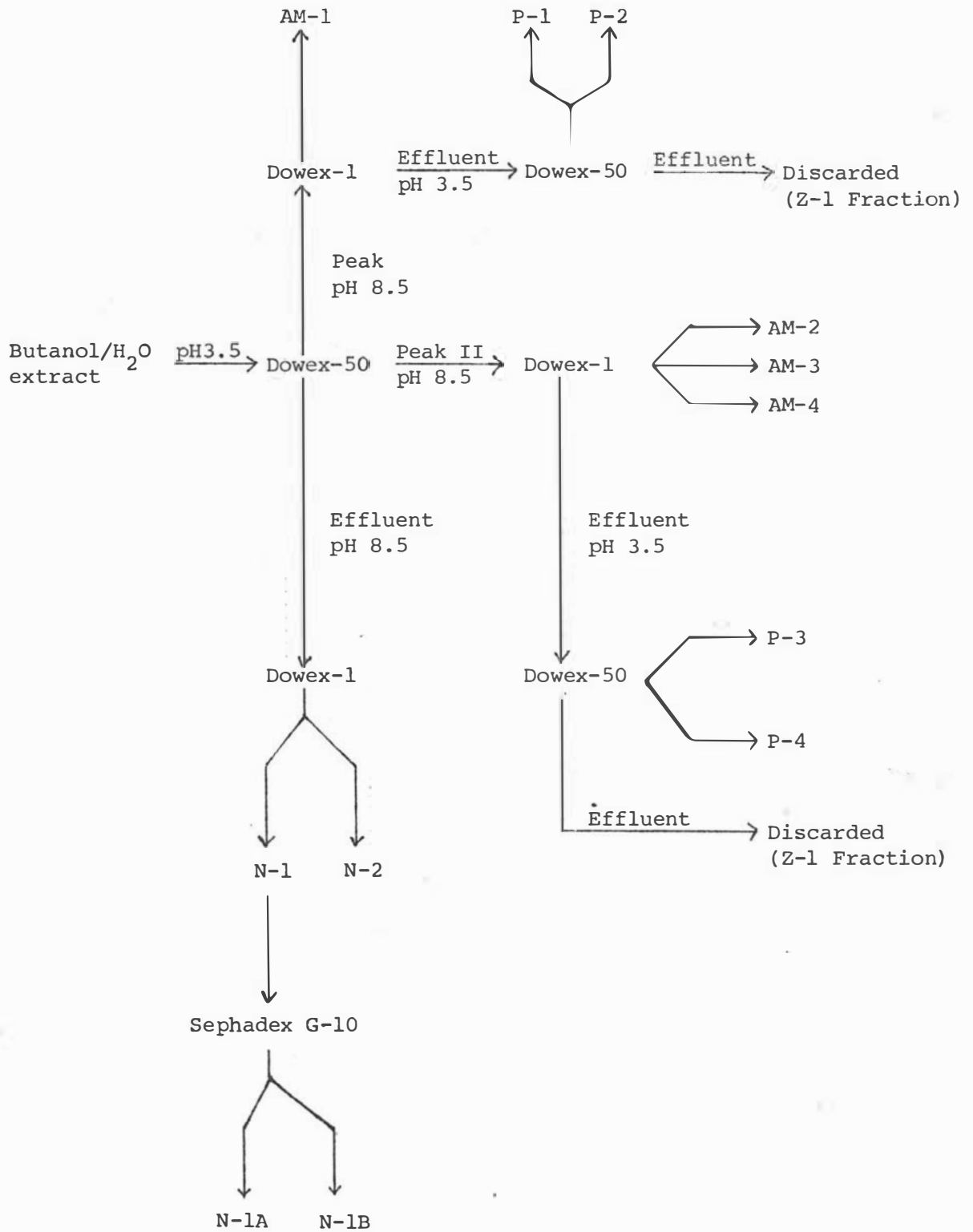
Using the yeast assay as described in section 3 the following results were obtained. (Table 4.3) The chromium concentration for each fraction was 192 pmoles per 2.5 cm³ of assay mixture, except for AM-4 (173) and P-1 (186). The rates for both sample and control are expressed as nanomoles of CO₂ evolved per minute, while the percentage enhancement is expressed as 100 x ((sample rate - control rate)/control rate). The specific enhancement is the enhancement per 100 pmoles of chromium while the purity is given as A₂₈₀ per 100 pmoles of chromium.

TABLE 4.3 BIOLOGICAL ACTIVITY OF CHROMIUM CONTAINING FRACTIONS OBTAINED FROM YEAST EXTRACT

Fraction	Sample Rate	Control Rate	Percentage Enhancement	Specific Enhancement	Purity
AM-1	21	17	+ 23.5	+ 12.2	2.41
AM-1	19	17	+ 11.7	+ 6.1	0.08
AM-2	17	22	- 22.7	- 11.8	0.73
AM-3	17	22	- 22.7	- 13.1	0.29
P-1	46	30	+ 53.3	+ 28.7	0.81
P-2	66	33	+120.0	+ 62.5	0.17
P-3	65	19	+232.0	+126.1	0.31
P-4	45	19	+136.8	+ 71.3	1.14
N-1A	30	26	+ 15.3	+ 8.0	4.93
N-1B	31	17	+ 82.4	+ 20.0	7.68
N-2	31	17	+ 82.3	+ 42.8	0.37

SCHEME 4.1

SCHEMATIC SUMMARY OF SEPARATION PROCEDURE



4.3.6 Percentage enhancement relative to P-3 chromium concentration.

Table 4.3 has shown fraction P-3 to be the most active chromium fraction. A concentrated sample of P-3 was prepared by freeze drying, and then redissolving the sample in a lesser volume of water. Activity assays were carried out on various chromium concentrations of the P-3 sample ranging between 0.0035 $\mu\text{g/g}$ to 0.23 $\mu\text{g/g}$ (an upper limit due to the solubility of P-3) and the results are shown in Figure 4.8. Also shown in this figure are the data obtained by Mirsky et al for a similar range of chromium concentrations in an active sample preparation from yeast extract. Their data has been recalculated to express the activity in terms of percentage enhancement of CO_2 production rather than the absolute CO_2 production rate given in their publication. Also the chromium concentration has been expressed in terms of the chromium concentration in the active sample and not that in the total activity assay mixture.

4.4 DISCUSSION

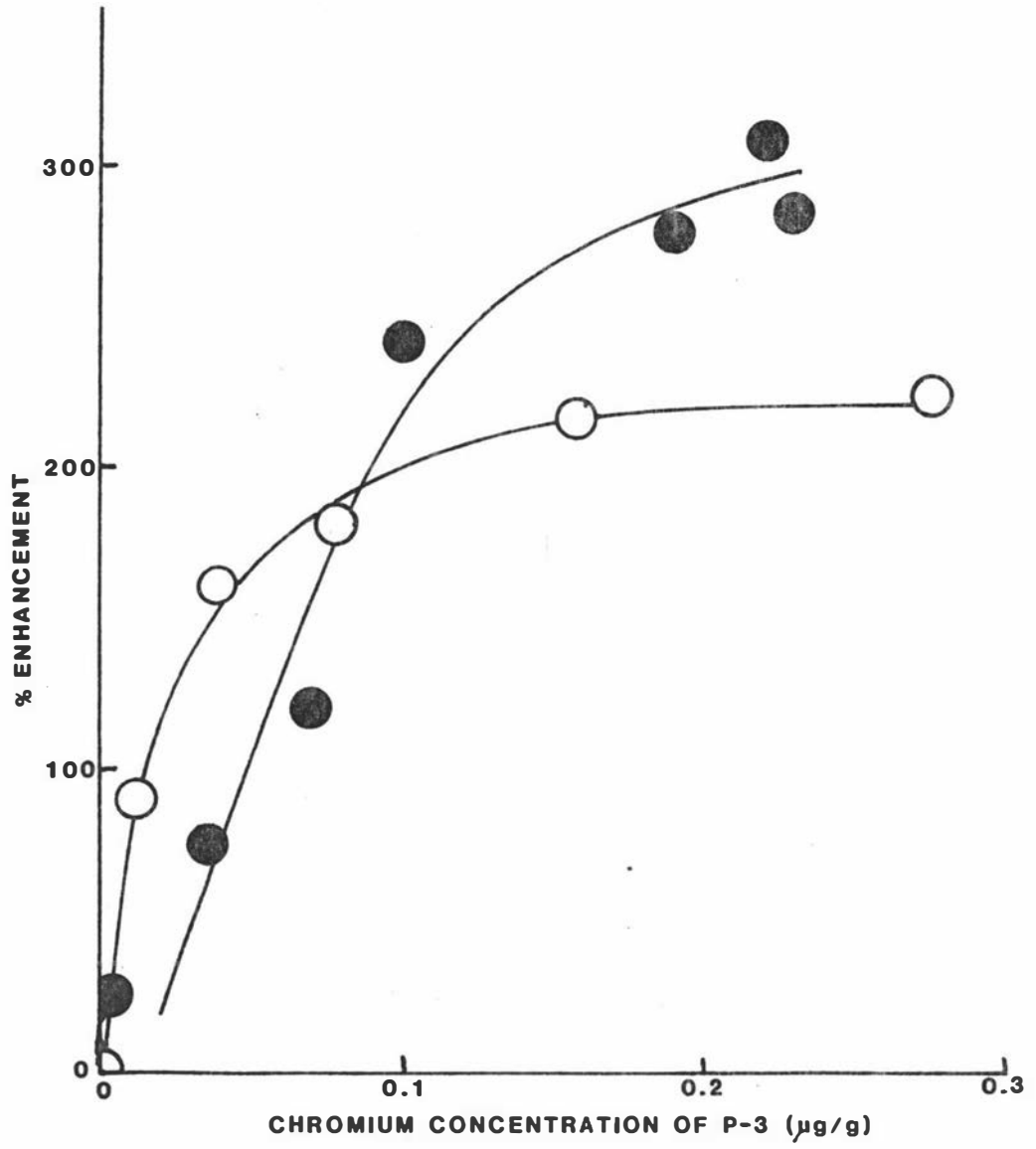
The use of a commercially available high chromium yeast extract has enabled adequate levels of chromium for detection by atomic absorption spectroscopy to be maintained throughout the isolation procedure and has enabled an isolation procedure to be developed. This isolation procedure has resulted in the resolution of the original chromium extract into 11 distinct fractions of varying yield and biological activity.

The Dowex ion-exchange columns used in this separation procedure were the only ones which bound significant amounts of anionic, cationic and amphoteric material. Whilst anionic material did bind to DE cellulose anion-exchange columns and may have been eluted from these with salt and pH gradients (section 2), comparison of the elution results from cellulose resins achieved in section 2 with these obtained here from Dowex 50 resins under comparable conditions, showed that only a very small amount of cationic material did in fact bind to CM cellulose cation-exchange resins.

FIGURE 4.8

Percentage enhancements recorded for a range of P-3 concentrations isolated as shown in Figure 4.5 (●—●), compared with the percentage enhancements recorded for a range of chromium concentrations of active material isolated by Mirsky et al (○—○).

FIGURE 4.8



The success of the scheme depends on the careful control of the pH gradients used to elute material from the Dowex ion-exchange resins. In particular it is necessary to be able to fractionate the material which binds tightly to the Dowex 50W-X12 cation exchange resin. To achieve this the column has to be neutralised as it is not possible to reduce the acidity of the loaded column simply by passage through it of even very high concentrations of sodium or ammonium chloride. Successful separations were only achieved when the pH gradients were generated in situ by passing Na_2HPO_4 through the column, using the intrinsic acidity of the loaded resin to provide the variable acidic buffer component. Gradients produced in this way were entirely reproducible.

As can be seen in Table 4.3 application of the separation procedure results in eleven apparently discrete and homogeneous chromium-containing fractions from the Merck yeast extract, a not too surprising result when the known complexing propensity of Cr (III) ions is considered (Rollinson et al (1967)). These fractions were further grouped into three categories based on their behaviour on the ion-exchange columns. The fractions which bind to Dowex 50 cation-exchange columns at pH 3.5 and also to Dowex 1 anion-exchange columns at pH 8.5 are designated amphoteric (AM), those which show cationic behaviour at pH 3.5 but do not bind to anion-exchange columns at pH 8.5 are classified as cationic (P) whereas those that do not bind to cation-exchange columns at pH 3.5 but do bind to anion-exchange columns at pH 8.5 are classified as anionic (N). It is of course possible that all the fractions are amphoteric but for discussion it is convenient to refer to them as described.

Fractions AM-1, AM-2, AM-3, and AM-4 comprised 6% (Table 4.2) of the total amount of extracted chromium and since they exhibited no biological activity (Table 4.3) they were considered as minor components of little interest.

The anionic green complex isolated in section 2 as the main complex associated with the chromium chloride-supplemented yeast showed a similar ion-exchange elution profile to that for the N-1 complex. The activity of the isolated

anionic green complex determined by the assay system described in section 3 resulted in a percentage enhancement of + 15 and a specific enhancement of 0.5. These values clearly indicate insignificant activity.

Many similarities between N-1 and the anionic green complex isolated in section 2 are apparent. Both fractions give a similar ion-exchange elution profile, both constitute the major chromium complex isolated from their respective sources, both are inactive with similar percentage enhancement values (+ 15.3 and + 15) and both separate into 2 fractions on gel filtration chromatography (N-1A, N-1B; high molecular weight anionic fraction, low molecular weight anionic fraction). Such results suggest that the two fractions are identical.

The major fractions in terms of total amounts of chromium are the anionic fractions N-1A, N-1B and N-2 which collectively make nearly 44% of the extractable chromium. While N-1A and N-1B are clearly biologically inactive, N-2 shows slight activity and hence possibly represents the first reported example of a negatively charged complex with glucose tolerance factor activity.

Since it seems generally accepted that the glucose tolerance factor is cationic (Toepfer et al (1977), Mirsky et al (1980)) the fractions of most interest with respect to the identity of the glucose tolerance factor are those classified as P-1, P-2, P-3 and P-4 (which are cationic at least at pH 3.5). Although none of these fractions could be considered to be pure it seems likely that P-2 and P-3 which had low values for the absorbance at 280 nm/100 pmoles of chromium are significantly more pure than P-1 or P-4 (Table 4.3). Estimates of the molecular weights given in Table 4.2 for the eleven chromium fractions show the spectrum of sizes to be expected if chromium binds to the various ligands which are present in the yeast and in the growth medium. In view of the known lack of biological activity of simple chromium (III) salts (Anderson (1980)) attention was focused on the larger molecular weight biologically-active fractions, which suggests that P-3 is the most likely candidate for identification with the glucose tolerance factor.

The activities determined for the limited range of P-3 chromium concentrations are shown in Figure 4.8. At chromium concentrations of fraction P-3 of less than 1.0 $\mu\text{g/g}$ a linear relationship between the percentage enhancement and the P-3 chromium concentration is apparent. At higher concentrations there appears to be some levelling off of activity which suggests a possible saturation effect.

Figure 4.8 also shows a similar plot of the variation in percentage enhancement with chromium concentration for an active sample as determined by Mirsky et al (1980). The chromium complex content of their active material is discussed in more detail later in this section but their described procedure is likely to produce an active sample with a number of cationic chromium fractions. Even allowing for the differences in the active samples and the slight differences in the method of activity determination the comparison of the two curves shows the two to be similar in shape. The similarities of the two curves raises the question as to whether the varying percentage enhancement level measured is due to the variation in the chromium concentration or due to some other factor. This question will be discussed in later sections.

The published procedures for the isolation of chromium complexes from yeast and yeast extracts can now be assessed in terms of the complexes isolated here. Schwarz and Mertz (1957) in their initial proposal of the existence of a glucose tolerance factor used either brewer's yeast or pork kidney powder as their basic source. They indicated the use of 6-12 fractionation steps but did not expand their description of the methods involved. Mertz (1969) in his review of the field makes mention of GTF being extracted and concentrated from brewer's yeast and pork kidney powder but he does not report details of the isolation and purification method used. Hence comparison of the methods for isolation used here and those used in the original GTF preparation is impossible.

The first published description of an attempt to isolate and purify GTF was undertaken by Votava et al (1973). They grew brewer's yeast on a medium containing chromium-51 and by using butanol- H_2O extraction combined with gel

chromatography isolated a single radioactive chromium-51 peak. This peak when chromatographed on cation (Dowex 50W resin) and anion (Dowex 1 resin) exchange columns and eluted with various concentrations of NaCl showed only anionic characteristics. Unfortunately the various pH values and conductivities at which the samples were loaded to these columns were not detailed. The one anionic complex which they did isolate would probably correspond to one of the major anionic fractions (N-1A or N-1B) isolated here. Under the elution conditions used by Votava et al the cationic (P) fractions and amphoteric (AM) fractions would probably remain bound to the various columns and hence remain undetected.

Hydrolysates of the anionic peak obtained by Votava et al when chromatographed using a silica gel method indicated the possible presence of the amino acids, leucine or isoleucine, proline, valine, alanine, serine and either glutamic acid or aspartic acid. As a crude test of the biological activity of the anionic complex, Votava et al tested the complex for its ability to be retained in male rats after a stomach-tubed dose. They found an enhanced retention over controls for which chromium-51 chloride was given as a stomach-tubed dose.

The major criticism of this preparation must be the poor chromium yield in terms of the amount of chromium in the anionic complex compared with that originally present in the growth medium of the yeast. Assuming a whole half-life decay period (27.8 days) between the addition of the chromium into the medium and the final measurement of the chromium-51 in the anionic complex as well as a radioactive counting efficiency of ca 1%, then only 0.04% of the radioactive chromium added to the yeast medium was isolated in the anionic complex. Even allowing for the fact that much of the remaining 99.96% of the chromium-51 must have remained in the growth medium the yield is still very low and demonstrates the inefficiency of their elution system.

The direct association of the amino acids (characterised by silica gel chromatography) with the chromium-51 must be criticised for the following reason. The total chromium

in the yeast growth medium was 4 μg and of this 0.04% was isolated in the anionic complex, then the total chromium isolated would have been $1.6 \times 10^{-3} \mu\text{g Cr}$ (31.3×10^{-3} nmoles Cr). Assuming all the material bound to this chromium was hydrolysed and chromatographed, for the amino acids to be present in a similar molar ratio to the chromium a detection limit for the amino acids of the same order of magnitude as the amount of chromium (31.3×10^{-3} nmoles) would be required. Normal detection limits for amino acids on silica-gel impregnated glass paper would be ca 0.05 μg (Kirchner (1978)) which assuming an average amino acid molecular weight of 140 corresponds to ca 357×10^{-3} nmoles of amino acid. Thus the minimum molar amount of amino acid that could have been detected would have corresponded to at least a ten fold excess over the number of moles of chromium-51. Therefore it seems likely that these amino acids were impurities and not (necessarily) complexed to the chromium as concluded by Votava et al.

Toepfer et al (1977) have published a procedure for isolating a biologically-active cationic fraction from brewer's yeast (S carlsbergensis) which has become the standard and much quoted source of GTF. In the preliminary steps an aqueous yeast extract was treated with activated charcoal from which material was eluted with NH_4OH and diethyl ether. After removal of the NH_4OH and ether, curiously the material was then hydrolysed by refluxing in HCl (5 M) for 18 hours. This hydrolysate was loaded onto a Dowex 50 W-X8 column and the active material was eluted in a broad peak with 0.1 M NH_4OH . Ammonium hydroxide when used as an elution agent in this laboratory resulted in all the bound chromium complexes being eluted as single unresolved peak. The elution reported by Toepfer et al is in agreement with this. Hence assuming that most of the chromium complexes present in the aqueous extract bound to and were eluted from, the activated charcoal then the broad peak eluted from the Dowex 50 column could contain 8 different chromium fractions (P-1, P-2, P-3, P-4, AM-1, AM-2, AM-3 and AM-4) and probably because of the severe hydrolysis conditions used, hydrolysis products of these fractions.

Toepfer et al attempted to chemically characterise the samples prepared in this way by using a variety of physical techniques such as ultra violet-visible spectroscopy and infra-red spectroscopy as well as by chemical analyses. From these studies they claimed to have detected the presence of glycine, glutamic acid, cysteine and nicotinic acid associated with the chromium.

However assuming that the brewer's yeast sample used had a normal chromium level of ca $\mu\text{g}/\text{gram}$ and allowing for a 100% retention of the chromium throughout the extraction and isolation procedure (which is unlikely) then the concentration of the chromium complex in the final eluate from the Dowex 50W-X8 column would have been about $3.3 \times 10^{-3} \text{ g/l}$. If the complex had a molecular weight of ca 400, the concentration of the complex would be approximately $4.3 \times 10^{-4} \text{ M}$. At this concentration of complex, if the attached ligands were nicotinic acid and amino acids, the maximum optical density at 262 nm would be about 8.0 whereas the optical density observed at this wavelength was in fact 160. Attempting to characterise the structure of what must therefore be rather impure samples is liable to lead to misleading results and the data obtained probably more correctly reflect the major impurities associated with the chromium complexes. Certainly the hydrolysis of the yeast extract would have resulted in an abundance of amino acids and nicotinic acid which could have contaminated the chromium fractions.

Kumpulainen et al (1978) have extracted both anionic and cationic chromium-containing fractions from brewer's yeast (S uvarum ex S carlsbergensis) grown on radioactive chromium (III) chloride and have also extracted chromium-containing fractions from the spent and sterile medium respectively. Unfortunately they do not explicitly report the pH at which they loaded the Dowex 50-X8 cation-exchange column. However if for example loading was at pH 6-7 their cationic fraction would contain P-1, P-2, P-3, P-4 and probably some of AM-1, AM-2, AM-3 and AM-4 as these fractions would not be resolved by elution with 0-2 M NH_4OH . The Sephadex QAE A-25 anion-exchange column used by these workers to separate anionic chromium-containing fractions does not allow a direct comparison with the results described here.

Mirsky et al have recently attempted to isolate GTF from yeast extract (Mirsky et al (1980)). The extract was first dialysed in order to separate high molecular weight components from GTF and other lower molecular weight material. However, no significant purification was obtained by this method during the course of the present work since the yeast extract seemed completely permeable to the 3500 molecular weight cut-off dialysis membrane. After the dialysis step Mirsky et al used a DEAE-II cellulose column and then a Dowex 50-X8 column but unfortunately they did not specify the sample pH and conductivity.

The DEAE column when eluted with an NH_4Cl gradient showed only background chromium levels so presumably the amphoteric and anionic peaks (AM-1, AM-2, AM-3, AM-4, N-1, N-2) had remained bound to the column. Elution of the Dowex 50 column was in two stages firstly with an NH_4Cl gradient during which no chromium peaks were eluted and then with 0.25 NH_4OH which was responsible for the elution of a single chromium peak. This peak would have contained all the cationic peaks (P-1, P-2, P-3, P-4).

A possible criterion for the purity of chromium fractions has been described in this section as the absorbance at 280 nm per 100 pmoles of chromium in the sample. None of the fractions isolated here (or from the other published procedures) could be considered pure by this criterion and must still be associated with extraneous material. All attempts to determine the structure of such impure samples must be viewed with suspicion.

4.5 CONCLUSION

The isolation of the eleven distinct chromium fractions described in this section, has enable for the first time the establishment of a solid basis for further work. Only by working with distinct, purified chromium complexes can characterisation results have any real meaning and unfortunately the work carried out to date in this field by other researchers has not involved such discrete chromium complexes and consequently the characterisations performed on these must be considered at the very least, with some skepticism.

Determination of the activity of the eleven isolated

complexes has enabled the elimination of amphoteric and anionic complexes from further consideration for glucose tolerance factor activity. It has confirmed the observations of Toepfer et al (1977) and Mirsky et al (1980) that the observed activity is due to a cationic complex or material. Unfortunately the purification of the cationic material in other published procedures has been achieved by the elution of the fractions with NH_4OH . Not only does this procedure elute several bound chromium fractions under a single unresolved peak but it also can cause an activating effect on fractions eluted in this way. This phenomenon will be further discussed in section 8.4.

The results reported in this section indicate that the active cationic complexes P-1, P-2, P-3, P-4 warrant further investigation with particular emphasis on the fraction with the highest activity, P3.

SECTION 5

THE ORIGIN OF CHROMIUM-CONTAINING FRACTIONS ISOLATED FROM YEAST.

5.1 INTRODUCTION

In section 4 it was shown that commercial yeast extract contained eleven apparently distinct chromium fractions. It seems most unlikely that each of these would have its own metabolic function in the yeast but rather that some are artefacts as a result of the chromium forming stable complexes with any available ligands present in the medium.

It was the aim of the work described in this section to use the column procedures established in section 4 to determine the origin of these chromium fractions. However it was necessary to be aware that not only could different growth conditions for the yeast lead to different chromium complexes but also that the conditions used in the purification scheme may alter existing complexes or create new chromium complexes.

In an attempt to resolve these questions the following experiments were carried out. Two chromium chloride-supplemented media were prepared, one with yeast and one without, both being shaken under aerobic conditions for about twenty-four days. These experiments were also repeated with chromium-51 instead of chromium chloride. The yeast-containing medium was centrifuged and the yeast extracted. The chromium fractions present in the following three solutions were then studied.

1. A medium solution which had never contained yeast and which will be referred to as supplemented medium. Any chromium complexes isolated from such a solution must be formed by reaction of the chromium with components of the medium or with column eluent reagents.

2. The medium solution which had contained the yeast and which will be referred to as spent supplemented medium. In addition to the chromium complexes present in the supplemented medium this solution could contain yeast-produced chromium complexes which have been transported across the cell membrane.

3. A solution containing all aqueous extractable chromium complexes from the yeast. Besides the chromium complexes formed in the yeast cell the solution may also contain chromium complexes which are produced in the medium and subsequently transported into the yeast cell.

With the information from such a study it was hoped to divide the chromium fractions into two groups. One group of complexes that arose simply as a result of the reaction of chromium with media components or column eluents and which cannot have any essential metabolic function in yeast (and presumably no activity in the yeast assay) (Group I) and another group of complexes (Group II) that are formed only in the yeast and occur only in the yeast or the spent medium.

5.2 METHODS AND MATERIALS

5.2.1 Yeast and media samples.

The chromium chloride-supplemented media either with yeast (2 l) or without yeast (1 l) were prepared as described in section 2.2.1c with the only ingredient omitted in the yeast-free media being the yeast. The yeast was harvested after 25 days in the supplemented medium.

A low-chromium sample of the yeast inoculum (a strain of S. cerevisiae) was prepared for the chromium-51-supplementation studies by a three-fold growth on the chromium-deficient medium prepared as described in section 3.1, except for the addition of the radioactive chromium-51. The amounts of chromium-51 used were 1 mCi for the yeast-free medium solution (250 cm³) and 3 mCi for the yeast-containing medium (500 cm³). Supplementation was also allowed to occur over a period of 25 days before harvesting by centrifugation.

5.2.2 Ion-exchange chromatography.

The procedure used for the isolation of chromium-containing fractions from the different starting materials was based on the yeast extract isolation scheme described in section 4 with Dowex 50W-X12 as the cation-exchanger and Dowex 1-X8 as the anion-exchanger.

For the chromium chloride-supplementation experiments there was always a much larger column loading volume than for the chromium-51 supplementation, hence the column volumes

ranged from 20 cm³ to 50 cm³ in the former case, and from 5 cm³ to 15 cm³ in the latter. Conditions used during the loading of these resins were a flow rate of less than 1 cm³/min, and a conductivity of the sample of less than 2000 μ mho. The Dowex 50W-X12 resin was loaded at pH 3.5 while the Dowex 1-X8 resin was loaded at pH 8.5.

The method of elution was as described in section 4 except that a less severe pH gradient was used. The elution procedures are summarized in Table 5.1.

TABLE 5.1 ELUTION CONDITIONS FOR ION-EXCHANGE CHROMATOGRAPHY.

<u>Resin Type</u>	<u>Elution Gradient or Solution</u>	<u>Relative Volumes of Eluent</u>
Dowex 50W-X12	(i) 0.2 M NaH ₂ PO ₄ until pH = 4.1	6
	(ii) Gradient 0.2 M NaH ₂ PO ₄ v 0.1 M Na ₂ HPO ₄	3 v 3
	(iii) Gradient 0.1 M Na ₂ HPO ₄ v 0.05 M Na ₃ PO ₄	3 v 3
	(iv) 0.05 M Na ₃ PO ₄	1
Dowex 1-X8	(i) Gradient 0.19 M Tris-HCl, pH 8.5 (2000 μ mho) v 0.19 M Tris-HCl + 0.16 M NaCl, pH 8.5 (10,000 μ mho)	3 v 3
	(ii) Gradient 0.19 M Tris-HCl + 0.16 M NaCl, pH 8.5 (10,000 μ mho) v 0.35 M Acetic acid + 0.19 M NaCl (10,000 μ mho)	3 v 3
	(iii) Gradient 0.35 M Acetic acid + 0.19 M NaCl (10,000 μ mho) v 0.07 M HCl (10,000 μ mho)	3 v 3
	(iv) 1.33 M HCl	1

5.2.3. Chromium analysis.

Analysis of chromium chloride-supplemented media was carried out by flame atomic absorption spectroscopy as described in section 2. Similarly chromium-51 radioactivity was measured on a Packard Auto Gamma Scintillation Spectrometer, Model 5285.

5.2.4 Activity.

Activity was measured for suitable samples by the standard method described in section 3.

5.2.5 Optical density at 280 nm, pH and conductivity measurements.

These were undertaken as described in section 2.

5.3. RESULTS

Table 5.2 lists the 6 different systems on which complete chromium complex isolation was undertaken.

TABLE 5.2 STARTING SOURCE SOLUTIONS FOR COMPLETE COMPLEX ISOLATION.

- (i) Chromium chloride-supplemented medium without yeast.
- (ii) Chromium chloride-supplemented spent medium.
- (iii) Chromium chloride-supplemented extracted yeast.
- (iv) Chromium-51-supplemented medium without yeast.
- (v) Chromium-51-supplemented spent medium.
- (iv) Chromium-51-supplemented extracted yeast.

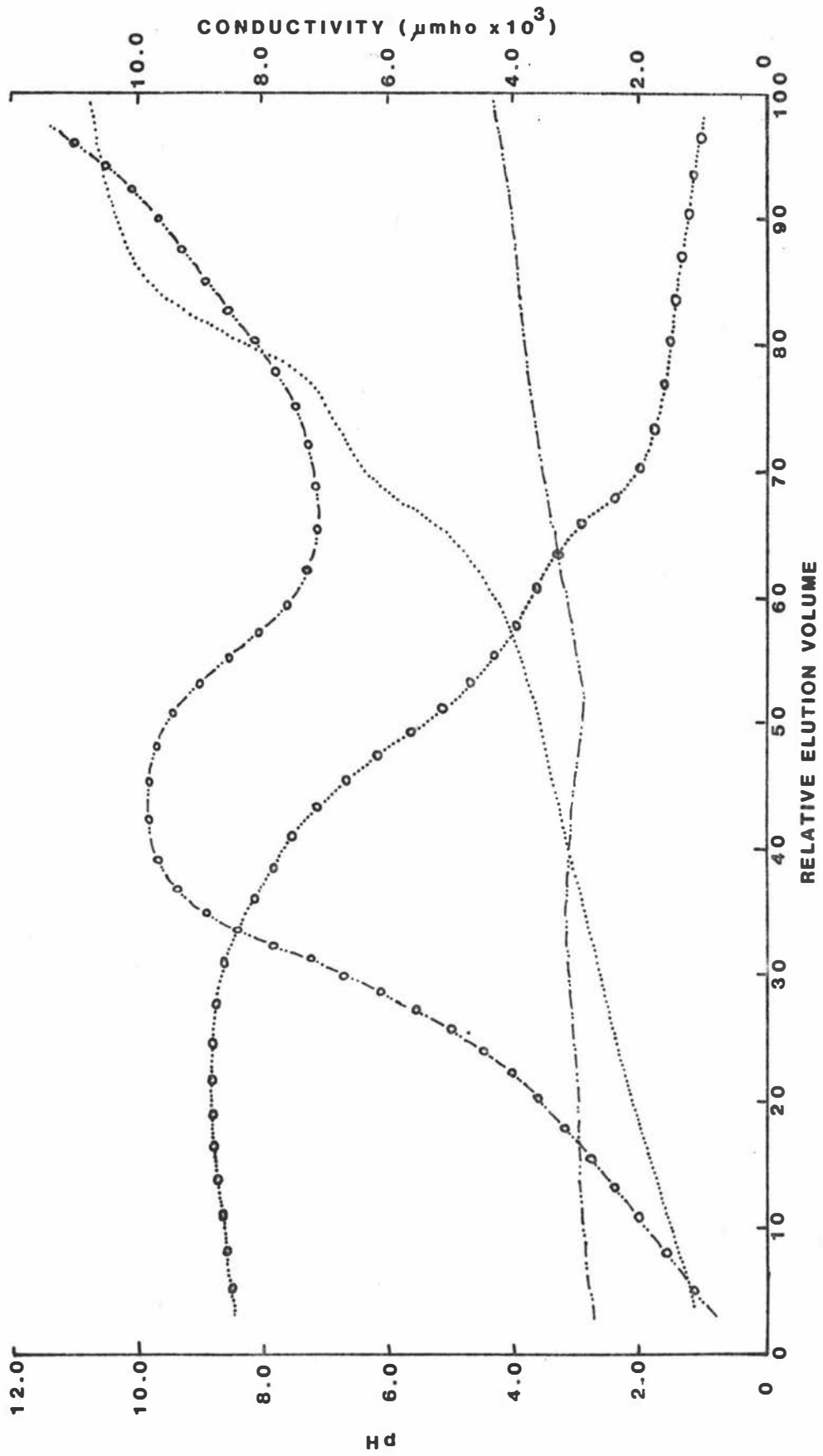
Some 34 different columns were required for these six different systems. Only the chromium peak positions are fully reported. Small variations in pH and conductivity did occur from one column to the next but an average profile for both the anion and cation-exchange columns is shown in Figure 5.1.

Throughout the isolations reported here direct comparison is made with the eleven complexes (P-1 to P-4, AM-1 to AM-4 and N-1 to N-2) isolated from yeast extract. During a column elution, if a peak isolated from the supplemented system was not directly aligned with a peak isolated from the yeast extract, or was a peak which was not present in the yeast extract isolation, the the peak was arbitrarily labelled by the addition or subtraction of a decimal number

FIGURE 5.1

Plot showing the standard elution profiles for pH and conductivity arising from the elution conditions described in Table 5.1 for both Dowex 50W-X12 resin (pH (.....), conductivity (---)) and Dowex 1-X8 resin (pH (.....), conductivity (---)).

FIGURE 5.1



to the subscript of the closest yeast extract peak. While the magnitude of the added or subtracted decimal fraction does indicate the peak position relative to other peaks it is not an indication of an exact peak position. For example, in Figure 5.3 (B, C and D), AM-1.5 is a label for peaks appearing to the right of the yeast extract peak, AM-1, in Figure 5.3 (A) while Figure 5.6 (C) P-3-.3 is a peak appearing to the left of the yeast peak, P-3, in Figure 5.6 (A). During the reporting and discussion of results for each column total chromium refers to the amount of chromium in the aqueous extract before the isolation procedure has commenced, while the individual percentages reported relate to the amount of this total chromium present in each fraction.

5.3.1 Supplementations with chromium chloride.

A summary of the chromium-containing peaks isolated from the various solutions obtained after the chromium chloride supplementation experiments is given in Table 5.3, together with those peaks isolated from yeast extract for comparison. These data are represented graphically in Figures 5.2, 5.3, 5.4, 5.5, 5.6 and 5.7. The directional arrows (\uparrow) in Table 5.3 show the shift in peak position which would be required to bring alignment of the supplemented peaks with a yeast extract peak. Alternatively where a yeast extract peak is absent the directional arrow indicates a peak shift which would result in alignment of a supplemented spent medium or an extracted yeast peak with a supplemented medium peak. Some of these peak shifts may be too large to be due to inter-column variations and may be discrete peaks in their own right.

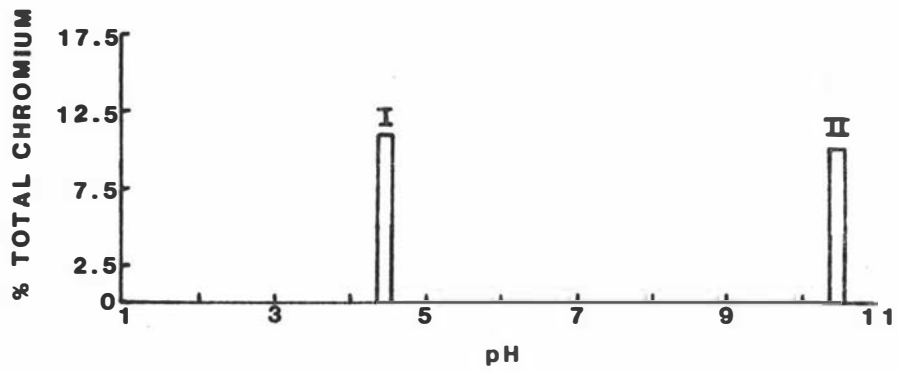
The six column isolation procedure which was the same as the elution procedure described in section 4 gives in summary, the following fractions.

- (i) Column I, Dowex 50W-X12, isolation of crude fractions I and II containing cationic and amphoteric material (Figure 5.2).
- (ii) Column II, Dowex 1-X8, isolation of amphoteric material (Part I) containing chromium fractions AM-1 to AM-1.9 (Figure 5.3).

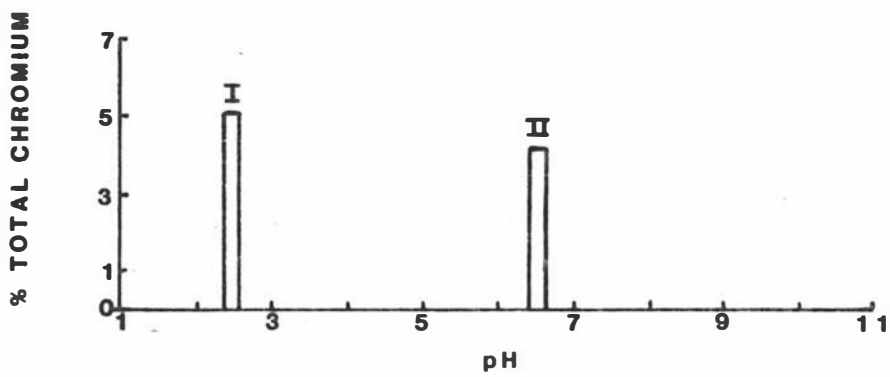
FIGURE 5.2 A,B,C,D.

The separation of starting sources into crude fractions I and II, containing cationic and amphoteric material by using Dowex 50W-X12 resin. A (yeast extract), B (supplemented medium), C (spent supplemented medium), D (extracted yeast). The percentage of total chromium in each peak shown by ().

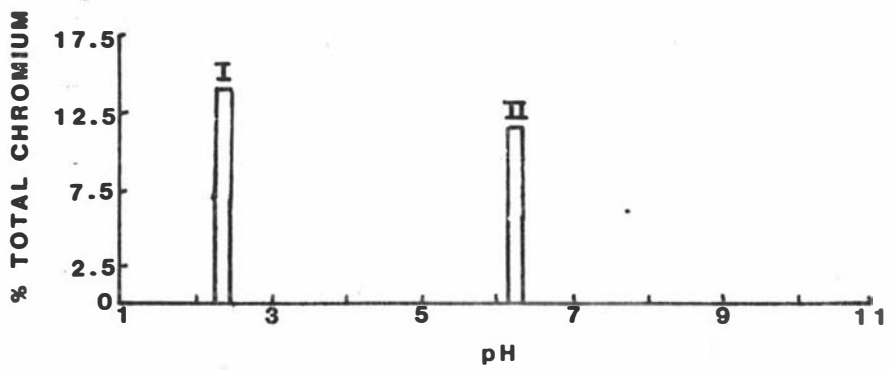
FIGURE 5.2



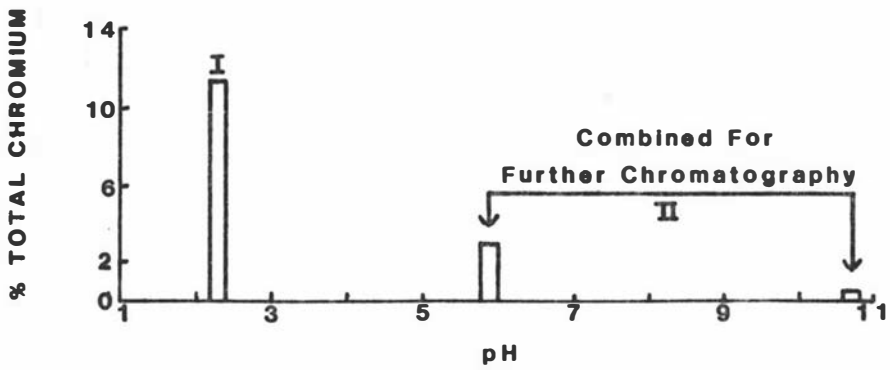
A



B



C



D

FIGURE 5.3 A, B, C, D.



The isolation of amphoteric fractions arising from the further fractionation of crude fraction I (Figure 5.2) by using Dowex 1-X8 resin. A (yeast extract), B (supplemented medium), C (spent supplemented medium), D (extracted yeast). The percentage of total chromium in each peak shown by () and the specific activity shown by ().

FIGURE 5.3

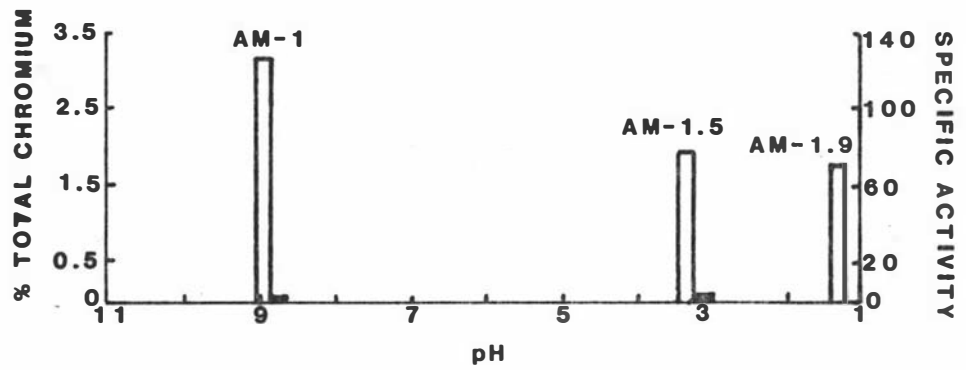
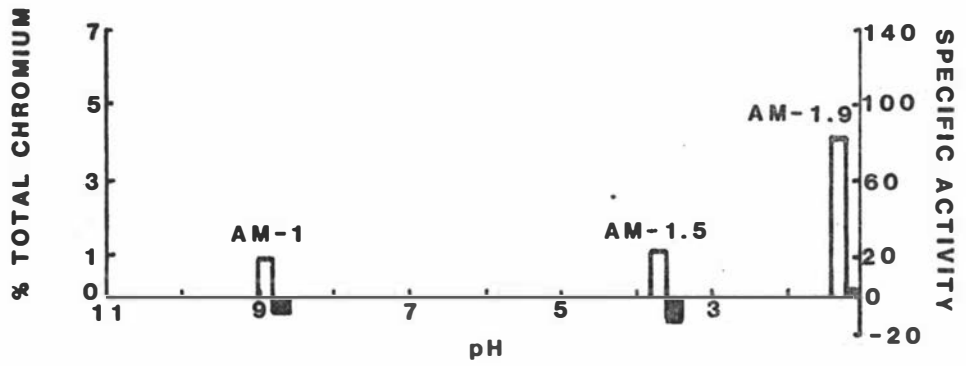
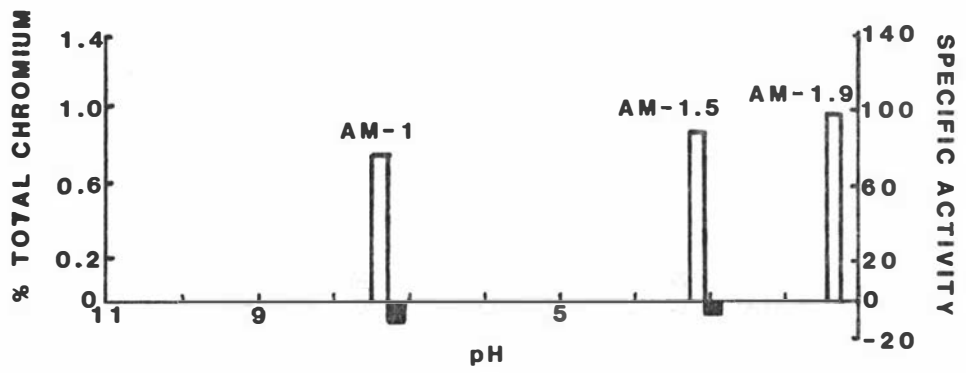
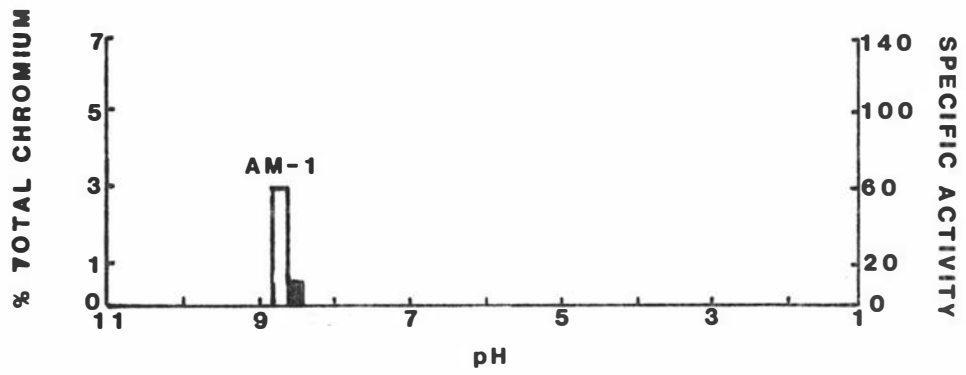


FIGURE 5.4 A, B, C, D.

The isolation of amphoteric fractions arising from the further fractionation of crude fraction II (Figure 5.2) by using Dowex 1-X8 resin. A (yeast extract), B (supplemented medium), C (spent supplemented medium), D (extracted yeast). The percentage of total chromium in each peak shown by (\square) and the specific activity shown by (\blacksquare).

FIGURE 5.4

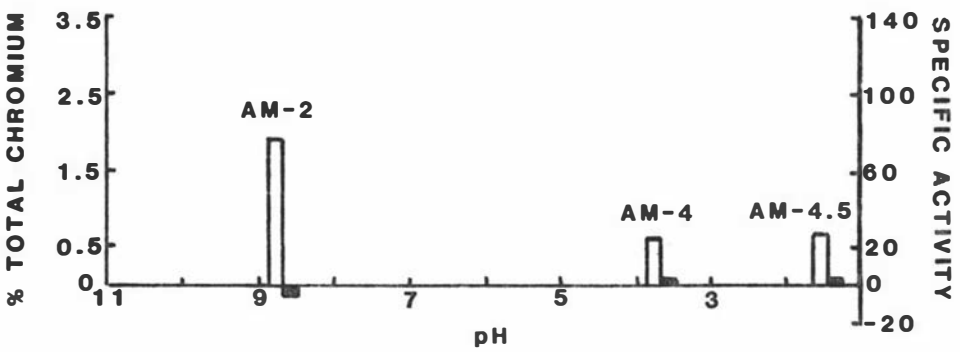
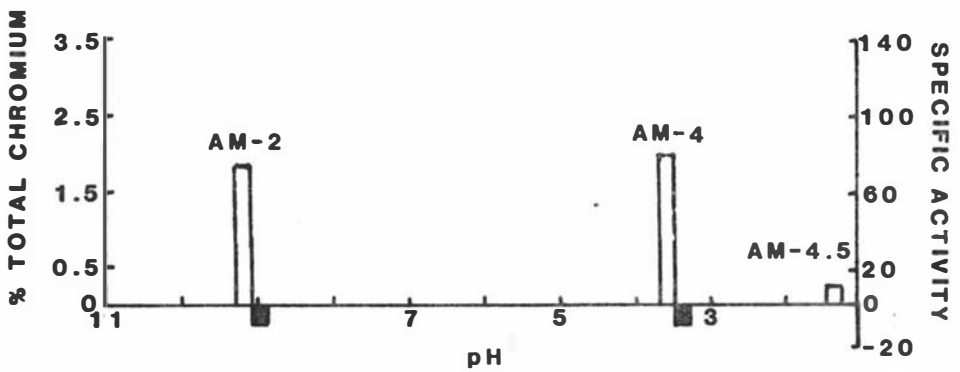
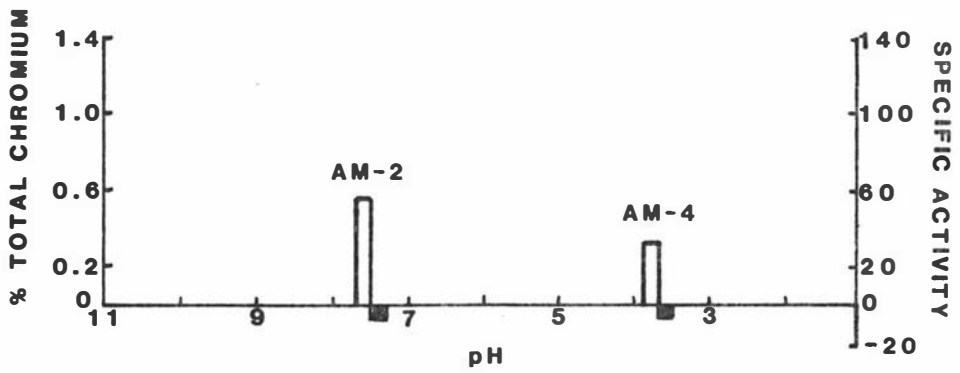
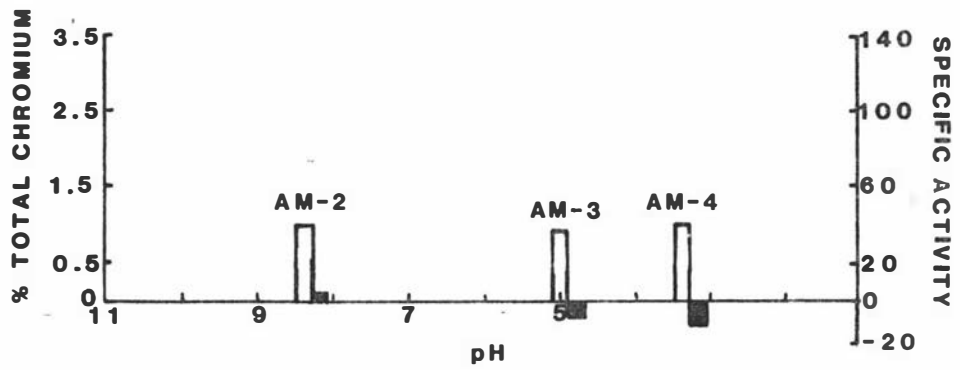


FIGURE 5.5 A, B, C, D.



The isolation of cationic fractions arising from the further fractionation of crude fraction I (Figure 5.2) by using Dowex 50W-X12 resin. A (yeast extract), B (supplemented medium), C (spent supplemented medium) D (extracted yeast). The percentage of total chromium in each peak shown by () and the specific activity shown by ().

FIGURE 5.5

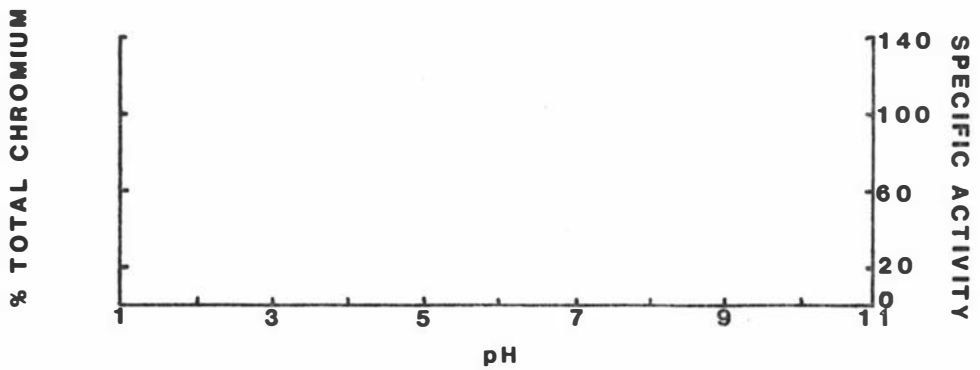
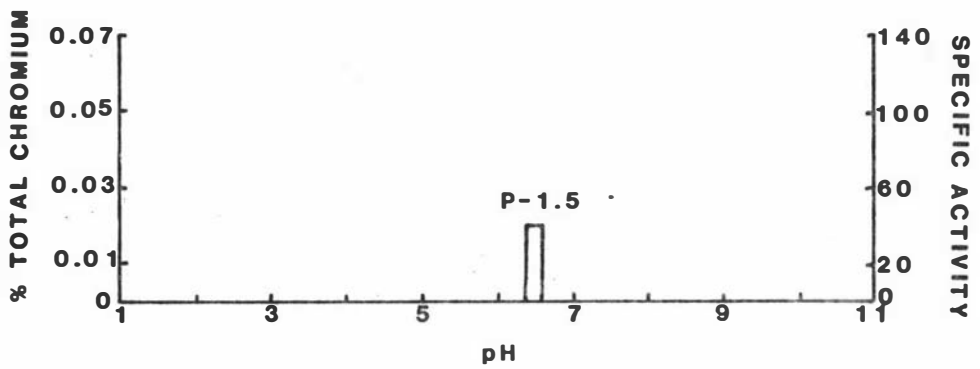
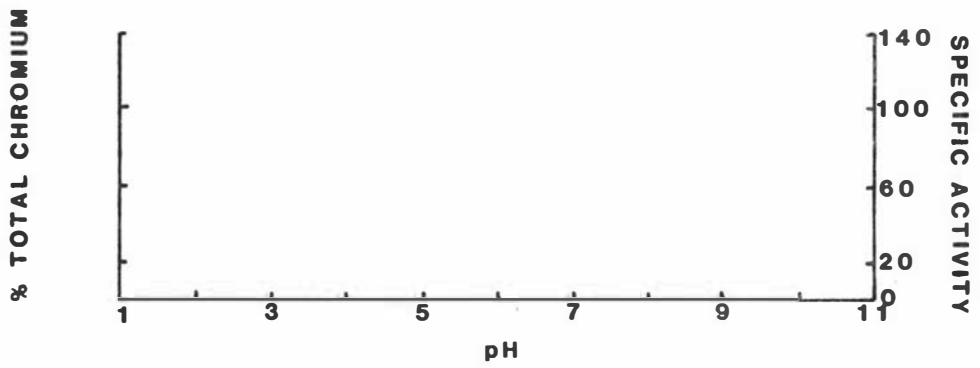
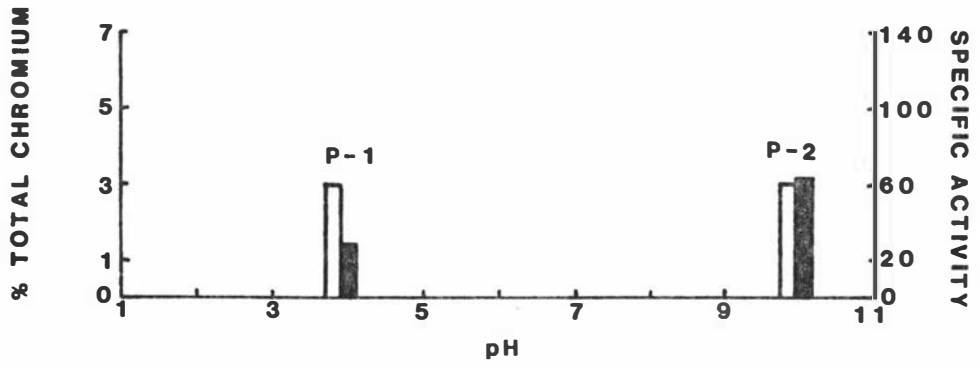


FIGURE 5.6 A, B, C, D.



The isolation of cationic fractions arising from the further fractionation of crude fraction II (Figure 5.2) by using Dowex 50W-X12 resin. A (yeast extract), B (supplemented medium), C (spent supplemented medium) D (extracted yeast). The percentage of the total chromium in each peak shown by () and the specific activity shown by ().

FIGURE 5.6

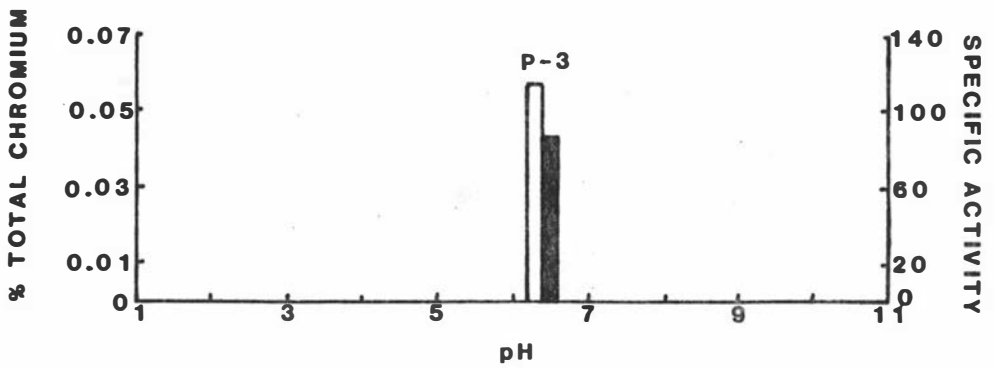
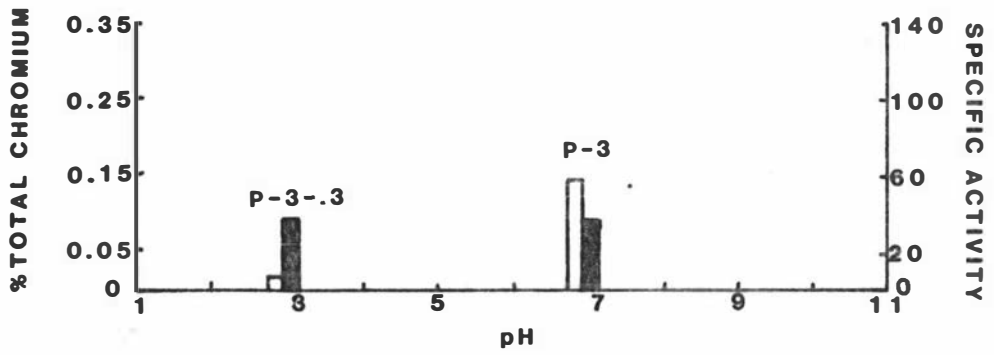
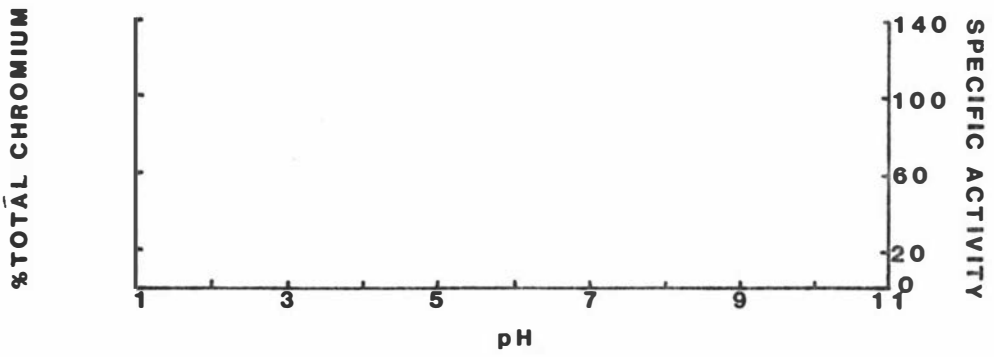
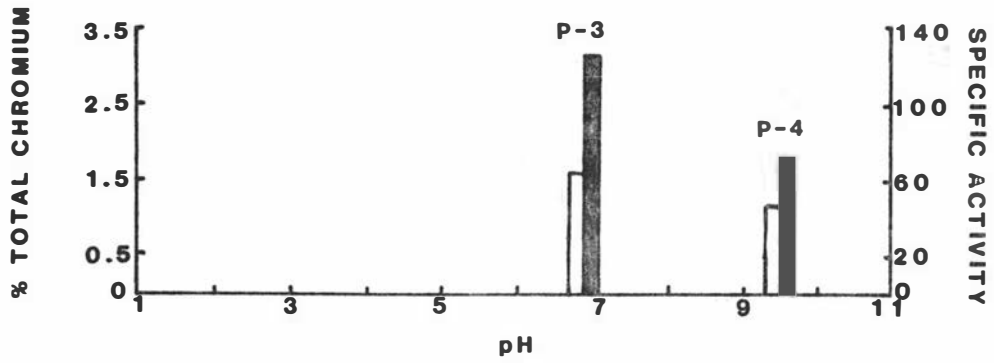


FIGURE 5.7 A, B, C, D.



The isolation of anionic fractions arising from the fractionation of the effluent from Column I by using Dowex 1-X8 resin. A (yeast extract), B (supplemented medium), C (spent supplemented Medium), D (extracted yeast). The percentage of the total chromium in each peak shown by () and the specific activity shown by ().

FIGURE 5.7

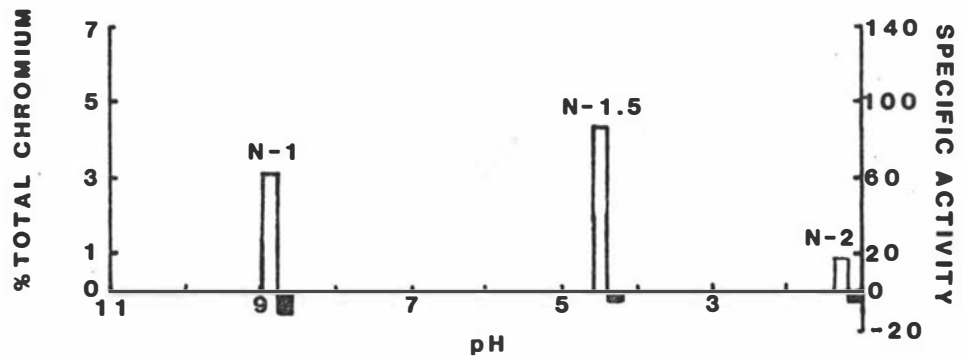
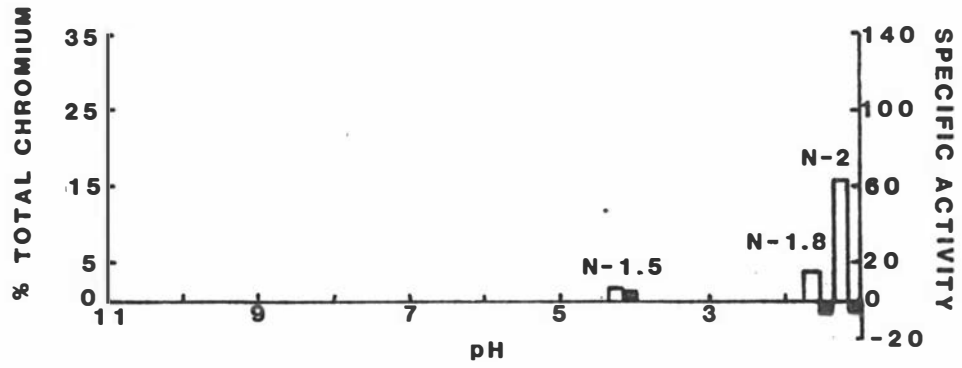
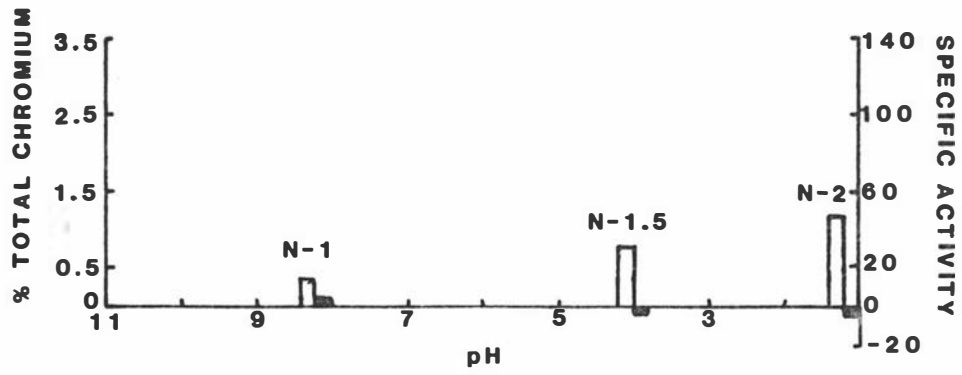
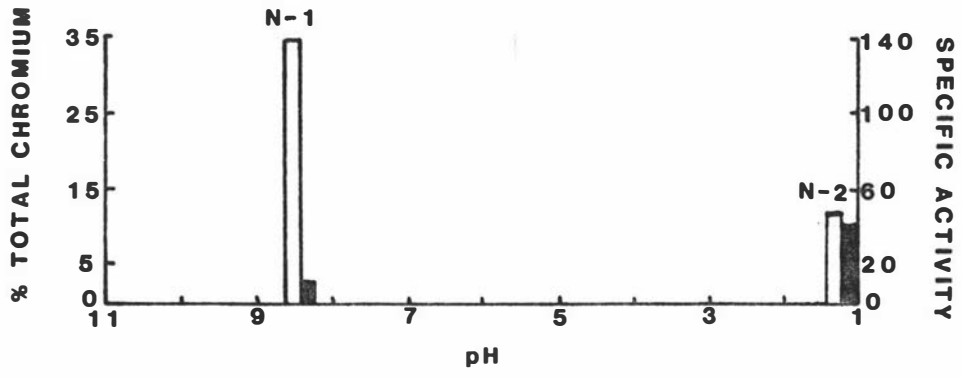


TABLE 5.3 CHROMIUM-CONTAINING FRACTIONS ISOLATED FROM
CHROMIUM CHLORIDE-SUPPLEMENTED MEDIA AND YEAST

Peak Description	Isolation Source				Graphical Representation (Figure)
	Yeast extract	Supplemented medium	Supplemented spent medium	Supplemented extracted yeast	
AM-1	X	X	X	X	5.3
AM-1.5		X	X	X	5.3
AM-1.9		X	X	X	5.3
AM-2	X	X	X	X	5.4
AM-3	X		↑	↑	5.4
AM-4	X	X	X ↑	X ↑	5.4
AM-4.5			X	X	5.4
N-1	X	X	↑	X	5.7
N-1.5		X	X ↑	X	5.7
N-1.8			X		5.7
N-2	X	X	X	X	5.7
P-1	X		↑		5.5
P-1.5			X		5.5
P-2	X				5.5
P-3-.3			X ↓		5.6
P-3	X		X ↓	X	5.6
P-4	X				5.6

- (iii) Column III, Dowex 1-X8 isolation of amphoteric material (Part II) containing chromium fractions AM-2 to AM-4.5 (Figure 5.4).
- (iv) Column IV, Dowex 50W-X12 isolation of cationic material (Part I) containing chromium fractions P-1 to F-2 (Figure 5.5).
- (v) Column V, Dowex 50W-X12 isolation of cationic material (Part II) containing chromium fractions P-3-.3 to P-4 (Figure 5.6).
- (vi) Column VI, Dowex 1-X8 isolation of anionic material containing chromium fractions N-1 to N-2 (Figure 5.7).

Each of the Figures 5.2 to 5.7 have been subdivided into A, B, C and D where A refers to the yeast extract isolation in section 4, B to the supplemented medium, C to the spent supplemented medium and D to the extracted yeast.

5.3.2 Supplementations with chromium-51

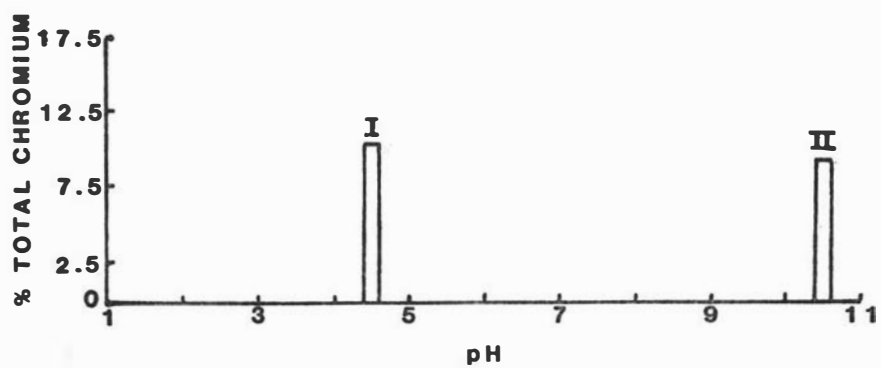
The results obtained from the chromium-51 supplementation experiments are shown in Table 5.4. The same six column procedure was used as described for the chromium chloride supplementation experiments with the results shown in Figure 5.8 (column I), Figure 5.9 (column II), Figure 5.10 (column III), Figure 5.11 (column IV), Figure 5.12 (column V) and Figure 5.13 (column VI). Again in these figures the label A refers to the yeast extract, B to the supplemented medium, C to the spent supplemented medium and D to the extracted yeast.

In the case of the chromium-51-supplemented medium the isolation procedure was incomplete because of the very low yield obtained for peak II from the elution of column I (Figure 5.8, B). To compensate for this deficiency, peaks arising from peak II from the elution of column I from the chromium chloride-supplemented medium have been included. These additional peaks would appear in Figures 5.10 (B) (no peaks would be transferred to Figure 5.12 (B)) and are shown in Table 5.4 by means of an \otimes .

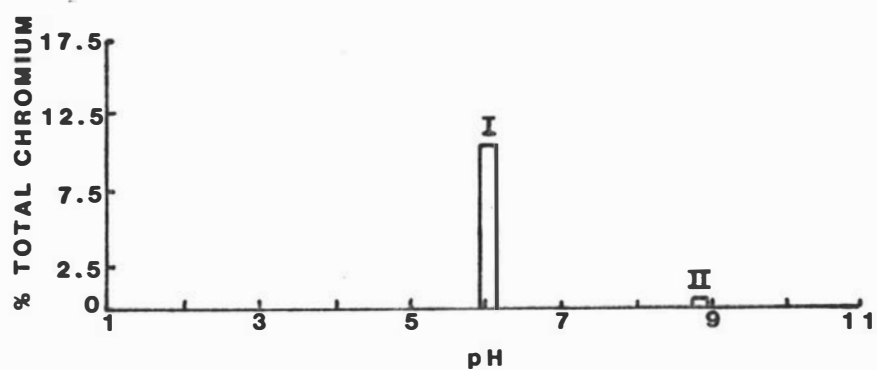
FIGURE 5.8 A, B, C, D.

The separation of starting source solutions into crude fractions I and II, containing cationic and amphoteric material by using Dowex 50W-X12 resin. A (yeast extract), B(supplemented medium), C (spent supplemented medium), D (extracted yeast). The percentage of total chromium in each peak shown by ().

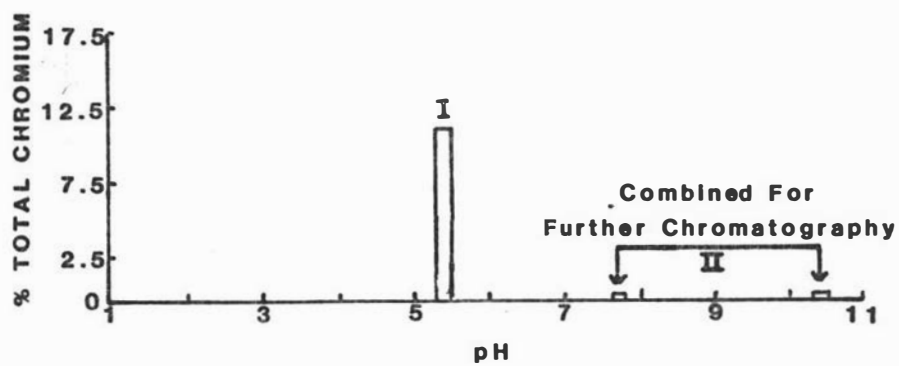
FIGURE 5.8



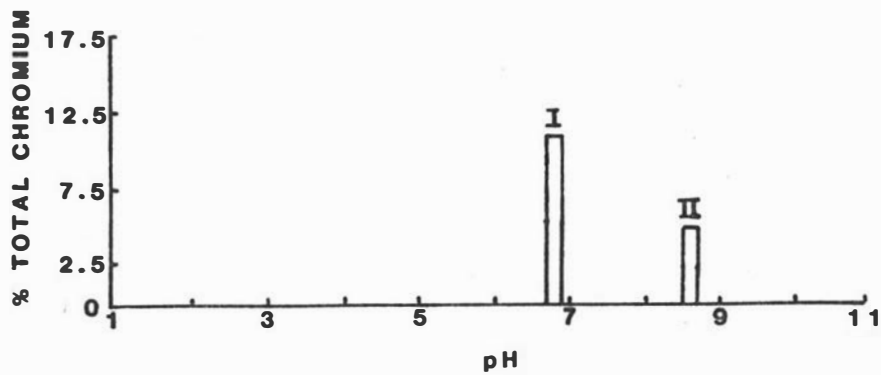
A



B



C



D

FIGURE 5.9 A, B, C, D.



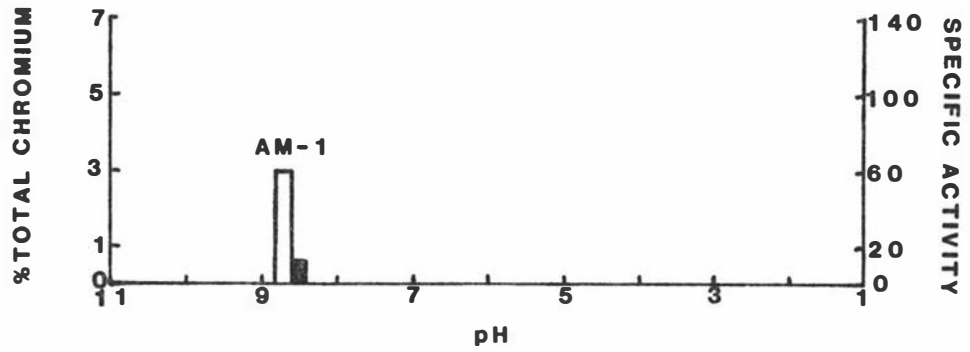
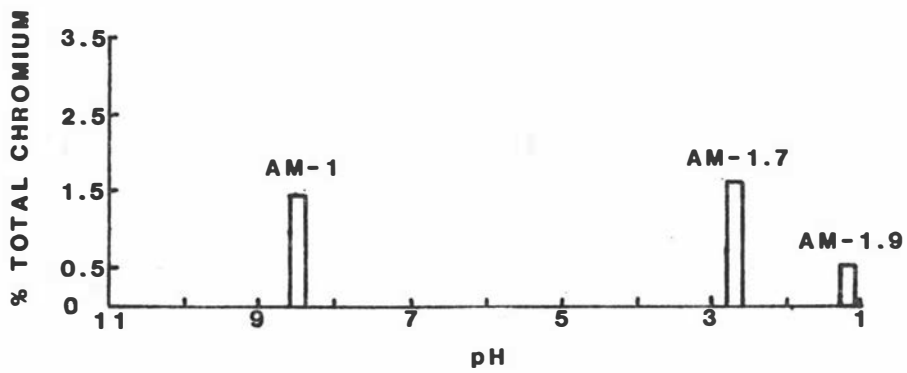
The isolation of amphoteric fractions arising from the further fractionation of crude fraction I (Figure 5.8) by using Dowex 1-X8 resin. A (yeast extract), B (supplemented medium), C (spent supplemented medium), D (extracted yeast). . The percentage of total chromium in each peak shown by () and the specific activity shown by ().

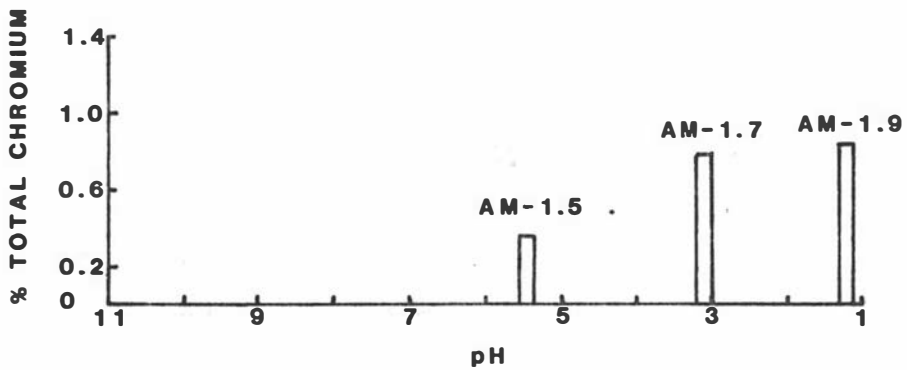
FIGURE 5.9



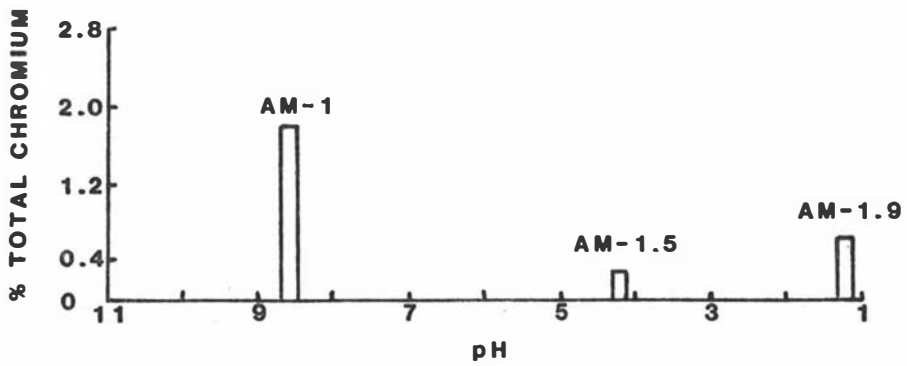
A



B



C



D

FIGURE 5.10 A, B, C, D.



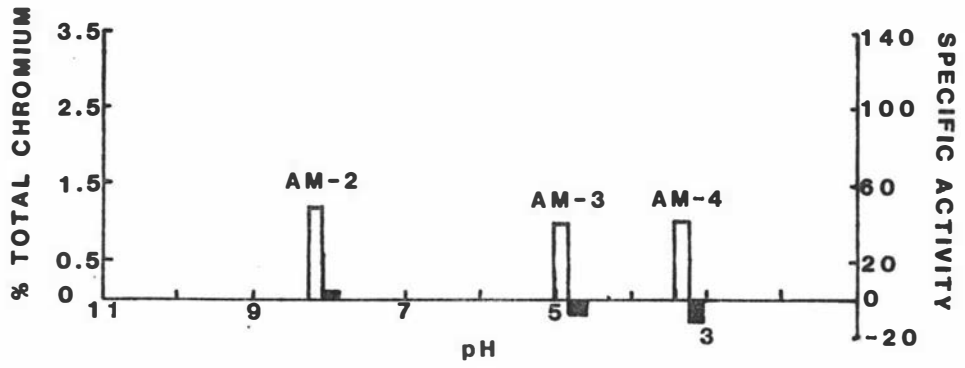
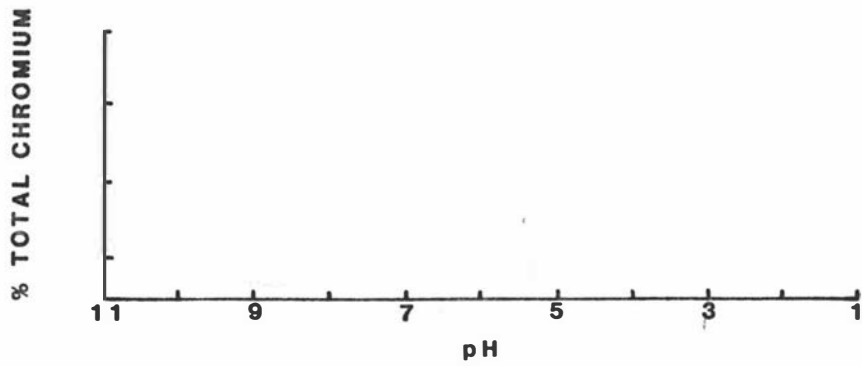
The isolation of amphoteric fractions arising from the further fractionation of crude fraction II (Figure 5.8) by using Dowex 1-X8 resin. A (yeast extract), B (supplemented medium), C (spent supplemented medium), D (extracted yeast). The percentage of total chromium in each peak shown by () and the specific activity shown by ().

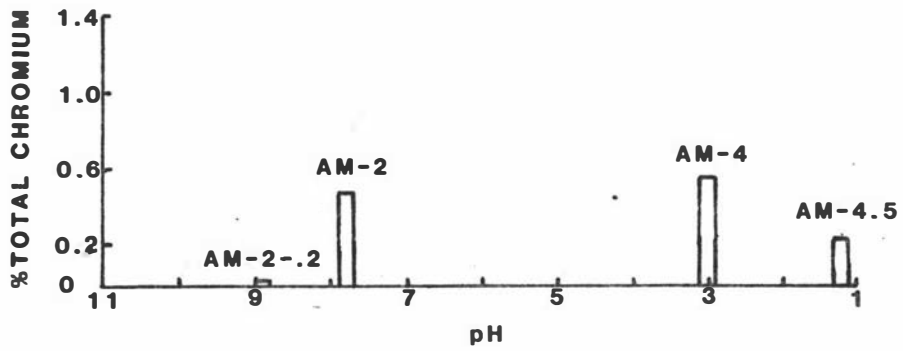
FIGURE 5.10



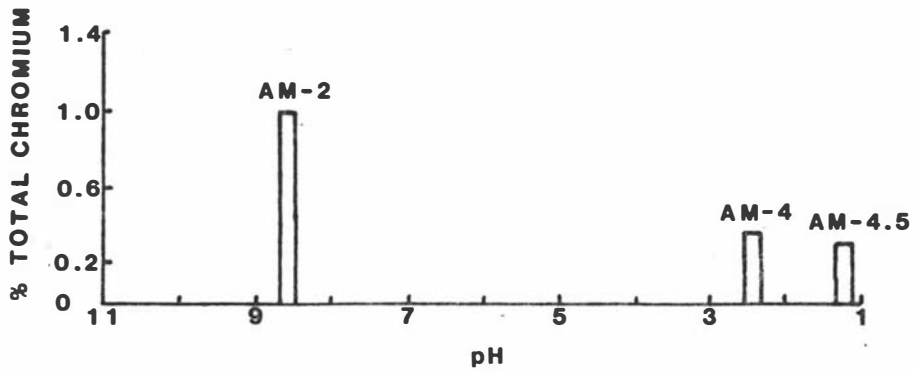
A



B



C



D

FIGURE 5.11 A, B, C, D.



The isolation of cationic fractions arising from the further fractionation of crude fraction I (Figure 5.8) by using Dowex 50W-X12 resin. A (yeast extract) B (supplemented medium), C (spent supplemented medium) D (extracted yeast). The percentage of the total chromium in each peak shown by () and the specific activity shown by ().

FIGURE 5.11

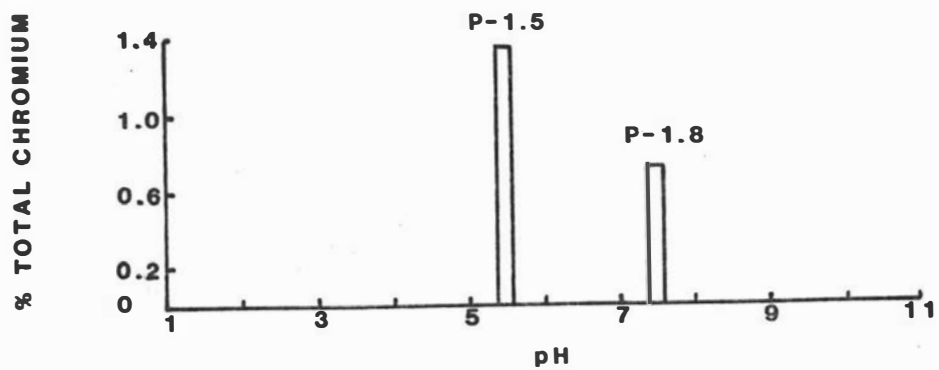
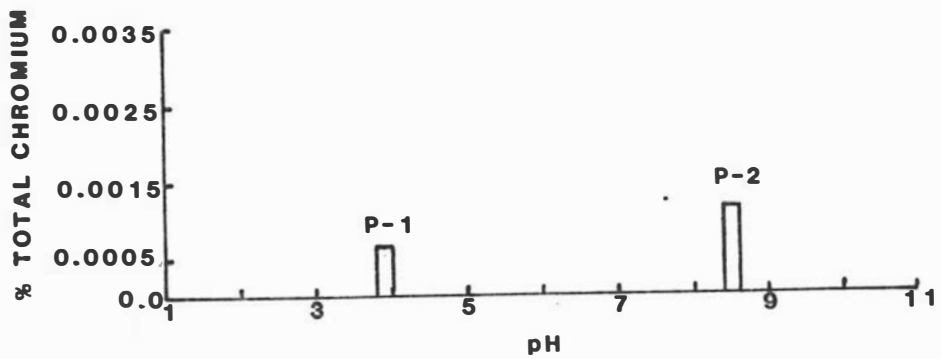
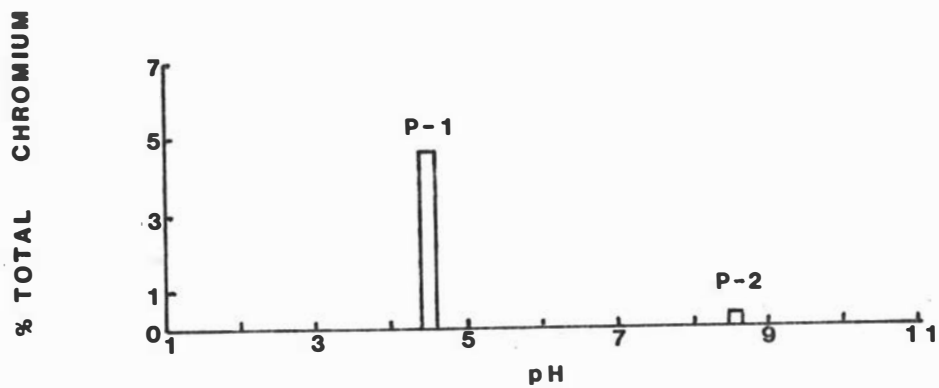
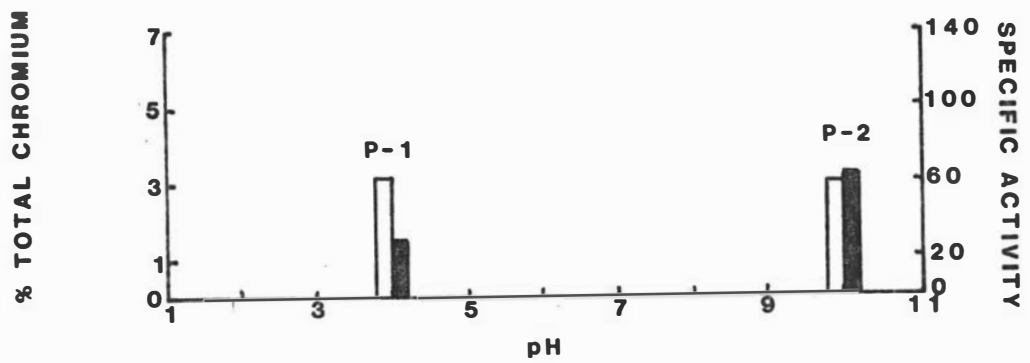


FIGURE 5.12 A, B, C, D.



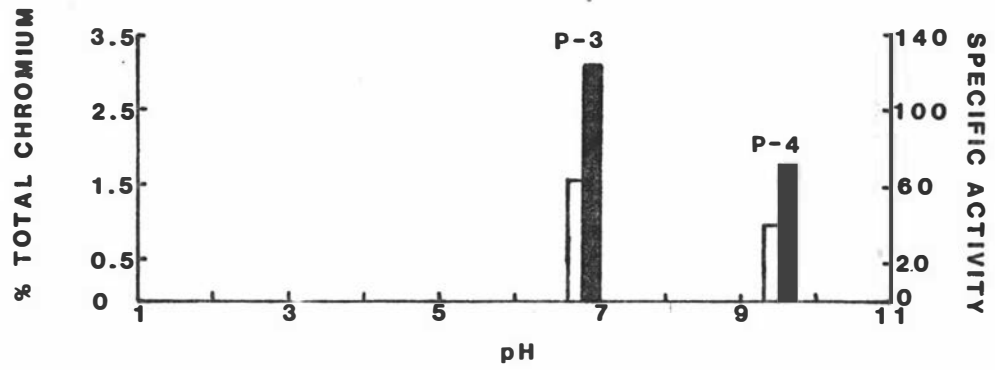
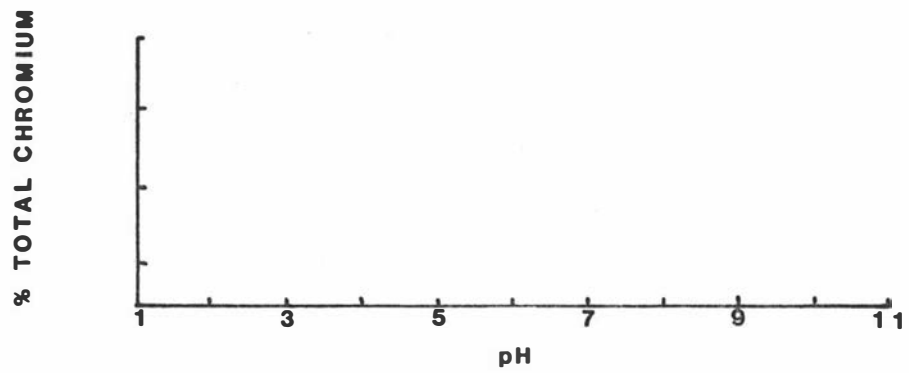
The isolation of cationic fractions arising from the further fractionation of crude fraction II (Figure 5.8) by using Dowex 50W-X12 resin. A (yeast extract), B (supplemented medium), C (spent supplemented medium) D (extracted yeast). The percentage of the total chromium in each peak shown by () and the specific activity shown by ().

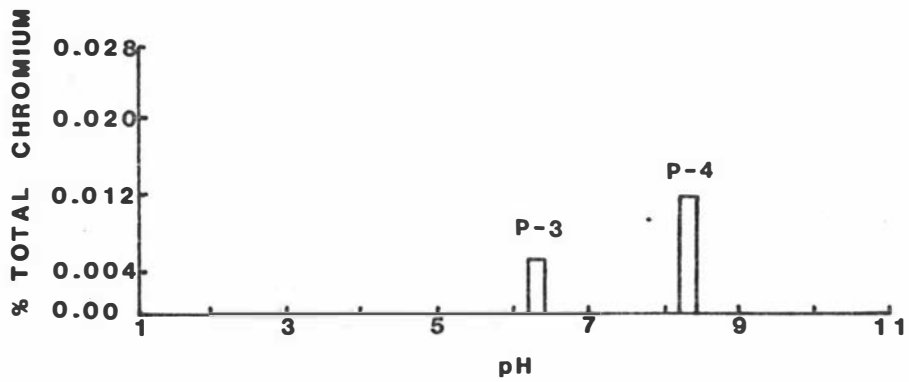
FIGURE 5.12



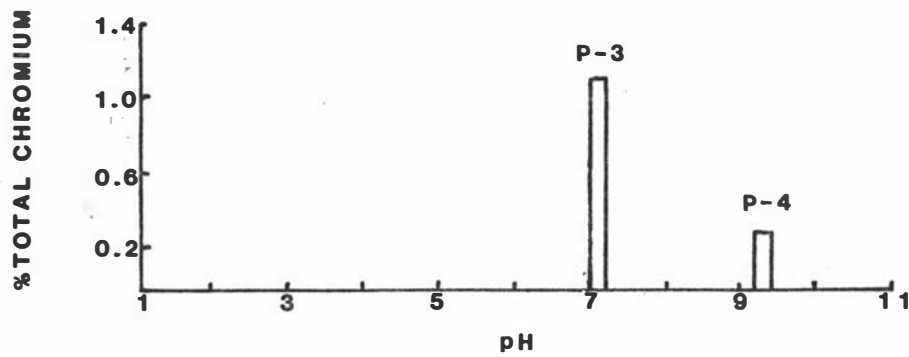
A



B



C



D

FIGURE 5.13 A, B, C, D.



The isolation of anionic fractions arising from the fractionation of the effluent from Column I by using Dowex 1.X8 resin. A (yeast extract), B (supplemented medium), C (spent supplemented medium), D (extracted yeast). The percentage of the total chromium in each peak shown by () and the specific activity shown by ().

FIGURE 5.13

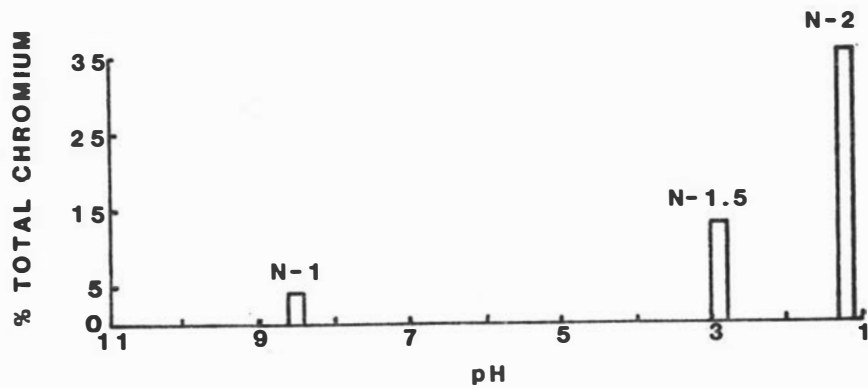
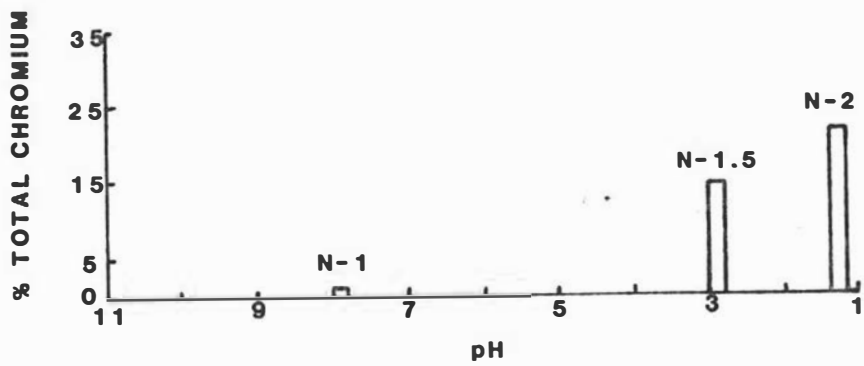
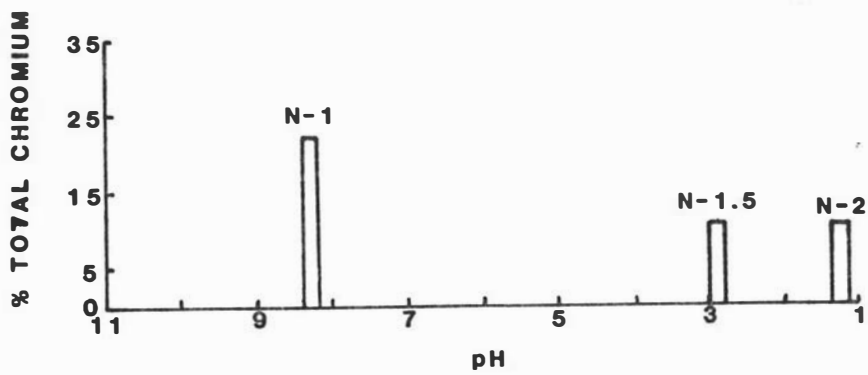
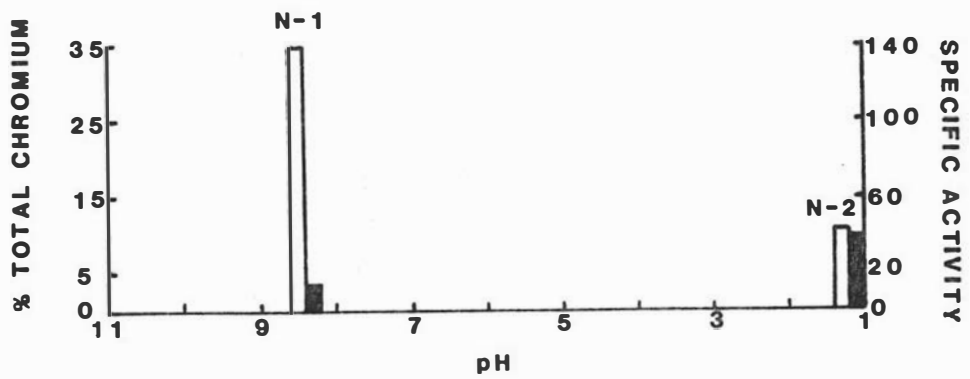


TABLE 5.4 FRACTIONS ISOLATED FROM CHROMIUM-51-SUPPLEMENTED MEDIA AND YEAST

Peak Description	Isolation Source				Graphical Representation (Figure)
	Yeast extract	Supplemented medium	Supplemented spent medium	Supplemented extracted yeast	
AM-1	X	X		X	5.9
AM-1.5			X	X	5.9
AM-1.7		X	X		5.9
AM-1.9		X	X	X	5.9
AM-2-.2			X		5.10
AM-2	X		X	X	5.10
AM-3	X	⊗			5.10
AM-4	X	⊗	X	X	5.10
AM-4.5			X	X	5.10
N-1	X	X	X	X	5.13
N-1.5		X	X	X	5.13
N-2	X	X	X	X	5.13
P-1	X	X	X		5.11
P-1.5				X	5.11
P-1.8				X	5.11
P-2	X	X	X		5.11
P-3	X		X		5.12
P-4	X		X		5.12

5.3.3 Activity.

Activities measured for the chromium peaks obtained from the chromium chloride supplementations are listed and compared with those obtained from the yeast extract, in Table 5.5. Graphical representation is also made in Figures 5.2, 5.3, 5.4, 5.5, 5.6 and 5.7. Some chromium chloride-supplementation peaks could not be measured because of either a low chromium level or a very high conductivity level arising from the very strong acid wash used as the final Dowex 1-X8 eluent. As discussed in section 3, both these conditions lead to invalid assay results. Assays could not be undertaken for chromium-51-supplemented systems since the molar amounts for chromium were far too low.

5.4 DISCUSSION.

5.4.1 Chromium chloride supplemented systems.

In the chromium chloride supplementation experiments for the isolations carried out on the two media solutions and on the extracted yeast solution, elution from column VI (Figure 5.7) gave three anionic fractions. At least some of these peaks must be produced by reaction of chromium with the medium and one of these presumably corresponds to the chromium-glucose complex which is discussed in section 2. For the spent supplemented medium (C) the complexes N-1.5 and N-1.8 do not align themselves exactly with the peaks from the supplemented medium (B) or from the extracted yeast (D), presumably because of inter-column variations. These anionic complexes (N-1, N-1.5, N-1.8) cannot have any essential metabolic function in yeast and are therefore categorised as Group I fractions.

The percentage of the total chromium isolated in each peak is represented by the peak heights drawn in Figure 5.2 to 5.7. As the total amount of chromium eluted from column VI varied markedly, with for example the yield from the spent supplemented medium (C) being only 15% of the chromium loaded compared to a more normal yield of 80%, it was difficult to make direct comparisons of peak heights between the different systems (Figure 5.7 A-D). This phenomenon was common to all isolations and not just for the anionic fractions. However in agreement with the findings in section 2, the proportion of anionic chromium eluted from column VI is high in relation

TABLE 5.5 SPECIFIC ACTIVITY OF FRACTIONS ISOLATED FROM CHROMIUM CHLORIDE SUPPLEMENTATIONS

Peak Description	Isolation Source				Graphical Representation (Figure)
	Yeast extract	Supplemented media	Supplemented spent media	Extracted yeast	
AM-1	+ 12.2	- 8.8	- 4.5	+ 4.8	5.3
AM-1.5		- 6.1	-12.3	+ 4.2	5.3
AM-1.9		#	+ 2.8	0.0	5.3
AM-2	+ 6.1	- 6.0	-10.4	- 1.1	5.4
AM-3	-11.8				5.4
AM-4	-13.1	- 1.1	- 9.8	+ 2.2	5.4
AM-4.5			#	+ 1.9	5.4
N-1	+ 8.0, \ddagger + 20 \ddagger	+ 0.9		- 8.2	5.7
N-1.5		- 2.0	+ 3.0	- 5.5	5.7
N-1.8			- 1.8		5.7
N-2	+42.8	#	- 4.8	- 1.6	5.7
P-1	-28.7				5.5
P-1.5			*		
P-2	+62.5				5.5
P-3-.3			+36.4		5.6
P-3	+126.1		+36.4	+82.0	5.6
P-4	+71.3				5.6

N.B. \ddagger value for N-1A, \ddagger value for N-1B

assay not undertaken because of the high acid content of the sample.

* assay not undertaken because of the low chromium concentration.

to the amounts of amphoteric and cationic chromium isolated in each of the three chromium chloride supplementations.

Two anionic peaks were observed for the yeast extract (A) and three for each of the chromium chloride-supplemented isolations (B,C and D). On gel filtration chromatography, the fraction N-1 for yeast extract separated into two peaks that were labelled N-1A and N-1B (section 4.3.4). It is possible that these peaks were separated by the improved ion-exchange chromatography elution in the supplementation experiments and are labelled as N-1 and N-1.5 using the nomenclature defined in this section. However no gel filtration experiments were carried out to confirm that these peaks were equivalent.

Numerous amphoteric peaks were obtained from each chromium chloride-supplemented system (Figures 5.3 and 5.4), although collectively they made up only about 10% of the total chromium. It is clear from a comparison of Figure 5.3 (B-D) and Figure 5.4 (B-D) that, with the exception of AM-4.5 all the amphoteric chromium peaks are artefacts resulting from reaction of chromium with the medium constituents (or arising from elution of the ion-exchange columns). The fact that these amphoteric fractions are found in the supplemented extracted yeast shows that they are readily taken up by the yeast cells during yeast growth. Thus these fractions can be classified as Group I complexes. When these peaks (AM-1, AM-1.5, AM-1.9, AM-2 and AM-4) are compared with the fractions isolated from commercial yeast extract (Figures 5.3 (A) and 5.4 (A)) an exact correspondence is not obvious. The reason for this is not clear but differences in the yeast growth conditions or in column parameters might be partly responsible. It could be that the supplemented peaks labelled AM-4 and AM-4.5 are in fact the same as the yeast extract fractions AM-3 and AM-4 (as suggested in Table 5.3). Whatever the reason for this apparent discrepancy, these amphoteric fractions for the yeast extract can still be classified as Group I complexes since none show any biological activity in the yeast fermentation assay.

Examination of the results from column IV and V for the elution of cationic fractions (shown in Figures 5.5 and

5.6) reveals that no cationic complexes are present in the supplemented medium and hence all cationic fractions from the supplemented systems can be labelled as Group II. Several cationic complexes (P-1.5, P-3-.3 and P-3) were isolated from the spent supplemented medium (Figure 5.5 (C) and 5.6 (C)) while only P-3 is found in the supplemented extracted yeast (Figure 5.6 (D)). Of the four fractions which were isolated from the commercial yeast extract in section 4 (Figure 5.5 (A) and 5.6 (A)), possibly all may be considered to originate from contact with the yeast and do not appear as medium artefacts.

A possible explanation for the P-1.5 and P-3-.3 peaks being found in the spent supplemented medium but not in the supplemented extracted yeast could be their low equilibrium concentrations within the yeast cell, thus not being detectable in the supplemented extracted yeast. However if they were easily diffusible through the cell wall, relatively high levels could accumulate in the spent-supplemented medium. Alternatively they might be formed outside the yeast cell with ligands excreted as waste from the yeast cell, but not present in the unused medium. P-3 must be either synthesised by the yeast and excreted into the spent supplemented medium or produced by reaction with excreted cell metabolites and taken up by the cell by diffusion. The cationic peaks P-1, P-2 and P-4 (Figure 5.5 (A) and 5.6 (A)) which were additional to the cationic peak, P-3, found in the supplemented-extracted yeast (Figure 5.6 (D)) could have been present as a result of the process of autolysis which could have altered the chromium fractions originally present in the yeast and in this case artificially increased the number of cationic fractions.

The overall amount of chromium eluted in the cationic fractions was low with a maximum of around 10% of the total chromium occurring in the yeast extract isolation (Figures 5.5 and 5.6 (A)).

The encouraging feature of the isolation of the cationic fractions was that P-3 was present in all isolations from yeast associated supplementations and in fact was the only peak retained by the yeast in the supplemented yeast extract. Thus P-3 at least can be classified as a Group II

complex.

5.4.2 Chromium-51-supplemented systems.

While the vastly different molar amounts of chromium in the chromium chloride and the chromium-51-supplemented systems might have been expected to cause quite different results, Table 5.3 and 5.4 show that there are still some areas of close agreement on comparison of similar column elutions.

This agreement is apparent in the elution of the anionic fractions shown in Figure 5.13 where each supplemented system (B-D) has three anionic peaks and consequently all can be classified as Group I. Again the variation in the percentage recovery of chromium of individual columns does not allow a detailed analysis of the proportion of the different chromium complexes. Generally though, the anionic peaks eluted occupied the major fraction of the total chromium.

Using the system for classifying fractions that was adopted for the chromium chloride supplementations the amphoteric peaks that are shown in Figures 5.9 and 5.10 that can be classified as Group I peaks are AM-1, AM-1.7, AM-1.9, AM-3 and AM-4 (assuming that peak II from the supplemented medium (Figure 5.8 (B)) realises both an AM-3 and an AM-4 peak labelled as \otimes on Table 5.4). The only peaks which can be classified as Group II were AM-2-.2 and AM-2.0 from the spent supplemented medium (Figure 5.10 (C)) and AM-2 from the extracted yeast (Figure 5.10 (D)).

The proportion of the total chromium-51 present in the amphoteric complexes was low. In all three chromium-51 supplemented isolations the combined chromium in all the amphoteric fractions accounted for less than 5% of the total. This fact and the wide variety of amphoteric fractions observed, suggests that most if not all of the chromium complexes are present through a random association with available ligand material.

The most noticeable feature of the cationic complex isolations shown in Figures 5.11 and 5.12 was the appearance of 2 peaks, P-1 and P-2 in the supplemented medium isolation (Figure 5.11 (B)), the first of which contained over 4% of

the total chromium. At this point there seems to be no obvious explanation for such a large peak, but their presence in the supplemented medium suggests that they too could be medium artefacts. The presence of these two cationic peaks in the supplemented medium complicated the previous proposal based on information from the chromium chloride supplementation that all cationic peaks could be classified as Group II. Now, however, allowing for the peak shifts marked with an arrow (\uparrow) in Table 5.4, both P-1 and P-2 were classified as Group I, while P-3 and possibly P-4 could still be classified as Group II.

As a proportion of the total chromium, cationic peaks generally accounted for a low percentage, with the maximum being ca 5% in the extracted yeast and supplemented medium. Very low amounts (ca 0.02%) were obtained from the supplemented spent medium and this may have indicated that the complexes here were the result of chromium complexing randomly with material excreted from the yeast cell. Comparison of these positive peaks with those isolated from the yeast extract showed reasonable positional agreement.

5.4.3 Activity.

All the anionic peaks isolated from the chromium chloride supplemented isolations were able to be assayed for activity, and these showed a range of percentage enhancements from -8.2 to +3.0 and were clearly inactive.

Only two of the amphoteric complexes AM-1.9 from the supplemented medium (Figure 5.3 (B)) and AM-4.5 from the spent supplemented medium (Figure 5.4 (C)) could not be assayed. The remainder of the complexes showed a range of activities from -12.3 to +4.8 and again were clearly inactive.

On analysis of the three cationic complexes which could be assayed, P-3-.3 and P-3 from the spent supplemented medium (Figure 5.6 (C)) and P-3 from the extracted yeast (Figure 5.6 (D)), a clear distinction from the amphoteric and anionic fractions was apparent. The specific activities ranged from +36.4 to +82.0 and consequently the three complexes all showed significant activity. The specific activity of P-1.5 from the spent supplemented medium could not be

determined because of the very low chromium level.

Activities of the complexes isolated from yeast extract when compared with those from the chromium chloride supplementations generally covered a wider range of activities. Because of the complete solubilization of the yeast extract before isolation was begun, it was likely that the concentration of soluble impurities capable of causing activity would have been higher than for an extracted yeast as in the chromium chloride supplementations. Consequently factors affecting the assay, either causing greater activity or lower activity were likely to be more predominant and hence have a larger effect in the yeast extract system.

5.5 SUMMARY AND ASSESSMENT OF EARLIER WORK

In summarising the range of chromium complexes isolated from the various sources, the following criteria were used to distinguish a yeast-produced, biologically active chromium complex(es) likely to be GTF, from other spurious complexes.

1. The complex must not have been produced by reaction of chromium with medium components or elution reagents. (Group I classification).

2. The complex must have been retained by the yeast on the assumption that in order to have some metabolic function it must be necessary to have the complex associated with the yeast cell. This criterion eliminated all complexes formed by random reaction with ligand material excreted from the yeast cell, or complexes formed within the yeast cell with such material and then excreted.

3. The complex must have shown activity in the standard assay. For the complex to have metabolic activity it must have been able to increase the metabolic function of the yeast, as indicated by an enhanced CO_2 production rate.

All anionic complexes have been classified as Group I. This classification was borne out by the lack of activity shown by any of the negative complexes and thus none of these complexes would be regarded as a GTF-like complex.

Of all the wide range of amphoteric chromium peaks, only AM-2 from the chromium chloride supplementation and AM-2-.2 and AM-2 from the chromium-51 supplementation were

classified as Group II. Both of these peaks do not meet the further requirements of a "GTF-like" complex in that AM-2-.2 was not retained by the yeast and was present only in the spent supplemented medium while AM-2 showed no activity from any of the isolations assayed.

Of the positive peaks, only P-3 met all three requirements for a "GTF-like" complex. The greatest activity seemed to occur in the complexes eluted from the Dowex 50W-X12 column VI, where both P-3 and P-4 were eluted from the yeast extract isolation, or similarly from the spent supplemented medium, P-3-.3 and P-3 (Figure 5.6).

In summary then the only peak to meet all the requirements for a "GTF-like" complex is P-3. If the second criterion of the complex being retained by the yeast was disregarded then P-4 could also be a complex with "GTF-like" activity. As both are eluted off the same Dowex 50W-X12 column (V), in further purification attempts both P-3 and P-4 were examined.

Of the different isolation procedures described to date only Kumpulainen et al (1978) have attempted to show the origin of their eluted chromium complexes. Growing yeast on a chromium-51-containing medium enabled these workers to isolate chromium complexes from three sources; sterile medium, medium after yeast centrifugation and yeast. To this point their work was similar to that described in this chapter. The basic difference in Kumpulainen's work compared with that described here, was in the use of only a single cation and anion-exchange column. The resins Dowex 50W-X8 and Sephadex QAE A-25, were used to fractionate their extracts from the different starting sources. Apart from their separation procedure which would bind both amphoteric and cationic-chromium on the same column, the eluents used were unsuitable for the reasons discussed earlier in section 4.4.

In agreement with the work described in this section, Kumpulainen has shown both cationic and anionic, and possibly amphoteric complexes existing in all three sources. A detailed comparison of the complexes described by Kumpulainen et al and those described in this chapter is not possible

because of the vastly different elution systems. Kumpulainen et al, using chromium-51 were not able to measure activity by any of the standard methods, but did attempt to show binding to insulin, though unsuccessfully. Their work is important in that in combination with the work described here, it is apparent that in yeast a moderate number of differently charged chromium complexes are present, some of which are products of chromium reacting with reagents in the growth medium.

SECTION 6

A SHORT PROCEDURE FOR THE ISOLATION OF CATIONIC FRACTIONS -APPLICATION TO BIOLOGICAL SAMPLES.

6.1 INTRODUCTION

As the most active fractions found from the isolation procedures described in sections 4 and 5 were all cationic, the remaining negative and amphoteric fractions were considered to be not relevant to the ultimate aim of isolating GTF. This conclusion is in agreement with the evidence published by several researchers that GTF is a cationic complex (Toepfer et al (1977) and Mirsky et al (1980)).

In order to isolate only the cationic complexes from the yeast extract it is not necessary to use the six column isolation procedure described in section 4. Instead at pH 8.5 all the amphoteric and anionic complexes will bind to an anion-exchange column and the cationic material in the effluent from this column will bind to a cation-exchange column. In section 4 two cation-exchange columns were used to elute four positive complexes but with the better elution procedures devised in section 5 it was hoped that the four separate cationic peaks could be resolved using a single cation-exchange column.

In principle such a "short isolation" procedure should allow a comparison to be made of the number and type of cationic fractions which could be isolated from the Merck high-chromium yeast extract and the Merck low-chromium yeast extract. It was hoped that this information would provide an insight into the way the presence of the different cationic chromium complexes in the yeast is affected by the levels of chromium available to the yeast.

When chromium was first proposed as a likely nutritional requirement, various workers published the chromium content of a variety of food-stuffs (Schroeder et al (1970) and Guthrie (1975)) and more recently, biological activity due to chromium-containing fractions extracted from various food-stuffs has been published by Toepfer et al (1973) and

Kumpulainen et al (1979). Precise analysis of chromium levels has proven difficult with different results being obtained from the analysis of identical samples by different methods (Alvarez (1979) and Parr (1977)). Greater reliability can probably be placed on the relative levels obtained in any one laboratory using the same analytical technique.

It is the aim here to select various samples which have been reported to have either a high chromium level or a high biological activity and subject these samples to an extraction procedure and determine the amounts of the cationic chromium-containing fractions by the short isolation procedure. These samples, if sufficient levels of chromium could be obtained, would then be assayed for activity. The samples chosen were; the two yeast extracts Merck high-chromium yeast extract and Merck low-chromium yeast extract, sage, black peppercorns, molasses, wheat bran, pork kidney powder and split green peas, of these samples, those that were reported to have high levels of chromium were; sage (Guthrie (1975)) molasses (Schroeder et al (1970)) and green peas (Schroeder et al (1970)). While containing relatively low levels of chromium, wheat bran (Roginsky et al (1971)) and pork kidney powder (Schwarz and Mertz (1957)) were reported to be good sources of GTF activity. Black peppercorns (Toepfer et al (1973)) and yeast (Mirsky et al (1980)) were reported to have high chromium levels and to be high in GTF activity.

It is of considerable interest and importance to ascertain whether the factor responsible for the reported biological activity is the same for all of these sources and identical with the cationic material isolated from the various brewer's yeast sources (sections 4 and 5). In particular the isolation of cationic chromium containing fractions from molasses (a product on which much of the commercially available yeast is grown) would necessitate reappraisal of the idea of a metabolically significant role for chromium complexes in glucose metabolism in yeast.

6.2 METHODS AND MATERIALS

6.2.1 Short isolation procedure for cationic fractions.

The ion exchange resins used were Dowex 1-X8 anion-

exchange resin and Dowex 50W-X12 cation-exchange resin. These were prepared as described in section 4. The aqueous extraction of chromium complexes from a 1:1 butanol-water system was omitted as this was previously shown to give only a very small purification.

Merck yeast extract (100g) was dissolved in ca 8 litres of distilled water and titrated to pH 8.5 with NaOH. Small adjustments were made to the volume to reduce the conductivity to 1000 μmho . This solution was loaded at a maximum speed of 4 cm^3/min onto a Dowex 1-X8 anion-exchange column with volume 250 cm^3 (4.8 cm diameter x 14 cm). The effluent from this column was then titrated to pH 4.0 and the volume adjusted with distilled water so that the conductivity was lowered to 1000 μmho . This sample was then loaded onto a Dowex 50W-X12 cation-exchange column (4.8 cm x 14 cm) again with a maximum speed of 4 cm^3/min . The alterations in the loading conditions of the columns from those used in section 4 consisted of a lower conductivity to ensure complete binding of the chromium complexes and a higher loading pH of the cation-exchange column to prevent precipitation of the low-chromium yeast extract which occurred at the previously used pH of 3.5. Only the cation-exchange column was eluted and the successive eluents used were:

- (i) NaH_2PO_4 (0.6M) until the pH of the effluent reached 4.3. The volume of this elution varied but was usually ca 1.7 litres.
- (ii) A gradient NaH_2PO_4 (0.6M, 200 cm^3) v Na_2HPO_4 (0.2M, 200 cm^3).
- (iii) A gradient Na_2HPO_4 (0.2M, 150 cm^3) v Na_3PO_4 (0.05 M, 150 cm^3).
- (iv) Na_3PO_4 (0.05M, 150 cm^3).

Fractions were collected and analysed for chromium, pH and conductivity as described in section 2. Activity and optical density at 280 nm were determined for the chromium peaks eluted.

6.2.2 Determination of chromium in biological materials.

Each of the selected biological samples, namely high-chromium yeast extract, low-chromium yeast extract, sage, black peppercorns, molasses, wheat bran, pork kidney powder and split green peas, was analysed by the wet ashing procedure described in section 2.2.2b

6.2.3 Methods of extraction.

As many of the biological samples had quite different characteristics, a variety of methods were used for cell cleavage or homogenisation of the material before aqueous extraction could proceed. All methods were chosen to be as mild as possible so as to minimise any changes in the naturally occurring complexes. Chromium analysis was usually undertaken on the aqueous extract to determine the percentage of the chromium that had been extracted. The methods adopted were as follows:

(i) Merck high-chromium yeast extract and low-chromium yeast extract. No homogenisation or cell cleavage was required as the two yeast extracts were readily soluble and were dissolved straight into distilled water.

(ii) Sage. The dried sage (20 g) was heated on a water bath in 250 cm³ of distilled water for 3 hours at 70°C. This mixture was then homogenised hot, in a Waring Blender at maximum speed for 5 minutes and then allowed to stand at 4°C for 3 days. The aqueous extract was then removed from the remaining solid material by centrifugation for 10 minutes at 4000 g.

(iii) Black peppercorns. The whole dried black peppercorns were initially ruptured by grinding with a mortar and pestle. They were then heated for 3 hours at 70°C in 250 cm³ of distilled water. The mixture was homogenised in a Waring Blender at maximum speed for 5 minutes while still hot and then after a further 250 cm³ of distilled water had been added, allowed to stand overnight at 4°C. The aqueous extract was obtained by removing the remaining solid material by centrifugation for 10 minutes at 4000 g.

(iv) Molasses. The molasses used was readily soluble in water and no prior treatment was required.

(v) Wheat bran. A method similar to that used for the dried sage was used in preparing an aqueous extract of wheat bran (100g). The wheat bran was heated in one litre of distilled water, homogenised and left to stand before the remaining solid material was removed by centrifugation.

(vi) Pork kidney powder. Pork kidney powder (18.8g) was stirred for 18 hours at 4° C in distilled water (200 cm^3). The sample was allowed to stand for 48 hours before being homogenised in a Waring Blender at maximum speed for 5 minutes. This treatment successfully dispersed coagulated material. The sample was again allowed to stand at 4° C for 18 hours before centrifugation at 4000g to remove the solid material. A concentrated sample of the aqueous extract was analysed for chromium.

(vii) Dried split peas. No extraction was attempted on split peas as the local variety obtained, contained insufficient chromium to make a successful extraction possible.

6.2.4 Isolation and activity of cationic complexes from biological materials.

The aqueous extracts obtained from the biological materials were subjected to the column procedure described in section 6.2.1. As sample size varied considerably, column sizes were altered accordingly as were the volumes of the eluents used. Chromium analysis, pH and conductivity measurements were carried out for each column as were activity and optical density measurements at 280 nm on the peak chromium tubes. All methods were as described in sections 2 and 3.

6.3 RESULTS

6.3.1 Short isolation procedure for cationic fractions.

The separation achieved for high-chromium yeast extract in the short isolation procedure revealed four chromium peaks as shown in Figure 6.1. Assuming a similar order of pH elution for the yeast extract as achieved in section 4, the four peaks can be labelled under the nomenclature used there from left to right in Figure 6.1 as P-1, P-3, P-2 and P-4. The pH and conductivity at which the peaks were eluted from the two column systems along with the levels of chromium obtained are given in Table 6.1.

FIGURE 6.1

The elution profile of cationic chromium containing fractions (————) from a Dowex 50W-X12 column eluted as described in methods. The fraction volume was 10 cm³. Conductivity (-----) pH (.....).

FIGURE 6.1

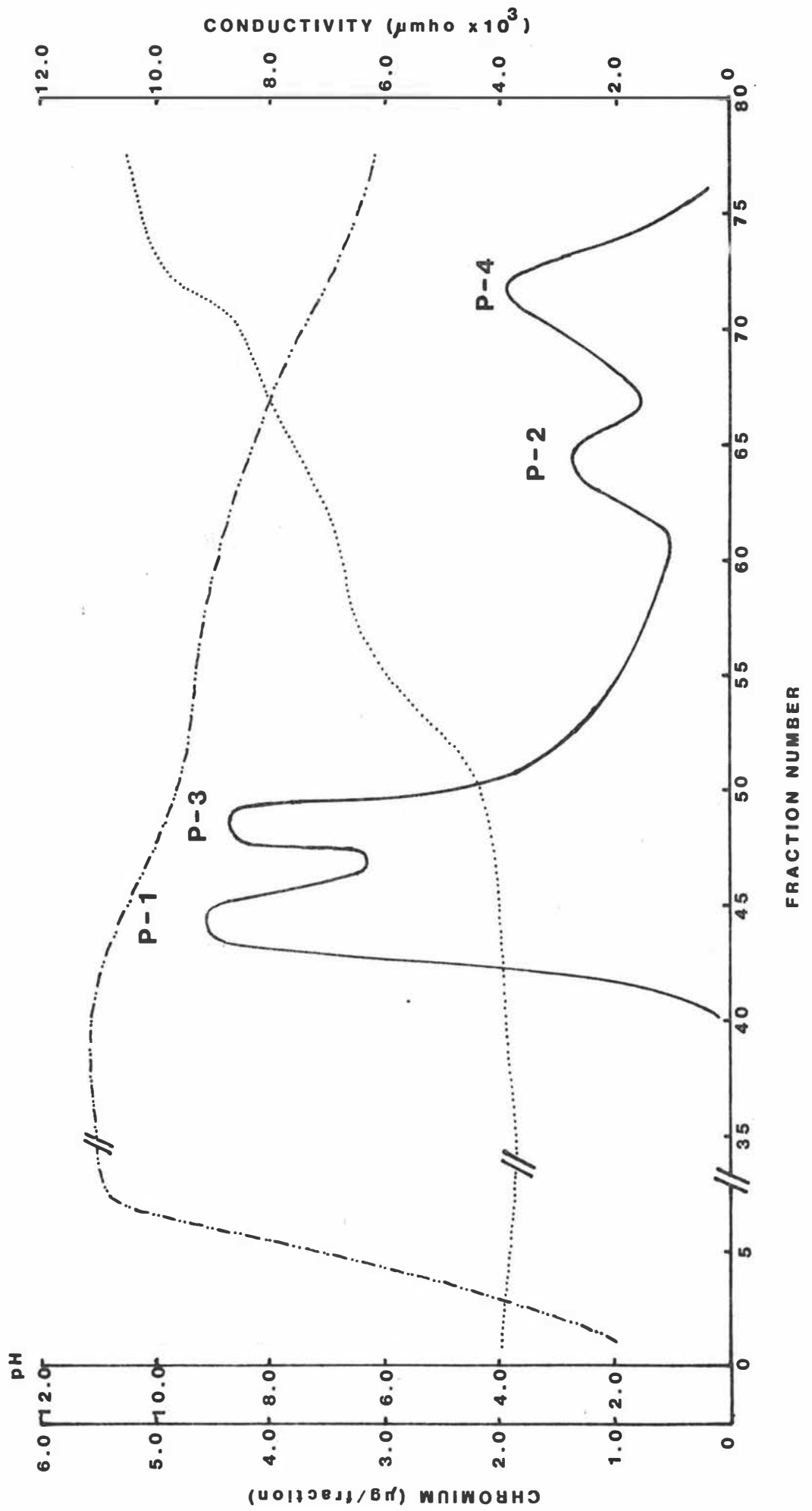


TABLE 6.1 COMPARISON OF THE ELUTION pH AND CONDUCTIVITY FOR THE CATIONIC FRACTIONS ISOLATED BY THE SIX COLUMN PROCEDURE (SECTION 4) AND THE SHORT ISOLATION PROCEDURE (SECTION 6).

<u>Cationic Fraction</u>	<u>Elution pH</u>	<u>Elution Conductivity ($\mu\text{mho} \times 10^3$)</u>	<u>Chromium (μg)</u>	<u>Elution Procedure (section)</u>
P-1	4.50	3.0	33	4
P-2	10.00	5.2	32	4
P-3	7.00	4.0	20.5	4
P-4	10.00	5.0	9	4
			Total	94.5
P-1	3.95	10.6	39	6
P-2	7.50	9.8	12	6
P-3	4.20	8.3	34	6
P-4	9.50	7.0	20	6
			Total	105

As the results reported in section 4 were for a 200g yeast extract preparation and the peak totals reported in Table 6.1 are for a 100g preparation the original values reported in section 4 have been halved.

6.3.2 Chromium levels in biological materials.

Chromium levels obtained by the wet ashing procedure from the various biological materials are shown in Table 6.2. Where possible, values reported by other workers (Toepfer et al (1973); Shroeder et al (1970) and Guthrie (1975)) are included for comparison.

TABLE 6.2 CHROMIUM LEVELS IN BIOLOGICAL SAMPLES

<u>Sample</u>	<u>Chromium ($\mu\text{g/g}$)</u>	<u>Toepfer et al ($\mu\text{g/g}$)</u>	<u>Schroeder et al ($\mu\text{g/g}$)</u>	<u>Guthrie ($\mu\text{g/g}$)</u>
Merck high-chromium yeast extract	11.2	-	-	-
Merck low-chromium yeast extract	1.15	1.17*	-	0.87 [‡]
Sage	2.0	-	-	4.01
Black peppercorns	0.84	0.38	-	3.91
Molasses	0.27	-	0.47#	-
Wheat bran	0.14	0.42	0.07	0.19
Pork kidney powder	0.11	-	-	-
Split green peas	<0.04	-	3.62	0.18

N.B. * Value refers to dried whole yeast

‡ Value refers to 'Marmite' yeast extract

Value refers to the maximum of a series of molasses samples analysed.

Other than the dried split peas all samples were considered to have sufficient chromium to warrant continuation with aqueous extraction.

6.3.3 Aqueous extractable chromium in biological samples.

The extraction procedures described in methods enabled the following amounts of chromium to be extracted into an aqueous solution (Table 6.3).

TABLE 6.3
 AQUEOUS EXTRACTABLE CHROMIUM IN BIOLOGICAL SAMPLES

<u>Sample</u>	<u>Chromium concentration</u> µg/g	<u>Weight of sample used</u> g	<u>Total chromium</u> µg	<u>Chromium extracted</u> µg	<u>Percentage extraction</u>
Merck high-chromium yeast extract	11.2	40.0	448	448	100%
Merck low-chromium yeast extract	1.15	42.0	48.3	48.3	100%
Sage	2.0	20.0	40	8.9	22.3%
Black peppercorns	0.84	50.0	42	- ‡	-
Molasses	0.27	72.0	19.4	19.4	100%
Wheat bran	0.14	100	14.0	9.2	66%
Pork kidney powder	0.11	18.8	2.1	1.5	71%

N.B. ‡ value not recorded.

6.3.4 Cationic chromium from biological samples.

Each biological sample from which chromium was extracted showed on separation by the short isolation procedure, the presence of cationic chromium. Variations occurred in column elution profiles but a standardised pH and conductivity profile is shown in Figure 6.2. This was established by comparing the total elution volume for each of the samples with a relative elution volume standard for all samples. The pH and conductivity profiles were an average representation of all the eluted samples. On this standardised profile the elution position of each cationic chromium peak is indicated by means of a bar. The height of the bar represents the amount of chromium that would have been isolated from a fifty gram sample of the starting material.


6.3.5 Activity and purity of biological samples.

Each of the cationic chromium peaks isolated from the different biological materials was assayed for biological activity. The results are shown in Figure 6.3. The amount of each sample assayed was chosen to contain 192 pmoles of chromium. To give some indication of the purity of each sample the optical density at 280 nm/100 pmoles of chromium was determined and is shown in Table 6.4. Enhancement and optical density at 280 nm/100 pmoles Cr values for the cationic complexes isolated in section 4 are included for comparison and to give an indication of the relative purification capabilities of each method.

6.4 DISCUSSION

The short isolation procedure described in methods is successful in its primary aim of isolating the previously identified cationic fractions P-1, P-2, P-3 and P-4 (Figure 6.1) from Merck high-chromium yeast extract by a far less time consuming method than that described in section 4. As can be seen from Table 6.1 the pH values at which the peaks were eluted are all shifted to lower values by varying amounts. Since elution occurs by a combination of pH and conductivity, the higher conductivities used in this short isolation procedure probably result in the observed elution at lower pH values. Comparison of the amounts of chromium

FIGURE 6.2

The relative elution positions are shown for the cationic fractions isolated from a range of biological samples () as described in methods. The peak heights represent the cationic chromium obtainable from a 50 g starting source. Standardised pH (.....) and conductivity (-----) profiles are shown.

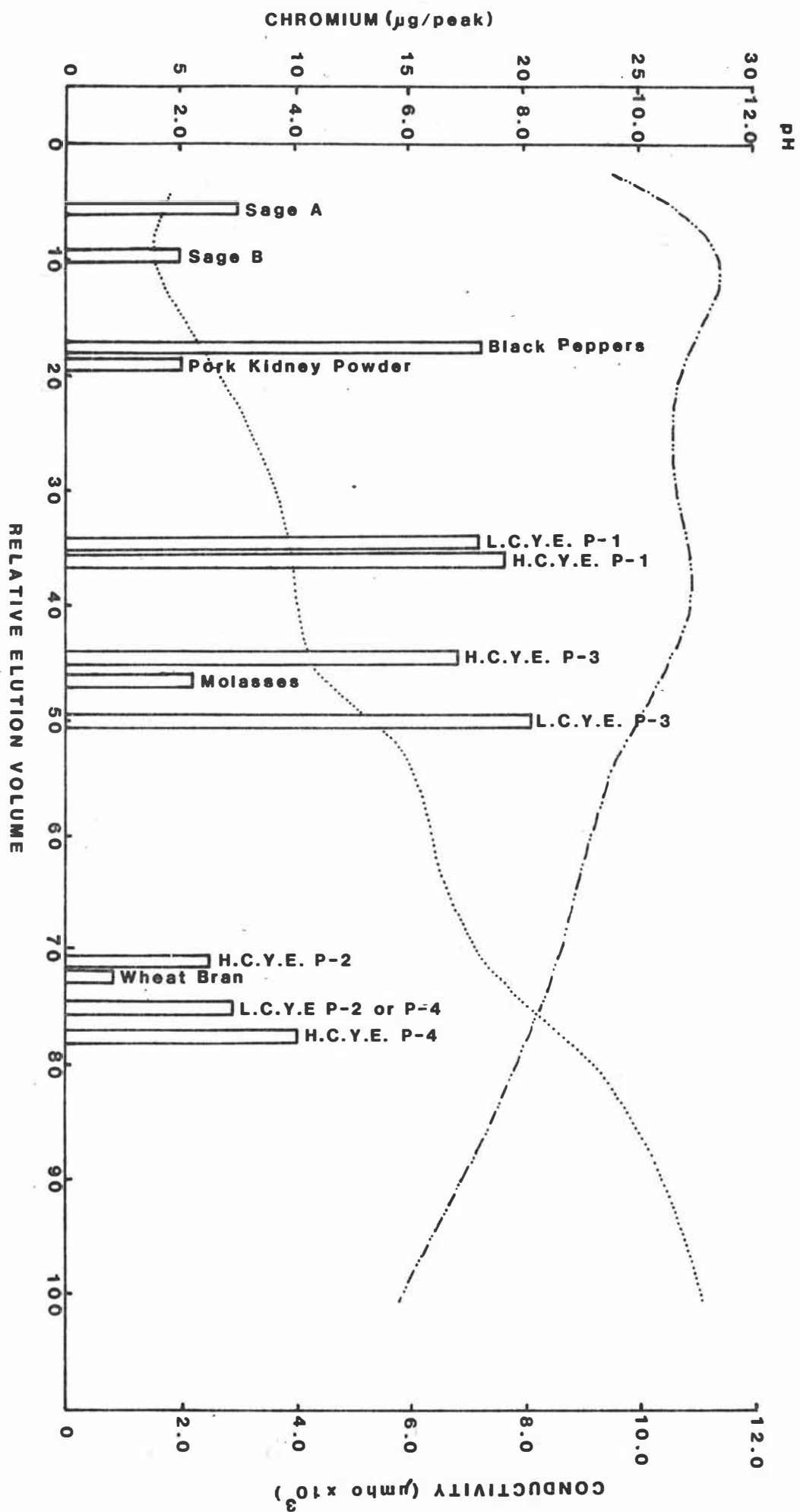



FIGURE 6.2

FIGURE 6.3

The percentage enhancements for the cationic fractions isolated in Figure 6.2 are shown at their elution pH (.....) by means of (). Each sample was assayed with a chromium concentration of 1 $\mu\text{g/g}$.

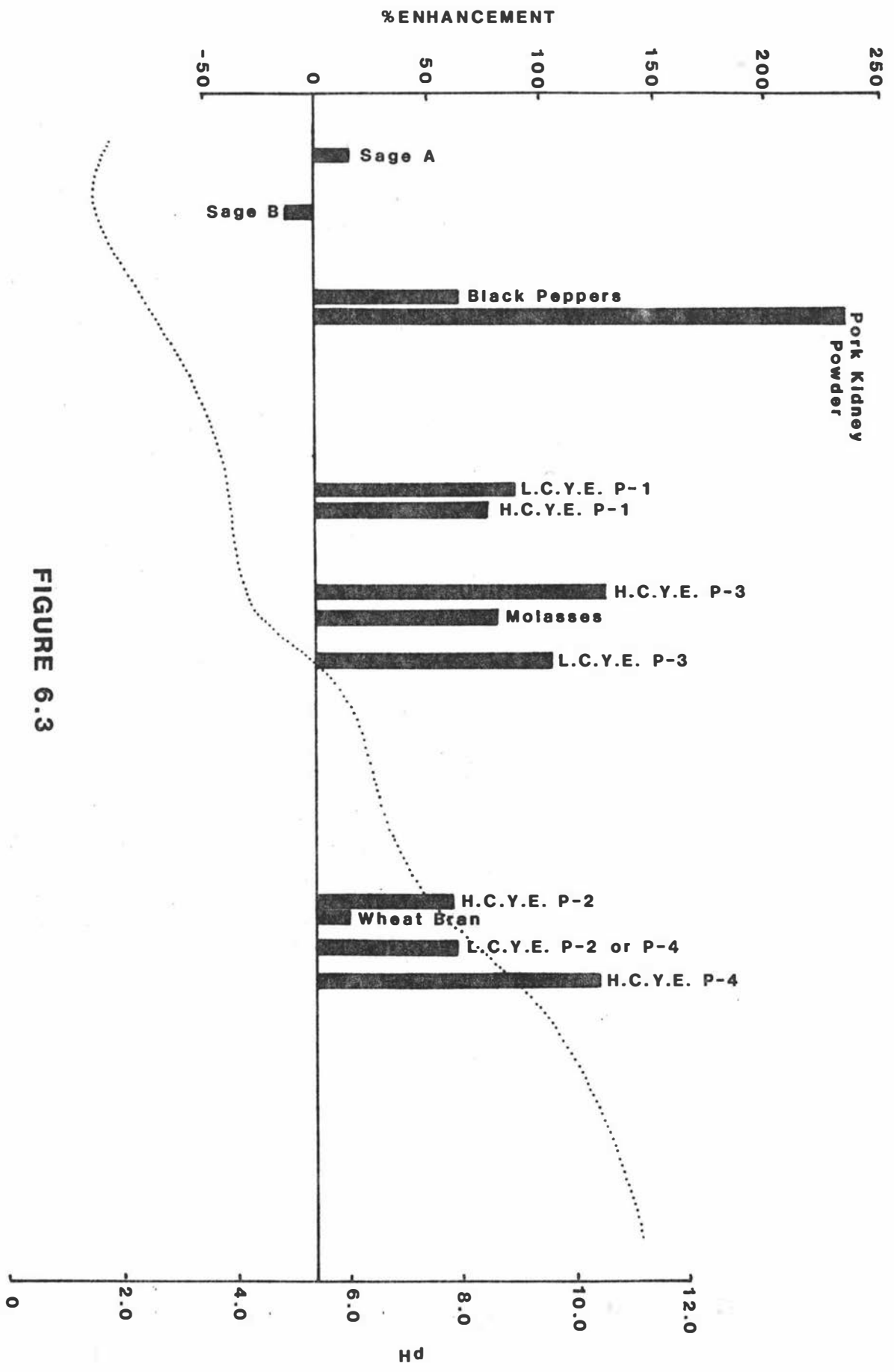


FIGURE 6.3

TABLE 6.4 ACTIVITIES OF BIOLOGICAL SAMPLES

Sample	Percentage Enhancement		Optical density at 280 nm/100pmoles Cr	
	Section 6 procedure	Section 4 procedure	Section 6 procedure	Section 4 procedure
Merck P-1	+ 74	+ 53.3	0.62	0.81
high- chromium yeast extract P-2	+ 59	+120	2.04	0.17
P-3	+129	+242	1.35	0.31
P-4	+124	+137	3.24	1.14
Merck P-1	+ 85		0.91	
low- chromium yeast extract P-2				
or P-4	+ 60		3.78	
P-3	+107		2.94	
Sage A	+ 16.3		4.5	
Sage B	- 12.7		2.23	
Black pepper- corns	+ 64		0.72	
Molasses	+ 85		0.29	
Wheat bran	+ 12.7		1.13	
Pork Kidney powder	+241		3.94	

isolated in each peak from high-chromium yeast extract by the section 6 procedure and the section 4 procedure shows some variation in levels namely : P-1 (+ 6 μg), P-2(-20 μg), P-3 (+ 13.5 μg) and P-4 (+ 11 μg). As the difference in the total amount of cationic chromium isolated by the two methods was only ca 5% it would appear that the reason for the variation in the amount of chromium in the individual peaks was that the section 6 isolation procedure had not resolved the peaks as well as the procedure described in section 4. Examination of the optical density at 280 nm given in Table 6.4 shows that the complexes isolated by the procedure in section 4 were generally of higher purity. Only P-1 when prepared by the short isolation procedure showed less absorbance at 280 nm. P-3, the peak found to be most active in sections 4 and 5 was far less pure by this criterion with the two values of $A_{280}/100$ pmoles of Cr being 0.31 (section 4 procedure) and 1.35 (short isolation procedure).

The activities for the cationic complexes isolated from high-chromium yeast extract shown in Figure 6.3 are generally much lower than the activities of comparable peaks isolated in section 4. P-3 still remains the most active peak but in the short isolation procedure the percentage enhancement was only slightly greater than one half of that determined from the section 4 procedure. The activity of P-2 was similarly reduced and P-4 to a lesser extent. P-1 still showed low activity but this was increased compared with that found from the section 4 procedure.

Since the eluted peaks obtained by the shortened section 6 procedure are not as well resolved as those from the section 4 procedure, the majority of the fractions are less pure or in the case of the P-3 peak have reduced activity. Thus the section 6 procedure has only a limited role in the purification of chromium containing fractions and is useful mainly as a rapid detection method.

The levels of chromium found in the biological samples tested (Table 6.2) were in good overall agreement with the results reported by Toepfer et al (1973), Schroeder et al (1970) and Guthrie (1975). Differences in sample type and

the methods of analysis would have resulted in the variations observed for similar samples from the different workers. Only the split green peas were found to have a chromium level which was too low for further experimentation. Except for the black peppercorns, which were not measured, the percentage of chromium which was extracted by water from each biological sample is shown in Table 6.3. In every case, including the black peppercorns the chromium levels were adequate to allow column chromatography to be carried out.

All the biological samples tested contained some form of cationic chromium. As can be seen from Figure 6.2 the elution of these fractions occurred at a wide variety of pH values, in fact chromium was eluted from pH 1.75 right through to pH 9.5.

Comparison of the peaks isolated from the high-chromium yeast extract and low-chromium yeast extract revealed certain similarities. (Figure 6.2). The elution of the first two low-chromium yeast extract peaks close to similar high-chromium yeast extract peaks enabled them to be labelled as low-chromium yeast extract P-1 (L.C.Y.E. P-1) and low-chromium yeast extract P-2 (L.C.Y.E. P-2). However instead of the two peaks labelled P-2 and P-4 eluted from the high-chromium yeast extract, a single peak bisecting these two peaks was observed for the low-chromium yeast extract. Whether this single peak was P-2 or P-4 or a combination of the two is not clear from Figure 6.2.

Examination of the percentage enhancements for the peaks isolated from high-chromium yeast extract and low-chromium yeast extract (Figure 6.3) showed that P-3 was the most active peak in both cases and the percentage enhancements for the two P-1 peaks were also in close agreement (+ 85, + 74). The P-2 peak isolated from the high-chromium yeast extract had a percentage enhancement of + 59 which suggests that the third peak isolated from the low-chromium yeast extract may in fact be P-2 since the percentage enhancement obtained for this peak (+ 60) was almost identical, but was considerably less than the percentage enhancement for P-4 (+ 124) obtained from the high-chromium yeast

extract analysis. If this analysis was correct P-4 would be absent from the low-chromium yeast extract.

The microgram amounts of chromium eluted in the respective peaks were similar for both high-chromium yeast extract and low-chromium yeast extract; P-1 (19.0, 18.0), P-3 (17.0, 20.5), P-2 (6.0, 7.0*) and P-4 (10.0, 7.0*) (*It was assumed that the third peak eluted from the low chromium yeast extract isolation was either P-2 or P-4). The comparative amounts eluted bore no relationship to the amount of chromium initially in the yeast extracts (448 μg and 48.4 μg). This suggests that the chromium contained in the low-chromium yeast extract was almost all cationic which in turn indicates that the anionic and amphoteric pools of chromium are formed only when an excess of chromium prevails.

This result is, however, in apparent disagreement with the results obtained from the very low chromium system in section 5 in which chromium-51 was used and the anionic complexes isolated comprised the major proportion of chromium. This could mean that there was a fundamental difference in the growth media of the yeast strains from the commercial low-chromium yeast extract and the S. cerevisiae strain used in section 5.

Taking into account the low percentage of cationic chromium isolated in the very low chromium system in section 5, the similar levels of cationic chromium observed here for the high-chromium yeast extract and the low-chromium yeast extract may mean that the cationic chromium fractions in the yeast extract are purely dependent on the medium on which it is grown and hence independent of any metabolic function in the yeast. Only from a detailed analysis of the various growth media used in the manufacture of yeast extract could this suggestion be substantiated. If in fact the cationic chromium levels were not related to the needs of the yeast, then this would be strong evidence against the proposal that GTF is a chromium-containing factor in yeast.

The range of percentage enhancements of the fractions isolated from the biological samples was well spread (Figure 6.3) with the Sage B peak registering a percentage enhancement of -12.7 while the fraction isolated from the pork

kidney powder being the most active with a percentage enhancement of +241.

Toepfer et al (1973) have reported a relative biological value for chromium complexes extracted from a range of food-stuffs. Of those analysed here the values for brewer's yeast, wheat bran and black peppercorns were reported. Comparison of these is shown in Table 6.5

TABLE 6.5 COMPARISON OF RELATIVE BIOLOGICAL ACTIVITY.

Sample	Percentage <u>enhancement</u>	Toepfer et al. Relative <u>biological value.</u> [#]
Wheat bran	+ 12.7	2.21
Black peppercorns	+ 64	10.21
Yeast extract or brewer's yeast	+129* (+242 [‡])	44.88

N.B. * maximum value for high-chromium yeast extract P-3 section 6 isolation procedure.

‡ maximum value for high-chromium yeast extract P-3 section 4 isolation procedure.

the rat fat pad assay was used in the determination of these values.

There is a surprisingly good correlation between the two systems, considering the vastly different methods of sample preparation and determination of biological activity, which is strong evidence that the yeast fermentation assay is measuring the same parameter as is the more commonly used rat fat pad assay.

As yet biological values for the chromium extracted from sage, molasses and pork kidney powder have not been reported in the literature, although pork kidney powder has been reported to be a good source of GTF (Mertz and Schwarz (1959)).

Of the fractions assayed for activity, the two peaks isolated from sage, A and B along with the wheat bran showed little or no activity and therefore the cationic chromium in these samples must be regarded as non-biological despite

literature claims that wheat bran is a source of GTF activity (Roginski et al (1971)). The three remaining non-yeast extract samples all showed activity; black peppercorns (+64), molasses (+85) and pork kidney powder (+241). The peak isolated from black peppercorns was eluted at a different pH value from the major, active yeast extract peak (P-3) and since it also has a lower activity, the chromium complex from the black peppercorns does not seem to be similar to the P-3 yeast extract fraction. The molasses peak isolated had a higher activity and more importantly was eluted very close to the P-3 fraction from the yeast extract. As much commercial yeast is grown on media containing molasses, the chromium complex isolated here may have been similar or identical to that contained in the yeast. This means that the P-3 fraction derived from the yeast extracts may in fact have been originally derived from the yeasts growth medium and hence may not be a metabolic product of the yeast. The fraction isolated from pork kidney powder showed the largest activity of any of the biological materials, including the complexes isolated from yeast extract (although it was about the same as the P-3 fraction obtained from yeast extract as described in section 4). This complex was eluted at a different pH to the yeast extract complexes and hence does not appear to be the same. The small sample size of the pork kidney powder fraction did not allow further characterisation work to be undertaken, but the activity reported here confirms the activity reported by Schwarz and Mertz (1957), who showed that pork kidney powder restored impaired glucose tolerance in rats to normal. The pork kidney powder fraction's activity is also important in that it shows a further correlation between the two assay systems discussed in section 3. Further work is needed to establish the exact nature of the active sample in the pork kidney powder fraction as the optical density at 280 nm level (Table 3.4) clearly indicates the presence of a large amount of non-chromium material.

6.5 CONCLUSION

It is clear from the results presented in this section that a wide range of cationic chromium fractions exist in samples which have been reported as sources of GTF and that these show varying degrees of activity in the yeast fermentation assay. For example, the active material in pork kidney powder and from commercial yeast extracts are significantly different in their origins and in their chromatographic behaviour on ion-exchange columns but show similar specific activities in the in vitro assay systems. Thus it does not appear possible to think of GTF as a single unique species but rather as a range of cationic materials possibly containing chromium. Given the impurity levels of the various isolated fractions it is quite conceivable that the observed activities are in fact due to some other as yet unspecified substance or substances and are not related to chromium at all.

The isolation of a moderately active cationic fraction, similar to P-3 from molasses presents somewhat of a problem since it raises the possibility that the presence of P-3 in commercial yeast extracts may be as a result of the same fraction being present in the growth medium of the yeast, rather than as a result of the yeasts metabolic activity. Results presented in section 5 on the other hand indicated that P-3 might result from yeast metabolic activity since it was not found in the supplemented medium. A possible solution to this dilemma could be that the molasses contains a cationic chromium fraction derived from the metabolic activity of appropriate sugar cane cells during which a complex is produced similar to that produced by yeast.

SECTION 7STUDIES OF PREINCUBATION OF YEAST AND CHROMIUM CHLORIDE7.1 INTRODUCTION

It is observed that simple chromium salts, unlike the chromium-containing fractions isolated from yeast extract, do not show any activity in the yeast assay (section 3.3.7) nor in any other bioassay which has been used to monitor for GTF activity (Mirsky et al (1980) and Anderson et al (1978)). Furthermore it has been claimed that if chromium salts are left for a long enough period of time, under the right conditions, the yeast converts it into a biological form which then shows biological activity in the test system.

The idea that biologically active chromium can be synthesised from so-called inorganic chromium by various species was applied to yeast by Burkeholder and Mertz (1967). They claimed that chromium (III) salts added to chromium-depleted yeast cultures caused an enhanced fermentation rate over controls after a 2 hour lag phase. This lag phase they concluded was possibly due to the time required for the yeast to synthesize the active chromium complex which when formed, accelerated the fermentation rate.

The synthesis of an active chromium complex from simple chromium (III) salts was first proposed by Mertz et al (1964) when working with rats raised on a low-chromium diet. They claimed that an intravenous injected dose of chromium (in the form of chromium chloride or as hexaurea chromium chloride) restored the previously impaired glucose removal rate of the rats to normal values after a period of two hours. They concluded that during this time the chromium was converted to a biological form which was necessary in order for glucose removal to occur at normal rates.

Similar studies have been carried out on diabetics in which some subjects showed an improved glucose tolerance after receiving oral doses of chromium (III) salts for periods of 15 to 120 days (Glinsmann and Mertz (1966)). This again was interpreted in terms of the conversion of the chromium into a biologically active form. Although the time scales for restoration of normal glucose tolerance in the rats and humans were vastly different, the different methods of

chromium supplementation (intravenous injection and oral dosage) could have caused this difference on the results. Similar results in which simple chromium (III) salts have been shown to have a biological effect after a period of time have recently been summarised by Anderson (1980).

In this section it is the aim to test this hypothesis that biologically-active chromium complexes can be formed from simple chromium (III) salts by yeast. Since the demonstration or otherwise of such an effect must ultimately have a bearing on whether or not chromium complexes isolated from yeast actually play a role in the normal functioning of the yeast.

7.2 METHODS AND MATERIALS

7.2.1 Yeast source.

As the effect attributed to the addition of simple chromium (III) salts had always been observed on chromium-deficient sources (Mertz et al (1964) and Burkeholder and Mertz (1967)), the yeast source to be used in these experiments was also made chromium-deficient. The yeast strain Saccharomyces ellipsoideus which had been rendered chromium-deficient by the growth procedure described in section 3.2.2 gave a wide range of fermentation rates in the assays, suggesting that it would be a suitable choice to test for increases in the fermentation rate after preincubation with chromium chloride to allow the formation of biologically active chromium complexes.

7.2.2 Preincubation of yeast in chromium chloride-containing phosphate buffer.

Chromium solutions ($\text{CrCl}_3 \cdot 6\text{H}_2\text{O}$, 10 cm^3) were made up in pH 5.75 phosphate buffer (0.063 M) at the following chromium concentrations: 0.0 $\mu\text{g/g}$, 0.1 $\mu\text{g/g}$, 1.0 $\mu\text{g/g}$ and 16.0 $\mu\text{g/g}$. Higher chromium concentrations could not be achieved as chromium phosphate precipitation occurred at levels of chromium higher than ca 16.0 $\mu\text{g/g}$. Sufficient yeast was added to the previously prepared chromium-containing buffer to give a final yeast cell concentration of 1.5×10^8 cells/ cm^3 at the appropriate times to give yeast samples that had been preincubated for 48 hours, 32 hours, 13 hours, 10 hours

and 1 hour respectively. At the completion of the preincubation period the concentrations of yeast cells were determined by the method described in section 3.2.3 and each sample in which the cell concentration varied from 1.5×10^8 cells/cm³ was diluted with the appropriate chromium-buffer solution.

Fermentation rates of the various yeast solutions were then determined by a method similar to that described for the assay system in section 3. The Warburg manometric method was used to monitor carbon dioxide production as described previously.

The following conditions were used:

FLASK: 2.0 cm³ of preincubated yeast sample in chromium-phosphate buffer.

SIDE ARM: 0.4 cm³ of glucose solution (0.126 M or 2.5% w/v)
: 0.1 cm³ of pH 5.75 phosphate buffer (0.063 M)

WATERBATH : temperature of 30° C.

Results of the fermentation rates for each of the chromium concentrations for each of the preincubation times were expressed in nmoles of CO₂/minute.

7.2.3 Preincubation of yeast in chromium chloride-containing defined media.

Chromium chloride solutions (10 cm³) were made up in defined media (section 3.2.3) at the following chromium concentrations 0.0 µg/g, 0.1 µg/g, 1.0 µg/g and 10.0 µg/g. Previously prepared chromium-deficient yeast was added at the appropriate preincubation times to give a final concentration of 1.5×10^8 cells/cm³. The preincubation times used were as follows: 49 hours, 35 hours, 17 hours, 12 hours and 1.5 hours. With the preincubation occurring in a medium solution, yeast cell multiplication was considerable for the longer preincubation times. Yeast cell concentration was measured for all samples and adjustments were made to bring each sample to the standard level of 1.5×10^8 cells/cm³ with the appropriate chromium-medium solution. Fermentation rates were measured manometrically in the Warburg apparatus (water bath, 30° C) by placing 2.0 cm³ of each solution in the flask, then purging for 3 minutes with O₂ free N₂ to ensure anaerobic fermentation. No side arm additions were needed since the medium solution in which

the yeast cells were preincubated contained sufficient glucose for fermentation. Fermentation rates were calculated in nmoles of CO_2/min . Yeast cell concentration was plotted against preincubation time to establish the effect of chromium chloride on the growth rate of cells.

7.2.4 Preincubation of yeast in chromium chloride-containing defined media with different carbohydrate components.

To determine the effect of different carbohydrate sources, the fermentation rate of yeast preincubated with chromium chloride solutions (10 cm^3) with a range of chromium concentrations ($0.0 \text{ }\mu\text{g/g}$, $0.1 \text{ }\mu\text{g/g}$, $1.0 \text{ }\mu\text{g/g}$ and $10.0 \text{ }\mu\text{g/g}$) were made up in defined media in which the only variable component was the carbohydrate source. These media were made up as described in section 3.2.4 with the following carbohydrate sources: glucose, galactose, fructose, sucrose and maltose. Chromium-deficient yeast was added to make the yeast cell concentration $1.5 \times 10^8 \text{ cells/cm}^3$. Each solution was preincubated for a period of 17 hours. Again there was considerable yeast cell growth, and adjustments to the yeast cell concentration were made prior to fermentation rate analysis by dilution to $1.5 \times 10^8 \text{ cells/cm}^3$ with the appropriate carbohydrate medium-chromium solution. Fermentation rates were determined as described in section 7.2.3. For each carbohydrate source the yeast cell concentration was plotted against the chromium concentration to determine the effect of chromium on the growth rate of the yeast in each medium, during a 17 hour preincubation.

7.2.5 Preincubation of yeast in chromium chloride-containing, carbohydrate-free, defined media.

To determine whether the basal rate of fermentation of chromium-deficient yeast was altered by chromium preincubation under conditions of carbohydrate starvation, the following solutions were prepared. Chromium chloride solutions (10 cm^3) in defined medium, as described in section 3, but without glucose were made up with the following chromium concentrations: $0.0 \text{ }\mu\text{g/g}$, $0.1 \text{ }\mu\text{g/g}$, $1.0 \text{ }\mu\text{g/g}$ and $10.0 \text{ }\mu\text{g/g}$. Chromium-deficient yeast was added at a preincubation time of 17 hours to produce a final yeast cell concentration of

1.5×10^8 cells/cm³. After the required 17 hour period, yeast cell concentration was determined and adjustments in cell concentration were made so that each concentration was again 1.5×10^8 cells/cm³. Basal fermentation rates were determined as described in section 7.2.3, again, with no side arm additions.

7.2.6 Preincubation of yeast in various chromium-containing yeast extract media.

Four defined media solution (10 cm³) labelled as A, B, C and D were made up as described in section 3.2.3 but with the following additions:

- A - no addition.
- B - 10% w/v low-chromium yeast extract.
- C - 10% w/v high-chromium yeast extract.
- D - 10% w/v high-chromium yeast extract plus chromium chloride to produce a final chromium concentration of 10 µg/g.

Chromium-deficient yeast was added at the appropriate times to produce concentrations of 1.5×10^8 cells/cm³. The preincubation times used were 46 hours, 25 hours, 17 hours and 1.0 hour. After the preincubation had been concluded, considerable yeast growth had occurred in the longer preincubations and again yeast cell concentration was determined and adjusted to 1.5×10^8 cells/cm³ with the appropriate medium solution. Fermentation rates were again monitored as described in section 7.2.2 with no addition from the Warburg flask side arm.

7.2.7 Miscellaneous activity assays.

(a) Effect of insulin.

Since insulin is essential in the rat fat pad assay and the assay using isolated adipocytes the effect of insulin both on its own and in combination with an active yeast extract fraction was determined. The standard assay described in section 3 was used with the following additions, of the samples to be tested, made from the side arm of the Warburg flask.

- (i) 0.1 cm³ of ca 0.7 µmolar Bovine insulin.
- (ii) 0.1 cm³ of active yeast extract fraction P-3 (chromium concentration of 0.1 µg/g).

- (iii) 0.1 cm^3 of ca $0.7 \text{ }\mu\text{molar}$ insulin and active yeast extract fraction P-3 (chromium concentration of $0.1 \text{ }\mu\text{g/g}$), premixed for 24 hours before assaying.
- (iv) 0.1 cm^3 of ca $0.7 \text{ }\mu\text{molar}$ insulin and active yeast extract fraction P-3 (chromium concentration of $0.1 \text{ }\mu\text{g/g}$), no premixing.

Activities were determined and described in the normal manner (section 3).

- (b) Effect of yeast extract-containing defined growth medium on the standard assay.

In the standard activity assay described in section 3 the yeast used in the assay has its final growth on a defined medium which was chromium free within the detection limits of the atomic absorption method used ($<0.04 \text{ }\mu\text{g/g}$). In this variation on the standard assay a defined medium was still used as the final growth medium but with the addition of various percentages of chromium-containing yeast extracts. All other conditions were as described for the standard activity assay in section 3. The following yeast extract additions were made to the defined medium. Chromium concentrations of the yeast extracts are shown in Table 4.1.

- (a) no yeast extract addition.
- (b) 4% w/v B.B.L yeast extract.
- (c) 2% w/v Oxoid yeast extract.
- (d) 2% w/v Difco yeast extract.

The fermentation rates of the yeasts grown on the various media were tested for blank fermentation rate by the addition of 0.1 cm^3 phosphate buffer (0.063 M) and for an active sample (chromium concentration = $0.1 \text{ }\mu\text{g/g}$) obtained from yeast extract.

7.3 RESULTS

7.3.1 Preincubation of yeast in chromium chloride-containing phosphate buffer.

Results of the fermentation rates for the four chromium concentrations at the five preincubation times chosen are shown in Table 7.1.

TABLE 7.1 FERMENTATION RATES OF YEAST AFTER PREINCUBATION OF YEAST IN CHROMIUM CHLORIDE-CONTAINING PHOSPHATE BUFFER.

Chromium concentration ($\mu\text{g/g}$)	Rate (nmoles CO_2/min)				
	Preincubation time (hours)				
	1	10	13	32	48
0.0	26.3	24.5	21.9	21.2	27.2
0.1	22.1	28.8	25.9	20.9	24.9
1.0	22.9	27.1	27.8	21.6	21.3
16.0	27.1	34.2	32.2	24.7	21.9

Figure 7.1 shows the comparison between increased fermentation rates due to preincubation carried out by Burkeholder and Mertz (1967) and those carried out here. The interpretation given to this data by Burkeholder and Mertz is discussed in section 7.4.

7.3.2 Preincubation of yeast in chromium chloride-containing defined media.

Using the method described in section 3.2.4 the yeast cell concentration for each of the chromium concentrations from each preincubation time was determined. Figure 7.2 shows yeast cell concentration graphed against the preincubation time for each chromium concentration as well as the blank with no chromium addition. Table 7.2 represents the fermentation rates in nmoles of CO_2/min for each of the chromium concentrations at each of the preincubation times.

FIGURE 7.1

The wide variation in the percentage of carbon dioxide evolution from yeast caused by the method of calculation (discussed in section 7.4) is shown by a comparison of the results reported by Burkeholder and Mertz (1967) with those obtained here. Burkeholder and Mertz (1967).

Chromium (III) pre-incubated for 16 hours/ unsupplemented controls (○————○).

Chromium (III), no pre-incubation/unsupplemented controls (○-----○).

Data obtained here.

16 µg/g chromium chloride pre-incubated for 32 hours/ unsupplemented controls (●————●).

0.1 µg/g chromium chloride pre-incubated for 48 hours/ unsupplemented controls (⊗————⊗).

16 µg/g chromium chloride, no pre-incubation/unsupplemented controls (●-----●).

FIGURE 7.1

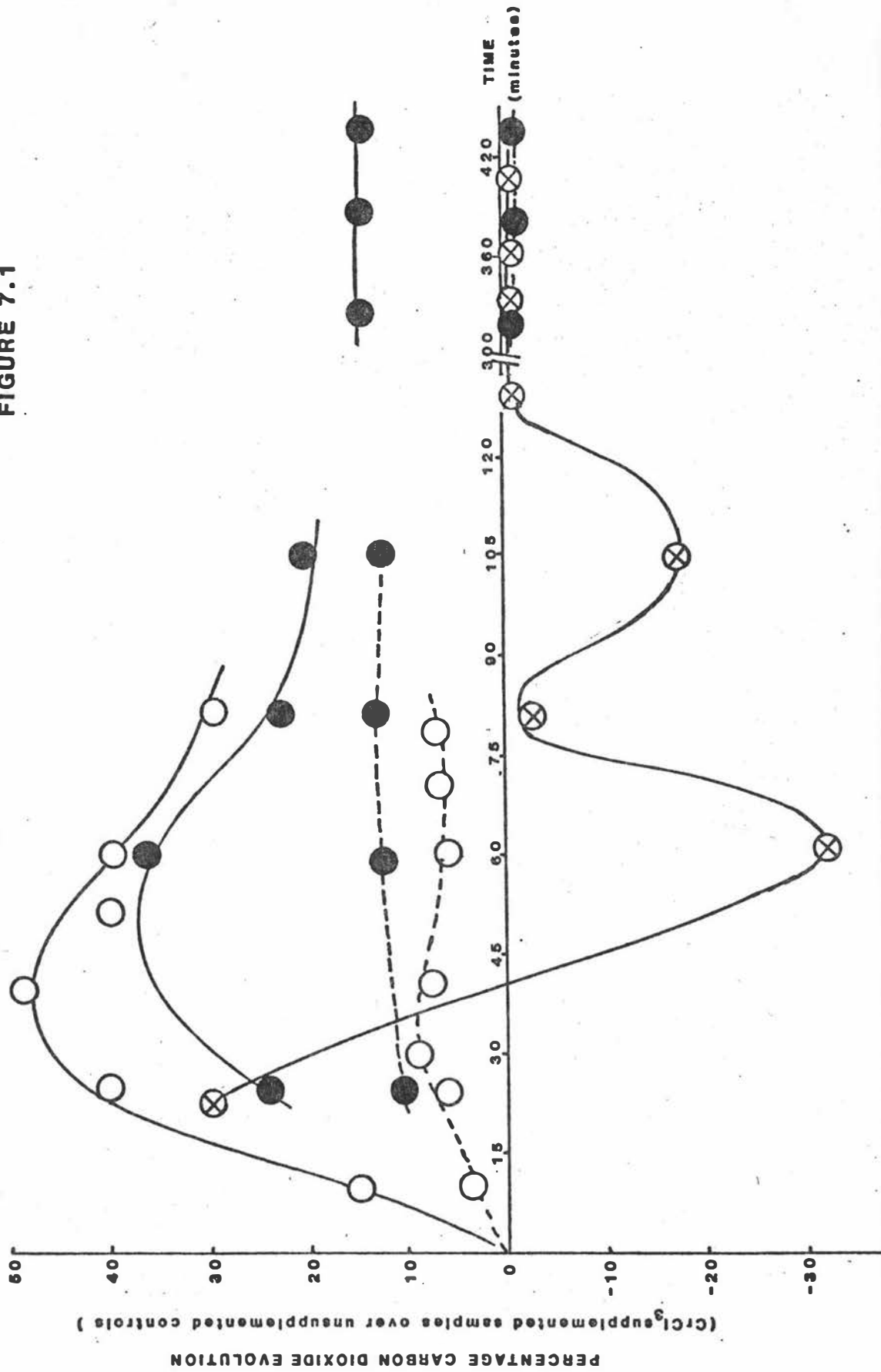


FIGURE 7.2

The variation in yeast cell concentration caused by pre-incubation of yeast over 48 hours in various concentrations of chromium chloride-containing defined media. 0.0 $\mu\text{g Cr/g}$ (—————), 0.1 $\mu\text{g Cr/g}$ (-----), 1.0 $\mu\text{g Cr/g}$ (- - - - -), 10.0 $\mu\text{g Cr/g}$ (.....).

FIGURE 7.2

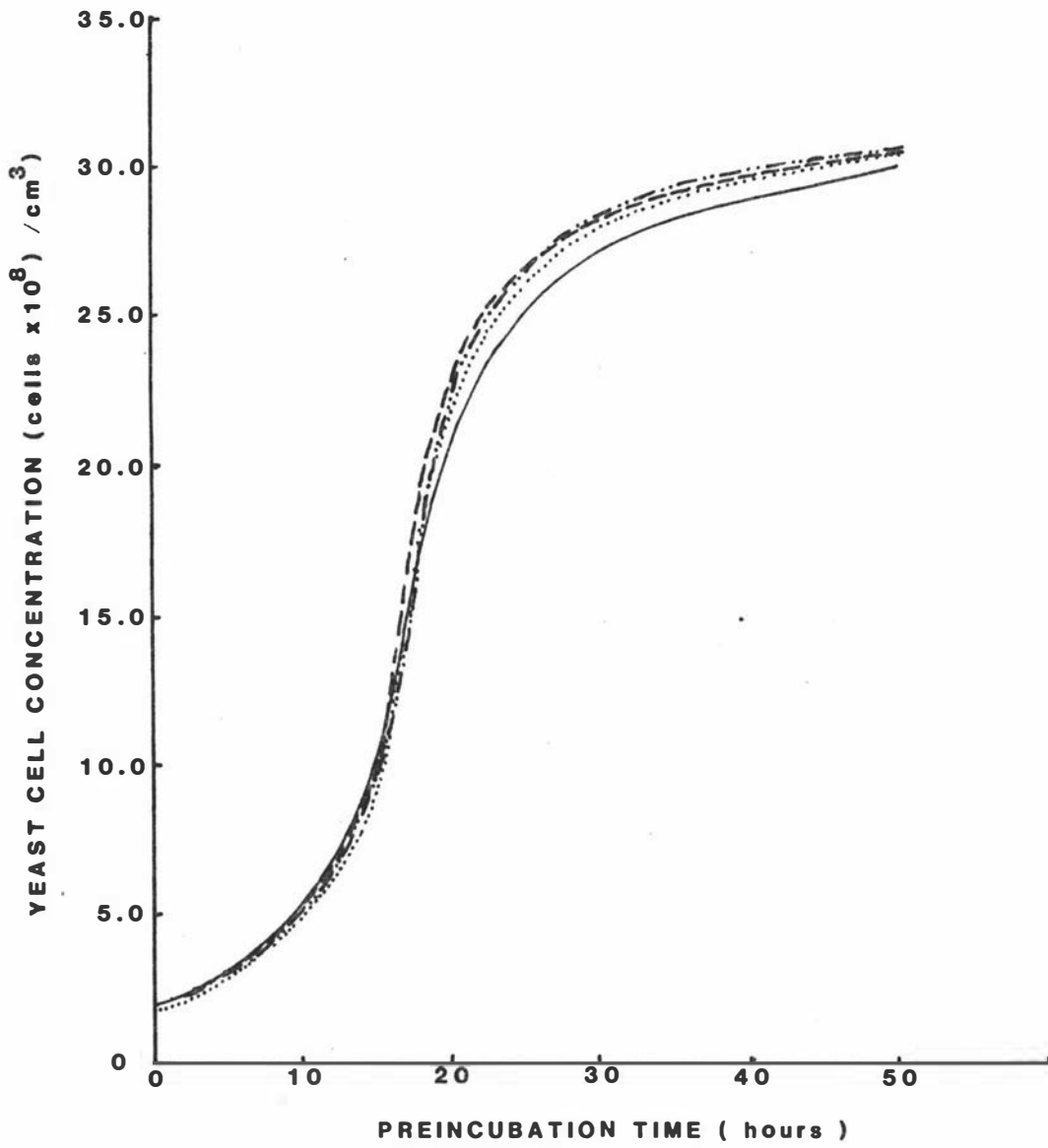


TABLE 7.2 FERMENTATION RATES AFTER PREINCUBATION OF YEAST IN CHROMIUM CHLORIDE-CONTAINING DEFINED MEDIA

Chromium concentration ($\mu\text{g/g}$)	Rate (nmoles $\text{CO}_2/\text{min.}$)				
	Preincubation Time (hours)				
	1.5	12	17	35	49
0.0	215	292	252	163	113
0.1	227	303	230	112	103
1.0	250	280	240	117	98
10.0	163	182	118	121	102

7.3.3 Preincubation of yeast in chromium chloride-containing defined media with different carbohydrate components.

The fermentation rates are shown in Table 7.3 for each of the chromium and yeast preincubations in the media solution comprised of the following carbohydrates: glucose, galactose, fructose, sucrose and maltose.

TABLE 7.3 FERMENTATION RATES AFTER A 17 HOUR PREINCUBATION OF YEAST IN CHROMIUM CHLORIDE-CONTAINING DEFINED MEDIA WITH DIFFERENT CARBOHYDRATE COMPONENTS.

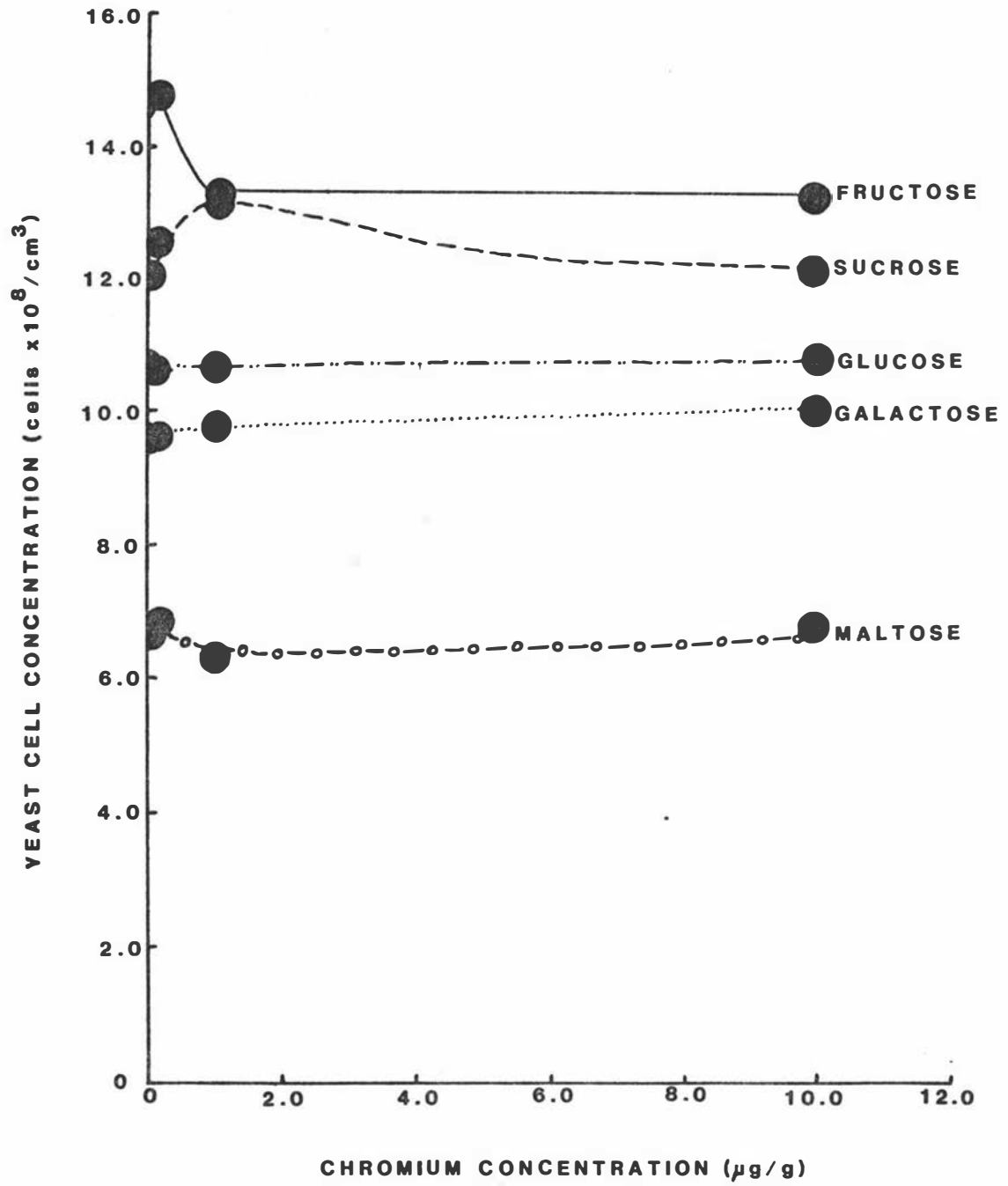
Chromium concentration ($\mu\text{g/g}$)	Rate (nmoles $\text{CO}_2/\text{min.}$)				
	Carbohydrate type				
	Glucose	Galactose	Fructose	Sucrose	Maltose
0.0	253	66	125	268	47
0.1	256	70	102	247	43
1.0	236	70	107	240	53
10.0	228	62	115	223	27

Figure 7.3 shows the variation of yeast cell concentration due to the chromium concentration for the 17 hour preincubation for each of the carbohydrate sources used.

FIGURE 7.3

Variation in yeast cell concentration caused by a 17 hour pre-incubation due to various concentrations of chromium chloride in defined media composed of various carbohydrates. Fructose (—————), Sucrose (-----), Glucose (-.....-), Galactose (.....), Maltose (-o-o-o-o-o-o-o-o-o-o-).

FIGURE 7.3



7.3.4 Preincubation of yeast in chromium chloride-containing, carbohydrate-free defined media.

Results to determine the basal rate of fermentation in the absence of any carbohydrate source were determined after a 17 hour preincubation time. The yeast cell concentration after the 17 hour preincubation along with the fermentation rate on the adjusted cell concentration solution are shown in Table 7.4.

TABLE 7.4 FERMENTATION RATES AFTER A 17 HOUR PREINCUBATION OF YEAST IN CHROMIUM CHLORIDE-CONTAINING, CARBOHYDRATE-FREE DEFINED MEDIA.

<u>Chromium concentration</u> ($\mu\text{g/g}$)	<u>Yeast cell concentration after 17 hour preincubation</u> (cells/cm ³)	<u>Fermentation rate</u> (nmoles CO ₂ /min)
0.0	2.25 x 10 ⁸	2.1
0.1	2.4 x 10 ⁸	1.5
1.0	2.25 x 10 ⁸	1.8
10.0	2.8 x 10 ⁸	1.7

7.3.5 Preincubation of yeast in chromium-containing yeast extract media.

Fermentation rates of the yeast, preincubated in the medium blank, and the three yeast extract-containing media, after the various preincubation times are shown in Table 7.5.

TABLE 7.5 FERMENTATION RATES AFTER PREINCUBATION OF YEAST IN DEFINED MEDIA CONTAINING DIFFERENT YEAST EXTRACT COMPONENTS.

Type of preincubation medium	Rate (nmoles CO ₂ /min)			
	Preincubation time (hours)			
	1.0	17	25	46
Blank	155	206	204	177
Low-chromium yeast extract	197	409	496	394
High-chromium yeast extract	197	236*	416	397
High-chromium yeast extract + CrCl ₃	146	383	383	219

N.B.* Low yeast growth occurred and consequently this value may not be significant.

Many of the rates shown in Table 7.5 were much faster than any others encountered previously with the Warburg apparatus. As a result of these rates being so fast the method of rate determination was perhaps near its limit and consequently larger errors will be associated with the faster fermentation rates.

7.3.6 Miscellaneous activity determinations.

(a) Activity of insulin in the standard activity assay.

The percentage enhancements calculated for the four samples detailed in methods 7.2.6a to determine the effect of insulin are shown in Table 7.6.

TABLE 7.6 ACTIVITY OF INSULIN IN THE STANDARD ASSAY.

<u>Sample</u>	<u>Percentage enhancement</u>
(i) 0.7 μ molar insulin	- 2.8
(ii) P-3 (Cr = 0.1 μ g/g)	+ 152
(iii) 0.7 μ molar insulin + P-3 (Cr = 0.1 μ g/g), premixed 24 hours.	+ 144
(iv) 0.7 μ molar insulin + P-3 (Cr = 0.1 μ g/g), no premixing,	+ 130

(b) Activities from the standard assay after yeast extract addition to the defined growth medium.

Results of the percentage enhancements of the fermentation rates for a blank and an active sample for the various growths on defined media, detailed in methods 7.2.6b, are shown in Table 7.7.

TABLE 7.7 PERCENTAGE ENHANCEMENTS OF BLANKS AND ACTIVE SAMPLES DETERMINED FROM YEASTS GROWN ON VARIOUS YEAST EXTRACT-CONTAINING MEDIA.

Yeast growth medium	Percentage enhancement from defined medium blank rate:	
	<u>Phosphate buffer blank</u>	<u>Active Sample</u>
Defined medium	0	+ 206
Defined medium + 4% w/v B.B.L. yeast extract	- 3.2	+ 254
Defined medium + 2% w/v Oxoid yeast extract	- 3.2	+ 254
Defined medium + 2% w/v Difco yeast extract	- 6.4	+ 303

7.4 DISCUSSION

The fermentation rates shown in Table 7.1 show no effect of either increased chromium concentration or increased preincubation time, all values lying within the range of values normally found for the blank assay described in section 3.3 (19 nmoles CO₂/min to 37 nmoles CO₂/min). Active samples have been shown to have percentage enhancements of up to 240% whereas the maximum for any of the chromium-yeast preincubations shown in Table 7.1 was only 47% (for the 16.0 µg/g chromium-yeast solution after preincubation for thirteen hours). Thus there is no evidence to support the formation of any biologically active chromium complex when the yeast is preincubated with chromium chloride in phosphate buffer.

Burkeholder and Mertz (1967) obtained the results shown in Figure 7.1 after a 16 hour preincubation. However the reported results were calculated as the percentage increase in CO₂ production of the chromium-supplemented sample over unsupplemented controls, and large errors arise in such calculations (especially during the early stages of fermentation) because a small difference is determined between two large numbers. The variability in the results which can be obtained from such a calculation can be shown by recalculating two selected pieces of data appearing in

Table 7.1. The 16 $\mu\text{g/g}$ chromium preincubated for 32 hours gave results similar to those of Burkeholder and Mertz, while for the 0.1 $\mu\text{g/g}$ chromium preincubated for 48 hours all the results calculated in this way, for times less than 100 minutes, showed a completely random behaviour. Such a random behaviour was consistent for the majority of the assays. The fermentation rates at longer assay times (>300 minutes) were however more consistent and perhaps more truly measure the effect that the chromium concentration and the preincubation time has on the fermentation rate. The values recorded for the two selected samples were: for the 16 $\mu\text{g/g}$ chromium, 32 hour preincubation a 14.5% increase, and for the 0.1 $\mu\text{g/g}$ chromium, 48 hour preincubation a 2.0% decrease. These were both insignificant variations when compared to the 240% increase obtained for an active P-3 sample.

Thus the results reported by Burkeholder and Mertz are more properly interpreted in terms of the large experimental errors in the early stages of the fermentation and there seems to be no evidence for the conversion of simple chromium (III) salts into a biologically active form on prolonged contact with the yeast.

Table 7.2 shows the fermentation rates for yeast preincubated in chromium chloride-containing media. It is apparent that all the rates, including the zero chromium blank, were considerably higher than those listed in Table 7.1 where the yeast preincubation was in phosphate buffer only. The magnitude of the increased fermentation rate (up to ten times) was surprising but may be explained in either or a combination of the following ways. In the case of the yeast preincubated in the medium, the yeast sample for assay was taken from an already fermenting sample and consequently a "fermentation lapse" did not occur in this method of assay whereas in the previous fermentations the yeast was taken straight from a phosphate buffer blank solution where little or no fermentation would have been occurring. Secondly, there was the difference in the method of the two assay systems. Whereas in the first case the preincubated yeast was assayed in phosphate buffer and

glucose, in the second case it was assayed in the defined medium itself. Both solutions contained glucose but the defined medium contained additional minerals and vitamins which may have caused the increased growth rate and fermentation rate. A detailed examination of the medium components with a view to ascertaining those which were capable of causing increased fermentation rates was not undertaken but all vitamins used (biotin, pantothenic acid, inositol, thiamine, nicotinic acid, pyridoxine and para-aminobenzoic acid) were regarded as necessary growth factors by Suomalainen and Oura (1971) and hence likely to cause elevated fermentation rates. Although direct comparison with the result in Table 7.1 was not possible comparison between the results given in Table 7.2 showed similar trends. Again there was a tendency towards lower fermentation rates both with increased chromium concentration and also longer preincubation times. The comparison of yeast cell concentration at each of the preincubation times, for the media containing the four different chromium concentrations, showed little or no alteration in cell concentration due to variation in the chromium concentration. Hence from the results expressed section 7.3.2 it would appear that chromium had little or no effect on the yeast cell concentration but had an inhibitory effect on the fermentation rate at higher chromium concentrations for preincubation times up to 49 hours.

The data expressed in Table 7.3.3 in which the fermentation rates of yeast preincubated in chromium chloride-containing media with various carbohydrate sources are shown, also showed high values. These were presumably due to either of the reasons proposed for the high fermentation rates seen in Table 7.2. Considerable variation in fermentation rates between carbohydrates was also observed but variation due to differences in chromium concentration was slight, again with the major effect being a decrease in rate at higher chromium levels. Certainly the decrease observed here was not as pronounced as that in Table 7.2. Figure 7.3 shows the variation of yeast cell concentration with chromium concentration after a 17 hour preincubation

for each of the carbohydrate sources. The nearly horizontal nature of the lines again suggested that although the different carbohydrates had an effect on yeast cell concentration, differences in chromium levels did not. It is clear though that the yeast growth is affected by the type of carbohydrate source present.

The data expressed in Table 7.4 showed that the basal fermentation rate of the yeast with no carbohydrate source at all was also unaffected by exposure to various chromium concentrations.

The fermentation rates shown in Table 7.5 again all show considerably higher rates than the average blank range of 19 nmoles CO_2/min - 37 nmoles CO_2/min for assays in phosphate buffer. More importantly, the preincubations in which the various yeast extracts were included in the medium, showed increased fermentation rates over the blank rates (defined medium only). Hence the preincubation in the yeast extract-containing media resulted in about a two fold increase in fermentation rate over control rate for yeast preincubated in the defined medium. Thus some material or factor in the yeast extracts is causing an enhancement over and above the accelerated blank rates brought about by preincubation in media which contain various growth factors.

The fermentation rates for the high-chromium yeast extract and low-chromium yeast extract are similar and suggest that each yeast extract contains similar quantities of the material causing the increased fermentation rates. Where chromium chloride has been added to the high-chromium yeast extract the fermentation rate values are generally lower, as observed previously, again indicating that preincubation with simple chromium (III) salts does not result in increased fermentation rates. Thus, these results are consistent with those reported earlier in this section. There does appear to be a time dependence in the attainment of the maximum rate of fermentation, with this maximum occurring at a preincubation time of 17-25 hours. Unfortunately no particular significance can be attributed to this phenomenon at the present time.

7.4.1 Miscellaneous activity determinations.

The rat fat pad assay which has been used extensively to determine the activity of biological chromium fractions (GTF) requires the presence of small quantities of insulin for optimum activity (reviewed section 3.1). This requirement has prompted researchers to speculate on a GTF-insulin interaction and some evidence has been found to support the claim in an in vitro situation (Campbell and Mertz (1963), McDonald and Belusko (1976)). Although insulin has not been reported to have any function within yeast it was considered worthwhile to assay it, along with an active P-3 fraction in the standard assay because of this proposed interaction. The results shown in Table 7.6 clearly show that insulin when assayed by itself had no effect on increasing the rate of CO₂ evolution and in combination with the active P-3 sample the enhancement recorded was less than that recorded for the active P-3 sample assayed on its own. These results confirm that the standard yeast assay has no requirement for insulin and are not supportive of the idea of a GTF-insulin interaction being essential for biological activity in all systems.

Results of assays conducted on yeast grown on various defined media with yeast extract additions are shown in Table 7.7. One of the major criteria for the standard assay has been the requirement of the yeast used in the assay to be chromium-deficient. Although the commercial yeast extracts used here have relatively low chromium concentrations, nevertheless growth of the yeast on these yeast extract-containing media prior to commencement of the assay would have enabled the uptake of chromium if it was in fact an essential requirement. Consequently fermentation rates determined in blank assays performed on the yeasts grown on media containing the yeast extract may have been expected to be higher than the blank assay performed on the yeast grown on defined medium in the absence of chromium. Similarly, the percentage enhancement observed for an active sample determined in an assay for which yeast was grown on yeast extract-containing media may be less due to the uptake of biologically active chromium from the yeast extracts. The results in Table 7.7 show that

neither of these expectations has been realised. The blank rates are similar for all the yeast samples and also, all the yeast samples show high percentage enhancements on addition of an active sample. Several possible explanations exist for these results.

(1) The extensive washing in distilled water after the yeast growth and before the yeast was used in an assay, in order to remove any adhering medium components, may have leached out the active material taken up by the yeast during its growth. This would indicate that by extensive washing yeast could be rendered chromium deficient.

(2) The yeast extracts used in the various growth media contained insufficient levels of biologically-active chromium to provide for the yeasts requirements so that when all the available chromium was taken up the observed fermentation rate was unaltered.

(3) These results could also be consistent with the non-existence of the glucose tolerance factor in yeast extract. In this case the observed percentage enhancements on addition of active sample would indicate that some external factor, not an integral part of the yeast cell was causing the increased percentage enhancement levels.

Considered overall the data expressed in this section suggests that if chromium chloride has any effect on yeast fermentation rates after preincubation, then this is an inhibitory one. No evidence was found to suggest that yeast could synthesise active chromium complexes capable of increasing the fermentation rate at least in the preincubation period used here of up to 48 hours. However there is clearly some factor in yeast extract which leads to enhanced fermentation rates over and above the effect of the essential growth factors required by yeast.

SECTION 8FURTHER FRACTIONATION AND ANALYSIS OF CATIONIC FRACTIONS8.1 INTRODUCTION

The isolation and determination of biological activity of all the chromium-containing fractions in commercial yeast extract has been carried out (section 4) and the origin of these fractions has been determined (section 5). The cationic fractions are the most active, particularly P-2, P-3 and P-4 but of these P-3 is the only fraction which fulfils the three criteria laid down for a biologically active complex. It is not produced by reaction with media or eluent reagents, is retained by the yeast and also it shows activity in the standard assay. Of the other cationic complexes P-4 fulfils all the criteria other than being retained by the yeast. These two fractions were therefore chosen for an attempt at further purification.

Since the results obtained in section 7 have cast some doubt on a functional role for chromium in yeast, the question arises as to whether the activity measured for the active fractions P-3 and P-4 is due to a chromium complex or to extraneous material. It is hoped that further purification of the fractions P-3 and P-4 might shed some light on this important question.

8.2 METHODS AND MATERIALS

8.2.1 Samples for further purification.

The samples selected for further purification work were:

(1) P-4 isolated from a high-chromium yeast extract prepared as described in section 4. (total chromium in the sample was 15 μg).

(2) P-3 isolated from a high-chromium yeast extract prepared as described in section 4 (total chromium in the sample was 20 μg).

(3) P-3 isolated from a low-chromium yeast extract prepared as described in section 6 (total chromium in the sample was 21 μg).

8.2.2 Ion-exchange chromatography.

Only Dowex 50W-X2 cation-exchange resin was used in the further purification of the chromium fractions. This was used because of its different binding properties compared with those of the Dowex 50W-X12 previously used. These differences were presumably brought about by the reduction in cross linkage in the resin to 2%. The resin was prepared in the H^+ form as described for the Dowex 50W-X12 resin in section 4. As the sample size was considerably reduced so too were the column volumes in comparison with the cation-exchange columns used previously. Sample size was always less than 500 cm^3 and a typical column would have a volume of 50 cm^3 and dimensions of $2.8\text{ cm} \times 8.0\text{ cm}$. The samples were loaded at pH 4.5 (titrated to this pH with HCl) with a conductivity of less than $5000\text{ }\mu\text{mho}$ at a speed of ca $2.0\text{ cm}^3/\text{minute}$. Experiments were carried out by loading samples at lower conductivity levels but the results obtained were similar which meant that it was more efficient to load at the higher conductivity level and thus avoid the need for extensive dilution of the sample.

A simple 2 step elution procedure was used which eluted the bound chromium in a single peak. The eluents used were:

(1) distilled water until the optical density at 280 nm had been reduced to near baseline levels.

(2) $0.05\text{ M Na}_3\text{PO}_4$ until the pH reached ca 11.8 and the optical density at 280 nm had been reduced to near baseline levels. The effluent from the column during loading of the sample as well as the eluted peaks were analysed for chromium and measured for pH and conductivity by the methods described in section 2.

8.2.3 Gel filtration chromatography.

Sephadex G15 gel filtration chromatography resin was used routinely for final purification. This resin had a molecular weight cut-off specified by the manufacturers of 1500, this giving a molecular weight range which was suitable for the separation required. The resin was prepared according to the manufacturers instructions. The column used had a volume of 450 cm^3 with dimensions of $2.6\text{ cm} \times 85\text{ cm}$. The

sample size for loading was always 5 cm³ or less. The column was run in distilled water and the conductivity, the optical density at 280 nm and the chromium levels of the fractions eluted were measured.

8.2.4 Activity.

Activity measurements for the eluted samples were performed by the standard assay procedure described in section 3.

8.2.5 Chemical characterisation measurements.

Chemical characterisation measurements involving infra-red spectra, visible and ultra-violet spectra, nmr spectra as well as mass spectroscopic data were carried out as described in section 2.

8.2.6 Activity caused by elution with ammonia.

Various samples both active and inactive, some containing chromium and some free of chromium (below detection limits of 0.04 µg/g) were bound to, and eluted from, Dowex 50W-X12 resin. The eluents used in all cases were varying concentrations of NH₄OH. Activity measurements as described in section 3 were made before and after elution to ascertain the effect which NH₄OH elution had on the activity of the samples.

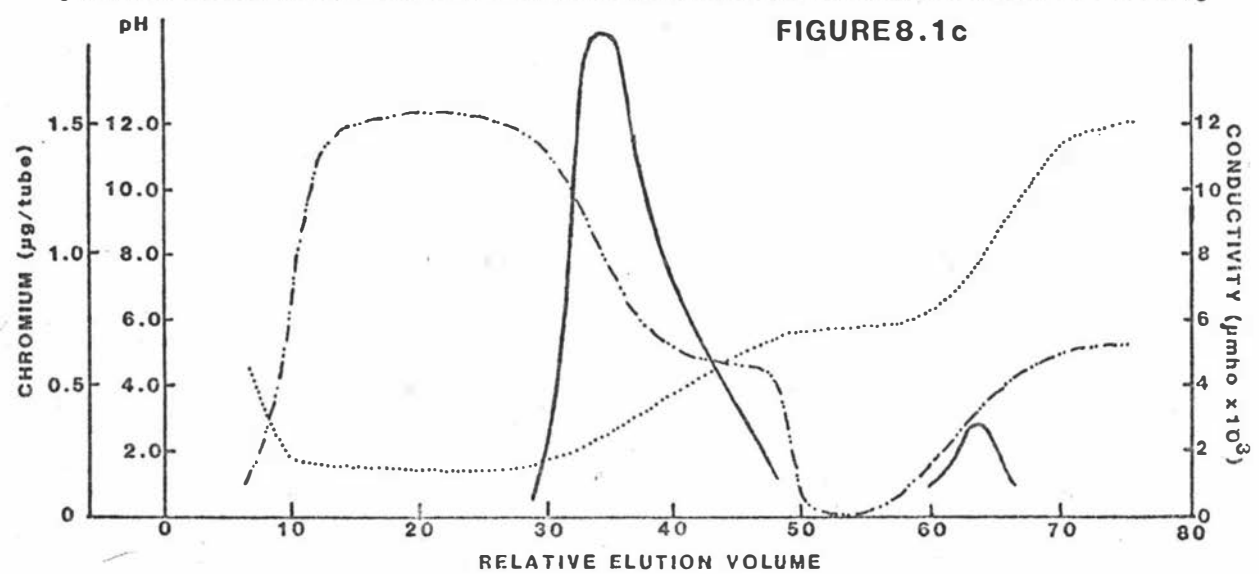
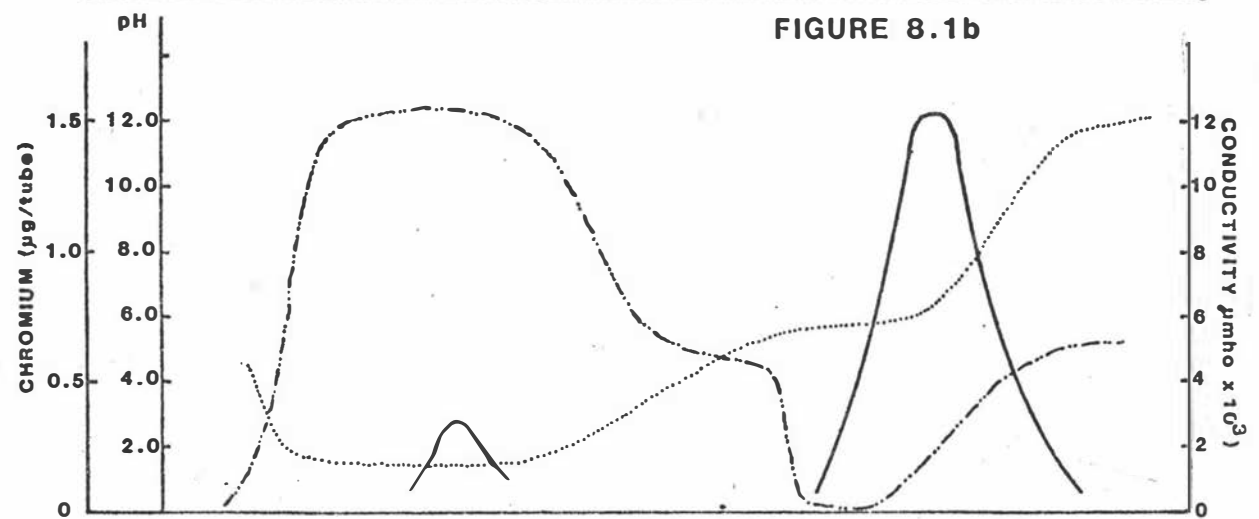
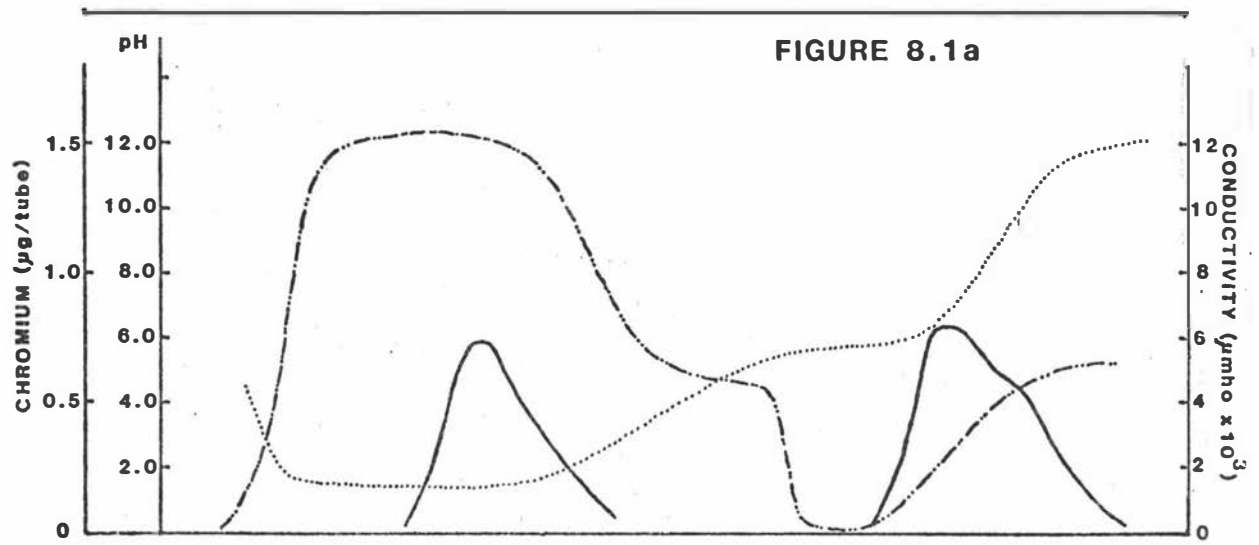
8.3 RESULTS

8.3.1 Ion-exchange chromatography.

The peaks obtained when the three samples (P-4 and P-3 from the high-chromium yeast extract and P-3 from the low-chromium yeast extract) were eluted as described in section 8.2.2 from a column containing the Dowex 50W-X2 resin are shown in Figure 8.1 a, b and c. In this figure to aid comparison a standard pH and conductivity elution profile has been calculated by averaging both the pH and conductivity profiles for each of the three elutions and plotting these on a uniform elution volume axis. Two chromium peaks were apparent from each elution, the first representing material either not binding or being eluted with the rise in pH as water was flowed through the column, while the second was eluted by the Na₃PO₄. The peak heights were variable for each of the three samples.

FIGURE 8.1 a,b,c

Each of the three figures shows an averaged pH profile (.....) and an averaged conductivity profile (-----) for elutions carried out from Dowex 50W-X2 resin. The tube volume collected was 10 cm³ and the chromium concentration is shown by (—————). 8.1a shows the separation of a P-4 fraction from high-chromium yeast extract, 8.1b shows the separation of a P-3 fraction from high-chromium yeast extract and 8.1c shows the separation of a P-3 fraction from low-chromium yeast extract.



Activities of these peaks were determined where the amount of chromium was sufficient and these are shown in Table 8.1 together with the optical density at 280 nm/100 pmoles of chromium.

TABLE 8.1 ACTIVITY OF FRACTIONS ELUTED FROM DOWEX 50W-X2.

<u>Chromium peak</u>	<u>Percentage enhancement</u> [‡]	<u>O.D at 280 nm/ 100 pmoles of Cr</u>
Sample (i) bound	+150	10.45
not bound	+ 26	0.29
Sample (ii) bound	+175	5.16
not bound	-	-
Sample (iii) bound	+ 91	2.22
not bound	+ 25	0.09

N.B. ‡ amounts of chromium where percentage enhancement has been determined were 192 pmoles.

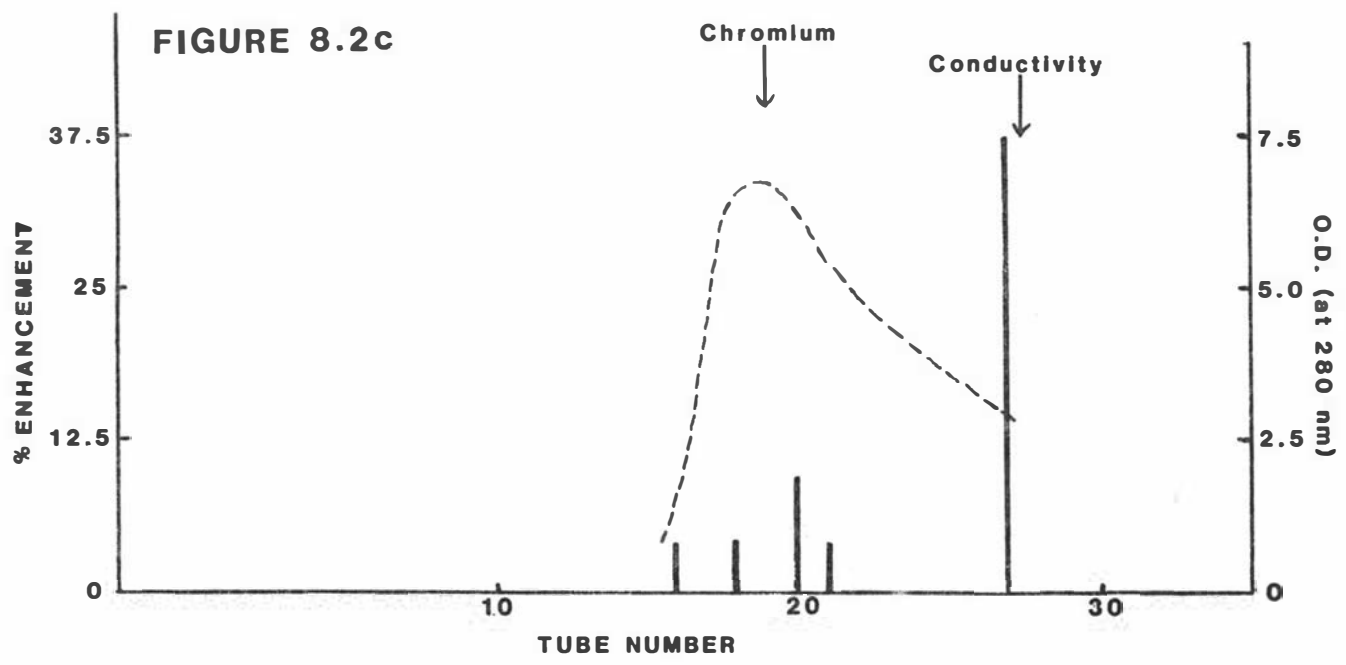
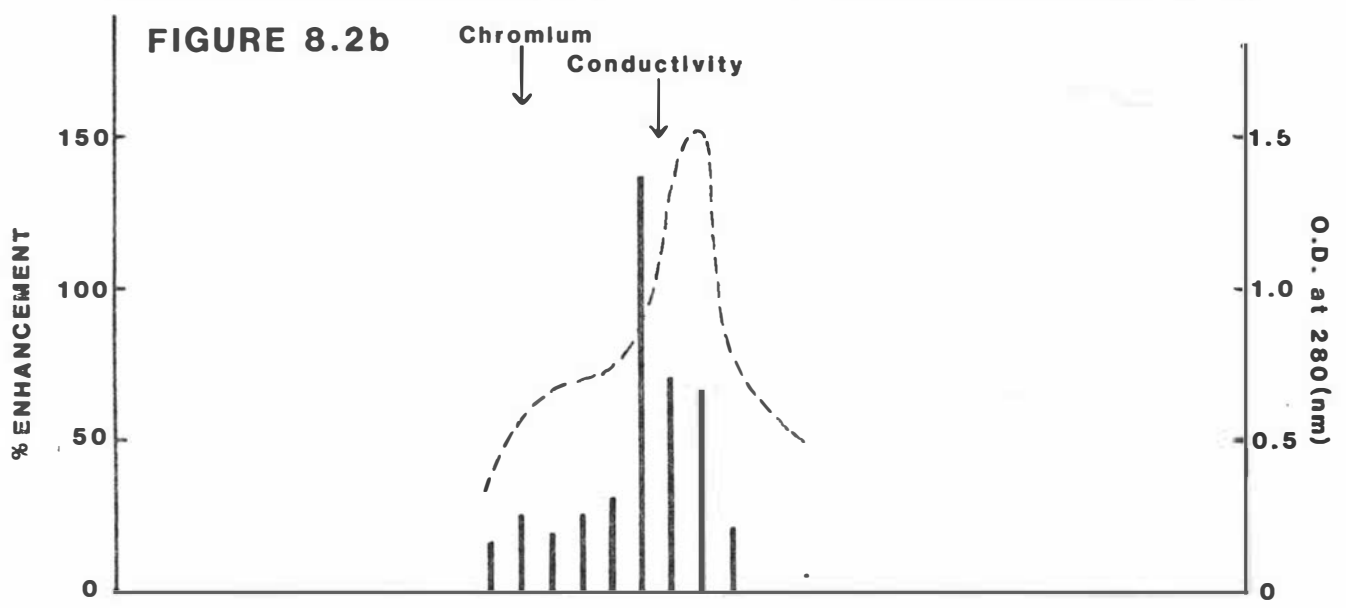
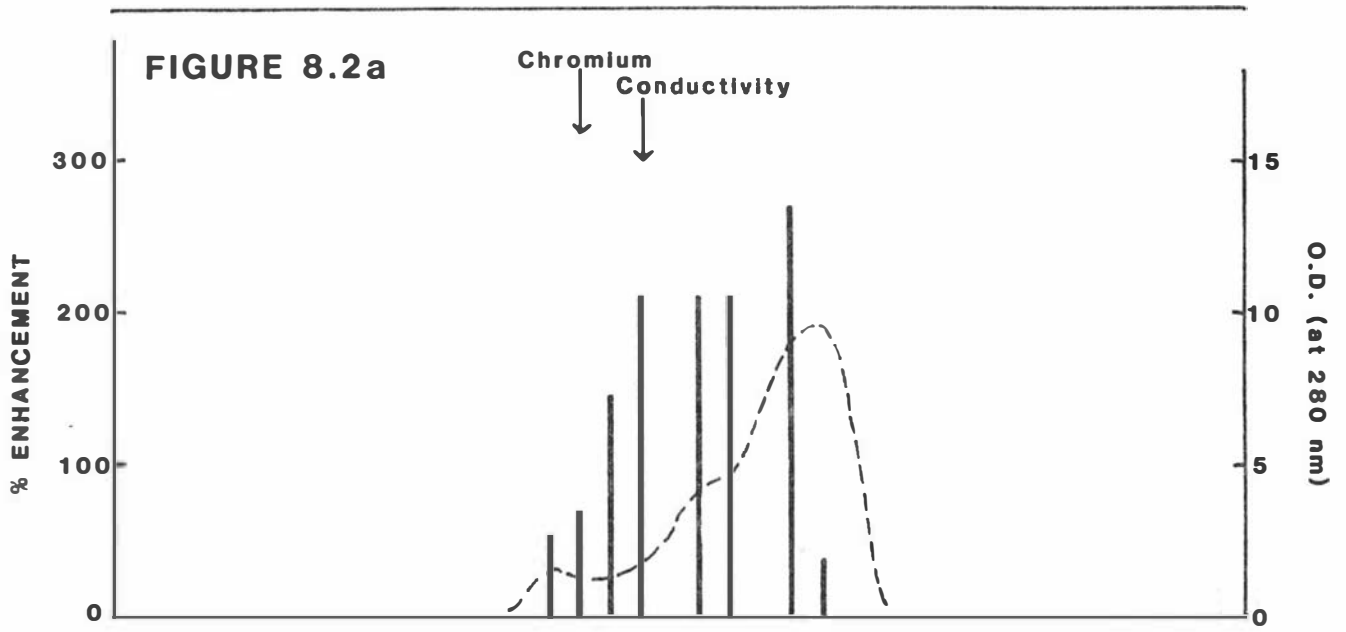
It is apparent from the data expressed in Table 8.1 that the active fractions remain bound to the Dowex 50W-X2 column. The disturbing feature is the large increase in the level of impurities as measured by the optical density at 280 nm for all the bound fractions. In each case the eluted bound fractions were bulked and freeze dried for gel filtration.

8.3.2 Gel filtration chromatography.

The fractions obtained after Sephadex G-15 gel filtration column chromatography on each of the three active samples from the ion exchange chromatography were measured for activity, chromium, conductivity and optical density at 280 nm. Figure 8.2 shows the profile of the optical density at 280 nm and the percentage enhancement at various points across the profile. The peak positions of the chromium and of the conductivity are marked with an arrow (↓). The bound chromium and conductivity gave single peaks. The optical density profile however was more complex. The activity profile was somewhat more spread but did show a definite peak. It is apparent from Figure 8.2 that in each case separation of the chromium peak from the main activity peak has occurred.

FIGURE 8.2a,b,c

The elution profiles are shown for each of the bound fractions from Figure 8.1 a,b,c on Sephadex G-25 gel filtration resin. The tube volume collected was 10 cm³, optical density at 280 nm (-----), percentage enhancement for individual tubes (|), chromium peak (↓), conductivity peak (↓). 8.2a shows the further separation of the P-4 fraction from high-chromium yeast extract, 8.2b shows the further separation of the P-3 fraction from high-chromium yeast extract, 8.2c shows the further separation of the P-3 fraction from low-chromium yeast extract.



8.3.3 Chemical characterisation of active fractions.

Fractions which had the highest activity (from the samples (i) and (ii) which were the P-4 fraction isolated from high-chromium yeast extract and the P-3 fraction isolated from a high-chromium yeast extract respectively) were freeze dried for further chemical analysis. From the data shown in Figure 8.2 a (P-4 high-chromium yeast extract) and b (P-3 high-chromium yeast extract) it is apparent that the activity peak for P-3 nearly coincided with the conductivity peak, while for P-4 the activity peak was displaced further to the lower molecular weight end of the elution profile. On freeze drying it was apparent that the solid white sample from the P-3 fraction was predominantly saltlike (which was consistent with the high conductivity of the sample) while P-4 showed the presence of an off-white, light, organic looking material. A flame test confirmed that most of the material was combustible. To determine whether the activity seen was due to salt or organic material 1 cm³ of each of the two samples was ashed as described in section 2.2.2b, then taken up in the same volume of pH 5.75 phosphate buffer (0.063 M). A standard assay was performed (section 3) on the ashed samples and these values were compared to those taken before the samples were ashed (Table 8.2).

TABLE 8.2 PERCENTAGE ENHANCEMENT OF SAMPLES BEFORE AND AFTER ASHING.

<u>Sample</u>	<u>Percentage enhancement</u>	
	<u>Before ashing</u>	<u>After ashing</u>
P-3	+ 117	8.3
P-4	+ 176	- 4.3

Both samples are clearly inactive after ashing.

As various workers have reported a loss of activity on drying (Shapcott et al (1977)) P-4 (the most active sample) was assayed for its retention of activity after freeze drying. The freeze dried sample gave a percentage enhancement of + 157 which was the same, within the variability of the measurement, as the percentage enhancement before freeze drying of + 176, hence demonstrating that freeze drying had not significantly altered the activity.

Chemical characterisation was undertaken predominantly on the relatively salt free P-4 sample by the methods of mass spectrometry, ^1H and ^{13}C nmr spectrometry, infra-red spectrophotometry and ultra-violet spectrophotometry. Limitations on the amount of P-4 did not allow elemental analysis to be undertaken. For the ultra-violet spectrophotometry the sample was tested at extreme ranges of acidic and basic pH as well as neutral pH to detect any shifts in peaks caused by the change in pH conditions.

The mass spectrum (Figure 8.3a) shows a molecular ion of mass/charge ratio of 137 with significant fragmentation peaks at 109, 108, 107, 77 and 30. The 137 peak was computer matched to a molecular formula of $\text{C}_8\text{H}_{11}\text{NO}$. Under acid and neutral conditions the ultra-violet spectrum (Figure 8.4a) shows peaks at 222 nm and 276 nm with a shoulder 282 nm. On change to alkaline pH the peaks shift to 239 nm and 295 nm respectively with the disappearance of the shoulder. The phenomenon is reversible on addition of acid to regenerate a neutral or acid pH. The shift of the peak from 222 nm to 239 nm suggests the removal of a proton from a bound -OH group while the shift of the peak from 276 nm to 295 nm suggests an alteration in the resonance structure. The spectra are in fact similar to those expected for phenol. The infra-red spectrum (Figure 8.4b) revealed the following major peak positions with the probable assignments given in Table 8.3.

FIGURE 8.3a

The mass spectrum obtained for the highest active P-4 (high-chromium yeast extract) fraction from the Sephadex G-25 column in Figure 8.2a.

FIGURE 8.3b

The mass spectrum obtained for the highest active P-3 (high-chromium yeast extract) fraction from the Sephadex G-25 column in Figure 8.2b

FIGURE 8.3a

P-4

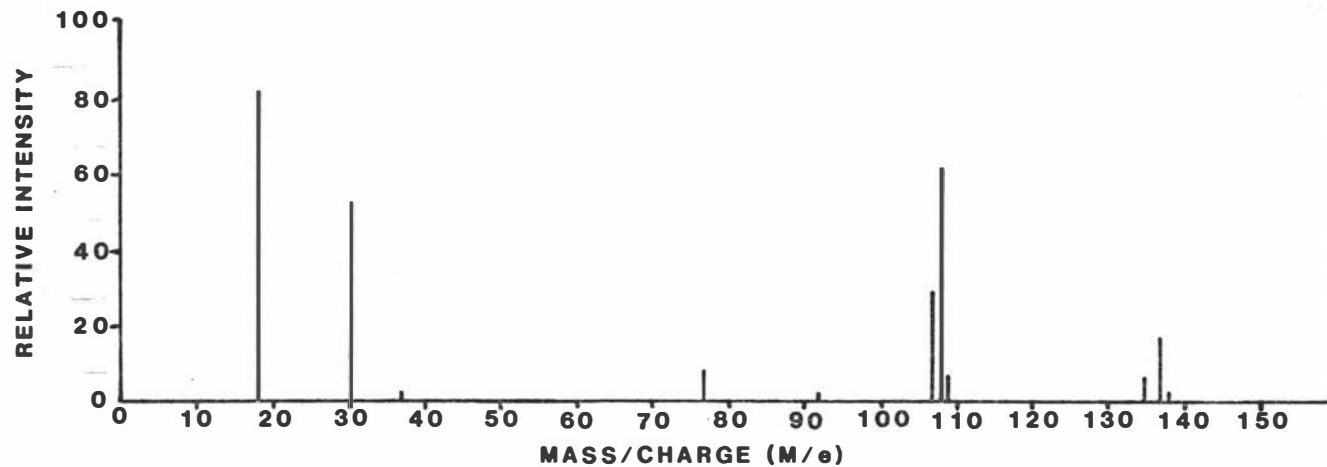


FIGURE 8.3b

P-3

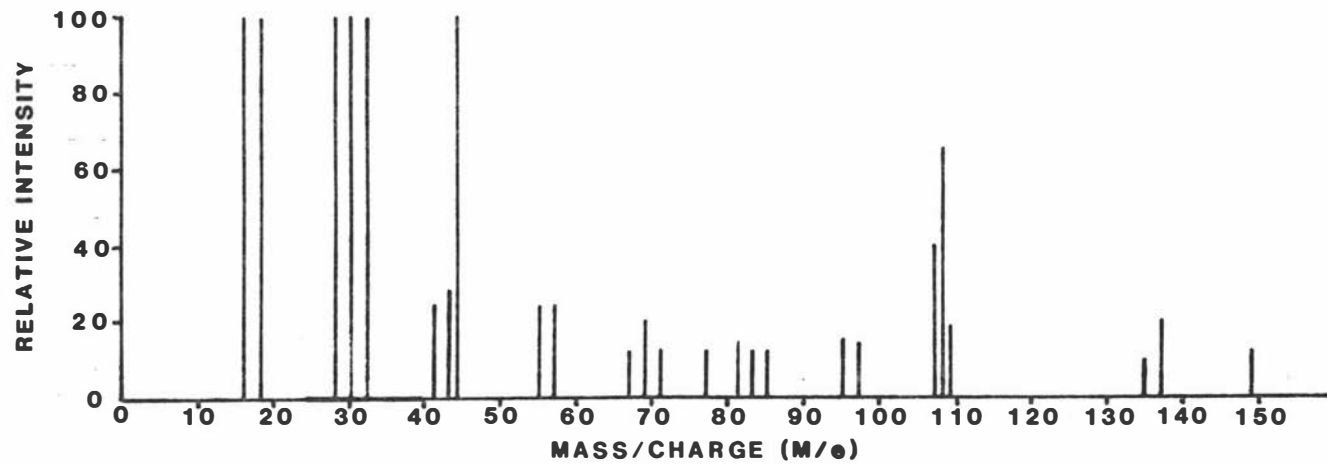


FIGURE 8.4a

The ultra-violet absorbance scan of the highest active P-4 (high-chromium yeast extract) fraction showing acid and neutral pH (—————) and alkaline pH (-----).

FIGURE 8.4b

The infra-red transmittance scan of the highest active P-4 (high-chromium yeast extract) fraction obtained by using a KBr pellet.

FIGURE 8.4a

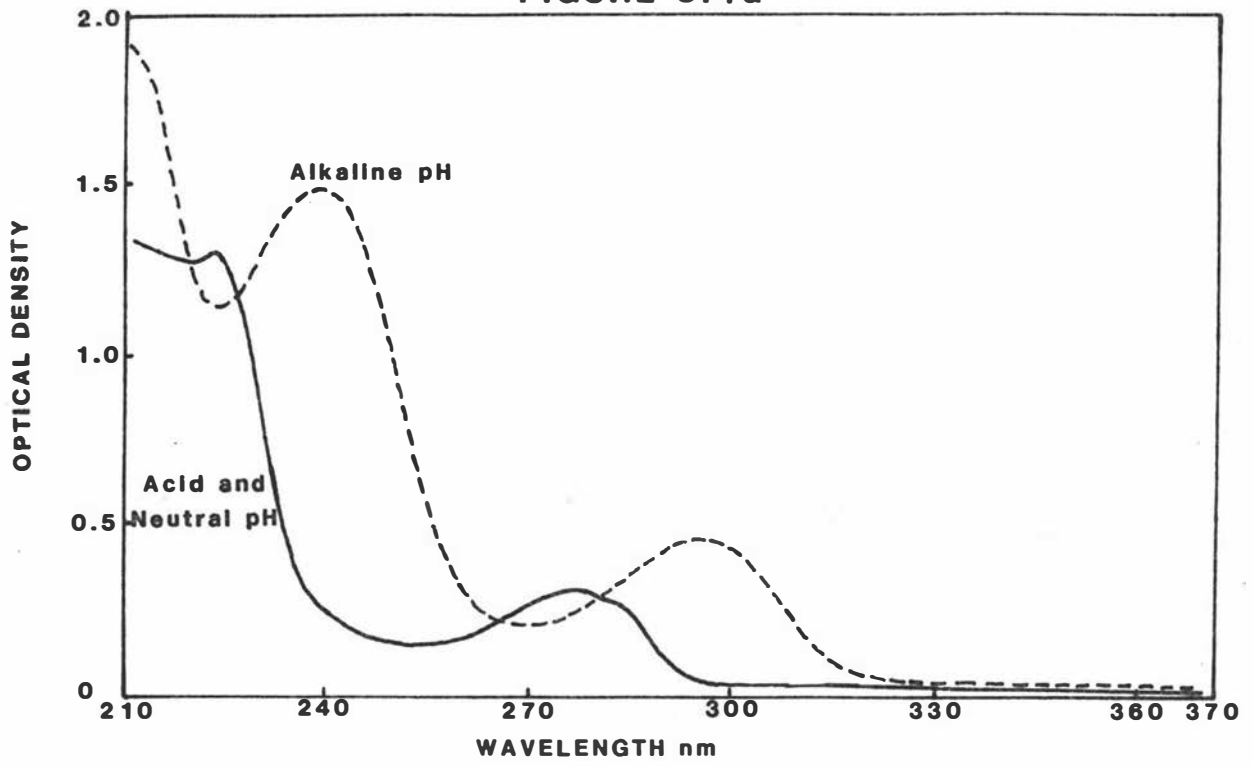


FIGURE 8.4b

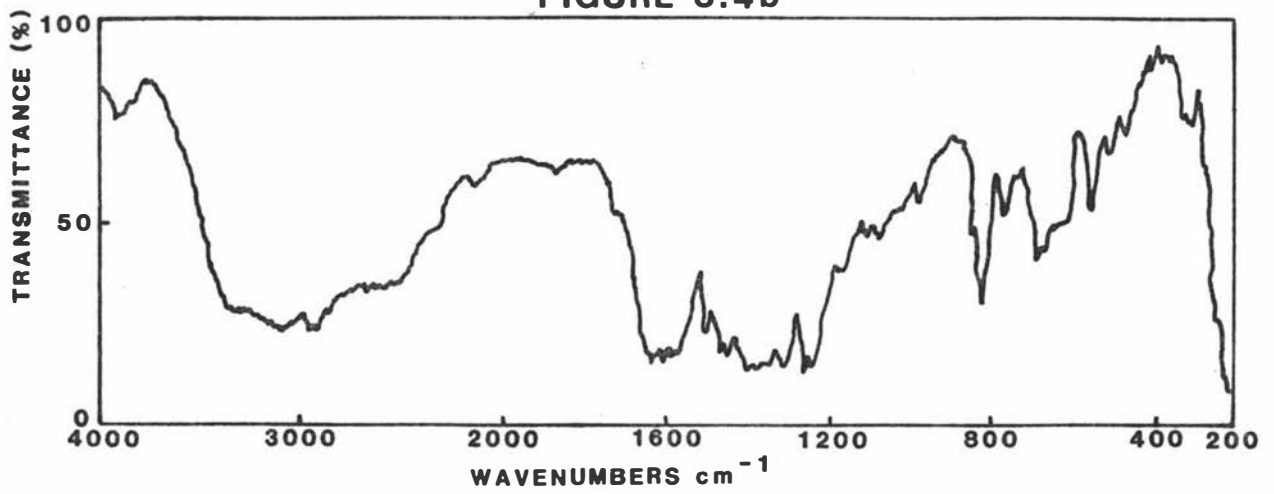


TABLE 8.3 ASSIGNMENTS OF MAJOR PEAKS FROM THE INFRA-RED SPECTRUM OF THE P-4 SAMPLE

<u>Peak description (wavenumbers)</u>	<u>Possible assignment</u>
Broad peak 3400-2500 centered at 3100	OH
Broad peak centered at 1600	NH
Sharp peak 1500	aromatic
Broad peak centered at 1350	phenol
Pair of peaks, 1240 and 1260	aromatic, possibly 1:4 substitution
Sharp peak 830	aromatic 1:4 substitution
Sharp peak at 560 and 510	amine or aromatic
Strong peak 200	amine-NH ₂

The ¹H nmr spectrum (Figure 8.5a) with the sample dissolved in D₂O showed a very strong H₂O band (omitted from the spectrum) and also three other distinct groups of peaks centered at 1.06, 2.42 and 6.39 ppm from the TPS reference signal. These peaks were given the following assignments 1.06 (no assignment possible), 2.42 (-CH₂-) and 6.39 (aromatic hydrogens showing a 1, 4 disubstitution pattern). The relative areas of these two peaks are approximately 1:1, thus if the aromatic region corresponds to 4 protons then the peaks in the -CH₂- region also correspond to 4 protons. The ¹³C spectrum (Figure 8.5b) showed four distinct peaks or groups of peaks centered at 34.82, 42.85, 118.41 (and 117.60) and 131.97 ppm from the TPS reference signal. These peaks were given the following assignments 34.82 (CH₂-N), 42.85 (>CH₂), 118.41 and 117.60 (aromatic carbons $\begin{array}{c} \text{-C} \\ \text{>} \\ \text{-C} \end{array}$ C-C) and 131.97 (aromatic carbons HO-C $\begin{array}{c} \text{C} \\ \text{<} \\ \text{C} \end{array}$). The two peaks in the -CH₂- region (34.82 ppm and 42.85 ppm) are consistent with two methylene groups, one of which is next to a benzene ring while the 3 peaks in the aromatic region can be assigned to 118.41, 117.60 and 131.97 ppm.

The combined information clearly shows that the unknown substance is predominantly parahydroxyphenylethylamine, more

FIGURE 8.5a

The ^1H nmr spectrum of the highest active P-4 (high-chromium yeast extract) fraction.

FIGURE 8.5b

The ^{13}C nmr spectrum of the highest active P-4 (high-chromium yeast extract) fraction.

FIGURE 8.5a

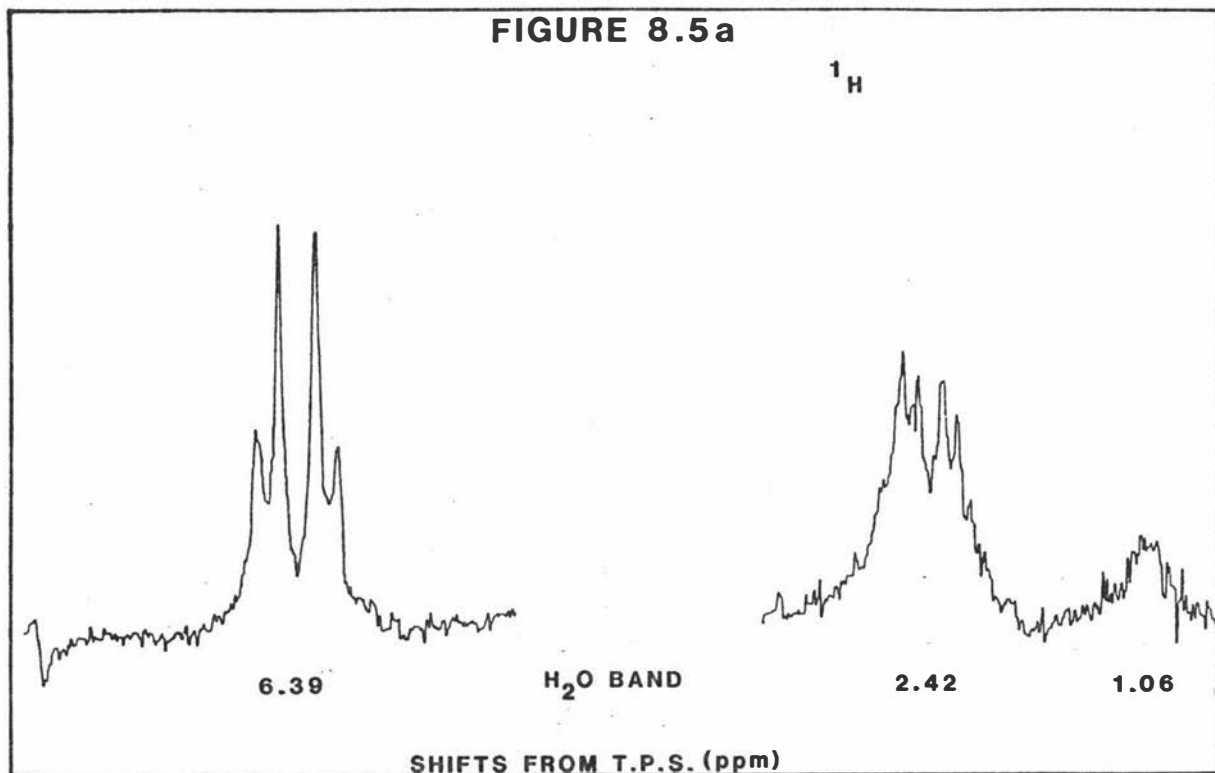
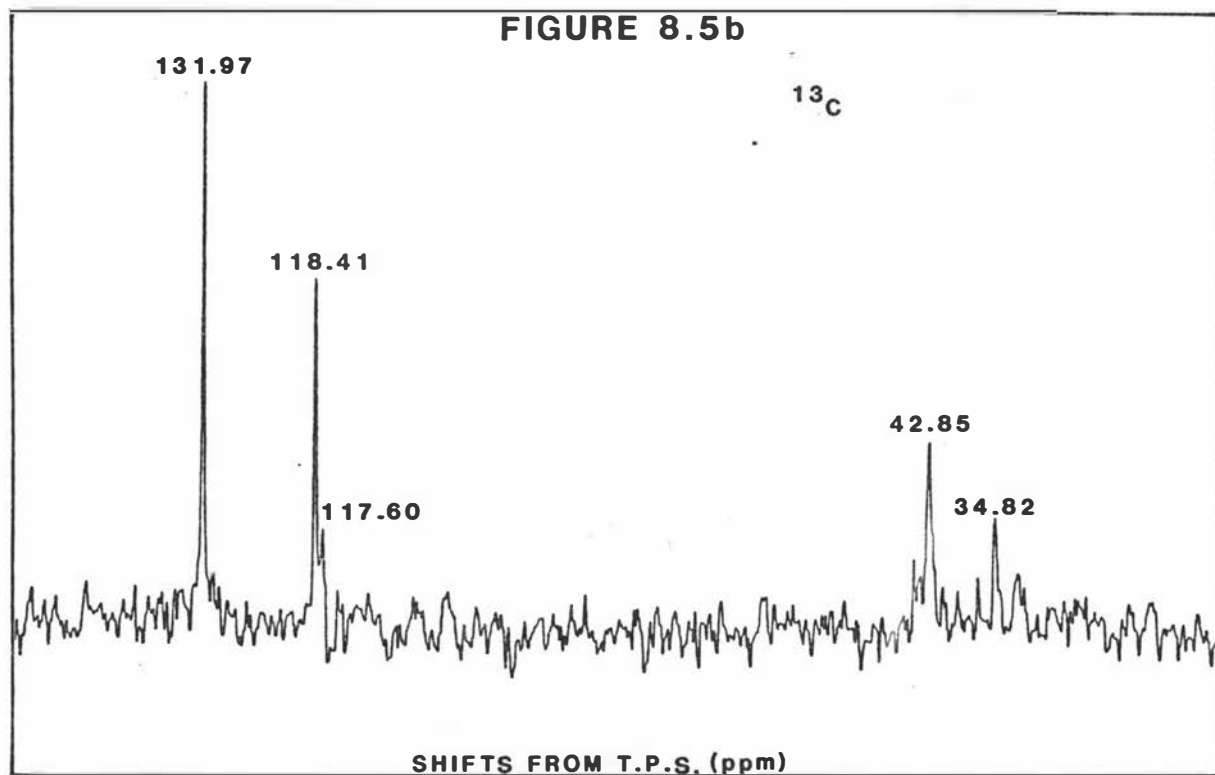
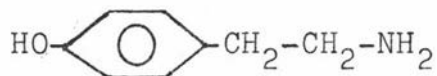


FIGURE 8.5b



commonly known as tyramine.



Examination of the mass spectrum of the P-3 sample (Figure 8.3b) revealed the presence of a considerable amount of low molecular weight material but also a substantial quantity of tyramine.

A sample of tyramine hydrochloride was used to prepare ultra-violet and infra-red spectra while the standard spectrum published by EPA/NIH Mass Spectral Data Base is presented for comparison along with the ultra-violet and infra-red spectra in Figure 8.6a,b and c. The melting point for the pure tyramine was reported to be 142° C (C.R.C. Handbook of Chemistry and Physics) while the measured range of the freeze dried material was 133-138° C.

8.3.4 Activity of tyramine (parahydroxyphenylethylamine).

Tyramine HCl was prepared in a series of aqueous solutions from which the activity was measured in the standard assay (section 3). The concentration range of tyramine chosen was 4.23×10^{-2} M to 2.8×10^{-4} M. The optical density at 280 nm was measured and the chosen range encompassed the optical density value at 280 nm of 11.6 of the freeze dried active sample. The measured percentage enhancements along with the optical density values are shown in Table 8.4 for each of the tyramine-HCl concentrations.

TABLE 8.4 ACTIVITY OF VARIOUS TYRAMINE CONCENTRATIONS

Concentration of tyramine (M x 10 ⁻³)	Optical density at 280 nm	Percentage enhancement
42.3	40.2	+10
25.3	24.1	- 6.6
16.9	16.1	+ 1.6
8.31	7.9	+ 5.0
4.16	3.95	- 6.6
1.66	1.58	+ 1.6
0.83	0.79	- 3.3
0.28	0.26	0.0

FIGURE 8.6a

The ultra-violet absorbance spectrum of a tyramine-HCl sample at acid and neutral pH (—————), and alkaline pH (-----).

FIGURE 8.6b

The infra-red transmittance spectrum of a tyramine-HCl sample obtained by using a KBr pellet.

FIGURE 8.6c

The mass spectrum published by EPA/NIH Mass Spectral Data Base for parahydroxyphenylethylamine (tyramine).

FIGURE 8.6a

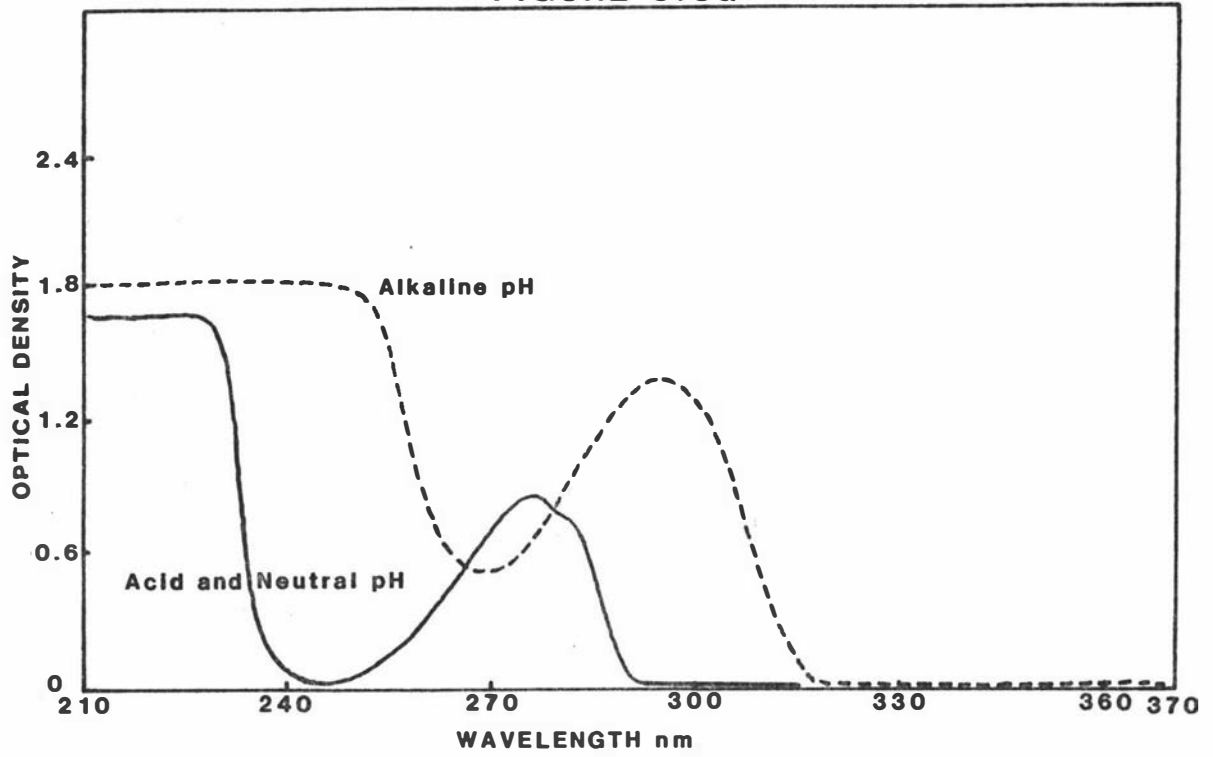


FIGURE 8.6b

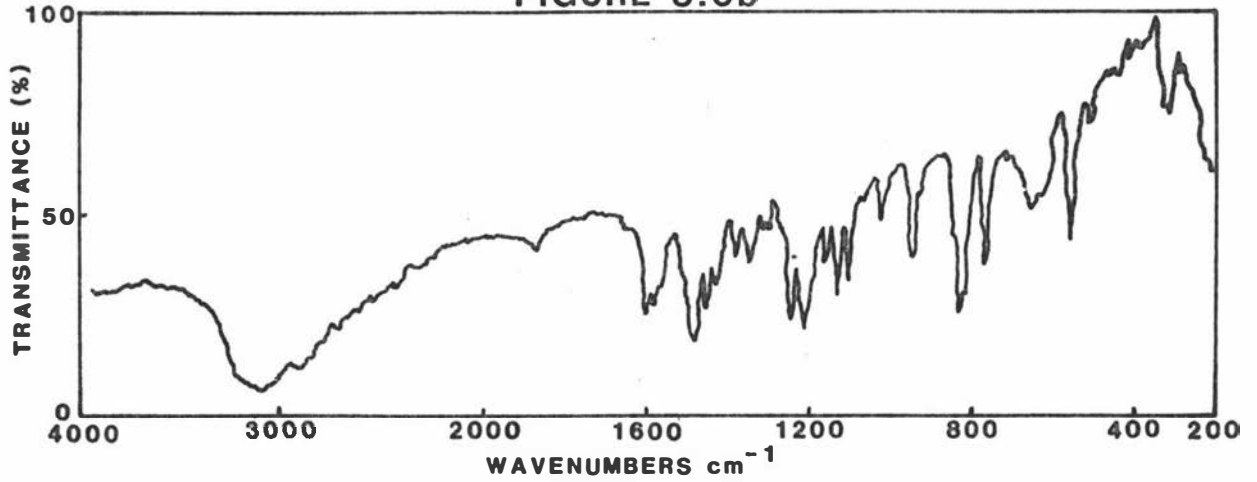
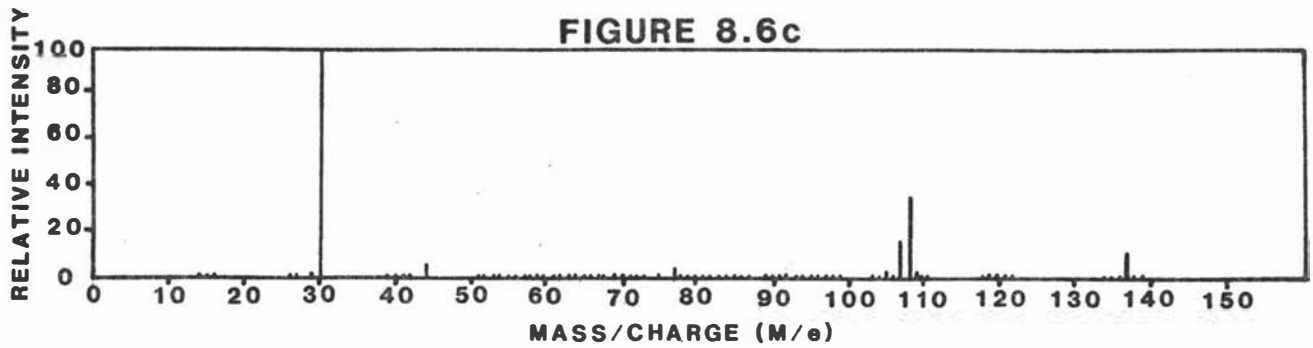


FIGURE 8.6c



These results show that the tyramine-HCl solutions are quite inactive and hence tyramine is not the material in the freeze dried sample which is responsible for the activity despite the fact that it constitutes more than 90% of the material in the freeze dried P-4 sample.

8.3.5 Further analysis and activity determinations.

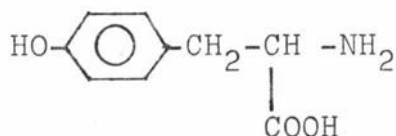
Because of the small amounts of each sample remaining, further examination was possible only by mass spectroscopy.

Two approaches were made, the first of which involved computer matching the unidentified mass/charge peak of 135 seen in Figures 8.3 a and b to phenylisothiocyanate.



The second approach involved acidifying the original freeze-dried material so as to convert any non-volatile salts present which would not produce a mass spectrum into their respective free acid groups. The mass spectrum given by this acid-treated sample again showed virtually only the tyramine spectrum with no additional peaks being observed.

Since tyramine may have been the degradation product of tyrosine and hence this may have been the active factor.



Activity assays were determined for saturated solutions of phenyl isothiocyanate, tyrosine and a solution composed of chromium chloride (5 µg/g) and tyramine (2.53 x 10⁻³ M) to determine whether a tyramine-chromium (III) solution was active. The percentage enhancements determined are shown in Table 8.5.

TABLE 8.5 ACTIVITY OF TYROSINE, PHENYLISOTHIOCYANATE AND A TYRAMINE-CHROMIUM (III) SOLUTION

<u>Sample</u>	<u>Percentage enhancement</u>
Saturated tyrosine solution	+39
Saturated phenyl isothiocyanate solution	-59
Chromium chloride-tyramine solution	-8.0

Although the saturated tyrosine solution does show some slight activity, clearly none of these samples can be the activating material in the P-4 freeze dried sample.

8.3.6 Activity caused by ammonia elution.

The effect of NH_4OH on the measured biological activity when used as the eluent for cation exchange Dowex 50W-X12 resin was examined in the following three systems.

(i) The activities were measured for two chromium chloride solutions each containing around $10 \mu\text{g/g}$ chromium, one of which had been bound to the cation-exchange column and eluted with $0.25 \text{ M NH}_4\text{OH}$.

(ii) The activities were measured for a P-3 solution obtained from high-chromium yeast extract with a chromium concentration of $0.15 \mu\text{g/g}$ before loading onto a Dowex 50W-X12 column and after elution with $0.5 \text{ M NH}_4\text{OH}$.

(iii) A moderately active P-3 sample obtained from high-chromium yeast extract which originally contained $0.1 \mu\text{g/g}$ of chromium was allowed to stand for 5 months at a near neutral pH during which time the chromium concentration was reduced to below detection limits, presumably by the effects of elution. This sample was loaded onto a Dowex 50W-X12 column and eluted with $0.25 \text{ M NH}_2\text{OH}$, again no chromium was detected. Activities were measured before and after the NH_4OH elution.

The activities obtained in each of the above three experiments are shown in Table 8.6. Addition of dilute NH_4OH ($0.25 \text{ M}, 0.1 \text{ cm}^3$) in the standard assay after an initial increase in the manometer level probably due to the release of NH_3 , caused almost complete inhibition of CO_2 production.

Attempts were made to determine the effect of the NH_4^+ ion on the fermentation rate in the standard assay. Some increase in the CO_2 production rate was observed for a $0.048 \text{ M (NH}_4)_2\text{PO}_4$ sample assayed as described in section 3. The percentage enhancement recorded for this sample was +54%. Results reported by Mirsky et al (1980) would suggest though that elution with NH_4^+ salts does not cause increased activity in eluted samples.

TABLE 8.6 ACTIVITY OF SAMPLES BEFORE AND AFTER ELUTION WITH NH₄OH

<u>Sample</u>	<u>Percentage enhancement</u>	<u>Percentage increase on NH₄OH elution</u>	<u>Eluent NH₄OH concentration</u>
Chromium chloride (10 µg/g)	+ 9	-	-
Chromium chloride (10 µg/g)	+ 32	+255	0.25 M
P-3 sample (high-chromium yeast extract, 0.15 µg/g)	+266	-	-
P-3 sample (high-chromium yeast extract, 0.15 µg/g)	+475	+ 79	0.5 M
High-chromium yeast extract sample (moderate activity 0.1 µg/g)	+111	-	-
High-chromium yeast extract sample after 5 month period (moderate activity Cr<0.04 µg/g)	+ 77	-	-
High-chromium yeast extract sample after 5 month period (moderate activity Cr<0.04 µg/g)	+313	+303	0.25 M

8.3.7 Activity relative to chromium concentration and optical density throughout the purification procedure.

Since the activity appeared to be separated from the chromium complexes on the gel filtration column in each of the three samples discussed in section 8.2.1, the activities for a P-4 preparation were determined at suitable points throughout a total preparation. Included here are isolation and purification steps described in this section and in section 4. These percentage enhancements were calculated as a function of chromium and of the optical density at 280 nm (Table 8.7).

TABLE 8.7 ACTIVITY OF P-4 RELATIVE TO CHROMIUM AND OPTICAL DENSITY (A_{280}) THROUGHOUT THE TOTAL PURIFICATION PROCEDURE.

Purification step	Percentage enhancement	Percentage [#]	Percentage
		enhancement 100 pmoles Cr	enhancement 1.0 O.D. at 280 nm
Merck high-chromium yeast extract.	+348	+176	15.6
Elution from Dowex 50W-X12 of Peak II	+228	+115	22.3
Effluent from Dowex 1-X8 column	+294	+ 99	50.2
P-4 peak	+136	+ 69	119
Peak eluted from Dowex 50W-X2	+91.4	+ 46	41
Sephadex G-15 separation	* + 70 ‡ +270	* + 22	‡ 25

N.B. # this has previously been defined as specific activity (section 2).

* values refer to the chromium peak.

‡ values refer to the optical density peak

8.4 DISCUSSION

Chromatography of the three chosen samples (a P-4 peak prepared from high-chromium yeast extract, a P-3 peak prepared from high-chromium yeast extract and a P-3 peak prepared from low-chromium yeast extract) on the Dowex 50W-X2 resulted in a separation of the chromium into two peaks in all three samples. All the chromium loaded was accounted for in the peaks eluted from the column for each sample but as can be seen from Figure 7.1 a,b and c the relative amounts of bound and unbound chromium are not the same for the samples. There seems to be no obvious reason for this difference but the variation in the levels of extraneous material (as reflected by the A_{280} values for the samples) may be effecting the binding of the chromium complexes.

Table 8.1 shows the activities determined for the two peaks obtained from each of the three samples (wherever chromium levels allowed). The important observation is that the activity remains associated with the bound chromium fraction. Where measurements were possible, the unbound peaks show low A_{280} values, well within the limits described in section 3 but also show low enhancement values in the activity assay. The disappointing feature of the bound fractions was the markedly increased A_{280} values which casts some doubt on the origin of the enhancement in the rate of CO_2 production assay.

Figure 8.2a shows the results of gel filtration chromatography carried out on each of the three bound fractions after freeze drying. The unexpected feature for each sample is the fact that the activity profile peak and the chromium peak do not coincide. This is the first piece of information which suggests that the chromium complexes isolated from the yeast extracts are not the major cause of the measured activity. The activity profiles for each of the three samples after gel filtration are quite dissimilar and in each case the highest activity does not correspond either to the conductivity peak or to the optical density peak. The considerably lower value of the enhancement for P-3 isolated from low-chromium yeast extract again confirmed the generally lower activity of material isolated from this

yeast extract source. For the P-3 and P-4 fractions isolated from high-chromium yeast extract, although the observed activity does not directly correspond with the optical density level at 280 nm, the higher activity observed for P-4 samples was accompanied by a higher optical density level.

The suggestion that the chromium complexes contained in the active fractions isolated from yeast extract are not the cause of the measured activity is further reinforced by the data shown in Table 8.7 where for the P-4 sample the percentage enhancement per 100 pmoles of chromium declines throughout the isolation and purification procedure. On the other hand the percentage enhancement observed relative to the optical density at 280 nm shows a maximum value after the isolation of the P-4 fraction from the Dowex 50W-X12 column. If indeed a discrete chromium complex were involved in these isolation and purification procedures then an increase or at least a constant level of percentage enhancement relative to the chromium level would be expected throughout the procedure. On the other hand the percentage enhancement relative to optical density at 280 nm would be expected to decrease throughout the procedure.

Characterisation work was carried out predominantly on the highest active fraction from P-4 since this was most free of salts. The data from Table 8.2 clearly show the activity to be lost on ashing and hence the active material is likely to be organic or at least destroyed at temperatures of 600° C. The data in the text show that on freeze drying 86% of the activity was retained, this value being just outside the error limits of 10% which could have been consistent with complete retention of activity.

The ultra-violet and visible absorption spectra of the sample obtained after freeze drying showed similarities to the spectrum for phenol, particularly in treatment with NaOH. Although the infra-red spectrum is somewhat difficult to interpret there is some evidence for the presence of a C-N bond, particularly the frequencies at 560 and 510 cm^{-1} . The combination of mass spectroscopy and nmr spectroscopy leave no doubt that the major component of P-4 is tyramine. The molecular ion peak at 137 was computer matched to the

formula $C_8H_{11}NO$ which on the basis of the absorption spectrophotometric results must contain the $HO-C_6H_4-$ group. The nmr spectra for both 1H and ^{13}C shows the presence of two methylene groups attached para to the phenolic-OH group which leaves an NH_2 group unaccounted for. Hence it can be concluded that the material is p-hydroxyphenylethylamine i.e. tyramine. The melting point of the sample confirmed this identification as did comparison with authentic spectra for tyramine (Figure 8.5 a, b and c). The absence of any extraneous peaks in the ^{13}C spectrum for the P-4 sample shows that it is substantially free from carbon containing impurities.

It is of interest to note that tyramine would be expected to behave like P-3 and P-4 on ion exchange chromatography since at pH 3.5 it will be positively charged and hence will bind to Dowex 50 ion exchange resins whereas at pH 8.0 it will be neutral since the pKa of the -OH group in tyramine will be approximately 9.0.

The percentage enhancement values shown in Table 8.4 clearly show however that over the range of tyramine-HCl concentrations determined (4.23×10^{-2} M to 2.8×10^{-4} M) tyramine shows no activity. As this range of concentrations encompassed the likely tyramine concentration for the P-4 sample it appears that the observed activity is not due to tyramine-HCl. At the pH of 5.75 at which the standard assay is conducted tyramine-HCl would exist primarily as the unprotonated amine and hence its predominant form would be that of the free tyramine found in the P-4 sample. The mass spectrum run on P-3 (Figure 8.3b) shows that tyramine is also the major organic component of this fraction but since tyramine itself is inactive there must be some other as yet unidentified material in both P-3 and P-4 which gives rise to the observed activity.

The difficulty of determining the chemical composition of an unidentified active material in a sample of only a few milligrams which is probably up to 90% tyramine is very great. Characterisation under these circumstances seemed possible only from the mass spectral data but the only further possibility suggested was the unlikely material, phenylisothiocyanate which was computer matched to the

unknown 135 mass/charge peak. Activity analysis of this along with tyrosine an amino acid of which tyramine was a possible degradation product again revealed no activity which would correspond to the enhancement seen for P-4.

8.4.1 Activity caused by ammonia elution.

The results in Table 8.6 show that elution from a Dowex 50W-X12 column using NH_4OH produces samples with increased activity. The increase in percentage enhancement of a chromium chloride sample after ammonia elution is only marginal and may not be a significant result. The other increases observed however are quite major with a P-3 sample containing 0.15 $\mu\text{g/g}$ Cr having its percentage enhancement increased from +266 to +475. This increase in activity was shown not to be dependent on chromium by the results given in Table 8.6. Here a moderately active sample from which all the chromium had been removed (within detection limits) presumably by elution showed a slightly lesser degree of activity after standing for the 5 month period. This sample though was still moderately active (+111 percentage enhancement). On elution from a Dowex 50W-X12 column with NH_4OH however the observed enhancement increased considerably. No chromium was detected in the eluted sample. Taken collectively these results clearly indicate that ammonia elution from a Dowex 50W-X12 column causes increases in activity both with and without the presence of chromium. These results also show that the activity measured from isolated high-chromium yeast extract fractions such as the one used here may not be associated with a chromium complex at all but may be associated with other material isolated with the chromium fraction. This material, in some way as yet unspecified, increases the percentage enhancement in the rate of CO_2 evolution when treated with NH_4OH .

Similar results to those described here were found by Kumpulainen et al (1979). Using the assay system described by Anderson et al (1978) these workers measured the activity of extracts from a range of biologically active materials including brewer's yeast and bovine liver. The biological activity of 50% ethanol and 0.1 M NH_4OH extracts of the samples was compared. In all cases the activity determined

from the 0.1 M NH_4OH extract was greater than the 50% ethanol extract and for the 2 brewer's yeast samples assayed the percentage increases were 95% and 81%. These values correspond to the increases observed in Table 8.6 for the yeast extracts of 79% and 306%. Hence in both assay systems NH_4OH either by extraction or by elution seems to have a very real effect on the measured biological activity of the sample.

In conclusion it appears that there is some material present in the fractions isolated from P-3 and P-4 which is causing the activity in the yeast fermentation assay, but this material does not contain chromium. There is also an important interaction between this material and NH_4OH which is not presently understood but it seems significant that for all reported GTF fractions, other than those studied in this thesis, NH_4OH has been used at some stage in the preparation. This includes as well the preparation described by Toepfer et al (1977) for the synthesis of biologically active chromium complexes. This activity does not seem to be due simply to the presence of NH_4OH or NH_4^+ ions since the percentage enhancements so far determined for these cannot account for the observed activity of the active samples.

Currently then the identity of the active factor (which may be GTF) is completely unknown and requires a further study before any comment about its identity can be made. It would be well to repeat this work in a laboratory with access to one of the other bioassays in order to see whether the results obtained here are general or only apply to the yeast fermentation assay. In the author's view it is likely that the results obtained in this thesis do apply generally and therefore chromium does not have any beneficial effects or importance in glucose metabolism and diabetes.

SECTION 9SUMMARY AND CONCLUSIONS DRAWN AS TO THE IDENTITY OF THE
GLUCOSE TOLERANCE FACTOR.

In the 24 years since the proposal for the existence of the glucose tolerance factor was first made, progress in the field has been slow. Very little more information about the chemical composition of the proposed factor is known now than was known in 1957. Rapid development in this field has been hindered, among other things, by the lack of an assay system capable of measuring true glucose tolerance factor activity for a large number of samples. Clearly assays based on the defined function of GTF (the restoration of impaired glucose removal rates in rats) would be most restrictive with regard to the total number of samples which could be routinely tested.

In the early stages of the work undertaken in this thesis an activity assay was not available, hence the direction of the work was aimed at isolating, purifying and characterising the major chromium fractions associated with whole yeast cells. The problems associated with detecting the very low natural levels of chromium in brewer's yeast proved an insurmountable obstacle to attempts to devise an isolation procedure for the various possible chromium complexes, since only the major fractions could be detected at all. Yeast was grown on a chromium-51-containing medium in an attempt to label the chromium complexes in the yeast so that even small amounts of these compounds could be monitored during the development of purification procedures. The medium was also supplemented with chromium chloride in order to try to increase the amounts of all the chromium-containing fractions in the yeast. Supplementation proved to be partially successful in increasing the levels of chromium which could be extracted from the yeast. This enabled an anionic complex from the yeast, to be isolated purified and identified as a chromium-glucose complex produced by interaction of chromium with the growth medium.

With the advent of a tested assay system provided by Professor Zvi Dori, for glucose tolerance factor activity

the screening of a large number of samples for activity became possible and with the availability of Merck yeast extract, with a relatively high natural chromium content, isolation and partial purification of chromium complexes became a possibility. The commercial yeast extract ensured the solubilization of all the chromium complexes in yeast, which overcame one of the main problems inherent in using whole yeast cells, since when these were extracted, varying percentage extractions were achieved, but never greater than 55%.

Having access to a suitable assay system and a convenient, commercially available starting material enabled the systematic isolation of all the chromium fractions in the yeast extract. The eleven fractions obtained are described in terms of their ionic characteristics on ion-exchange columns under the elution conditions used. Their activity was determined from the standard assay in which the increased rate of CO_2 production for an active sample was compared with the rate of CO_2 production for a phosphate buffer control during fermentation under anaerobic conditions. On the basis of this isolation scheme several important conclusions emerge:

(1) There are a large number of chromium fractions with a diverse range of ionic forms isolatable from yeast (eleven fractions in total; 3 anionic, 4 cationic, 4 amphoteric and at least 1 neutrally charged which was not isolated).

(2) The cationic fractions were all active to varying degrees while the amphoteric and anionic fractions show low activities (with the possible exception of the moderate activity shown by the N-2 fraction).

(3) All fractions were still associated with quite high levels of extraneous material as indicated by the generally yellow appearance of most of the fractions and the moderately high optical density levels at 280 nm for all fractions. Clearly further purification was required before a meaningful chemical characterisation could be attempted.

The isolation scheme enabled the origin of the various isolated chromium fractions to be investigated. From a

comparison of the fractions isolated from supplemented medium, spent supplemented medium and extracted yeast it is apparent that most of the isolated complexes are products of reaction with either the medium or elution reagents. These findings suggested three important criteria to be fulfilled before any complex can be considered as a true glucose tolerance factor. These are:

- (1) the isolated fraction must not be a product of reaction with the medium or elution reagents.
- (2) the fraction must be retained by the yeast.
- (3) The fraction must show activity in the standard yeast assay.

On the basis of these criteria only one fraction, P-3, emerges as a potential candidate for identification with the glucose tolerance factor. Fraction P-4 satisfies the first and third criteria, but does not meet the second since it does not appear to be retained by the yeast. These two fractions were the only ones which warranted further purification and investigation.

Various reports have appeared concerning the activity of chromium-containing fractions isolated from various biological materials of nutritional importance. To investigate a range of these by the six column procedure described in section 4 would clearly have proved to be very time consuming. The short isolation procedure described in section 6 was therefore used and enabled the rapid isolation of cationic fractions from a range of biological materials to be achieved. A wide range of cationic fractions are obtained from the materials chosen all with different elution profiles. This indicates that the cationic chromium complexes in the different biological materials are not the same. This is most apparent for the samples showing the highest activities since pork kidney powder and P-3 (from the high chromium yeast extract) are eluted at quite different positions from the cation-exchange column. It is therefore apparent that the glucose tolerance factor activity at least as determined by the yeast assay does not result from a single chromium-containing species.

Since brewer's yeast is supposedly the best source of GTF it is reasonable to assume that it would play some important role in the yeast's biochemistry or growth. Consequently it would be expected that some chromium complex would be found to be an integral part of the yeast cell. However comparison of the levels of cationic chromium isolated from high-chromium yeast extract, low-chromium yeast extract (which were similar), and the very low amount found in the chromium-51 yeast system used in section 5 mitigate against the idea that a cationic chromium complex is necessary for the normal metabolism of glucose by yeast or for yeast growth.

Results from pre-incubations of chromium chloride and yeast in the various media and buffer solutions indicate that yeast is not able to form any biological complexes from chromium chloride during its growth phase which are capable of increasing its fermentation rate (at least over the 48 hour pre-incubation period used). However pre-incubation of yeast in a medium containing yeast extract does show an increased fermentation rate. This enhancement is similar to that observed when P-3 is added to the yeast fermentation assay in phosphate buffer without any pre-incubation. On the basis of these results it is unlikely that the enhancement can be due to the presence of chromium complexes. In fact during the isolation of P-3 from crude yeast extract the activity relative to a fixed amount of chromium actually declines.

During the course of this work the yeast used in the standard assay was rendered chromium deficient through 20 to 30 growth cycles by the methods described in section 3.2.2. This yeast, however, still survived and grew at apparently the same rate for the last assay growth as for the first. This again shows that chromium does not play an essential role in the metabolism and growth of yeast and thus can be of little significance to it.

The results reported in section 8 clearly support this conclusion since on further purification of P-3, the factor which causes the activity in the yeast assay was found not to contain chromium. Unfortunately the identity

of the material which does cause the activity remains completely unknown. Obviously more work needs to be done to collect and identify the true active factor rather than inactive chromium complexes.

A consideration throughout this work is the question of whether the yeast assay is in fact measuring the same activity as do the other reported assays such as the rat fat pad assay. However a good indication that the two assay systems do in fact measure the same activity is provided by the close correlation between the results published by Toepfer et al and those demonstrated here on what are apparently similar biological materials.

Although it must be conceded that the in vitro rat fat pad and isolated adipocyte assays might respond in different ways to the chromium complexes isolated in this thesis, in view of the preceding discussion it would seem unlikely that material which shows every evidence of being incidental to the yeast, could be related to the glucose tolerance factor. However P-3 and P-4 should be tested in a laboratory which has the facility to perform these assays. Also the material obtained from pork kidney powder (as described in section 7) should be subjected to further investigation.

The results obtained in the present work do support the existence of a factor which can be isolated from yeast extract and which is capable of increasing the fermentation rate of yeast under anaerobic conditions. This factor has several properties in common with the supposed glucose tolerance factor defined by Schwarz and Mertz (1957). Both factors are isolated from yeast or yeast extract and both factors are cationic. These similarities raise the possibility that the two factors are identical. The one apparent point of disagreement is the fact that the active factor isolated here clearly does not contain a chromium atom. The suggestion that the glucose tolerance factor does not contain a chromium atom must be examined in the light of previously published literature.

To date all effects described for GTF preparations from yeast extract could equally well be consistent with

the existence of a non-chromium containing factor. Since no preparation of GTF produced thus far is a pure chromium complex, extraneous material must obviously be present and could account for the effects now commonly ascribed to chromium. It is within this extraneous material that the glucose tolerance factor is probably located.

The effects attributed to supplementation of chromium (III) salts must be critically examined. Where human patients with disorders involving hyperglycemia have been treated with either short or long term supplementation of chromium salts, widely conflicting results have been obtained. Some researchers have reported results of doubtful significance showing improvement in a hyperglycemic condition (Hopkins et al (1968), Levine et al (1968)) while others have reported the effect of chromium supplementation on such a condition to be nil (Carter et al (1968), Sherman et al (1968)). Certainly at this point in time no researcher has been able to propose unequivocally that chromium supplementation is capable of causing improvement in a hyperglycemic condition in humans.

Much of the evidence supporting the involvement of chromium in the glucose tolerance factor has arisen from supplementation of chromium salts on rats fed a low-chromium diet of American Torula yeast. A consistent result obtained by workers using this procedure has been that the impaired glucose removal rate of rats has been restored to normal by chromium (III) supplementation (Mertz and Schwarz (1959) Mertz et al (1965)). In all cases however, the glucose removal rates have been calculated in terms of the reduction of excess blood glucose (total blood glucose - fasting blood glucose) rather than the more accepted method of measuring the reduction in the total blood glucose. Woolliscroft and Barbosa (1977) have challenged the results obtained by the researchers using the reduction of excess blood glucose method and further state that differences observed in glucose removal rates from such calculations may result from a methodological artifact.

Further contradiction has arisen from a report published by Gubler et al (1975) in which they have observed

that rats grown on the chromium-deficient diet of *Torula* yeast while becoming chromium deficient do not develop the symptom described by Schwarz and Mertz (1957) of an impaired glucose removal rate. (The method of determination of the glucose removal rate was not stated.)

Taken collectively these results must cast extreme doubt firstly, as to whether chromium is involved with the GTF as defined by Schwarz and Mertz (1957), and secondly, as to whether the factor exists at all.

In conclusion the results reported in this thesis clearly indicate that chromium has no biological significance in yeast. There is however an unidentified non-chromium containing factor in yeast capable of causing an increased fermentation rate in yeast. As the previous work conducted on the effects of chromium salts is based on somewhat shaky experimental evidence and the work undertaken in this thesis has shown several direct correlations of the yeast assay with other alternative 'GTF' activity assays, the proposal can be made that the unidentified non-chromium containing factor in yeast and GTF, as defined by Schwarz and Mertz (1957), are in fact equivalent. This non-chromium containing complex is capable of increasing the fermentation rate of yeast and by implication possibly has an effect on the glucose removal rate of rats which have been grown on an American *Torula* yeast diet.

It is apparent that considerable work has yet to be done. Isolation and purification schemes must be designated to monitor activity rather than chromium. Only by such methods can the identification of the true glucose tolerance factor be made and only then will the true effect of GTF be determined.

APPENDIX ISUPPLIERS OF CHEMICALS

Acetic acid	May and Baker (M & B)
Acrylamide	British Drug Houses (B.D.H.)
Ampholyte solution	L K B (Sweden)
Ammonia	M & B
NH_4Cl	Koch Light
$(\text{NH}_4)_2\text{SO}_4$	B D H
$\text{Ba}(\text{OH})_2$	B D H
Biogel P-2	Bio-Rad
Biotin	Sigma
Black peppercorns	Sanitarium
H_3BO_3	M & B
Brewer's yeast	'Lion' Breweries Fermentation Industries
Butan-1-ol	B D H
$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	Riedel de Haen (R de H)
Ca-pantothenate	Sigma
Citric acid	M & B
Chromium-51 (chloride)	Amersham
$\text{CrCl}_3 \cdot 6\text{H}_2\text{O}$	R de H
$\text{Cr}(\text{NO}_3)_3$	B D H
$(\text{Cr})_2(\text{SO}_4)_3$	B D H
$\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$	B D H
L-cysteine-HCl	B D H
Dowex 1-X8	Bio-Rad
Dowex 50W-X2	Bio-Rad
Dowex 50W-X12	R de H

Ethanol	B D H
Ethanolamine	B D H
$\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$	B D H
Folic acid	Sigma
Fructose	M & B
Galactose	M & B
Glucose monohydrate	M & B
L-glutamic acid	Sigma
Glycine	M & B
HCl	Ajax
Inositol	Difco
Maltose	M & B
$\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$	R de H
MnCl_2	M & B
NN' methylene bisacrylamide	B D H
Molasses	Q.M.C. (Australia)
α naphthol	M & B
Ninhydrin	B D H
HNO_3	Ajax
Phenol	B D H
P_2O_5	B D H
Polyethylene glycol	B D H
Pork kidney powder	Sigma
KCl	M & B
$\text{K}_2\text{Cr}_2\text{O}_7$	Koch Light
KH_2PO_4	R de H
K_2HPO_4	R de H
KI	M & B
$\text{K}_2\text{S}_2\text{O}_8$	B D H

Pyridoxine -HCl	B D H
Sage	Sanitarium
Sephadex G10	Sigma
Sephadex G25	Sigma
Split green peas	Sanitarium
Sodium acetate	M & B
NaCl	M & B
NaH_2PO_4	R de H
Na_2HPO_4	R de H
$\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$	Baker
Na_3PO_4	R de H
Na_2SO_4	M & B
Sucrose	Baker
H_2SO_4	Ajax
NNNN' tetramethylethylene- diamine	B D H
Thiamine-HCl	Sigma
Tris (hydroxymethyl) aminomethane	Sigma
Vitamin B12 (cyanocobalamin)	Sigma
CM 23	Whatman
DE 23	Whatman
Wheat bran	Sanitarium
Yeast extract	Merck Oxoid B B L Difco Sanitarium (Marmite)
Yeast nitrogen base without amino acids	Difco
ZnCl_2	M & B

REFERENCES

- Alvarez, R., Wolf, W. and Mertz, W. (1979), Chromium in Nutrition and Metabolism (Editors D. Shapcott and J. Hubert), 85-93.
- Anderson, R.A., Brantner, J.H. and Polansky, M.M. (1978), Journal of Agriculture and Food Chemistry, 26 1219-1221.
- Anderson, R.A., Polansky, M.M. and Brantner, J.H. (1979), Chromium in Nutrition and Metabolism (Editors D. Shapcott and J. Hubert), 181-187.
- Anderson, R.A. (1981), The Science of the Total Environment, 17, 13-29.
- Angyal, S.J. (1973), Carbohydrates in Solution (Editor R.F. Gould), 106-120.
- Behne, D. and Diehl, F. Nuclear Activation Techniques in the Life Sciences (IAEA-SM-157/11), 407.
- Bellusco (1977 thesis), Evidence for chromium-insulin interaction. (Order No. 77-22, 331).
- Burkeholder, J.N. and Mertz, W. (1967), Proceedings of the 7th International Congress on Nutrition, V, 701-705.
- Campbell, W.J. and Mertz, W. (1963), American Journal of Physiology, 204, 1028-1030.
- Carter, J.P., Kattab, A., Abd-El-Hadi, K., Davis, J.T., El Gholmy, A. and Patwardhan, V.N. (1968) The American Journal of Clinical Nutrition, 21(3), 195-202.
- Christian, G.D., Knoblock, E.C., Purdy, W.C. and Mertz, W. (1963), Biochimica et Biophysica Acta, 66, 420-423.
- Davidson, I.W.F. and Blackwell, W.L. (1968), Proceedings of the Society for Experimental Biology and Medicine, 127, 66-70.

- Davidson, I.W.F. and Burt, R.L. (1973), *The American Journal of Obstetrics and Gynaecology*, 116(5), 601-608.
- Doisy, R.J. (1963), *Endocrinology*, 72, 273-278.
- Evans, G.W., Roginski, E.E. and Mertz, W. (1972), *Federation Proceedings of the Federated American Societies For Experimental Biology*, 31(1), 264.
- Freund, H., Atamian, S. and Fischer, J.E. (1979), *Journal of the American Medical Association*, 241(5), 496-498.
- Ghafghazi, T., Maghbareh, A. and Barnett, R. (1979), *Toxicology*, 12, 47-52.
- Ghafghazi, T., McDaniel, M.L. and Lacy, P.E. (1980), *Diabetologia*, 18, 229-232.
- Glinemann, W.H., Feldman, F.J. and Mertz, W. (1966), *Science* 152, 1243-1245.
- Glinemann, W.H. and Mertz, W. (1966), *Metabolism*, 15(6), 510-520.
- Gubler, C.J., Izatt, R.M., Eatough, D.J., Murdock, D.M. and Christensen, J.J. (1975), *Proceedings of the 10th International Congress on Nutrition, (Kuoto, Japan)*, 566.
- Gurson, C.T. and Saner, G. (1971), *The American Journal of Clinical Nutrition*, 24, 1313-1319.
- Gurson, C.T. and Saner, G. (1978), *The American Journal of Clinical Nutrition*, 31, 1158-1161.
- Guthrie, B.E. (1975), *New Zealand Medical Journal*, 82, 418-424.
- Hopkins, L.L., Ransome-Kuti, O. and Majaj, A.S. (1968) *The American Journal of Clinical Nutrition*, 21 203-211.

- Jeejeebhoy, K.N., Chu, R.C., Marliss, E.B., Greenberg, G.R. and Bruce-Robertson, A. (1977), *The American Journal of Clinical Nutrition*, 30, 531-538.
- Kienle, K.H., Ditschuneit, H.H., Opferkuch, R. and Seeling, W. (1979), *Chromium in Nutrition and Metabolism* (Editors D. Shapcott and J. Hubert), 189-197.
- Kirchner, J.G. (1978), *Thin layer chromatography* (2nd Edition), (N.Y. Wiley).
- Kraszeski, J.L., Wallach, S. and Verch, R.L. (1979), *Endocrinology*, 104, 881-885.
- Kumpulainen, J., Koivistoinen, P. and Lahtinen, S. (1978), *Bioinorganic Chemistry*, 8(5), 419-429.
- Kumpulainen, J.T., Wolf, W.R., Veillon, C. and Mertz, W. (1979), *Journal of Agriculture and Food Chemistry*, 27, 490-494.
- Kumpulainen, J., Anderson, R.A., Polansky, M.M. and Wolf, W.R. (1979a), *Chromium in Nutrition and Metabolism* (Editors D. Shapcott and J. Hubert), 79-84.
- Levine, R.A., Streeten, D.H.P. and Doisy, R.J. (1968), *Metabolism*, 17(2), 114-125.
- Liu, V.I.K. and Morris, J.S. (1978), *The American Journal of Clinical Nutrition*, 31, 972-976.
- Mathur, R.K. and Doisy, R.J. (1972), *Proceedings of the Society for Experimental Biology and Medicine*, 139, 836-838.
- Mertz, W. and Schwarz, K. (1959), *American Journal of Physiology*, 196, 614-618.
- Mertz, W., Roginski, E.E. and Schroeder, H.A. (1965), *Journal of Nutrition*, 86, 107-112.

- Mertz, W., Roginski, E.E. and Reba, R.C. (1965a), American Journal of Physiology, 209, 489-494.
- Mertz, W. (1969), Physiological Reviews, 49(2), 163-239.
- Mertz, W. and Roginski, E.E. (1971), Newer Trace Elements in Nutrition (Editors W. Mertz and W.E. Cornatzer), 123-153.
- Mertz, W. (1975), Nutrition Reviews, 35(5), 129-135.
- Mirsky, N., Weiss, A. and Dori, Z. (1980), Journal of Inorganic Biochemistry, 13, 11-21.
- Onkelinx, C. (1977), American Journal of Physiology, 232(5), E478-E484.
- Orstein, L. (1964), Annals of the New York Academy of Science, 121, 321.
- Parr, R.M. (1977), Journal of Radioanalytical Chemistry, 39, 421-433.
- Pekarek, R.S., Hauer, E.C., Rayfield, E.J., Wannemacher, R.W. and Beisel, W.R. (1975), Diabetes, 24, 350-353.
- Preston, A.M., Doisy, R.J., Preston, M. and Freeman, J.N. (1976), Journal of Nutrition, 106, 1391-1397.
- Rabinowitz, M.B., Levin, S.R. and Gonick, H.C. (1980), Metabolism, 29(4), 355-364.
- Rollinson, C.L., Rosenbloom, E. and Lindsay, J. (1967), Proceedings of the 7th International Congress on Nutrition, V, 692-697.
- Roginski, E.E. and Mertz, W. (1969), Journal of Nutrition, 97, 525-530.
- Roginski, E.E., Toepfer, E.W., Polansky, M.M. and Mertz, W. (1971), Federation Proceedings of the Federated American Societies for Experimental Biology, 30(1), 238.

- Schroeder, H.A. (1966), *Journal of Nutrition*, 88, 439-445.
- Schroeder, H. (1968 review), *The American Journal of Clinical Nutrition*, 21(3), 230-244.
- Schroeder, H.A., Nason, A.P. and Tipton, I.H. (1970), *Journal of Chronic Diseases*, 23, 125-142.
- Schwarz, K. and Mertz, W. (1957), *Archives of Biochemistry and Biophysics*, 72, 515-518.
- Schwarz, K. and Mertz, W. (1959), *Archives of Biochemistry and Biophysics*, 85, 292-295.
- Shapcott, D. (1980), *Diabetologia*, 19, 164.
- Sherman, L., Glennon, J.A., Brech, W.J., Klomberg, G.H. and Gordon, E.S. (1968), *Metabolism*, 17(5), 439-442.
- Suomalainen, H. and Oura, E. (1971), *The Yeasts*, Volume 2 (Editors A.J. Rose and J.S. Harrison), 3-74.
- Tipton, I.H. and Cook, M.J. (1963), *Health Phys.*, 9, 103.
- Tipton, I. H. Schroeder, H.A. and Perry, H.M. (1965), *Health Phys.*, 11 403-451.
- Toepfer, E.W., Mertz, W., Roginski, E.E. and Polansky, M.M. (1973), *Journal of Agriculture and Food Chemistry*, 21(1), 69-73.
- Toepfer, E.W., Mertz, W. Polansky, M.M., Roginski, E.E. and Wolf, W.R. (1977), *Journal of Agriculture and Food Chemistry*, 25, 162-166.
- Tuman, R. W. and Doisy, R.J. (1977), *Diabetes*, 26, 820-826.
- Tuman, R. W., Bilbo, J.T. and Doisy, R.J. (1978), *Diabetes*, 27, 49-56.

Votava, H.J., Hahn, C.J. and Evans, G.W. (1973), Biochemical and Biophysical Research Communications, 55, 312-319.

Wise, A. (1978), American Medical Association Journal, 240(19), 2045-2046.

Woolliscroft, J. and Barbosa, J. (1977), Journal of Nutrition, 107, 1702-1706.