THE DETERMINATION OF CHROMIUM IN HUMAN SERUM AND URINE.

by

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Abstract.

The Determination of Chromium in Human Serum and Urine.

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A critical evaluation of the published data for chromium levels in serum and urine shows major discrepancies, indicating that further work to establish normal values for these parameters is necessary.

Methods have been developed for the determination of total protein-bound, and alpha-2-globulin-bound, chromium in serum, and chromium in urine.

The sample pretreatment for serum is based on concurrent protein precipitation and dehydration using propan-2-ol for the total protein-bound chromium, and 0.5M hydrochloric acid in propan-2-ol for the alpha-2-globulin-bound metal. The precipitates are washed in propan-2-ol, then in toluene. Urine aliquots equivalent to 20 umol creatinine are dried at 75 ^{0} C. Acetic acid (plus 7.5% v.v. sulphuric acid) and 1,1,1,5,5,5-hexafluoropenta-2,4-dione are added to the serum precipitates and urine residues. The chromium in the specimens is converted to the beta-diketonate at 75 ^{0} C, and the complex extracted with petroleum spirit. The excess diketone is removed by washing with phosphate buffer. The chromium is back-extracted with ammonia in EDTA solution and, after an evaporation step, dissolved in ammonium acetate solution.

Atomic absorption spectrometry with electrothermal atomisation is used to measure the chromium, and because of the matrix simplification achieved, background correction is not necessary.

The mean results on serum from normal subjects were 0.11 ug/L for total protein-bound chromium, and 0.07 ug/L for alpha-2-globulin-bound chromium. The detection limit was 0.03 ug Cr/L for both serum parameters. The mean normal value for urinary chromium was 0.44 ug/ 10 mmol creatinine, with a detection limit of 0.05 ug Cr/ 10 mmol creatinine. The analytical relative standard deviations for the three parameters at the above levels were : 7%, 9% and 13% respectively.

The serum chromium parameters did not show a significant response to a glucose challenge.

Precautions against sample contamination were taken, and techniques for reagent purification, and equipment cleaning to a high standard were developed.

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Abbreviations used.

AAS.	Atomic absorption spectrometry.
EDTA.	Ethylenediaminetetra-acetic acid.
ETA.	Electrothermal atomisation.
GTF.	Glucose tolerance factor. (see chapter II, section 2.4.).
Hfacac.	1,1,1,5,5,5-Hexafluoropenta-2,4-dione.
OGTT.	Oral glucose tolerance test.
RSD.	Relative standard deviation.
SD.	Standard deviation.
Tfacac.	1,1,1-trifluoropenta-2,4-dione.

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CHAPTER I.

TRACE METALS IN HUMAN NUTRITION AND DISEASE.

This chapter is an introduction to trace metals and in particular to those aspects which are important to the clinical chemist. The primary consideration is whether or not the determination of a particular trace metal in a sample from a patient under investigation has any value. That is : will the result of the trace metal measurement influence the treatment of the patient to his or her advantage? The analytical result of a determination will clearly need to to be of acceptable precision and accuracy. Furthermore, reliable normal results are necessary for comparative purposes. Elements which are either essential for normal metabolism or important toxic agents are clearly possible candidates for useful measurements.

1.1. TRACE METALS.

Trace elements are defined by IUPAC (1) as those present at less than 100 mg/L. Table 1.1 lists trace metals in order of decreasing reported concentrations in human serum or plasma. The data in the table are taken from the 1980 review "Normal levels of Trace Elements in Human Blood Serum or Plasma." by Versieck and Cornelius (2). The original results from the review have been processed to present a balanced report free from distortion by a few extreme values. The overall means listed for particular metals are the average of the reported means after the exclusion of outliers (outliers being defined as values outside three standard deviations). Reiteration was carried out until the data being processed were free from outliers. The values excluded were in all cases high outliers. Furthermore the ranges of the processed data for each metal were divided into ten equal intervals and the modal decile determined. The median, modal decile (numbered in ascending order) and percentages of reported means within the modal decile are also presented in table 1.1. Iron was not included in Versieck's review, presumably because iron determinations have reached a reasonable degree of reliability, and the normal range is no longer in dispute. However, iron has been added to the table for comparative purposes, the reported values are from "Biochemical Values in Clinical Medicine" (3).

The mean normal value reported by different workers should be in

general similar. Clearly data presented for the establishment of normal levels should not be taken from isolated ethnic groups, closed societies on unusual diets, populations exposed to high environmental levels of a particular metal, or other unrepresentative population samples. Furthermore homeostatic mechanisms exist for the control of essential trace metal concentrations. The tabulated results were all reported to be from apparently healthy adults and the number of subjects sampled by the majority of contributing analysts was such that a small range of values would be confidentially expected. The wide scatter seen for the majority of metals could not be explained by biological variation but clearly illustrates the general unsatisfactory state of trace metal determinations on human serum and plasma. The modes for all trace metals for which adequate data were available in Versieck's review, were with one exception, at the lowest decile. This markedly skew distribution is consistent with contamination of the sample being the major problem.

Two pairs of metals, one pair present in relatively high concentrations, the other pair being in the ultratrace region, illustrate the influence of element availability for comtamination purposes on the ease of an accurate analysis. The first pair to be considered are zinc and copper.

Zinc determinations appear to be less satisfactory than would be expected from the comparatively high overall mean concentration. The reported means vary from 840 to 3090 µg/L. However 73% of the results are between 840 and 1290 µg/L. The major problem appears to be contamination of the samples. Serum zinc samples are particularly susceptible to contamination before separation because of the high zinc content of all cells, including thrombocytes. The zinc concentration in erythrocytes is 12 times that in plasma, and that in leucocytes is 25 times that in erythrocytes. Reimold et al (4) investigated contaminations interfering with the determination of plasma zinc and found polystyrene tubes, vacutainers and glass tubes to be unsatisfactory for both sample collection and storage. Polypropylene tubes with polythene stoppers were acceptable. Furthermore, the non-water wettable plastic surface if used with a suitable anticoagulant should help to prevent zinc release from lysed thrombocytes. However Reimold did find anticoagulants from some sources to have significant zinc concentrations. Furthermore many paper tissues

commonly used for wiping pipette tips were found by Reimold to have a high zinc content. The mean normal plasma zinc was found by Reimold to be 877 µg/L.

TABLE 1.1.

Ranges of reported means of trace metal concentrations in human serum or plasma.

Metal.	Concentration µg/L. Mean.	Median.	Range.	Decile of mode.	% in mode.	Mean n.
Zinc.	1285	1965	840 - 3090	1	51	77
Copper.	1130	1131	540 - 1721	5	39	74
Rubidium.	193	221	52 - 390	Insuff. data.		30
Aluminium.	102	202	3.72 - 400	1	62	83
Tin.	57	67	30 - 103	Insuff. data.		124
Arsenic.	39	96	1.07 - 190	1	71	20
Nickel.	20	32	1.6 - 621	1	43	41
Chromium.	17	28	0.14 - 55	1	30	61
Molybdenum.	13	17	0.58 - 34	Insuff. data.		55
Vanadium.	12	29	0.02 - 57	1	57	43
Cobalt.	11	36	0.00 - 72	1	71	40
Manganese.	11	17	0.54 - 34	1	35	43
Silver.	9.3	13	0.68 - 25	Insuff. data.		45
Mercury.	5.7	7.9	1.8 - 14	1	50	24
Antimony.	2.5	2.9	0.52 - 5.2	Insuff. data.		35
Caesium.	1.0	1.0	0.74 - 1.3	Insuff. data.		47
Iron.	1375		790 - 1960			

Note. 1. The range quoted for iron is the biological range.

2. Mean n. is the mean number of subjects sampled by the workers reporting a normal mean value for a particular trace metal.

The reported normal mean values for serum or plasma copper levels are more consistent than the zinc values. The range of reported normal means is 850 to 1720 µg/L if a single low value of 540 µg/L is omitted, and 75% of the means are between 850 and 1200 µg/L. Furthermore, in contrast to the marked skew distribution of the zinc values with the mode at the lowest decile, the reported copper means have a normal distribution with the mode at the 5th decile. The inference must be that

there are no serious contamination problems associated with the collection of samples for serum or plasma copper determinations.

The "ultra-trace" pair of metals selected for discussion are caesium and chromium. Caesium has been chosen because it is the only metal in this class with reasonably consistent reported means. The risk of contamination with caesium during sample collection and storage is minute, since the element is amongst the least ubiquitous in the environment. However the caesium concentration in eryrthrocytes is about 6.5 times that in serum (Versieck et al (5)) therefore haemolysis or delayed separation of the plasma will produce an elevated result.

The contrasting metal of the second pair is chromium, the trace metal of interest in this study. The range of reported means is very large, 0.14 to 55 µg/L, and the markedly skew distribution with the mode at the lowest decile as shown in figure 1.1 indicates that contamination is the major problem. Versieck (6) demonstrated that gross chromium contamination can result from the use of stainless steel needles for sample collection (chromium determinations will be discussed in detail in chapter III).

Two points emerge from the above discussion. The first is that the ratio of a particular metal in the environment, available for sample contamination, relative to the normal plasma concentration is more important than the latter factor alone, in determining analytical difficulties. The second factor is the possibility of contamination from the cellular elements where high intracellular analyte concentrations are present. However, the data reported for caesium confirm that this second factor can be controlled if sufficient care is taken. The frequency distribution of Cu, Zn and Cr within the deciles are illustrated in figure 1.1. The data from table 1.1 were used for this figure and Cs was omitted because insufficient data were available. The normal distribution of the Cu values contrast with the skew distribution of the levels reported for the other two metals.

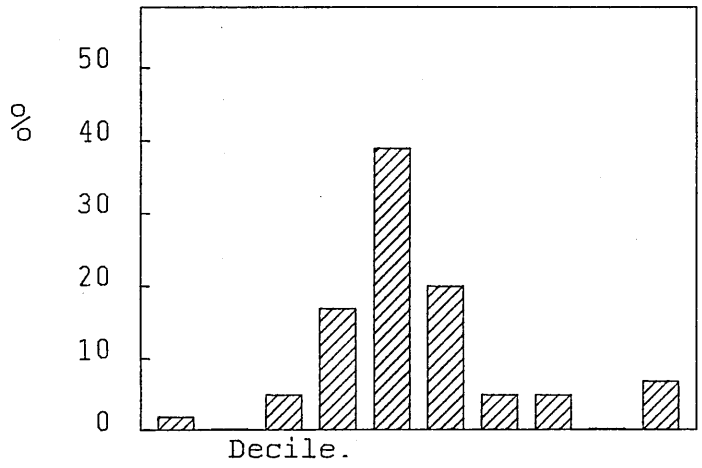
1.2. ESSENTIALITY.

Aggett (1) considered the following to be the criteria for trace element essentiality. The first is that deficiency is associated with reproducible defects which respond to the re-introduction of the element. Essential trace elements should be present in relatively constant concentrations throughout life. Concentrations of

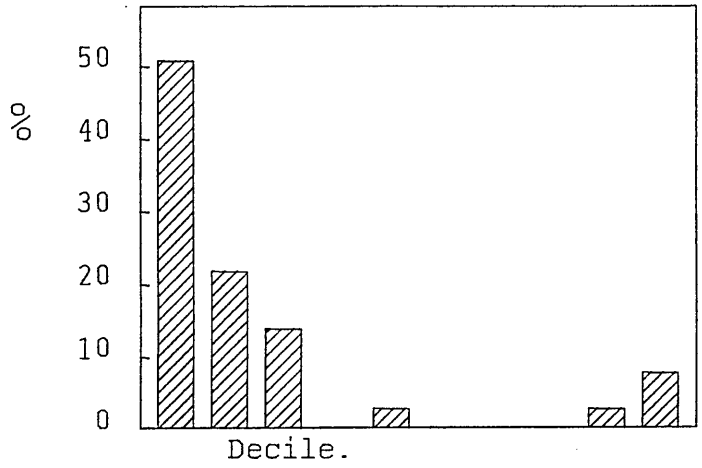
FIGURE 1.1.

Percentage in each decile.

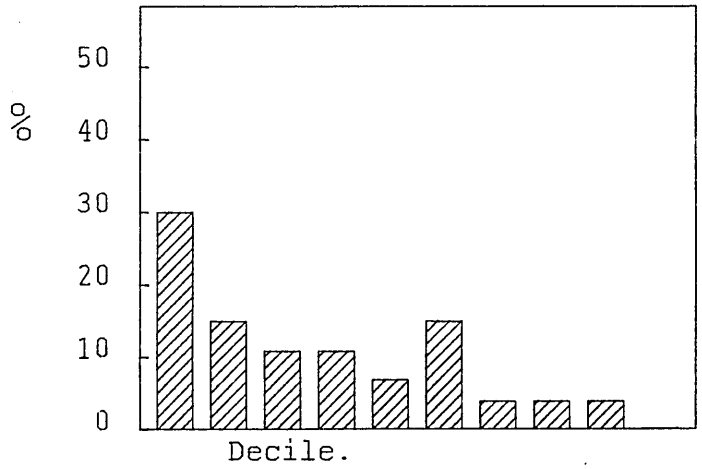
COPPER.



ZINC.



CHROMIUM.



non-essential trace elements have a skewed distibution within a population, whilst those of essential elements follow a normal distribution because homeostatic mechanisms exist for essential elements.

Essential trace elements must by definition be physiologically active at low concentrations. They are constituents or activators of enzymes, co-enzymes and hormones, all species which are active at low concentrations. The mechanisms by which metals function in enzyme systems is listed in table 1.2.

TABLE 1.2.

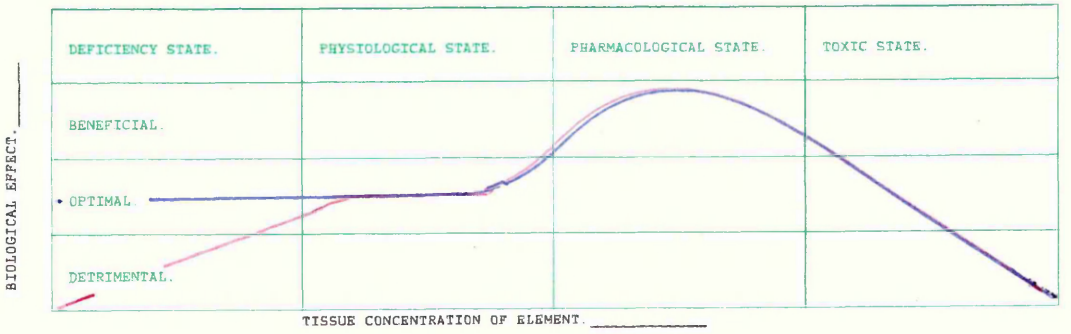
Mechanism of metal function in enzyme systems.

1. Direct participation in catalysis.
2. Combination with substrate to form an intermediate complex, upon which the enzyme acts.
3. Formation of a metalloenzyme that binds the substrate in an enzyme-metal-substrate or enzyme-metal-coenzyme-substrate complex.
4. Combination of metal with a reaction product to alter equilibrium.
5. Maintenance of the quaternary structure of the enzyme.

The relative body burden of an element tends to correspond to the environmental availability. Many non-essential elements are in fact present in higher concentrations in serum than essential ones. For example aluminium, an abundant environmental element not proven to be essential, is present at much higher concentrations than chromium, which is generally accepted as an essential element. The "spectrum of biological effects", figure 1.2, illustates several important points.

FIGURE 1.2.

The spectrum of biological effects. (Aggett (1)).



Red, essential trace element.
Blue, non-essential trace element.

Figure 1.2 illustrates that essential trace elements are potentially toxic, and that concentrations of a trace metal modestly above the optimal may be beneficial under certain circumstances. For example chromium supplementation has been reported to have a beneficial effect on lipid metabolism, however other workers have not been able to confirm this. Nevertheless there are sound theoretical grounds for a beneficial effect if chromium is accepted as an essential component of an insulin cofactor. The evidence for beneficial chromium, and the toxicity of chromium are both examined in chapter II, section 2.3.

Table 1.3 lists the evidence for essentiality in humans of the trace elements for which the concentrations in serum or plasma are reported in table 1.1. The possible value to the clinical chemist of reliable determinations, assuming these to be available, are also indicated. Seventeen metals are listed, of these seven have importance as toxic substances, and five are postulated as possibly being involved in trace element deficiency states in human subjects.

TABLE 1.3.

Evidence for essentiality, and value of assays, in humans.

Metal.	Evidence for essentiality.	Value of assays.
Al.	A regulatory mechanism has been documented (Versieck et al (2)). Two enzyme systems have been shown to be Al dependent : 1. Reaction beween succinate dehydrogenase cytochrome-c in vitro (Underwood (7)). 2. Al forms chelates with pyridoxal amino acid Schiff bases which act as model transaminases (Schroeder (8)).	Al is known to be toxic in the presence of renal failure. Dialysis patients particularly at risk. From: 1. Al contaminated dialysis solutions. 2. Al compounds given to prevent the accumulation of phosphate. Serum Al greater than 100 µg/L said to indicate high probability of Al deposits in bone (Danks et al (9)). But many workers report normal values higher than this.
Sb.	No known biological function in living organisms.	Antimonials shown to be of value in tropical medicine (Versieck et al (2)). Sb levels possibly of value in therapeutic monitoring.

TABLE 1.3. continued.

Metal	Evidence for essentiality.	Value of assays.
As.	Evidence has been presented that As may be an essential trace element (Versieck et al (2)).	Toxicology only at the present time.
Cs.	No known vital function.	No value.
Cr.	Evidence for involvement in carbohydrate metabolism, as an essential component of "glucose tolerance factor", an insulin cofactor generally accepted.	Controversial. Lack of reliable reference levels for serum/plasma or urine. Reports of response to a glucose challenge even in normal subjects also contradictory.
Co.	The element serves its paramount established biological function as a component of vitamin B12.	Competitive protein binding assays of vitamin B12 using ^{57}Co are reliable and valuable.
Cu.	Essential, present in many enzymes and proteins. Enzyme activities dependent on Cu include: 1. Cytochrome oxidase. 2. Superoxide dismutase (cytosolic). 3. Caeruloplasmin (93% of plasma Cu on average). 4. Lysil oxidase. 5. Dopamine hydroxylase. 6. Ceramide galactosyl transferase. 7. Ferrioxidase.	Two inherited disorders of Cu metabolism occur in humans. 1. Menkes' syndrome. 2. Wilson's disease. Cu determinations on serum and urine, and caeruloplasmin activities are all valuable.
Fe.	Essential constituent of the respiratory proteins: 1. Haemoglobin, about 75% of the total iron.	Iron deficiency is the most common disease in the world today (Golden and Golden (10). Caused by:

TABLE 1.3. continued.

Metal	Evidence for essentiality.	Value of assays.
Fe. continued.		
	2. Myoglobin. 3. Cytochromes, minute fraction of total iron. About 25% in storage as: 1. Ferritin, iron core surrounded by a protein shell. Ferritin circulates in the serum and extracellular fluid in equilibrium with: 2. Haemosiderin, insoluble iron store in the reticuloendothelial system. Transferrins, iron transport proteins.	1. Inadequate iron ingestion. 2. Inadequate absorption. 3. Increased iron loss. Measurement of serum ferritin appears to be the earliest indicator of iron depletion. Serum iron does not fall until later, when iron deficient erythropoiesis develops. Ferritin determinations also give the earliest detection of iron overload. Treatment can be initiated minimising damage to the liver and other organs (Halliday (11)).
Mn.	Hurley (12) states: 1. The skeleton and otoliths are susceptible to Mn deficiency. 2. Superoxide dismutase found in mitochondria contains Mn. 3. Mn deficiency results in abnormal mucopolysaccharide synthesis, alterations in the integrity of membranes and to abnormal brain function. The enzyme activities generally accepted as being Mn dependent are: 1. Glyosil transferase. 2. Arginine pyruvate carboxylase.	Only one reliable case of Mn deficiency documented. A subject receiving a purified "chemically defined" diet designed to establish the adult need for vitamin K. The patient was unable to correct his depressed prothrombin levels when given vitamin K until Mn was restored to his diet (Underwood (13)). Tanaka (14) and Papavasiliou (15) both found whole blood Mn levels below normal in about one third of children with convulsive disorders of unknown cause however all the children were on anticonvulsant therapy.

TABLE 1.3. continued.

Metal	Evidence for essentiality.	Value of assays.
Hg.	Essentiality for man not proven.	Toxicology purposes only. Neutron activation analysis is only suitable for the determination of total Hg. Refined atomic absorption methods can distinguish between organic and inorganic Hg. The principal excretory route for organic Hg is via the bile into the faeces as methyl-Hg-cysteine, (Vallee (16)).
Mo.	Mo is essential as a constituent of xanthine aldehyde and sulphide oxidases (Aggett (1)).	No known deficiency cases in man.
Ni.	Essentiality for man not proven, although deficiency states in animals well documented Mertz (18)). A Ni containing macroglobulin has been found in human serum, but function is unknown (Sundermann (21)).	In view of the concern about Ni toxicology, expressed by Sundermann (17), reliable reference concentrations are needed for the general population so that accurate determinations on exposed subjects can be interpreted.
Rb.	No evidence that Rb is essential.	The close physico-chemical relationship of Rb to K has stimulated biological interest in Rb. Fieve et al (19,20) report that there is some evidence that Rb has unique neurophysical characteristics.

TABLE 1.3. continued.

Metal	Evidence for essentiality.	Value of assays.
Ag.	No evidence that Ag is essential for living organisms.	Toxicology only but Ag is not important in this field.
Sn.	Schwartz et al (22) produced evidence that Sn was an essential element for some animals in 1970. No definite proof of essentiality in man.	Toxicology only.
V.	The balance of opinion is probably that V is essential, but no definite proof. No V metalloprotein known. Golden and Golden (10) state that homeostatic control is exerted over V, and that the metal does not accumulate with age. Cantley et al (23) and Karlish et al (24) have produced evidence that vanadate inhibits the Na pump.	Golden and Golden (10) state that the V content of human diet appears to be considerably below that required by some animals. They conclude that the low serum V concentration in patients with nutritional oedema may represent a natural human deficiency. Dimond et al (25), reports that V salts are relatively non toxic to man.
Zn.	Zn is one of the most important trace metals, over 50 Zn metalloenzymes have been identified. All key metabolic pathways have Zn metalloenzymes.	Acrodermatitis enteropathica is a rare congenital disorder of Zn absorption. The disease is readily cured by giving Zn sulphate. All patients for whom data are available have very low serum/plasma Zn levels.
	Williams et al (26) and Kirchgessner et al (27), both report that when an animal dies from Zn deficiency the metalloenzymes and the	The incidence of marginal Zn deficiency, and the value of Zn determinations for the diagnosis of this condition are

TABLE 1.3. continued.

Metal	Evidence for essentiality.	Value of assays.
Zn continued.		
	tissue Zn content have hardly changed. Zn is unique amongst trace metals in that there does not appear to be a functional body store of this essential element. Virtually all the Zn is locked away in bone or protein, this is responsible for the rapid onset of symptoms on a Zn deficient diet.	both controversial subjects. Solomons (28) believes that Zn (and Cu) deficiency states probably represent an under recognised segment of human nutrition problems. Solomons also states that the simple determination of total Zn (or Cu) alone is insufficient for assessing the metal status of an individual.

1.2.1. Regulation.

The metabolism of the essential trace elements is usually adjusted by a homeostatic mechanism to maintain body burdens and concentrations near the optimum levels. The uptake from food and the excretion rate may both be used for control. For example Elinder (29) reports that for iron and zinc regulation takes place in the gastrointestinal tract, absorption rising under deficiency conditions and falling when stores are adequate. The uptake of copper, in contrast, is stated by Elinder to be always high, status being regulated by an effective and rapid biliary excretion.

The measurement of serum iron and the determination of iron status have reached a reasonable degree of reliability. The involvement of iron in a single metabolic process, respiration, undoubtedly helps in the determination of status. Iron status regulation is therefore a useful comparative model for other metals. The factors affecting iron absorption are obviously relevant to many other metals, despite the fact that regulation may not be by absorption. Limited absorption, or particularly favourable absorption conditions can clearly override the homeostatic mechanism, leading to deficiency or overload respectively. The conditions resulting from failure of the regulatory mechanism are also relevant, because chromium is one of several metals

carried by the iron transport protein transferrin. Furthermore a high incidence of diabetes mellitus is seen in one type of iron overload, idiopathic haemochromatosis, and Sargeant et al (30) postulated that the high saturation of transferrin seen in this condition could result in chromium deficiency.

1.2.1.1. Iron status regulation.

The absorption of iron is susceptible to conditions in the intestinal lumina, particularly to pH and to the presence of oxidising or reducing agents. A low pH increases absorption because of the higher solubility of iron, especially Fe(III). the presence of reducing agents favours absorption because Fe(II) is absorbed about three times as efficiently as Fe(III).

Reimold et al (4) gives the following account of iron absorption. The normal intake of iron averages about 20 mg per day, but only a small fraction of this is absorbed, since an adult male maintains iron balance on an absorption of about 1 mg per day. However females during the reproductive years absorb about 1.5 mg daily to balance menstrual losses. The absorptive behaviour of the intestinal mucosa changes under conditions of abnormal iron status in an attempt to achieve homeostasis. However under deficiency conditions the amount of dietary iron that can be absorbed is limited by its bioavailability. Absorption rises only to about 4 mg daily under conditions of depletion, and falls only to 0.5 mg per day in overload states. In areas where the populations subsist on cereal diets containing little ascorbic acid or meat, iron is less available, and the ability to increase absorption is even more restricted.

There is a limited loss of iron from the body. In the normal adult male the total excretion is from 0.5 to 1.0 mg per day according to Moore (31). The bulk of the iron is lost in the faeces where it is derived from the intracellular iron of exfoliated epithelial cells and erythrocytes. The urinary loss is reported by Dagg et al (32) to be less than 0.1 mg daily. When the total body iron content is raised, iron excretion is somewhat increased. This is mainly due to the fact that each desquamated epithelial cell contains slightly more iron. Astaldi et al (33) claims that under conditions of iron overload macrophages packed with iron pass into the intestine.

1.2.2. Deficiency.

A number of nutritionists consider that absolute deficiencies of

trace elements are unlikely to occur except among subjects who have rare inborn errors of metabolism, metabolic alterations secondary to protein-energy malnutrition, primary disease or stress, and those receiving total parenteral nutrition for prolonged periods. The only reasonably convincing cases of Cr deficiency reported are from this last category, that is prolonged total parenteral nutrition, and this will be discussed in chapter II.

1.2.2.1. Dietary variables.

O'Dell (34) states that there are major discrepancies in the literature as regards the definitions of bioavailability, some investigators equating bioavailability with absorption. O'Dell defines bioavailability as the proportion of nutrient in food which is absorbed and utilised. Utilisation is the process of transport, cellular assimilation and conversion to a biologically active form or forms. The dietary variables affecting the bioavailability of trace elements are given in table 1.4 below.

TABLE 1.4.

Dietary variables affecting the bioavailability of trace elements.

A. Extrinsic factors.

1. Age or maturity of food.
2. Processing
3. Dietary components.

i) Protein. Snedeker (35) showed that a high animal protein diet significantly increased the apparent absorption of Zn.

ii) Carbohydrate. Fields et al (36) reported that replacement of starch by sucrose reduced the hepatic and renal Cu of rats fed on a low Cu diet.

iii) Fibre. Davies (37) reviewed the effects of dietary fibre. He was unable to draw firm conclusions.

iv) Phytate and oxalate. O'Dell et al (38) demonstrated that phytate decreases Zn bioavailability in experimental animals. Kelsay (39) showed that foods such as spinach, which are rich in both fibre and oxalate decrease Zn balance.

4. Minor dietary components.

Considerable evidence has accumulated that a dietary excess of one trace element may have a detrimental effect on the absorption of another particularly if the latter is present at a minimal level. O'Dell (34) reports the following interferences:

TABLE 1.4. continued.

Fe(II), Pb and possibly Sn reduce Zn.

Zn, Cd, Ag and Mo reduce Cu absorption.

B. Intrinsic factors.

1. Chemical speciation.

McKenzie (40) regards chemical speciation as the more important of the two intrinsic factors. Some differences can be explained on the basis of aqueous or lipid solubility. As a general rule organics are better absorbed than inorganics.

2. Dietary concentration.

Frolich (41) supports the Norwegian policy of having a very restrictive fortification policy for foods because of the interactions of some elements, and the possibility of overloading some subjects.

Sandstead (42) states: "A factor that probably protects people in industrialised societies from the occurrence of greatly distorted trace element relationships is the variety of foods consumed from a variety of sources. Poor people and members of agrarian societies may have less protection in this regard. In the latter instance, food from local farms may be the major dietary constituents. When this occurs, persons living on the farms may be subjected to undesirable soil/plant interactions similar to those affecting livestock."

1.2.2.2. Genetic defects leading to deficiency.

Genetic defects leading to trace element deficiences are extremely rare.

1. Menkes' kinky hair syndrome.

This condition was first described in 1962 by Menkes et al (43) as an x-linked recessive disorder. Danks et al (9) reported that patients with Menkes' syndrome were Cu deficient and that the primary defect was probably one of intestinal malabsorption. Nooijen et al (44) produced evidence that Cu accumulates in various tissues particularly kidney and duodenal mucosa, thus Menkes' syndrome cannot be one of simple Cu deficiency. Furthermore, in agreement with the identification of the Cu binding protein as a metallothionein, it was found that Zn, Cd and Hg were also trapped in these tissues. Table 1.5 compares the Cu levels in Menkes' syndrome with normal values (Danks (45)).

TABLE 1.5.

Copper levels in Menkes' syndrome compared with normal values.

Tissue.	Copper units.	Normal baby 6 to 12 months.	Menkes' syndrome.
Serum.	µg/L.	700 to 1525	127 to 381
Liver.	µg/g dry wt.	50 to 120	10 to 20
Brain.	"	20 to 30	1 to 7
Kidney.	"	10 to 20	240
Duodenum.	"	7 to 29	50 to 90

2.Wilson's disease.

Wilson's disease or hepatolenticular degeneration is an inherited metabolic disorder that affects Cu metabolism. The fundamental disturbances in Wilson's disease are:

i) A gross reduction in the rate of incorporation of Cu into caeruloplasmin.

ii) A considerable reduction in the biliary excretion of Cu.

The copper levels in Wilson's disease are compared with normal levels in table 1.6 below (Danks (46)).

TABLE 1.6.

Copper levels in Wilson's disease compared with normal.

	Normal adult.	Wilson's disease.
Serum caeruloplasmin mg/L.	200 to 400	0 to 200
Total serum Cu mg/L.	700 to 1525	191 to 635
Non caeruloplasmin Cu as a % of total Cu.	5 to 10	> 10
Urinary Cu µg/24 hrs.	< 40	100 to 1000

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3. Achroderma enteropathica.

Achroderma enteropathica is a rare congenital inherited disorder of Zn absorption. The disease is readily cured by the administration of Zn sulphate. All patients for whom data are available have very low serum/plasma Zn levels.

4. Deficiency of transferrin.

A subject with apparent atransferrinaemia was reported by Heilmeyer et al in 1961 (47). The subject had severe iron deficiency,

and required regular blood transfusions. However although both parents had low transferrin levels they were not iron deficient.

The analytical results reported for the subjects with the known inborn errors involving trace metal metabolism illustrate both the need to carry out determinations on several types of specimen and the potential value of fractionation procedures.

1.2.2.3. Excessive loss.

Excessive trace element loss may follow various conditions. The more common ones include burns, kidney disease, cirrhosis, diuresis and the therapeutic administration of chelating agents.

The broad spectrum determination of trace element status for subjects in this group would clearly be of value. Adequate control of status should lead to some improvements in prognosis and quality of life, in those cases where significant deficiencies exist.

1.3. DETERMINATION OF TRACE METAL STATUS.

The criteria given by Kirchgessner (27) for the diagnosis of trace element deficiency are listed in table 1.7 below.

TABLE 1.7.

Criteria for the diagnosis of trace element deficiency.

1. Characteristic and sensitive for the particular trace element.
2. Indicate latent and early stages of imminent deficiency.
3. Give some clues to the causal factors.
4. Afford easy sampling of the analytical material.
5. Simple and robust analytical methods.

These criteria can be approached for only one trace metal, iron, at the present time.

1.3.1. Choice of material for analysis.

Determinations are usually carried out on whole blood, serum/plasma, urine or hair. The containers used may contaminate any of these samples with metals. Zn is a particular problem and Reimold et al (4) reports that the rubber sealing rings used with many sample containers, especially glass ones are especially troublesome.

1.3.1.1. Blood.

Whole blood, serum or plasma are the samples used for most trace

element determinations, as blood is a convenient readily available material. However there are some special problems.

Blood may be contaminated during the sampling procedure. The stainless steel needles can contribute Cr, Mo, Fe and Ni. When the analyte to be measured is not equally distributed between the blood cells and the plasma whole blood results are influenced by the "pack cell volume". As mentioned in 1.1 serum/plasma are misleading for elements like Zn which are present in much higher concentrations in the cells if haemolysis occurs, or if the serum/plasma is not separated from the cells without an unreasonable delay. Plasma is superior to serum for early separations, but anticoagulants may contain the elements to be measured, as discussed earlier with respect to Zn determinations. The anticoagulant may react with the metal to be measured and this could affect the result. For example, a sample collected using EDTA as anticoagulant would be unsuitable for determinations after a fractionation procedure, if the original metal distribution was disturbed.

The time at which the specimen is taken and the position of the patient may be important. For example a patient adopting a recumbent position experiences a flow of extracellular fluid into the blood vessels leading to a change in many components of the blood plasma. Samples taken after a meal may reflect trace element absorption and be significantly higher than fasting samples, or a trace element containing species may respond to post prandial changes. The last factor is especially pertinent to Cr determinations, particularly after fractionation procedures because the proposed insulin cofactor containing Cr would reasonably be expected to respond to blood glucose changes. In addition to the above changes diurnal variations may occur.

1.3.1.2. Urine.

Urine has the advantage of being readily available in relatively large volumes, and the collection system can be very simple, especially for males, minimising contamination problems. The meaningful parameter is the excretion relative to a fixed amount of creatinine. The urinary concentration is only useful for the detection of gross toxic states. The urine volume and the physical size of the subject under investigation must be considered in all other studies.

1.3.1.3. Hair.

Hair trace element levels can provide useful historical

information according to some workers. Mertz (48) suggested that hair is a "meaningful and representative" tissue for the analysis of Zn, Cu and Cr. Amador et al (49) reported that patients with acrodermatitis enteropathica had low concentrations of Zn in their hair. However, contamination from the environment, and from applied cosmetic preparations, is a major problem. Hildebrand et al (50) concluded that because of cosmetic treatment, the effects of which could not be eliminated by the commonly used sample preparation procedures, hair could not be expected to indicate the concentrations of the intrinsic metals. There is general agreement that trace metal determinations on hair are of little value in the acute situation.

1.3.1.4. Other materials.

Analysis of soft tissues obtained by biopsy may provide more information than can be obtained from blood and urine. However it is obvious that good clinical grounds would be necessary to justify the collection of such samples. This category of sample has been used mainly for the investigation of excessive exposure to toxic metals. Sequestering tissues are usually sampled. For example, Ulucci et al (51) advocated renal biopsies for Cd studies.

1.3.1.5. Interpretation of results.

The interpretation of results may be difficult for any sample type. Thus blood, serum/plasma and urine all give an indirect estimate of intracellular trace element content. Plasma carries newly absorbed trace elements as well as those being transported to their target organs. The concentrations of some elements, or fractions of an element may be under tight homeostatic mechanisms and remain virtually unchanged until the body reserves are almost exhausted, as indicated in table 1.2 for Zn.

1.3.2. Fractionation.

Fractionation to determine the amount of trace element present in the form from which it derives its essentiality (usually a metalloenzyme, coenzyme or hormone) may help to overcome some of the interpretation problems discussed above. However, the extra processing involved in such fractionation procedures gives an increased chance of loss or gain of the element, and of course greater sensitivity is needed. Nevertheless the greater percentage change in a fraction with a higher fractional contribution from the essential species could compensate for this, giving a more sensitive measurement of status.

Speciation is the ultimate goal, however more modest fractionation procedures may be useful. The trace element distribution over various binding entities is clearly an important topic. The trace elements in plasma are mainly present in three forms. A labile exchangeable fraction bound to albumin, a group bound to low molecular weight substances such as amino acids, metabolites and drugs, and the essential form, which is usually incorporated in specific metalloproteins and is usually relatively inert to exchange of the trace element component. Table 1.8 lists the fractionation techniques used at the present time.

TABLE 1.8.

Fractionation techniques.

Basis.	Technique.
1. Molecular weight.	i) Gel chromatography. ii) Ultrafiltration.
2. Complex stability.	Addition of a complexing agent followed by solvent extraction.
3. Solubility.	i) Precipitation with chemicals. eg. The determination of the Zn bound to albumin after the precipitation of alpha-2-macroglobulin with polyethylene glycol (Giroux et al (52)). ii) Precipitation with antibodies. eg. The determination of the Cu bound to albumin after the removal of caeruloplasmin.
4. Indirect methods.	i) Determination of a biological property. eg. Caeruloplasmin measured using its oxidase activity. This test is widely used in the diagnosis of Wilson's disease (see 1.2.2.2.) ii) Immunological techniques. eg. The Radioimmunoassay measurement of ferritin. The best test for the determination of iron status, giving early and reliable detection of overload or deficiency, as reported earlier in table 1.3.

The last category in table 1.8 "indirect methods", is probably the one which will be most developed in the future, both the techniques listed

in this category being important.

Williams (53), Fiabane et al (54) and Halstead et al (55) all considered the low molecular weight complexes to be important, because it is well established that intestinal absorption, reactions in blood plasma, deposition into tissues, passage through the blood brain barrier, renal and biliary excretion, and even the synthesis of metalloproteins, all involve low molecular weight complexes of the transition metal ion. The complexing agents are usually amino acids.

For obvious reasons the determination of trace elements after fractionation has been applied to the trace elements present in higher concentrations, for example Zn, Cu and Fe. The value of the fractionation procedures in use at the present time for Cu and Fe have been reported in table 1.8. The Zn content of serum is almost entirely bound to protein with 50 to 60% bound to albumin and 30 to 40% bound to alpha-2-macroglobulin, and a variety of methods have been used to separate these two fractions. However the involvement of Zn in nearly all organs and metabolic pathways render determinations of total serum Zn, and of the Zn present in these two major fractions, of limited value.

Danks et al (56) has suggested that the measurement of an appropriate metalloprotein before and after the administration of a physiological replenishment dose of metal should distinguish low levels due to metal deficiency from those due to other mechanisms. Low initial levels that do not change after treatment would indicate low apoprotein production due to genetic variability or other factors. Defects in the absorption of the metal would also prevent a response, eg in Menkes' syndrome. The underlying principle is that deficiency of a metal places a constraint upon the formation of active holo-metal-enzyme, but a moderate excess of metal does not superinduce production of active enzymes. Danks states that it seems logical that this should be true for metallo-enzyme, as opposed to metal transport or binding proteins, which might be expected to be super inducible.

1.3.4. The Assessment of Chromium Status.

The determinations of total serum Cr, GTF Cr and free GTF Cr are clearly the initial targets to aim for.

A chromium analogue of the iron storage substance ferritin could exist, and measurements of this by immunoradiometric techniques would then probably provide the most reliable guide to chromium status.

1.4. CONCLUSIONS.

1.4.1. Reference Values.

The normal levels of a small minority of trace metals in human serum/plasma have been established with reasonable confidence. Two principal parameters appear to be relevant to the analytical problems when determining a particular trace element level. The first is concentration, the second is the availability of the metal in the laboratory environment, containers, sampling equipment and process materials.

A low concentration exacerbates matrix and contamination problems, a high availability factor increases contamination problems.

Metals present in very low concentrations and with high availability factors for example chromium and nickel would be expected to present special problems in the development of reliable determinations. The sampling procedure, which is very difficult for the analyst to control for routine samples, presents particular problems for these two metals, because of the ubiquitous stainless steel syringe needle. The only practical answer to this problem is to develop techniques not involving the measurement of the trace element itself, for assessing the metal status of subjects, especially in the routine clinical chemistry laboratory.

1.4.2. Essentiality.

The characteristics which make a particular trace metal a difficult analyte, will also produce problems in essentiality studies. A very low plasma concentration implies a very low dietary requirement and gross deficiency is then difficult to produce experimentally, if there is a relatively high environmental availability. Furthermore, natural deficiency is unlikely to occur, except under very exceptional conditions.

The demonstration of a metal dependent enzyme system is a possible route to proof of essentiality.

The three metals nickel, chromium and vanadium are all present in very low concentrations in human plasma, and have relatively high environmental availability. Furthermore no enzyme systems dependent on any of these three trace elements have been found. Chromium is the only one of the three to be virtually universally accepted as essential, although there is some support for the other two. Nevertheless the

evidence for chromium as an essential trace metal is perhaps not entirely convincing. The evidence for chromium as an essential trace metal is discussed in chapter II.

1.4.3. Status.

Trace metal status is clearly easier to define from laboratory tests when the metal under consideration is involved in a single metabolic process, or where one is markedly predominant. The diagnosis of iron status is an example. The only completely reliable indication of trace metal deficiency is still the careful observation of the clinical response to supplementation of the nutrient under investigation, under controlled conditions.

1.4.4. Chromium.

The reference range for serum/plasma chromium is very uncertain, and very little work has been carried out on serum chromium fractionation. The lack of a consensus value is understandable, given the ultratrace concentration and high availability mentioned earlier.

The establishment of reliable reference ranges for serum/plasma and urine chromium are necessary for two purposes. The first is for the interpretation of accurate determinations on subjects exposed to possibly toxic quantities of chromium. The second purpose is for investigations into the assessment of chromium status by laboratory tests. The fact that chromium essentiality appears to depend only on a single molecular species, which acts as an insulin cofactor is an advantage. However a chromium analogue of the iron storage protein ferritin, if one exists would be the ideal analyte.

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CHAPTER II.

REVIEW OF CHROMIUM AS A TRACE METAL IN HUMAN PHYSIOLOGY.

Chromium is important in human physiology both as an essential trace metal and as a serious health hazard to workers in certain industries. The toxicity of chromium must be discussed here because of its relevance to chromium supplementation.

2.1. TOXICITY OF CHROMIUM.

Cr is used extensively in many industries. Hatherhill (1) reports that Cr(VI) is capable of crossing oral and pulmonary membranes and perhaps skin as well. Cr(III) is stated to have a markedly reduced ability to cross membranes.

2.1.1. Mutagenicity of chromium.

Mutagenic activity by Cr has been demonstrated in both bacteria and rodents.

2.1.2. Evidence for mutagenicity.

Hatherhill (1) reports that Cr workers have significantly higher incidences of lung cancer, with relatively few reports of cancer in other tissues, he refers to references (2) and (3).

2.1.2.Mechanism of mutagenic activity.

Levis and Majone (4) tested Cr(III) and Cr(VI) compounds of varying solubilities in cell cultures. The observations made in the experiments are given in table 2.1.

Table 2.1.

Observations by Levis et al on mutagenicity and carcinogenicity tests.

1. Cr(III) compounds have low activity because of strong binding to the cell mebrane, with resultant poor penetration.
2. Highly soluble Cr(VI) compounds such as K or Na dichromate show low activity in live animals because of fast spread and rapid reduction mainly by erythrocytes which are readily penetrated by Cr(VI).
3. Cr(VI) compounds of moderate solubility such as Ca chromate or Zn chromate hydroxide, have high mutagenic activity.
4. The cells most at risk are ones which cannot readily reduce Cr(VI) in the cytoplasm, before contact is made with the nucleus. Cr(III) produced by reduction of Cr(VI) in the cytoplasm tends to be

TABLE 2.1. continued.

retained by the cell membrane.

5. Some Cr(III) compounds tested appeared to be active because of contamination with Cr(VI).
6. There is probably very little oxidation of Cr(III) to Cr(VI) in biological systems.

Jennette (5) reported that the chromate anion mimics the sulphate ion and crosses the cell membrane on the sulphate transport system.

The mutagenic activity of chromium appears to depend on the penetration of the cell membrane by Cr(VI) on the sulphate transport system. The Cr(VI) must then avoid reduction to Cr(III) by the microsomal fraction of the cytoplasm and gain access to the nucleus. The Cr(VI) is then reduced at the nucleus and the Cr(III) produced interfers with DNA synthesis.

2.1.3. The diagnosis of chromium toxicity.

Toxicological monitoring can be carried out in two complimentary ways. The first is environmental monitoring, defined as the direct qualitative and quantitative assessment of exposure by measuring the harmful agents present in the working environment. The second, biological monitoring, is the indirect qualitative and quantitative assessment of exposure of a group or of an individual to noxious agents present in the workplace.

The objectives and requirements of biological toxicology monitoring are given in table 2.2 below.

TABLE 2.2.

Objectives and requirements of biological toxicology monitoring.

1. Determining the presence and extent of human health hazards from exposure to the toxic metal.
2. Establishing reference base lines of concentration ranges in the general population for the metal being considered.
3. Establishing dose response relationships.
4. Correlating toxic metal concentrations with sources of contamination and determining risks to defined target populations.
5. Determining trends in the toxic metal concentrations in humans.

A consistent picture emerges from the Cr toxicology literature. The important points are that contamination of the air in the working environment by Cr(VI) can lead to high Cr body levels because of the ability of Cr(VI) to cross the membranes lining the respiratory tract. Furthermore Cr(VI) compounds of moderate solubility appear to be the most dangerous because they can generate high Cr levels at the cell nucleus. However significant Cr(III) absorption takes place only via the gastrointestinal tract, about 0.2% of ingested inorganic Cr(III) being taken up.

Plasma and urinary total Cr levels appear to have limited value, and may be useful only for comparative work on workers exposed to similar Cr compounds and under similar conditions.

Lewalter et al (6) investigated the use of Cr levels in isolated erythrocytes for toxicological monitoring purposes. The authors reported that Cr(VI) taken up by erythrocytes are stored in the cells for the rest of the cells' life span. Subjects were found to vary in their "plasma reduction capacity" (ability to reduce Cr(VI) in their blood plasma) and thus to reduce the intracellular Cr burden. The interindividual differences observed were considered to be genetically determined. The conclusions of the author was that the "isolated erythrocyte" Cr level was a useful biological monitor for exposure to highly soluble chromates. However, more work was needed with low solubility and insoluble chromates before firm conclusions about these could be drawn.

2.1.4. Conclusions, chromium toxicity.

The measurement and strict control of the Cr(VI) concentration in the workplace atmosphere appear to be the primary safety measures to be undertaken. A biological monitoring technique which takes account of individual variations in susceptibility and does not depend on the measurement of Cr is needed. The test should be analogous to the determination of erythrocyte levels of 5-aminolaevulinic acid dehydratase (ALAD) and free protoporhyrin for the detection of lead poisoning. Low (ALAD) and high free protoporphyrin levels that are not due to exposure to Pb are found in some subjects. However this is not a disadvantage for "industrial" monitoring purposes as these individuals are clearly not suitable for work involving exposure to Pb. The "plasma reduction capacity" mentioned earlier could be a useful screen test to eliminate individuals who are less suitable for work which exposes them

to Cr.

The conclusion to emerge with respect to Cr supplementation from this necessarily brief and limited discussion of Cr toxicology, is that ingestion of extremely modest, that is less than 250 µg per day of Cr(III) for a limited time is acceptable. The tests to be carried out over the supplementation period must be of well proven reliability to justify the exercise. However the administration of Cr supplements to pregnant subjects should be avoided until more information about human Cr metabolism is available.

2.2. ESSENTIALITY OF CHROMIUM.

Mertz and Schwartz (7) were the first to produce evidence that Cr is an essential trace element. In 1959 they observed that rats fed on a diet of Torula yeast, which is deficient in Cr, developed impaired glucose tolerance.

Investigations depending wholly on Cr determinations will not be presented in this chapter, because chromium measurements on biological fluids, appear to be too inconsistent for reliable conclusions to be made from them.

2.2.1. Essentiality in Animals.

The evidence that Cr is an essential trace metal for animals comes almost entirely from rats maintained on an artificial low Cr diet.

2.2.1.1. Artificial low chromium diet.

Mertz and Schwartz as mentioned above were the first in this field. They observed that rats fed on a Torula yeast-sucrose diet developed glucose intolerance which was improved by Cr(III) salts alone of the 40 elements tested, although, Mn(II) did produce a slight benefit.

Schroeder (8) produced similar evidence to Mertz and Schwartz in 1966. However, by preventing airborne contamination Schroeder maintained more strictly controlled conditions producing a greater degree of glucose intolerance. Nevertheless he did report a number of qualifications on the evidence supporting Cr as an essential trace metal. Schroeder acknowledged that a diet free from Cr was not available. Therefore a marginal or moderate state of deficiency only had been induced in the rats. Furthermore the characteristic renal lesions of human diabetes mellitus were not detected on microscopic

examination of the tissues. Nevertheless a disturbed glucose metabolism simulating mild maturity onset diabetes of man was observed. The evidence against Cr deficiency as the cause of the glucose intolerance is not insubstantial. Schroeder noted that the diet was undoubtedly deficient in nutrients other than Cr. And that male rats supplemented with vitamin E exhibited glycosuria whether or not Cr was fed, possibly as a result of the high sucrose diet.

Davies et al (9) fed rats on a commercial Teklad diet containing 30% Torula yeast as the sole protein source, plus 60% sucrose with fat, minerals and vitamins. The diet was stated by the manufacturers to have a Cr content of 0.12 µg /g. However Davies found two batches of the Teklad diet to have a Cr levels of about 0.47 and 0.78 µg Cr/g. Commercial rat cubes contained about 0.82 µg Cr/g. Nevertheless the rats fed on the Torula yeast diet showed growth retardation as reported by both Mertz and Schroeder, referred to earlier. Adipocytes were isolated by Davies from rats fed on commercial cubes and from rats fed on the Teklad diet. However only the adipocytes from the rats fed on the Teklad diet showed the responses to yeast fractions in a test with suboptimal insulin concentrations that is generally accepted as indicating "Cr deficiency". This test is discussed later in the GTF section of this chapter. Davies found that Cr supplementation of the Teklad diet did not improve the growth rate, or alter the behaviour of the adipocytes. Furthermore evidence was produced by Davies indicating that the high sucrose content of the Teklad diet was responsible for the poor growth and abnormal adipocytes.

2.2.1.2. Rate of absorption of glucose in Channa punctatus.

Sastry and Sunita (10) studied the effects of Cd and Cr on the rate of absorption of glucose from the intestine of the snakehead fish, Channa punctatus.

All concentrations of Cd decreased the rate of glucose transport. However the authors found to their evident suprise that Cr increased the glucose absorption rate at all concentrations examined. The highest rate of absorption occurring at 0.001mM Cr. Increases above this level gradually decreased the absorption rate.

2.2.2. Essentiality in man.

The only real evidence supporting Cr as an essential trace metal in man comes from three subjects, all women on long term total parenteral nutrition. There is perhaps a very modest support from

investigations into the protein calorie malnutrition of infancy occurring in certain deprived areas and also from certain iron overload conditions.

2.2.2.1. Subjects on total parenteral nutrition.

Jeejeebhoy et al (11) thoroughly documented the case history of a female aged 40 years who had been receiving total parenteral nutrition for five years. The patient showed an unexpected 15% weight loss and peripheral neuropathy. An intravenous glucose tolerance test showed reduced glucose clearance. Moreover a respiratory quotient of 0.66 indicated that fat was being used as a major energy source. The patient showed a limited response to insulin therapy. However 250 µg of Cr daily as the chloride, added to the infusate restored glucose metabolism to normal within two weeks. The glucose intake had to be reduced over the next 5 months to prevent the patient becoming overweight. A maintenance dose of 20 µg Cr per day was sufficient to keep the patient well for the next 18 months.

Freund et al (12) reported a second case of apparent severe Cr deficiency. The glucose intolerance developed after 5 months of total parenteral nutrition following complete bowel resection. The patient a woman aged 45 years went into a hyperglycaemic hyperosmolar nonketotic coma. Concomitantly a metabolic encephalopathy developed. Insulin therapy produced a limited improvement only, with erratic glycosuria. However Cr supplementation of 150 µg Cr chloride per day was started after 7 months. Within a few days insulin was not needed to control the blood glucose. Furthermore the encephalopathy cleared completely and the patient started to gain weight.

The third case report, by Brown et al (13), was very similar to the other two. The patient lost weight on a regimen on which she had previously maintained a stable weight as in the other two cases, but unlike the earlier subjects this patient did not have clinical evidence of neuropathy or encephalopathy. The glucose intolerance began after about 7 months of total parenteral nutrition, very similar to the 5 months reported by Freund et al. Jeejebhoy's patient had been on "TPN" for 3.5 years before glucose intolerance developed. However, the chromium content of the nutrition regimen received by this patient may have changed over this long period, although clearly any change that occurred was unplanned. All three patients responded after 3 to 5 days of chromium therapy and exogeneous insulin was then no longer required.

The plasma Cr levels reported in the three cases above show the usual inconsistencies. Jeejebhoy reported blood levels to be 0.55 µg Cr/L with a normal range of 4.9 to 9.5 µg/L. Freund reported serum Cr levels 50 µg/L for the patient in his report, a normal range of 50 to 90 µg/L was quoted. The plasma Cr level reported on Brown's patient was 1.0 µg/L before treatment, with a reference range of 18 to 38 µg/L. However, the follow-up plasma Cr levels remained unchanged at 1.0 µg/L after 1, 3 and 12 months of chromium supplementation, despite the subjects return to normal glucose tolerance.

2.2.2.2. Protein-calorie malnutrition of infancy.

Gurson et al (14) reported that a single dose of Cr increased the glucose removal rate in 9 out of 14 cases of protein-calorie malnutrition in Turkey.

Hopkins et al (20) reported that Cr deficiency was important in the development of disorders of carbohydrate metabolism in protein-calorie malnutrition in both Nigeria and Jordan. Whereas defective utilization of glucose in protein-calorie malnutrition in Egypt could not be attributed to a lack of Cr.

Carter et al (16) also found no evidence of Cr deficiency in protein-calorie malnutrition in Egypt.

2.2.2.3. Idiopathic haemochromatosis.

Sargent et al (17) postulated that patients with haemochromatosis had reduced Cr retention due to exclusion of Cr by Fe at metabolic binding sites. The iron transport protein transferrin also transports Cr, and is highly saturated by Fe in idiopathic haemochromatosis. The diabetes seen in some haemochromatosis patients was postulated by Sargent to be possibly caused by Cr deficiency, rather than by deposition of Fe as haemosiderin at the sites of insulin production in the pancreas. Sargent administered ^{51}Cr(III) to 5 normal males and to 11 patients with idiopathic haemochromatosis. The retention of ^{51}Cr was measured with a whole body counter for 8 months and blood levels measured for 40 to 80 days. Sargent found that the zero time intercepts of the slopes of both the whole body retention curves, and of the blood clearance curves were significantly different. Moreover the data on patients after Fe depletion were not significantly different from the data derived from normal subjects.

Lim et al (18) carried out a similar study on 16 subjects, 5 normals and 11 patients with varying degrees of haemochromatosis. The

clearance of Cr from different body organs, and from the whole body was reported to be more rapid in the patients with "fully developed" haemochromatosis.

The two papers above are both consistent with the hypothesis that Cr absorption and transport, are sensitive to the degree of saturation of transferrin by Fe. The iron overload patients showing an increased incidence of diabetes mellitus are all of types with a high degree of Fe saturation of transferrin. However heavy haemosiderin deposits in the pancreas are associated with a high transferrin saturation.

Bothwell et al (19) commenting on diabetes and haemochromatosis states that it was assumed in the past that the diabetes was due to damage to the islets of Langerhans, and low levels of insulin have been demonstrated in some patients supporting this supposition. However it is apparent that other factors unassociated with tissue damage by Fe deposits are involved. The prevalence of diabetes among first degree relatives of diabetic patients with haemochromatosis is much higher than among the first degree relatives of non diabetic haemochromatotics. The diabetes seen in the relatives of the diabetic haemochromatotics is of the maturity onset type with high levels of circulating insulin. The hepatic damage seen in the type of iron overload under discussion may also play a part as cirrhosis is associated with both insulin resistance and hyperglucagonaemia.

Heilmeyer et al (20) thoroughly documented the case history of a 7 year old child with congenital atransferrinaemia. The parents of the subject both had reduced serum transferrin levels and the child had no detectable transferrin. The subject suffered from severe iron deficiency anaemia with iron deposits in many tissues, including the pancreas. The iron deposits presumably resulted from the many blood transfusions, which had been given at three monthly intervals since birth. However the report contained no reference to any disturbance of glucose metabolism. The regular blood transfusions alone would not supply sufficient Cr on the basis of the report on the distribution and binding of Cr by Lim et al (18), see table 2.4 in section 2.5. Moreover the parents were not reported to have shown any evidence of glucose intolerance although, as reported above, they both had low transferrin levels. One must conclude that transferrin does not have the same vital role in the useful transport of Cr that it has with Fe.

The diabetes seen in idiopathic haemochromatosis is clearly not fully understood, perhaps Cr deficiency may be a factor, in a condition with a varying spectrum of contributing causal factors.

2.2.2.4. Marginal chromium deficiency.

Investigations into the effects of Cr supplementation on subjects with possible marginal Cr deficiency, have usually involved groups of elderly individuals with relatively mild glucose intolerance, of the non-insulin requiring type. The maturity onset diabetic produces normal, or above normal quantities of insulin, and is a popular choice as a possible marginal Cr deficiency subject.

A variety of tests have been chosen to determine if a response to Cr supplementation has occurred. However tests of glucose tolerance are common to all investigations. The change in plasma lipids, and in the insulin response to a glucose challenge, have also been monitored by some workers. The total cholesterol/high density lipoprotein cholesterol ratio together with the glycosylated haemoglobin levels, are probably the ideal parameters for objectively assessing if supplementation has been of real benefit to the recipient.

Table 2.3 below lists reported responses on subjects with possible marginal Cr deficiency to Cr supplementation. It can be seen that the reported responses are inconsistent and even contradictory, to such an extent that no reliable conclusions are possible.

2.2.2.5. Marginal chromium deficiency in pregnant women.

The glucose intolerance found in pregnancy was first reported by Hurwitz et al (27) in 1942, and the possibility that increasing demand for Cr by the foetus, could lead to maternal Cr deficiency, on a diet of borderline sufficiency, has been a popular hypothesis for the glucose intolerance of pregnancy.

Knopp et al (28) reporting on metabolic adjustments in normal and diabetic pregnancy, posed the question: whether the foetus acts solely as a parasite draining fuels from the mother, or whether the mother adapts to augment the supply of energy fuels to the foetus? The authors concluded that there is a maternal "push", as well as a foetal "pull", and the changes in carbohydrate and lipid metabolism seen in "normal" pregnancy is adaptive, enabling a greater push of nutrients to take place during the peak foetal fuel requirements of the third trimester. Clearly completely reliable analytical data on Cr levels would be difficult to interpret, even if such data were available.

TABLE 2.3.

Responses of subjects with possible marginal chromium deficiency to chromium supplementation.

i) Key to code used.

Subjects.

A. Normal.

B. Intermediate glucose tolerance, in the normal/diabetic border.

C. Maturity onset diabetic, diet controlled.

D. Maturity onset diabetic, taking oral antidiabetc medication.

E. Maturity onset diabetic, on insulin.

F. Juvenile onset diabetic, on insulin.

G. Diabetic, classification not stated.

Supplementation.

Cr. Cr admininstered as Cr chloride.

YB. Brewers' yeast, high GTF content.

YBE. Brewers' yeast extract, low GTF content.

YT. Torula yeast.

Response parameters.

GT. Glucose tolerance.

FBS. Fasting blood sugar.

HbG. Glysolated haemoglobin as fraction of the total haemoglobin. Glysolation of haemoglobin occurs in the erythrocyte in the peripheral circulation during the 120 day life span of the cell. HbG is a good indicator of the long term integrated plasma glucose level.

IL. Insulin levels following a glucose challenge.Increased sensitivity to insulin would be a positive response.

FIL. Fasting insulin level.

Cpept. Insulin C-peptide is secreted with insulin in equimolar amounts but is cleared from the circulation more slowly. Consequently the molar ratio of insulin to Cpept. in plasma changes in response to the stimulation and suppression of insulin secretion. This test is useful in detecting insulin secretion in patients on insulin medication.

Chol. Total serum cholesterol.

HDL. High density lipoprotein cholesterol.

C/HDL. Total serum cholesterol/HDL cholesterol ratio. Widely used as a risk factor for coronary thrombosis. Low ratios favourable.

TGL. Triglycerides.

TABLE 2.3. continued.

ii) Data.

Reference.	Supplementation.	Subjects.	Response.
Glinsmann et al. (21).	Cr 60 µg/day. 15 to 120 days.		
	24 days.	F(1).	GT. No change.
	18 days.	D(1).	GT. Improved.
	140 days.	C(1).	GT. Improved.
	73 days.	E(1).	GT. No change.
	133 days.	D(1).	GT. No change.
	15 days.	D(1).	GT. No change.
Levine et al. (22).	Cr 150 µg/day. 120 days.	B(4).	GT. Improved. IL. No change.
	120 days.	B(6)>	GT. No change. IL. No change.
Rabinowitz. et al. (23).	Cr 150 µg/day.	F(21).	All categories of subjects showed no significant changes in :- FBS. Chol. TGL. GT. FIL. Cpept.
	YB 6 µg Cr/day.	C(9).	
	YBE 18 µg Cr/day.	D(11).	
The three above supplements provided about equal amounts of Cr after correction for bioavailability. Placebo <0.4 µg Cr/day. All subjects received 3 of the 4 supplements.			
Vinson et al. (24)	Yb 218 µg Cr/day.	A(6).	HbG. No change.
	6 months.	B(5).	HbG. Improved. C/HDL. Improved.
		E(7).	HbG. No change. C/HDL. No change
		D(5).	HbG. No change. C/HDL. No change.

TABLE 2.3. continued.

Reference.	Supplementation.	Subjects.	Response.	
Hunt		D(22).	Both categories	
et al.	YB, YT as control.		showed no	
(25).		C(17).	significant	
			change in :-	
			FBS.	
			HbG.	
			FIL.	
			Chol.	
			HDL.	
			TGL.	
Martinez		B(8).	GT.	Improved.
et al.	Cr 200 µg/day.		IL.	Improved.
(26).		A(13).	GT.	No change.
	70 days.		IL.	No change.

The vast majority of pregnancies are uneventful for both mother and child indicating that significant Cr deficiency cannot be common. Chromium supplementation in pregnancy could not be encouraged without much better evidence than has been produced at the present time.

2.3. BENEFICIAL CHROMIUM.

The normal level of Cr may not be the optimal. Evidence has been presented by some workers indicating that Cr supplementation of the diet can have beneficial results in man and in animals.

2.3.1. In Animals.

Abraham et al (29) investigated the effects of Cr supplementation on cholesterol induced aortic plaques in rabbits, and concluded that Cr supplementation had a significant effect on the regression of these plaques.

The effects of Cr supplementation on rabbits fed on the very high cholesterol diet used by Abraham cannot be accepted as a reliable guide to the effects of Cr supplementation on humans on a normal diet. However, the results do indicate that Cr may be involved in lipid metabolism in mammals

2.3.2. Beneficial Effects in Man.

Atherosclerosis is a major health problem in the western world.

Insulin and hence Cr, if we accept GTF as an insulin cofactor, is very much involved in lipid metabolism. LDL (low density lipoprotein) and triglycerides favour the development of atheroscleosis. In contrast HDL (high density lipoprotein) exerts a protective effect. Insulin has a lipogenic activity and if GTF enhances other effects of insulin but not its lipogenic effect then higher GTF levels could be beneficial. Diabetics tend to have high lipid levels with a consequent increase in atherosclerosis.

Riales et al (30) investigated the effect of Cr chloride supplementation on glucose tolerance and serum lipids including HDL cholesterol on a group of healthy adult men. Cr chloride was used instead of yeast to eliminate the possibility that any effects could be ascribed to some other factor present in yeast. In this controlled double blind study of 23 men aged from 31 to 60 years the test group ingested 200 µg Cr per day, for 5 days of each week, for 12 weeks. The Cr supplementation was reported to have resulted in a significant increase in HDL cholesterol, a decrease in body weight and a trend towards decreased triglycerides. Total cholesterol and LDL cholesterol did not change significantly.

Offenbacher et al reported in 1980 (31) an improvement in glucose tolerance and plasma cholesterol after a supplementation study with brewer's yeast. However, a control group given Torula yeast also showed significant improvements in plasma cholesterol levels. The subjects were a group of healthy elderly individuals in a retirement home. Offenbacher et al repeated and extended the study in 1985 (39). The subjects of this second investigation were from the same area of New York city, and of similar age, 63 to 93 years, socioeconomic level and ethnic origins as the first group. The same health criteria were used to select the subjects in both studies. The 23 individuals in this second study were randomly assigned into one of three supplementation groups:

i) Brewer's yeast, 5 µg Cr per day (cf 10.8 µg Cr/day in 1st study).
ii) Cr chloride, 200 µg Cr per day.
iii) Placebo.

The authors reported that no significant changes occurred in glucose tolerance , insulin, cholesterol or triglycerides in any of the three groups in this second study. Three possible explanations were considered for the different findings in the two studies :

i) Some substance in the yeast other than Cr was responsible for at least part of the improvement in the first study.

ii) Too little yeast Cr was fed in the second study.

iii) The free living group had adequate levels of dietary Cr and hence good Cr status at the begining of the study.

A beneficial effect from Cr supplementation has not been conclusively demonstrated.

2.4. GLUCOSE TOLERANCE FACTOR (GTF).

Mertz (33) reported the first biologically active compounds known to contain Cr in 1957. This compound was considered to be a dietary agent required for the maintenance of normal glucose tolerance in rats, and consequently it was called "Glucose Tolerance Factor" (GTF).

The term GTF appears to be associated with two characteristics. The first to be described was a high dietary availability. Oberleas (34) reports that absorption of Cr from a normal diet ranges from 0.5 to 2%. In contrast absorption of Cr from GTF may rise to 25%. The second characteristic of GTF is the ability to enhance the effects of suboptimal concentrations of insulin on the oxidation of glucose. Adipocytes from the epididymal fat pads of diet induced glucose intolerant rats are usually used. The stimulation of the rate of carbon dioxide production from glucose in a Cr deficient yeast bioassay system is also used, and as it is easier to perform it is often used for screening purposes.

A number of Cr containing compounds have been reported to have one or both the above characteristics. "Substances showing GTF activity" appears to be a better term than GTF, to avoid the implication of a single molecular species. A single molecular species may dominate the GTF activity in human plasma, and if this can be demonstrated the compound could be referred to as "Human GTF".

Toepfer et al (35), and Mirsky et al (36), isolated substances with GTF activity from yeast. The former reported that the active material contained Cr, nicotinic acid, glycine, glutamic acid and cysteine, and that reacting these compounds together in vitro produced a mixture of Cr complexes with GTF activity. Cooper et al (37) investigated a mixture of the above compounds and also found that the

products showed GTF activity. Cooper and Toepfer concluded that the niacin-Cr-niacin axis was important for GTF activity.

Davies et al (9) reported the isolation of two small Cr free amino compounds with GTF activity from yeast. These substances, later identified as ornithine and a substituted lysine, both increased the effect of 10 μU of insulin to that of about 200 μU, when 70 μg of either were added, in the adipocyte test.

2.4.1. Mode of Action of GTF.

Christian et al (38) postulated that the formation of a ternary complex between Cr, the A chain disulphide of insulin, and membrane sulphydryl groups facilitates the initiation of the hormone action. In support of this Simonoff (39) reported that polarographic studies have led to the hypothesis that GTF participates in a ternary complex with insulin and a membrane receptor site. Furthermore, Anderson et al (40) reported that a Cr containing fraction, isolated from a synthetic GTF mixture, was found to bind strongly to porcine insulin.

Mirsky et al (36), however, produced evidence that GTF influences the transport of sugar to cells. GTF isolated from yeast was found to increase carbon dioxide production from glucose, fructose, mannose and galactose, by several yeast strains, when grown on a Cr depleted medium. The increased production from galactose, which is known to differ from glucose in the initial metabolic steps, together with the ineffectiveness of GTF on a cell free extract, and the results of a Michaelis plot for carbon dioxide production, support the hypothesis that GTF influences the transport of sugar to the cells.

Potter et al (41) using the hyperglycaemic clamp technique developed by DeFronzo et al (42), reported that Cr supplementation of glucose intolerant older people, resulted in improved glucose utilisation, and increased beta cell sensitivity to glucose. However tissue sensitivity to insulin was not significantly changed, and an assay of erythrocyte insulin receptors failed to detect any significant change. Potter's findings are consistent with increased glucose transport after Cr supplementation.

The increased rate of glucose absorption reported earlier in Channa punctatus (10) after exposure to Cr, also supports increased transport of glucose as a mode of action of GTF.

The increase in insulin sensitivity reported by some workers would favour the hypothesis of an insulin GTF complex. However, many

investigators have found no significant change in insulin levels after Cr supplementation, even in subjects who have shown improved glucose tolerance.

2.5. DISTRIBUTION AND BINDING OF CHROMIUM.

Lim et al (18) investigated the kinetics of Cr(III) in the human body using a whole body scintillation counter. The Cr in the plasma was found to be 95% protein bound in equilibrium with 5% unbound or bound to small molecules. The plasma Cr was reported to be in equilibrium with three clearly defined tissue compartments, with fast, medium and slow transfer rates. Each imaged organ appeared to contain varying proportions of each compartment. Table 2.4 gives details of the three compartments, a plasma level of 0.1 µg Cr/L was assumed. The slow compartment was considered to be a storage compartment, whilst the medium compartment could represent a short term borrowing pool. The kidney was reported to clear the unbound plasma Cr. A daily excretion of about 0.11 µg/day was calculated for the derived model.

TABLE 2.4.

The distribution of chromium in the human body.

Compartment.	Half life.	Tissue.	Total Cr µg.
Fast.	0.5 to 12 hours.	Adipose and muscle.	0.13
Medium.	1 to 14 days.	About equal in adipose, muscle liver and spleen.	0.8
Slow.	3 to 12 months.	Liver and spleen.	24
Plasma.			0.3

Graf-Harsanyi et al (43) investigated the distribution of Cr in a sample of lyophilised animal serum. The proteins were separated using gel-filtration, measured using conventional spectrophotometry at 280 nm. The Cr concentration was determined using atomic absorption spectrophotometry with electrothermal atomisation. Two chromium containing fractions were found. One was in the high molecular weight range, in the macroglobulin group. The second fraction was found among proteins of low molecular weight, such as albumin and transferrin.

2.5.1. Transferrin.

The iron transport protein transferrin has two Fe binding sites, and is

normally maintained at 30% saturation, so that spare sites are available. The two sites although similar are not identical. Harris (44) reports that the Fe site preference is pH dependent, but only one site will bind Cr. Mn and Cu are also bound by transferrin. The release of Fe by transferrin is very dependent on pH. Bates et al (45) found the half life to be 14 hours at pH 7.5, whereas at pH 4.1 the half life was 2 seconds.

Cr in blood plasma appears from the references cited to be present in three pools. The smallest pool, in which the Cr is bound to small molecules represents about 5% of the total, and is susceptible to excretion by the kidney. The other two pools consist of protein-bound Cr. In one of these pools the Cr is bound to relatively low molecular weight proteins, transferrin predominating. The remaining Cr is bound to alpha-2 macroglobulin. The transferrin bound Cr should be readily released in acid solutions.

2.6. ASSESSMENT OF CHROMIUM STATUS.

The only conclusive test for Cr deficiency at the present time is the response to supplementation using inorganic Cr, and only subjects with gross deficiency can be unequivocably diagnosed.

The relative response following a glucose challenge has been proposed and investigated by many workers for the establishment of Cr status. However this parameter depends on the determination of Cr levels in plasma/serum and or urine, and is discussed in detail in chapter VIII.

2.7. CONCLUSIONS.

The three cases of gross Cr deficiency in subjects on long term parenteral nutrition, represent the only convincing evidence for Cr essentiality, certainly in humans and arguably in mammals. The three patients appeared to respond to inorganic Cr, and the inadvertent co-administration of an identical essential nutrient to all three subjects is improbable. The time interval before the development of Cr deficiency symptoms is consistent with the data in table 2.4.

The reports on rats maintained on a low Cr diet are not so convincing. There is some doubt about the Cr content of the diet, and evidence that the unquestionably high sucrose content of the diet may have been responsible for the observed glucose intolerance.

The contribution of marginal Cr deficiency as a significant factor in glucose intolerance, must be considered as not proven in the three classes of subjects, maturity onset diabetics, pregnant women and haemochromatotics, investigated.

Maugh (46) reported that insulin levels are normal or high in maturity onset diabetics, whereas insulin receptors are reduced. Investigations of Cr supplementation to maturity onset diabetics have failed to produce consistent reports of increased insulin sensitivity, or increased binding of insulin to cell receptors.

The absence of clear evidence that Cr supplementation has a remedial action on individuals with mild glucose intolerance does not preclude beneficial effects on normal subjects. Clearly a metabolic abnormality not due to Cr deficiency, could exert a controlling effect not susceptible to Cr supplementation.

The reliable demonstration of a beneficial effect from Cr supplementation in humans, clearly requires a large scale long term clinical trial, and there are possibilities of marginal harmful effects. Laboratory tests would have only a minor role in this investigation. Inorganic Cr, not yeast would have to be used to eliminate the effects of other factors present in yeast.

2.8. THE PROPOSED INVESTIGATION.

The determination of Cr in plasma/serum or urine is not an easy task, that is clear from the lack of agreement on a normal range. The "probable normal range" has been steadily revised downwards and the true level, is probably near the detection limit attainable at the present time, when the matrix problems are taken into account. The diagnosis of chromium deficiency even if Cr levels are a reliable guide, must depend on the ability to identify values below the normal range; a very exacting demand. The response to a glucose challenge of the four parameters below may be a more realistic investigation to undertake, and a response would itself, be some evidence of an involvement by chromium in glucose metabolism. Analytical techniques for the determination of these parameters are necessary before their value in the establishment of chromium status can be investigated.

Parameters.

1. Total protein-bound serum chromium.
2. Alpha-2-globulin-bound serum chromium.

Parameters. continued.

3. Free serum chromium.

4. Urine chromium, this presumably gives an indirect, time integrated, measurement of the free serum chromium level, and is probably a more realistic goal, as the free serum chromium level is anticipated to be very low.

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CHAPTER III.

REVIEW OF CHROMIUM DETERMINATIONS ON HUMAN SERUM/PLASMA AND URINE.

3.1. SERUM AND URINE CHROMIUM CONCENTRATIONS REPORTED IN THE LITERATURE.

3.1.1. Serum/Plasma Chromium.

Table 3.1 below lists a selection of the published values for serum/plasma chromium, together with the analytical technique used.

TABLE.3.1.

Plasma or serum chromium concentration µg/L.

YEAR.	ANALYTICAL TECHNIQUE.	MEAN.	RANGE.	NOTES.	REFERENCE.
1956	Spectrograph.	22	7 to 52		(1).
1956	Spectrograph.	185	82 to 308		(2).
1959	Spectrograph.	25	16 to 39		(3).
1960	Spectrograph.	28	9 to 56		(4).
1962	Spectrograph.	55	10 to 390		(5).
1966	Spectrograph.	171	/		(6).
1966	Air/hydrogen flame AAS.	28	23 to 34	MIBK extraction after wet ashing and oxidation to Cr(VI).	(7).
1967	NAA.		0.7 to 3.2		(8).
1967	Air/hydrogen flame AAS.	30	11 to 66	MIBK extraction after wet ashing and oxidation to Cr(VI).	(9).
1968	Air/hydrogen flame AAS.	23.2	/	Used method directly above.	(10).
1969	GC.ECD.	447	40 to 1440	tfacac extraction after acid digestion.	(11).
1971	Spectrograph.	28	<10 to 260		(12).
1972	AAS/ETA.	5.1	3.1 to 7.2	Wet ash pretreatment.	(13).
1972	NAA.	9.3	/		(14).
1972	NAA.	10.3	/		(15).
1972	GC.ECD.	13.5	2.7 to 24	tfacac extraction after acid digestion.	(16).
1972	NAA.	45	14 to 77		(17).
1972	AAS/ETA.	4.7	/	Wet ash pretreatment. Al needles used.	(18).

TABLE.3.1. continued.

YEAR.	ANALYTICAL TECHNIQUE.	MEAN.	RANGE.	NOTES.	REFERENCE.
1974	AAS/ETA. D2 bkgr.	1.6	/	Direct injection. Stds. in dextran.	(19).
1974	Emission argon/silver arc.	3.1	/		(20).
1974	Chemiluminescence.	150	/	Wet ash pretreatment.	(21).
1975	AAS/ETA.	<0.5	/		(22).
1975	AAS/ETA.	43	/	Kansas City, Missouri.	(23).
		12	/	Kansas City, Kansas.	
1975	Emission Electrical plasma.	20.5	/		(24).
1976	GC.MEED.	7.2	3.6 to 9.9	Dry ash, tfacac ext.	(25).
1978	NAA.	0.16	0.04 to 0.35	Samples taken using polypropylene cannula.	(26).
1978	AAS/ETA. WI bkgr.	0.14	/	Wet digestion in fused silica tube.	(27).
1978	NAA.	1.7	/		(28).
1978	NAA.	6.0	/		(29).
1979	NAA.	0.45	/		(30).
1979	AAS/ETA. WI bkgr.	0.075	/	Pretreatment- oxidation nitric acid/hydrogen peroxide. Stainless steel needle used!	(31).
1979	AAS/ETA.	8.2	/		(32).
1979	AAS/ETA.	0.7 (serum).	1.0 to 1.5	Ash pretreatment. (Li heparin plasma).	(33).
1980	AAS/ETA.	2.9 (whole blood).		Direct injection	(34).
1980	AAS/ETA. U hc bkgr.	2.0	/	Direct injection dil.1/3 Triton X soln.	(35).
1980	AAS/ETA. D2 bkgr.	4.6	4.4 to 6.1	Direct injection. Method of ref. (19).	(36).
1981	AAS/ETA. D2 bkgr.	1.6	/	Direct injection Method of ref. (19).	(37).
1982	NAA.	1.0	/		(38).
1983	AAS/ETA. WI bkgr.	0.12	/	Direct injection. Plastic catheter for samples.	(39).
1984	AAS/ETA.	3.0 (whole blood).		Pretreatment- wet oxidative digestion.	(40).

TABLE.3.1. continued.

YEAR.	ANALYTICAL TECHNIQUE.	MEAN.	RANGE.	NOTES.	REFERENCE.
1984	AAS/ETA.	0.12	/	Siliconised needle plus PVC tubing for samples.	(41).
1984	AAS/ETA. Zeeman bkgr.	/	10.6 to 31.8		(42).
1984	AAS/ETA. WI bkgr.	0.11	/	Pretreatment- dry ash. Siliconised needle plus PVC tubing for samples.	(43).
1984	PIXE.	8.5	7.6 to 9.4	MIBK extraction after wet ashing and oxidation to Cr(VI).	(44).
1985	AAS/ETA.	0.27	0.09 to 0.63	Pretreatment- partial digestion with bacterial protease. Siliconised needle plus PVC tubing for samples.	(45).
1985	AAS/ETA. WI bkgr.	0.56	0.012 to 1.0	Direct injection.	(46).

Abbreviations.

AAS	atomic absorption spectrometry.
ETA	electro thermal atomisation.
GC	gas chromatography.
ECD	electron capture detector.
MEED	microwave excited emission detector.
NAA	neutron activation analysis.
PIXE	proton-induced X-ray emission.
bkgr	background correction.
D2	deuterium lamp.
WI	tungsten iodide lamp.
MIBK	methyl isobutyl ketone.
tfacac	1,1,1-trifluoro-2,4-pentanedione.

3.1.1.1. Discussion.

The obvious trend is towards lower mean values. The mean value in the decade starting in 1960 was 126 µg Cr/L, falling to 16 µg/L in the next decade and to 1.9 in the period from 1980 to 1985. AAS/ETA has

become the most popular technique and the two techniques AAS/ETA and NAA are the only ones to have produced reported mean values below 5.0 µg Cr/L. In the period from 1980 to 1985 ten of the eleven results reported were obtained using AAS/ETA and five of the mean values quoted were less than 1 µg Cr/L. Furthermore, three of the five mean values below 1 µg Cr/L were in the 0.1 to 0.2 µg/L range and one of the other two values was only slightly higher at 0.27 µg Cr/L.

A major cause of falsely elevated serum/plasma values appears to be contamination with extraneous Cr during the sampling procedure, and this will be discussed in detail in chapter VII. The published values of less than 0.3 µg Cr/L were, with two exceptions quoted by workers who reported taking stringent precautions against sample contamination during specimen collection.

Three authors reported mean levels of less than 0.3 µg Cr/L prior to 1980, and one (Versieck et al (26)) used a polypropylene cannula for sample collection. However Vanderlinde et al (31) stated that a conventional stainless steel needle was used for the collection of samples which gave a mean serum chromium level of 0.075 µg/L, and Kayne et al (27), in default of any specific details for sample collection presumably also used conventional equipment. Stainless steel needles from different sources and even different batches from a particular source may vary as sources of extraneous chromium: perhaps Vanderlinde and Kayne were just lucky.

The four workers who reported mean values of less than 0.3 µg Cr/L in the period from 1980 to 1985, avoided contamination at venepuncture from the conventional stainless steel needle by using a plastic cannula, or combination of a short siliconised steel needle attached to a length of PVC tubing.

The four authors above, who reported values of less than 0.3 µg Cr/L, all used Perkin Elmer 5000 atomic absorption spectrophotometers equiped with HGA 500 furnaces. The important characteristic of this model appears to be the background correction capability, both tungsten iodide and Zeeman facilities are available. Three workers Anderson et al (41), Kumpulainen et al (39) and Veillon et al (43), used tungsten iodide whilst Offenbacher et al (45) used Zeeman. Kumpulainen used direct injection, and background correction must have been obligatory. Offenbacher used an unusual pretreatment process, partial digestion with proteolyic enzymes derived from bacteria, whilst Veillon used the

more traditional dry ash with magnesium nitrate, however both still found background correction was necessary. The last two teams of investigators, Offenbaker and Veillon enjoyed the luxury of Class 100 clean areas for their pretreatment processes. Anderson may have used direct injection, he certainly did for urines but no details are given or referred to for serum samples.

Simonoff et al (44) reported the high reference value of 8.5 µg Cr/L and claimed that losses of Cr as a volatile component during processing, and or the measuring process, was responsible for the lower values quoted in the literature. The evidence for and against the existence of a volatile chromium component in biological materials, and premature losses during processing and or at the ash stage of the ETA cycle are discussed in section 3.4.1.

The main conclusions from the discussion above are that blood samples must be collected using a plastic cannula, and if AAS/ETA is used, then an instrument equipped with tungsten iodide or Zeeman correction is probably needed.

3.1.2. Urine Chromium.

Table 3.2 below lists a selection of the published values of Cr levels in urine, together with the analytical techniques used.

TABLE.3.2.

Urine chromium concentrations µg/L.

YEAR.	ANALYTICAL TECHNIQUE.	MEAN.	RANGE.	NOTES.	REFERENCE.
1969	GC.ECD.	36.5	4 to 196	tfacac extraction after acid digestion.	(11).
1972	AAS/ETA.	5.2	2.6 to 10.6	Wet ash using perchloric acid.	(13).
1973	AAS/ETA.	11.7	3.0 to 38	Direct injection.	(47).
1978	AAS/ETA. WM. bkgr.	0.5	0.2 to 0.7	Continuum source echelle monochromator AAS.	(48).
1978	AAS/ETA. WI. bkgr.	1.1	0.2 to 2.2	Acid digestion.	(27).
1979	AAS/ETA. D2. bkgr.	8.5	/	Direct injection.	(49).
1979	AAS/ETA. WI. bkgr.	0.4	/	Acid digestion.	(31).
1980	AAS/ETA. D2. bkgr.	0.79	/	Hydrogen diffusion flame shield supplement to argon.	(50).

TABLE.3.2. continued.

YEAR.	ANALYTICAL TECHNIQUE.	MEAN.	RANGE.	NOTES.	REFERENCE.
1980	AAS/ETA.	0.48	/	Low temp. ash & atomisation.	(34).
1981	AAS/ETA. WI. bkgr.	0.15	/	Direct injection. Values confirmed using:- i) stable isotope GC. MS. ii) CEWM-AAS.	(51).
1982	AAS/ETA.	0.72 (20 to 35 yrs.) 0.39 (47 to 69 yrs.)		Direct injection.	(52).
1983	AAS/ETA. D2. bkgr.	0.8	0.2 to 2.4	Low temp. atomisation.	(53).
1983	AAS/ETA. WI. bkgr.	0.13	/	Direct injection.	(39).
1983	ICPES.	1.0	/	Selective preconcentration using chelating resins.	(54).
1983	AAS/ETA. WI. bkgr.	0.11	/	Direct injection.	(55).
1984	AAS/ETA. D2. bkgr.	0.5	/	Direct injection.	(56).
1984	AAS/ETA.	4.9	/	Wet oxidative digestion.	(40).
1985	AAS/ETA. WI. bkgr.	0.31	0 to 0.77	Direct injection.	(57).

Abbreviations.

AAS atomic absorption spectroscopy.
ETA electrothermal atomisation.
GC gas chromatography.
ECD. electron capture detector.
WM. wavelength modulation.
WI. tungsten iodide lamp.
D2. deuterium arc lamp.
bkgr. background correction.
MS. mass spectrometry.
CEWM. continuum source echelle monochromator wavelength modulated.
ICPES. inductively coupled plasma emission spectroscopy.

3.1.2.1. Discussion.

The trend to lower values is not so marked for urine chromium levels compared with those for serum/plasma. The averages of the mean values from table 3.2. for the inclusive five year periods 71 to 75, 76 to 80 and 81 to 85 are respectively, 6.2, 2.3 and 1.0 µg Cr/L. Chromium determinations on urine have been virtually exclusively by AAS/ETA.

Direct injection techniques are clearly more suited to urine samples than to serum/plasma specimens, because of the relatively simple matrix of the former, and much work has been done to overcome background absorption problems . The use of relatively low atomisation temperatures, with a carefully controlled ash treatment has been claimed to produce satisfactory results, even with deuterium arc background correction, by Halls and Fell (53), Routh (50) and Brodie and Routh (56).

Nomiyama et al (34), and Ping et al (52) claimed that with suitable furnace parameters background correction was not needed. Veillon et al (51), Kumpulainen et al (39) and Morris et al (57) used a tungsten iodide lamp for background correction. Veillon and Kumpulainen appear to have a significantly lower mean than the other workers. Anderson et al (55) using the technique developed by Veillon et al, also quoted a very low value, however for obvious reasons urine values in µg Cr referred to a given creatinine excretion are more comparable.

The lack of comparable data, that is µg Cr referred to a given creatinine excretion, is a handicap. The work of Veillon et al (51) and Anderson et al (55) indicates that urine chromium excretion must be less than 2 µg/day. Veillon reported that as only 0.5 to 1 % of chromium in the diet was absorbed (radiotracer experiments), and as no reasonable diets in the U.S. could supply more than 100 µg Cr/day, then urinary excretions above 2 µg/day, certainly on U.S. residents, were improbable. Anderson reported a mean excretion of 0.22 µg Cr/day and verified the urinary values by three independent AAS/ETA methods and by a stable isotope gas chromatography/mass spectrometer procedure.

Firm conclusions cannot be made from the above data, but if the U.S. dietary figures are correct, then the low values reported by Anderson, Kumpulainen and Veillon for urinary Cr are favoured. The

techniques using deuterium arc background correction, even with low temperature atomisation, and the direct injection methods with no background correction, must then be suspect.

3.2. ANALYTICAL TECHNIQUES USED.

A large number of analytical techniques have been used for the measurement of Cr in plasma/serum and urine, but only three have demonstrated a low enough detection limit for determinations below 1 µg/L. These techniques are atomic absorption with electrothermal atomisation (AAS.ETA), neutron activation analysis, (NAA) and stable isotope dilution mass spectrometry (IDMS). The most widely used by far is AAS.ETA, 85% of reports on plasma/serum, and 91% of reports on urine, published since 1980, have used this technique. The relatively low cost and high throughput of AAS/ETA make this the method of choice, and it is the technique to be used in this study. The three above techniques are discussed below, AAS/ETA in detail the other two very briefly, as although the results obtained are of considerable significance the equipment used has very limited availability so far as the clinical chemist is concerned. The IDMS and NAA methods used by Veillon and Versieck respectively included a dry ash preliminary step, but both Veillon and Versieck investigated losses of "volatile Cr" during ashing procedures, and used conditions they considered to be acceptable. The work they carried out in this field is included in section 3.4.1.

3.2.1. Stable Isotope Dilution Mass Spectrometry.

Veillon et al (58) used this method as a reference method for the simpler direct injection AAS/ETA technique (51).

Lyophilised urine samples were ashed in an oxygen plasma discharge and the Cr extracted as the trifluoroacetylacetone chelate. Samples were spiked with ^{50}Cr and the isotope ratio measured by combining gas chromatography-mass spectrometry, using dual ion monitoring. The reported Cr level on a human urine pool was 0.32 µg/L, with a standard deviation of 0.019 (n = 4). The urine pool was also subjected to Cr determination using the novel continuum source, echelle monochromator, wavelength-modulated AAS described in section 3.3.1.1. The results by this later method agreed within experimental error, with a mean of 0.34 µg Cr/L, and a standard deviation of 0.1 µg/L.

3.2.2. Neutron Activation Analysis.

Versieck et al (26), reported a mean serum Cr value of 0.16 µg/L, and this was the only mean value below 1 µg Cr/L measured by NAA reported in the table.

Lyophilised serum samples corresponding to 1 mL of serum were irradiated, after dry ashing, in a nuclear reactor for 12 days at a high neutron flux rate. A decay period of 30 days was followed by a selective separation of Cr as chromyl chloride. The gamma spectra were measured using a germanium/lithium detector. The blood samples were collected using a polypropylene catheter into high purity quartz tubes, and the latter were used throughout the procedure.

The results obtained are very useful as reference values but clearly the method is not one that can be widely used.

3.3. AAS WITH ELECTROTHERMAL ATOMISATION (AAS/ETA).

Flameless AAS is claimed by the leading instrument manufacturers to have a sensitivity for Cr of about 1 to 10 pg, corresponding to 5 to 50 µL of a solution containing 0.1 to 1.0 µg Cr/L. However this is true only for dilute aqueous solutions, but not for complex biological matrices.

3.3.1. Matrix effects.

Analytical interferences in AAS/ETA can be severe even for elements and matrices that are normally interference free with flame AAS. Matrix effects are particularly severe with biological samples because of their complexity. Background effects constitute a major source of error, but other interference effects can cause problems. Physical effects can affect the "spread" of the injected material within the graphite tube, and this will influence the analytical response. Matrix dependent chemical effects may cause an apparent enhancement or depression of the response, these may be classified as stable compound effects, volatile compound effects and vapour phase interferences.

3.3.1.1. Background correction.

Salt particles and smoke are particularly troublesome for chromium measurements on biological samples. Background absorption is much greater in the UV range and background correction is rarely required above 430 nm. The most sensitive resonance line for Cr measurements is 357.9 nm, low enough for significant interference by

background absorption and too high a wavelength for a deuterium arc, the usual background correction source to provide adequate intensity.

Guthrie et al (58) conclusively demonstrated in 1978, that AAS/ETA instruments in use at that time were simply measuring background absorption of urinary components. Deuterium lamps were used as the background correction continuum source, and these had insufficient intensity at the wavelength used to measure Cr in both plasma and urine. The Cr lamp must be run at a low intensity to balance the deuterium arc and a high gain setting is then needed. Halls and Fell (53) stated that the magnitude of the interference was increased with increasing gain used on the spectrometer, thus the use of background correction could increase the interference. However Halls and Fell found that reducing the atomisation temperature to 2400 ^{0} C completely eliminated the problem providing the urine was diluted with an equal volume of water before sampling. Routh (50) also claimed to have successfully used a deuterium arc for background correction in urine Cr determinations. Routh also used a low atomisation temperature, 2300 ^{0} C. However he further reduced the background absorption, by using a hydrogen diffusion flame as a supplement to the inert gas flow through the graphite furnace. Routh claimed to have reduced the background absorbance to 1/3rd of its original value using this technique. However, as reported earlier, there is some evidence that these low temperature atomisation methods are not satisfactory.

A number of instrument modifications have been developed to overcome the inadequacies of the deuterium arc system.

Kayne et al (27) used a quartz-halogen lamp to give greater intensity at the Cr resonance line. Commercial instruments are now available with quartz-halogen lamps built in for background correction in the visible region.

Thompson (35) used the 358.5nm line of a uranium hollow cathode lamp as the background correction source for plasma Cr measurements.

Guthrie et al (59) used the novel continuum source, echelle monochromator wavelength-modulated AAS developed by Harnly et al (60). This system is claimed to have the benefits of double beam and background correction with a much simpler optical system.

Zeeman and Smith-Hiefjte background correction systems are available on commercial instruments. The currently available background systems all give reduced sensitivity, but differ in other

characteristics.

3.3.1.1.1. Comparison of background correction systems.

Broad comparisons only are possible, as the different background correction systems have not been used under identical conditions. However continuum source background correction systems which use two lamps will give optimum performance only when both lamps are carefully aligned.

i) Sensitivity.

All background correction systems give reduced sensitivity. Continuum source (deuterium arc or tungsten iodide) correction requires a beam splitter and suffers an attendant 50% light loss, nevertheless this system has the highest sensitivity of any background correction system. The Zeeman system gives reduced sensitivity due to the light loss at the polarizer and because splitting is not usually complete, so that there is some analyte absorption included in the background measurement. The reduction in sensitivity using Smith-Hieftje correction is caused by some analyte atomic absorption occurring during the high current pulse. Zeeman and Smith-Hieftje are probably similar in terms of sensitivity. Intensity of the source is the limiting factor in the continuum source echelle monochromator wavelength-modulated AAS. The xenon source used by Harnly et al, has low intensity in the UV region and maximium output at 480nm. The sensitivity using this instrument for Cr appears from Harnly's report to be better than that given by the Zeeman system, but lower than that found with conventional AAS using a tungsten iodide correction source.

ii) Ability to handle high background absorbance.

The continuum source echelle monochromator wavelength-modulated AAS developed by Harnly et al is reported to be capable of handling a background absorbance of 3.0 units. Sotera and Kahn (61) report that the deuterium arc system can correct for background absorbances as high as 1.5 units at the Cr 357.9nm line in instruments of good optical design. Perkin Elmer claim that their Zeeman 5000 system can correct for up to 2 units of background absorbance, and Instrumentation Laboratory state that the Smith-Hieftje system on their IL Video 11 and IL Video 22 instruments can handle up to 3 units of background absorbance.

iii) Structured background.

The continuum source system (deuterium arc or tungsten iodide)

cannot cope with structured background. The other systems discussed above are reported by their principal protagonists to be able to correct for structured background.

iv) Spectral overlap.

Spectral interferences are rare in AAS, but the absorption band of some other element can overlap the emission line of the hollow cathode lamp characteristic of the analyte in some cases. The continuum source echelle monochromator wavelength-modulated AAS has the best reported performance in this field. Zander et al (62) claimed correction for spectral overlaps with differences as small as 0.003 nm. Perkin Elmer report satisfactory correction with lines 0.02 nm apart for their Zeeman 5000 system, whilst Instumentation Laboratory can only manage 0.05 nm for their Smith-Hieftje equipment. Deuterium arc and tungsten iodide systems are of course unable to cope with spectral overlap.

v) Linearity of the corrected curve.

The Zeeman system is the poor performer in this field, because as the concentration of the analyte increases, the sidebands broaden and begin to absorb progressively more of the perpendicular component of the source radiation. This analyte absorbance is seen as background by the correction system, and is subtracted, causing the working curve to bend over and eventually to reverse direction, according to Fernandez et al (63). The usable range is therefore limited to about 0.5 absorbance units, which is ample for Cr determinations on serum/plasma or urine.

The calculation of absorbance for the wavelength modulated system is not easy, but Harnly et al (60) claimed to have developed a circuit.

vi) Cost.

The continuum source echelle monochromator wavelength-modulated AAS is probably the most expensive, as the monochromator must be of very high quality, and the electronics are complicated. The high production costs are presumably responsible for the absence of a commercial instrument of this type. The Zeeman system is also expensive. However the Smith-Hieftje equipment is even cheaper than a deuterium arc.

vii) Conclusion.

The important characteristics for Cr determinations on

biological fluids are probably sensitivity and ability to handle high background absorbances, because of the low analyte concentrations and smoke generating properties of the samples. The data in tables 3.1 and 3.2 indicates that the tungsten iodide system is probably the most suitable, assuming that serum and urine Cr levels are below 0.3 µg/L.

3.3.2. Chemical Effects.

The depressed response by Cr in the presence of chlorides, which is attributed by some workers to the loss of Cr as the volatile halide, is discussed and investigated in chapter V, section 5.3.3.

3.4. PRETREATMENT PROCESSES.

The 5 techniques listed in table 3.1 which report values of less than 0.3 µg Cr/L for serum samples, include two by direct injection, one partial digestion using bacterial proteolytic enzymes, one dry ash and one wet ash. The reported urine levels below 0.3 µg Cr/L in table 3.2 are all derived from direct injection techniques.

The main controvery in the pretreatment field appears to be concerned with the loss of a volatile chromium fraction.

3.4.1. Volatility Losses.

Simonoff et al (44), found that dry ashing at 500 ^{0} C resulted in apparent loss of up to 50% of the original Cr, but conceded that the lost Cr could be retained in acid insoluble residues.

Shapcott et al (64) reported in 1977 that losses of Cr occurred from serum, liver , kidney and muscle when ashed at temperatures greater than 750 ^{0} C. However no losses of exogenous ^{51}Cr added to control tissues were found. The volatile Cr was attributed to "glucose tolerance factor". In contradiction to this Shapcott (65) stated in 1979 that no loss of ^{51}Cr from spiked urine and serum heated to 1000 ^{0} C occurred. The contradiction is that Shapcott in the later report appears to be accepting the ^{51}Cr spike as a reliable guide to losses of the native Cr.

Jones et al (66) reported no losses of ^{51}Cr from endogenously labelled brewers yeast with oven drying, lyophilisation, or wet digestion even with high levels of chloride at up to 800 ^{0} C.

Versieck et al (26) investigated the volatilization during dry ashing rigorously, in the dry ashing stage of the serum Cr determination procedure using NAA which produced a mean value of 0.16 µg Cr/L. No losses of radioactive Cr were recorded from serum dry

ashed at up to 450 ^{0} C. The serum was from patients who had received ^{51}Cr intravenously, and from rats fed for one week with ^{51}Cr(III) and ^{51}Cr(VI). Versieck also dry ashed organic material from the National Bureau of Standards (NBS). NBS bowens kale, orchard leaves and bovine liver were reported to show no losses after dry ashing at 450 ^{0} C. Finally, NBS brewer's yeast showed no losses when dry ashed at 800 ^{0} C.

Simonoff et al considered that the tests carried out by Versieck were not conclusive as : "inorganic Cr is only slowly converted to a volatile (organic) form, the loss of which would not be detected by the foregoing, short term tests."

The tests carried out by Versieck appear to be reliable and the evidence for the structure of "GTF" certainly does not suggest a volatile compound, particularly in the presence of available oxygen. The loss of volatile Cr at the ash stage of the ETA cycle, which is discussed and investigated in chapter V, section 5.3.3., under conditions in which volatilization is more probable, appears not to take place.

3.4.2. Effectiveness of the Pretreatment Process.

The two aims of the pretreatment process for Cr determinations by AAS/ETA on serum or urine are to produce a solution in which the Cr is concentrated, relative to the original sample, and is suitable for measurement without the need for background correction, using ideally, primary standards for calibration.

The two methods for serum Cr determinations giving values below 0.3 µg Cr/L in which a pretreatment process was used, Veillon et al (43) and Offenbacher et al (45) both required background correction at the AAS/ETA stage. The process used by Offenbacher et al resulted in a dilution (1/2) of the original sample, and the method of additions was required for calibration. Veillon et al did not specify the volume of solvent used to dissolve the ash residue, and hence no concentration factor can be deduced. Veillion used bovine serum for the calibration standards.

3.5. DETECTION LIMIT.

There is no universally accepted definition of the "detection limit" of an analytical process, and many workers do not quote one, or give sufficient data for a relative value to be calculated by applying a standard formula. However, the 4 techniques for serum/plasma Cr

determinations giving mean values of <0.3 µg Cr/L, referred to below, gave broadly similar data as listed in table 3.3.

TABLE 3.3.

Data from four serum/plasma Cr techniques.

Reference.	Serum/plasma Cr µg/L.		Blank Cr µg/L.		Detection
	Mean.	Range.	Mean.	Range.	limit.
(26).	0.16	0.04 to 0.35	0.048	0.03 to 0.07	/
(39).	0.12	/	/	/	0.05
(43).	0.11	<0.05 to 0.29	0.02	/	0.05
(45).	0.27	0.09 to 0.63	0.04	/	/

It can be seen that the relationship between the detection limit and the normal range is in all cases, unfavourable for the confident identification of samples with levels below normal.

3.6. CONCLUSIONS.

The wide scatter of reported means for chromium levels in serum/plasma and urine confirm that severe problems are associated with these determinations.

The ability to confidently identify chromium levels significantly below the lower limit of the reference range requires that considerable improvements in the detection limit are made. The considerable advances that have been made in background correction systems have clearly contributed to published work in the last few years and have certainly narrowed the uncertainty around the normal range. However no background correction system is entirely satisfactory at the present time.

A major cause of falsely elevated serum/plasma values appears to be contamination with extraneous chromium during the sampling procedure. The published values of less than 0.3 µg Cr/L were, with two exceptions, quoted by workers who reported stringent precautions against sample contamination during specimen collection.

The available evidence indicates that the mean serum/plasma chromium level is certainly less than 0.3 and probably close to 0.15 µg Cr/L.

The lack of comparable data for urines, that is µg Cr referred to a given creatinine excretion is a handicap. The work of Veillon et

al (51) and Anderson et al (55) referred to earlier, indicated that urine chromium excretion must be less than 2 µg/day.

3.7. THE PROPOSED INVESTIGATION.

The information derived from the topics discussed in this chapter for the proposed investigation indicates that both serum and urine Cr levels are probably very low, <0.3 µg Cr/L for the former and a probable excretion of less than 1 µg/day for the latter. The methods developed, besides having the appropriate detection limit and sensitivity for the above low levels, should be such that loss of Cr as a volatile component of the original samples, cannot occur.

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CHAPTER IV.

THE DEVELOPMENT OF A METHOD FOR THE DETERMINATION OF CHROMIUM IN PLASMA/SERUM.

4.1. THE GENERAL DESIGN OF THE METHOD.

The principal features governing the design of the method are the very low concentration of chromium anticipated, and the extreme complexity of the matrix. A highly selective extraction producing a concentrated final solution with the minimum contamination by other serum constituents is required. The minimum number of stages and reagents should be used , and the ratio of sample volume to reagent volume must be as large as possible, to minimise the addition of extraneous chromium. The reagents used should be available with very low chromium levels, and be suitable for purification by relatively simple techniques. The final solution must be suitable for graphite furnace AAS without background correction. Simple pretreatments must be developed to separate the protein-bound chromium from the unbound, and to remove the transferrin-bound fraction from the total protein-bound metal.

The most promising technique to achieve a large selective concentration step is solvent extraction, using ideally an alkane or as second choice an arene. The vast majority of the constituents of human serum are too hydrophilic to be extracted by petroleum spirit. Cholesterol is the only major serum component that is soluble in this solvent, but it is virtually non-extractable unless the lipoproteins are severely denatured (Abell et al (1)). The vast majority of drugs are too polar to be extracted by petroleum spirit alone. The addition of a small amount of a more polar solvent such as hexanol is needed to extract the majority of the drugs commonly measured in the clinical chemistry laboratory. Fatty acids are extracted from acidified serum by alkanes but under the acid conditions used to remove the transferrin bound chromium the fatty acids are removed to waste at this preliminary stage. A second advantage of petroleum spirit is the low concentration of chromium in this solvent.

The two beta diketones 1,1,1-trifluoropenta-2,4-dione (tfacac) and 1,1,1,5,5,5-hexafluoropenta-2,4-dione (hfacac) have been used for chromium determinations on serum as reported in chapter III, and

although the chromium levels found using these materials appear to be too high, these beta-diketones are the obvious choice to produce alkane soluble complexes from ultra low concentrations of Cr(III). The best path to isolate the chromium in a form suitable for AAS/ETA is probably by back extraction from a high boiling point petroleum spirit, using a volatile acid or base to minimise the solute concentration. Hydrochloric acid and ammonia are obvious choices as acid and base respectively, as both these are readily volatilised from the extract and are relatively easy to purify.

The protein precipitation pretreatment can be combined with the dehydration needed prior to the reaction with hfacac by using propan-2-ol. The dehydration step is required with hfacac because this reagent reacts with water to form a hydrate which is a weak complexing agent compared with the parent beta-diketone. The decantation and subsequent evaporation of the supernatant provides a simple separation of the non protein-bound chromium.

The ease with which iron can be removed from transferrin by dilute acid has been reported earlier (2.53). The transferrin-bound chromium, together with the non protein-bound chromium, can probably therefore be removed by precipitating the serum proteins under dilute acid conditions.

The experimental work in this and subsequent chapters is outlined in the main text and reported in more detail in an experimental section under references with the prefix "E" at the end of the appropriate chapter, which is denoted by the first digit of the reference number, for example, the details of procedure CDI are recorded in E401, at the begining of the experimental section of this chapter. The experimental details are, for the sake of brevity, recorded as "standard procedures" wherever possible. For example the serum chromium determination pretreatment processes have been recorded as CDI, CDII and CDIII, representing the procedures used at three main stages in the continuous development of the adopted method which is CDIII. The between main stage experiments are reported in terms of deviations from the appropriate standard procedure.

4.2. MONITORING THE RECOVERY OF THE CHROMIUM.

The obvious choice for monitoring the recovery of the Cr(III) present in the sample is ^{51}Cr(III). A demonstration that the added

chromium exchanges with the native element is essential, and ideally the added chromium should represent less than 1% and certainly less than 10% of the native metal.

The first experiment demonstrated that added Cr(III) readily exchanged with the native element at 60° C, and rather slowly at 37° C. In this experiment radioactive chromium as both chromium chloride (0.38 µg Cr/L) and crude GTF (preparation, Toepfer et al,(2)) (0.48 µg Cr/L) was added to two aliquots of a human serum pool. The percentage of radioactive chromium precipitated by propan-2-ol and by 0.6M perchloric acid from the two pools using procedure CD1 (E401) was measured both before and after heat treatment at 37° C and 60° C. The 37° C pool was sampled after 24 and 72 hours and the 60° C pool after 1 hour. The propan-2-ol precipitates from all samples contained 98% ± 1 of the radioactive chromium. However the perchloric acid precipitated widely disparate amounts from the "cold" chloride and GTF pools, but increasing heat treatment brought these fractions together as the radioactive chromium was redistributed. The redistribution of the radioactive chromium is illustrated in figure 4.1.

The two cold labelled serum pools were subjected to electrophoresis using the standard electrophoresis conditions described in the experimental section (E404). The fractions of the radioactive chromium extracted by the perchloric acid closely matched the percentages bound by the beta-globulins, presumably by the transferrin component of this serum protein fraction. The results are given in table 4.1 below.

TABLE 4.1.

Fraction of ^{51}Cr extracted by perchloric acid compared with that bound by the beta-globulins.

		Perchloric acid extracted.		Beta-globulin bound.	
	n.	Mean.	RSD.	Mean.	RSD.
Chromium chloride spike.	4	74%	3.3%	76%	5.1%
Crude GTF spike.	4	28%	1.8%	27%	6.6%

t tests confirm no significant difference between perchloric acid extracted and beta-globulin-bound fractions.

A human serum pool was spiked with radioactive Cr(III) chloride at three different concentrations in a third experiment. Aliquots of the three labelled pools were then heated at 60° C for 30 minutes to speed up the equilibration process. Samples of the three pools both before and after the heat treatment were then subjected to electrophoresis using the standard conditions (E404). The results are illustrated in figure 4.2. A substantial fraction of the radioactive chromium bound to beta-globulin in the cold aliquot, migrated on heating to the alpha-2-globulin fraction, in the pool with the lowest concentration of added chromium. However no significant changes occurred on heating the pools with the higher concentrations of radioactive chromium.

The three experiments described above confirm that added chromium rapidly exchanges with the native element at 60° C. This ready exchange indicates that added radioactive chromium can give a meaningful guide to the recovery of the native element during the processing preceding the AAS chromium determinations. The size of the spike to be added in the final recovery checks must be decided in the light of the chromium levels found in the test samples. The agreement between the percentage of chromium extracted by 0.6M perchloric acid and that bound by the beta-globulin fraction supports the susceptibility of transferrin-bound chromium to removal by dilute acids. However this is reported in more detail in the section on protein precipitation.

4.2.1. The Distribution of Chromium in Human Serum Protein Fractions.

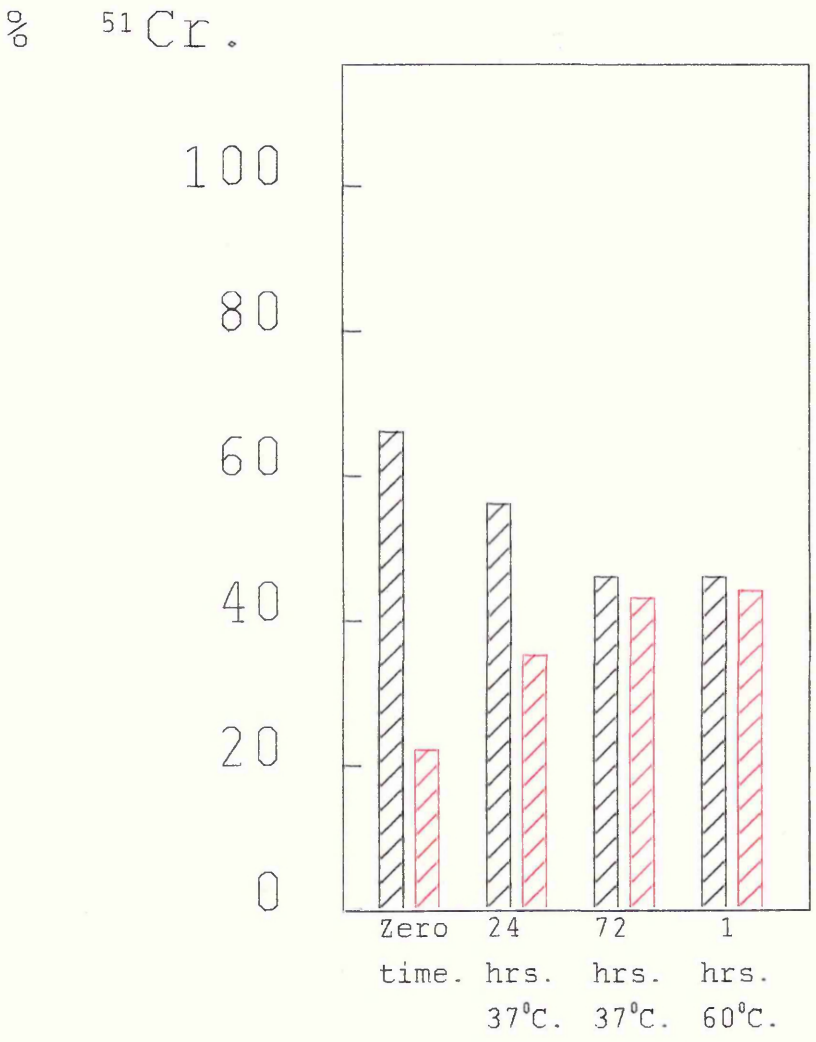
The chromium distribution results from the electrophoresis experiments described above are given in table 4.2 below.

The three major chromium containing fractions were albumin, alpha-2-globulin and beta-globulin. The chromium binding species in the beta-globulin fraction is presumably transferrin. The agreement between perchloric acid extractable and beta-globulin-bound percentages, and the capacity of this relatively small fraction to accept 80% of the 38 µg Cr/L spike, as reported in table 4.2 above, support this.

The marked differences shown by the "cold" and "hot" aliquots of pool A were not reflected in the other two chromium chloride spiked pools. The redistribution from beta-globulin to alpha-2-globulin, and to a lesser extent to albumin, presumably cannot take place on a similar percentage basis at the higher chromium levels because a

FIGURE 4.1.

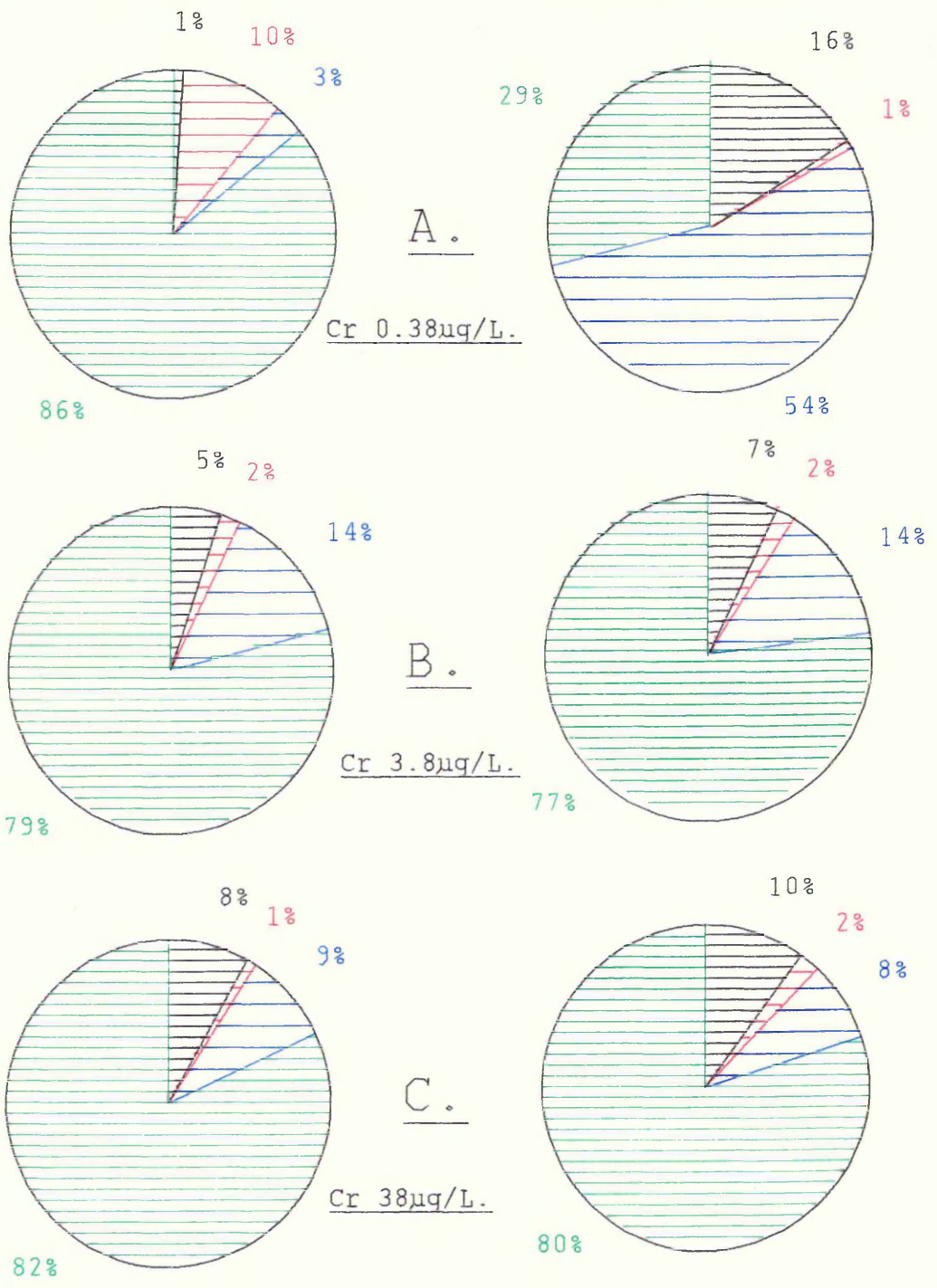
Comparison of the percentage ^{51}Cr precipitated by 0.6M perchloric acid before and after equilibration.



Black "GTF".
Red $CrCl_3$.

FIGURE 4.2.

Chromium distribution profile in serum proteins before and after equilibration.



Black albumin.
Red alpha-1-globulin.
Blue alpha-2-globulin.
Green beta-globulin.

limited number of binding sites are available. The crude GTF added to the serum at a similar chromium level to that in pool A showed relatively high binding to albumin and especially to alpha-2-globulin, without equilibration.

TABLE 4.2.

Distribution of ^{51}Cr in Human Serum Protein Fractions.

	Chromium chloride spikes.						GTF spike.
µg Cr/L.	(A) 0.38		(B) 3.8		(C) 38		0.48
	Cold.	Hot.	Cold.	Hot.	Cold.	Hot.	Cold.
Albumin.	1%	16%	5%	7%	8%	10%	30%
Globulins.							
Alpha-1.	10%	<1%	2%	2%	1%	2%	<1%
Alpha-2.	3%	52%	14%	14%	9%	8%	43%
Beta.	86%	27%	78%	74%	79%	77%	27%
Gamma.	1%	5%	1%	2%	2%	3%	<1%

Cold = no heat treatment.

Hot = heat equilibration (60 ^{0} C for 1 hour).

The above observations provide some evidence that alpha-2-globulin and or albumin may have some functional binding sites for human GTF and Kallee (3) reported that alpha-2-macroglobulin binds insulin and is probably responsible for the physiological transport of this hormone, with which "GTF" is often associated.

The observation by Graf-Harsanyi et al (4) discussed in chapter II that the protein-bound chromium in animal serum is mainly present in two protein fractions , namely transferrin and alpha-2-globulin is in general supported by the electrophoresis results above. The chromium precipitated under dilute acid conditions has been labelled "alpha-2-globulin—bound chromium" in this work, as this appears to be the major chromium containing component of the precipitate.

4.3. FLOW CHARTS.

Two flow charts have been introduced at this early stage to ensure that both the sample pretreatment process and the development of this process can be easily followed.

4.3.1. Flow Chart of the Pretreatment Process Developed.

Figure 4.3 is a flow chart for procedure CDIII, the method used

for the serum chromium determinations the results of which are reported and discussed in chapter VIII.

4.3.2. Development Flow Chart.

The development process outlined in figure 4.4 is the theme of this chapter. The extended description of the development process has been broken down into the same stages and follows the same order as given in the flow chart. The actual development process followed a reiterative pattern of course, and included contamination control and developments in the AAS/ETA area. Contamination control and AAS/ETA optimisation are described in later chapters.

4.4. SPECIMEN VOLUME.

The sample volume was progressively increased from 250 µL through 1000 µl to 5000 µL as evidence for the very low concentration of chromium in human serum accumulated. The volumes of the reagents used were not increased in proportion and a steady increase in the sample derived Cr to reagent blank was achieved. A fall in Cr extracted from the surfaces of the containers used, relative to the sample Cr should also theoretically result from the increase in sample volume. All volumes used were finally reduced by 20% to increase the pipette "freeboard" to help in contamination control, enabling pipettes to be used at 80% of their maximimum capacity, an acceptable compromise between precision and contamination goals. The large sample volume is clearly a disadvantage, but is put into perspective by the problems associated with obtaining an uncontaminated blood specimen which involves discarding, for chromium determination purposes the first 30 mL of blood after venepuncture with a stainless steel needle.

4.5. PROTEIN PRECIPITATION.

A reagent miscible with both petroleum spirit and water, and capable of precipitating the serum proteins, was required for the determination of the total protein-bound chromium. The miscibility with petroleum spirit was necessary for the penetration of the precipitated protein mass by the hydrophobic solvent. Propan-2-ol was an obvious choice and was found to be suitable both for total protein-bound chromium determinations and, plus 0.5M hydrochloric acid for alpha-2-globulin-bound chromium measure

Perchloric acid was initially used as the protein precipitant

FIGURE 4.3.

Method CDIII flow chart.

Serum specimen.

1. Protein precipitation. using:- i) propan-2-ol for total protein-bound Cr. ii) 0.5M HCl in propan-2-ol for alpha-2-globulin-bound Cr.	Non protein-bound Cr to waste.
Roller mixer 15 min.	Non protein-bound Cr, albumin-bound Cr and transferrin-bound Cr to waste.
2. Wash, using propan-2-ol.	Dehydration and removal of unwanted Cr fractions completed.
Shake to resuspend.	
3. Wash X 2, using toluene.	Removal of propan-2-ol, as not suitable for $Cr(hfacac)_3$ conversion of serum Cr.
75 ° C 15 min.	
4. Add, acetic/sulphuric acid and hfacac.	Conversion of serum Cr to $Cr(hfacac)_3$.
75 ° C overnight.	
5. Add, petroleum spirit.	Selective extraction of $Cr(hfacac)_3$.
Roller mixer 4 hrs.	
6. Wash, 2.2M K_2HPO_4.	Removal of excess hfacac as hydrated derivative.
Roller mixer 1 hr.	
7. Wash, water.	Completes removal of excess hfacac, and ensures no neutralisation of NH_3 by phosphate.
Roller mixer 1 hr.	
8.Back extraction, using 5M NH_3 plus EDTA.	$Cr(hfacac)_3$ to $Cr(OH)_6^{3-}$.
Roller mixer overnight.	
9. Evaporation, of NH_3 and water.	$Cr(OH)_6^{3-}$ to CrEDTA.
80 ° C, desiccator.	
10. Dissolve Cr(EDTA), using 0.5M NH_3 and 0.7M acetic acid.	
80° C, vortex, 10 min.	

Chromium in solution suitable for AAS/ETA.

FIGURE 4.4.

Development flow chart (sheet 1).

1. Specimen volume (4.4).

i) 250 µL.

ii) 1000 µl.

iii) 5000 µL.

iv) 4000 µL.

Increasing specimen : reagent ratio. Test derived Cr : blank Cr increased. Final scale down by 20% to increase pipette "freeboard" to help in contamination control.

2. Protein precipitation (4.5).

a) TOTAL CHROMIUM.

i) Methanol.

ii) Propan-2-ol.

Very bulky precipitate, not easily washed, and only 85% Cr pptd. Propan-2-ol ppt. superior, 98% Cr pptd.

b) "GTF" CHROMIUM.

i) 0.6M perchloric acid.

ii) 0.5M HCl in propanol.

0.5M HCl in propanol gives better recovery, dehydrates and precipitates, easier to purify.

3.Complexing agent (4.6).

i) tfacac.

ii) hfacac.

hfacac gives higher recovery, and is easier to purify.

4.Conditions for complex formation (4.7).

i) Dried ppt. plus toluene.

ii) Ppt. in propanol plus toluene.

iii) Ppt. washed free from propanol with toluene.

iv) Toluene plus acetic acid containing 7.5% v.v. sulphuric acid.

Development to higher and more reliable recoveries. Increasingly robust method.

FIGURE 4.4. continued.

Development flow chart (sheet 2).

5. Extraction of $Cr(hfacac)_3$ from the protein mass (4.8).

i) Toluene.

ii) Petroleum spirit hot. — Specificity improved.

iii) Petroleum spirit cold.

6. Removal of excess hfacac (4.9).

i) Water only.

ii) 1M K_2HPO_4 . — Improved efficiency of excess hfacac extraction, no significant increase in the loss of Cr.

iii) 1M K_2HPO_4 then water.

iv) 2.2M K_2HPO_4 moderated by acetic/sulphuric acid added earlier, then water.

7. The back extraction process (4.10).

i) NH_3 then solution in 0.001M perchloric acid after evap. — Improved solubility after evaporation.

ii) NH_3 plus EDTA, then dissolve in 0.1M acetic acid after evaporation.

iii) NH_3 plus EDTA, then dissolve in 0.5M NH_3 ,0.7M acetic acid after evaporation. — Improved AAS/ETA characteristics.

in the alpha-2-globulin-bound chromium series but was found to give variable recoveries. Dilute (0.5M) hydrochloric acid in propan-2-ol was substituted, and only one wash in propan-2-ol was then required because of the concurrent precipitation and dehydration then possible.

The suitability of 0.5M hydrochloric acid in propan-2-ol was demonstrated using an equilibrated human serum pool spiked with 0.074 µg/L of radioactive chromium. The changes in the fraction of chromium precipitated was investigated with respect to the mixing time before separating, the concentration of hydrochloric acid and the relative volume of reagent volume to sample. Aliquots of the pool were subjected to precipitation with 0.6M perchloric acid and to electrophoresis for comparative purposes. The losses during three washes with propan-2-ol after protein precipitation with propan-2-ol and 0.5M HCl in propanol were monitored. Method CDII (E402) was followed except for the tests with perchloric acid, with this reagent procedure CDI (E401) was used. The results of these experiments are summarised in table 4.3.

TABLE 4.3.

i) Mixing time of precipitation varied, 0.5M HCl in propanol.

Time.	Mean.	S.D.
15 min.	75.8	3.01
Overnight.	73.7	2.01

n = 4

t test indicates no significant difference.

ii) Three washes with propan-2-ol.

	Percentage radioactive Cr removed.			
Precipitant.	Precipitation.	Wash 1.	Wash 2.	Wash 3.
Propan-2-ol.	3.3	0.4	0.4	0.3
0.5M HCl in propan-2-ol.	18.8	5.4	2.0	0.7

n = 4

iii) Four concentrations of HCl in propan-2-ol.

	Percentage of radioactive Cr precipitated.			
	0.1M	0.2M	0.5M	1.0M
Mean.	86.6	83.8	75.8	73.5
RSD.	0.7	1.1	1.3	1.8

n = 4

TABLE 4.3. continued.

iv) Comparison of % ^{51}Cr extracted by 0.5M HCl in propanol with that extracted by 0.6M perchloric acid and with the % ^{51}Cr bound to beta-globulin.

	Percentage radioactive chromium.		
	0.5M HCl.	Perchloric.	Beta-globulin.
Mean.	24	29	25
RSD.	4.1	4.9	2.0

n = 4

t tests confirm no significant difference between the three means.

4.5.1. Protein Fractions Precipitated by 0.5M Hydrochloric Acid in Propan-2-ol.

A number of human serum samples were processed using method CDIII (E403) and the supernatants, from the precipitation stage with 0.5M hydrochloric acid in propan-2-ol, were subjected to total protein and albumin determinations. The methods used for the measurements of these two parameters are given in the experimental section under reference E407. The total protein and albumin concentrations of the original serum samples were also determined. A total of eight samples were tested.

The total protein and albumin determinations both confirmed that precipitation with 0.5M hydrochloric acid in propan-2-ol is selective in precipitating globulins, leaving albumin in solution. The albumin present in the supernatants was compared with that in the original serum samples, and the mean ratio was 96%, confirming that little albumin was precipitated. The total protein content of the supernatant fluids as a percentage of that in the original sample was compared with the original serum albumin concentration, expressed as a percentage of the total protein. The mean figure was found to be 97%, indicating that virtually all the protein not precipitated under the conditions used belongs to the albumin fraction.

4.5.2. Iron Concentrations in the Supernatants.

The iron concentrations of the supernatant fluids after the precipitation of the alpha-2-globulin-bound chromium using method CDIII (E403) was also determined.

The supernatants were diluted with 0.1M acetic acid (1:11) and

the iron concentrations measured using AAS/ETA as described in the experimental section (E408). The iron levels in the serum samples were measured by a standard spectrophotometric procedure using a centrifugal analyzer: this method is outlined in E408. Twelve human serum samples were tested and the data were subjected to a t test which indicated that there was no significant difference between the two means.

4.5.3. Protein Precipitation, Summary.

In summary, propan-2-ol and 0.5M hydrochloric acid in propan-2-ol appear to be useful protein precipitation reagents for the determination of total protein-bound serum chromium and alpha-2-globulin-bound chromium respectively. The treatment with 0.5M hydrochloric acid in propan-2-ol appears to be selective for precipitating the alpha-2-globulin-bound chromium. The other two major chromium binding protein fractions, albumin and beta-globulin make no contribution to the precipitate, as albumin is not precipitated and the metal bound to beta-globulin is eluted into the supernatant. Relative volumes of reagent to sample of 2:1 followed by a single wash with propan-2-ol gives reproducible precipitates sufficiently dehydrated to allow penetration of the protein mass by a hydrocarbon solvent. The propan-2-ol wash continues both the dehydration process and the removal of unwanted chromium fractions initiated in the protein precipitation step. The wash of the alpha-2-globulin-bound chromium precipitate also completes the removal of fatty acids released by the acid precipitant. The fatty acids if not removed at this stage are extracted by the hydrocarbon solvent leading to emulsion formation and interference at the wash and back extraction stages.

The total protein-bound chromium appears to represent about 96% of the total element, which agrees with the value quoted by Lim et al (4) of 95%, referred to in chapter II.

The modest change in the percentage chromium extracted by hydrochloric acid in propan-2-ol over the acid concentration range 0.1 to 1.0M supports the precipitation of a species fundamentally different from that which bound the extracted metal in the untreated serum. The agreement noted earlier between the fraction of chromium bound to the beta-globulin as measured after electrophoretic separation and the chromium extracted by 0.6M perchloric acid was demonstrated again, and found to be not significantly different from that extracted by 0.5M hydrochloric acid in propan-2-ol. These observations are consistent

with the extraction of transferrin-bound chromium by 0.5M hydrochloric acid in propan-2-ol, and the use of this reagent for a simple separation of the alpha-2-globulin-bound chromium from the transferrin bound element.

4.6. COMPLEXING AGENT.

The two beta-diketones 1,1,1-trifluoropenta-2,4-dione (tfacac) and 1,1,1,5,5,5-hexafluoropenta-2,4-dione (hfacac) have been successfully used to extract chromium from biological fluids, as reported earlier.

Savory et al (5,6) reported that it was necessary to completely destroy all organic compounds present in the sample before quantitative isolation of the chromium in serum using tfacac could be achieved.

An experiment using serum diluted 1 in 4 with phosphate buffer at pH 6.5 confirmed Savory's observation that serum components interfered, at least in aqueous solution. Negligible recoveries were given by the diluted serum although good recoveries were recorded on the phosphate buffer in the absence of serum, even with high Fe(III) concentrations (4 times that in normal serum).

The technique of precipitating the protein-bound chromium with propan-2-ol, followed by drying the isolated precipitate involves the minimum pretreatment. The two beta-diketones were compared in recovery tests on the dried propan-2-ol precipitates from 250 µL of serum spiked with radioactive chromium as the chloride at a concentration of 0.6 µg/L. The volume of propan-2-ol used was 2.5 mL and the precipitates were dried at 95 ^{0} C for 1 hour. The reagents added and the results obtained are listed in table 4.4 below. The tubes were thoroughly vortexed after adding the reagents and then heated for 24 hours at 95 ^{0} C. The tubes were then cooled to minus 20 ^{0} C, then 1000 µL of petroleum spirit was added and the chromium extracted on a roller mixer for 2 hours. The radioactive chromium was determined as described in E405 in the experimental section.

The results clearly demonstrated the superiority of hfacac: this diketone produced recoveries approximately double those recorded for tfacac under similar conditions.

TABLE 4.4.

Comparison of recoveries using two beta-diketones.

Beta-diketone.	% Cr recovered. hfacac.	tfacac.
1. 150 µL dichloromethane plus 150 µL diketone.	84	46
2. 250 µL diketone.	88	/
3. 500 µL diketone.	/	33
4. 1000 µL diketone.	/	35

4.6.1. The volatility of chromium as the hfacac complex.

Si-Jung Yeh and Chin-Nan Ke (7) reported that the volatility of chromium as the hfacac complex was responsible for the poor precision recorded when using hfacac for chromium determinations. The possibility of variable losses occuring during the analytical process because of the volatile nature of the chromium complex was therefore investigated, in a manner relevant to the process under development.

The chromium complex is only exposed to evaporation in solution in petroleum spirit and at ambient temperature. The experiment carried out was therefore designed to measure chromium losses under these conditions. Radioactive chromium as the hfacac complex was dissolved in petroleum spirit (120 to 160 ^{0} C b.pt.). Aliquots, of 2000 µL volume were then distributed into 12 x 75 mm polypropylene tubes. Four tubes were capped as reference tubes, and four tubes were left uncapped in a fume cupboard with the fan operating for 24 hours. The radioactive chromium was then determined (E405). The test series of tubes were then left for a further 24 hours uncapped in the fume cupboard as before. The petroleum spirit in the test series was then made up to the original volume of 2000 µL before measuring the radioactive chromium. The results are given in table 4.5 below.

The volatility of the chromium complex when dissolved in petroleum spirit appeared to be relatively low and this observation led to an experiment to purify hfacac by distillation from petroleum spirit. The purification process is given in detail in chapter VII and demonstrated that only 0.28% of the added radioactive chromium was present in the distilled hfacac.

Table 4.5.

Retention of Cr hfacac complex in pet. spirit at ambient temps.

Retained radioactive chromium as a % of the mean reference value.

	24 hours.	48 hours.
Mean.	101.8	99.13
SD.	0.9574	2.425

The mean count was 75352, and the mean background was 351.

Statistical count RSD 0.36%.

The results discussed above confirm that reasonable handling at ambient temperatures should not result in significant evaporative losses of chromium, as the hfacac complex from solutions in petroleum spirit.

4.7. CONDITIONS FOR COMPLEX FORMATION.

The early experiments on serum used dried protein precipitates, and the temperature used at the drying stage was investigated using aliquots of a human serum pool spiked with either chromium chloride or crude GTF, at radioactive chromium concentrations of 1.36 or 0.48 µg Cr/L respectively. The samples were processed using method CDI (E401) except that one series were dried at 37 ^{0} C and a duplicate series were dried at 78 ^{0} C. The procedure was terminated after extracting the chromium beta-diketone complex into petroleum spirit and the radioactive chromium recovery determined at this stage. The results of the experiment are listed in table 4.6 below. The results of the experiments clearly demonstrated that the lower temperature drying was superior, especially for the perchloric acid precipitates, as charring occurred with these at the higher temperature.

TABLE 4.6.

Chromium recoveries on dried protein precipitates.

	Percentage radioactive chromium.					
	Propan-2-ol.			0.6M perchloric acid.		
	Precipitated.	Recovered.		Precipitated.	Recovered.	
		37 ^{0} C.	78 ^{0} C.		37 ^{0} C.	78 ^{0} C.
Chromium chloride.	99	98	73	25	98	3
Crude GTF.	98	74	10	49	99	6

The optimum temperature for the complex formation step was investigated in a similar experiment to the one above, but only the chromium chloride spiked serum was used and the precipitates were all dried at 37 ^{0} C. The recoveries were compared over the temperature range 80 through 100 and 120 to 140 ^{0} C, at the complex formation stage. The protein precipitates after extraction of the beta-diketone Cr complex with petroleum spirit were dried and dissolved in 1000 µL of 5M sodium hydroxide to determine the percentage chromium not converted to the acetylacetonate. The radioactive chromium in the sodium hydroxide solution was determined using the usual technique (E405). The results of this experiment are summarised in table 4.7 below.

TABLE 4.7.

Optimisation of the complex formation temperature.

Temperature ^{0} C.	% ^{51}Cr retained in the protein precipitate.			
	Propan-2-ol.		0.6M perchloric acid.	
	i.	ii.	i.	ii.
80	2	2	1	2
100	3	2	2	3
120	19	10	19	58
140	30	9	69	66

The results clearly demonstrated that the lower temperatures were much superior. The poor performance at the higher temperatures may be due to increased leakage of the volatile beta-diketone at higher vapour pressures, and this would also be consistent with the poor precision. The greatly increased charring of the organic material at the higher temperatures is possibly an important factor for the alpha-2-globulin-bound chromium recoveries.

The avoidance of the drying stage with the associated possibilities for contamination of the open vessels, and the inevitable time delay, especially at lower temperatures was seen as a clear advantage. The experiment described below was carried out to determine the minimum amount of hfacac that would give acceptable recoveries with a sample volume of 1000 µL. However "wet" precipitates were used and 0.5M hydrochloric acid in propan-2-ol was used as the protein precipitant for the alpha-2-globulin-bound chromium, and therefore a number of developmental changes are illustrated. The method used was

essentially CDII (E402) except that only 1000 µL of sample was processed, and the wash to remove the excess beta-diketone was with 5 ml of water for 1 hour, and no phosphate buffer solution was used. The volumes of hfacac were 50 through 100 to 250 µL, and 1000 µL of toluene without acetic acid were added with the diketone. The residual protein masses were dissolved in 1000 µL of 5M sodium hydroxide solution and 1000 µL of both petroleum spirit and the water wash solution were reserved to determine the chromium distribution. The results are summarised below in table 4.8. The sample used was a human serum pool spiked with chromium chloride at 1.0 µg Cr/L and equilibrated at 60° C for 30 minutes.

TABLE 4.8.

Distribution of chromium, "wet" precipitates processed.

i) Radioactive chromium precipitated.

	Propan-2-ol.	0.5M HCl/propanol.
Initial precipitate.	96.1%	50.5%
After propan-2-ol wash.	95.9%	42.0%

ii) Distribution of radioactive Cr as a % of that precipitated.

a) Propan-2-ol precipitates (total protein-bound Cr).

	Vol. hfacac.	Residue.	Water wash.	Pet. spirit.	Ammonia.	Total.
1.	50 µL	60%	11%	<1%	23%	94%
2.	100 µL	22%	8%	2%	56%	88%
3.	250 µL	2%	5%	4%	84%	95%

b) 0.5M HCl in propan-2-ol (alpha-2-globulin-bound Cr).

	Vol. hfacac.	Residue.	Water wash.	Pet. spirit.	Ammonia.	Total.
1.	50 µL	2%	7%	1%	68%	78%
2.	100 µL	3%	5%	2%	65%	75%
3.	250 µL	2%	5%	1%	68%	76%

The minimum quantity of hfacac needed for the total protein-bound chromium stream appeared to be 250 µL. However the conversion to the acetylacetonate was satisfactory in the alpha-2-globulin-bound series even with 50 µl of beta-diketone. The addition of a toluen soluble acid to the precipitates from the total protein-bound chromium series with the hfacac, appeared to be a possible way to improve the recoveries obtained with small volumes of diketone. The high conversion to acetylacetonate in the alpha-2-globulin-bound chromium stream was

presumed to be due to the lower pH in this series. Acetic acid was chosen as the toluene soluble acid, and improvements in the conversion of chromium to the beta-diketone derivative in the total protein-bound chromium series were seen. The addition of sulphuric acid (7.5 % v.v.) to the acetic acid resulted in further improvements. Sulphuric acid was added because it is a strong acid, free from water, and is miscible with a toluene, acetic acid mixture of the composition used.

The "missing" chromium in the alpha-2-globulin-bound stream was due to the poor separation of the aqueous and organic phases in this series, this in turn was caused by the failure of the water wash to remove the acidic substances, probably fatty acids generated during the processing of this series.

4.8. THE EXTRACTION OF THE CHROMIUM COMPLEX FROM THE PROTEIN MASS.

The precipitates in the total protein-bound chromium stream are in the form of leathery discs which are less readily penetrated by solvent than the loosely aggregated particulate precipitates produced by the 0.5M hydrochloric acid in propanol. The former were therefore used for investigations into the extraction of the chromium acetylacetonate from the protein mass.

A human serum pool was spiked with radioactive chromium at a concentration of 0.56 µg Cr/L. Aliquots of the serum pool were then processed for total protein-bound chromium using standard method CDII (E402) up to and including step 4. The chromium hfacac complex was then extracted with 10 mls of either toluene or petroleum spirit (140 to 160 ^{0} C b.pt.), the details of the extraction conditions and results are listed in table 4.9 below.

TABLE 4.9.

Comparison of toluene and pet. spirit as extraction solvents.

		Percentage ^{51}Cr extracted.				
		Roller mixer.	Oven 75 ^{0} C.			
		Ambient T, 4 hrs.	1 hr.	2 hrs.	4 hrs.	Overnight.
Toluene.	(mean).	79.0	78.5	79.3	79.5	85.5
	(S.D.)	1.7	1.9	2.4	1.7	1.2
Pet. spirit.	(mean).	87.0	81.3	82.8	81.6	85.0
		0.7	1.7	1.3	2.1	1.4

n=4. Statistical count RSD = 0.6%.

The recoveries were measured using technique E405 on 1000 µL samples of the solvents. Petroleum spirit at ambient temperature was chosen, on the basis that less extraction of unwanted material should theoretically result.

4.9. REMOVAL OF EXCESS BETA-DIKETONE.

The removal of excess beta-diketone , which would tend to accompany the chromium analyte through the process, was initially recognised as a necessary stage in the procedure.

4.9.1. Problems caused by excess hfacac.

Two problems were anticipated, interference with the back extraction, by neutralisation of the base used, and interference at the electrothermal atomisation stage by smoke production. However an unexpected interference causing gross diminution of the chromium signal at the electrothermal atomisation stage was found. The interference proved difficult to eradicate as even a 3% retention of the excess hfacac resulted in severe interference.

4.9.2. Water wash.

The suitability of a water wash for the removal of the excess beta-diketone was investigated using 250 µL of hfacac in a mixture of 8 mL of petroleum spirit and 1 mL of toluene. Aliquots of the solution were washed with 4 mL of water on a roller mixer for a variable length of time ranging from 5 to 120 minutes. A second experiment was carried out in which an aliquot of the solution was subjected to three 24 hour sequential washes with 4 mL of water. The hfacac extracted was determined by titration with M sodium hydroxide using phenolthalein as indicator. The 100 % reference was 250 µL of hfacac in 4 mL of water. The results are listed in table 4.10 below.

TABLE 4.10.

Percentage hfacac extracted by water washes.

A) Variable wash time.		B) Three sequential 24 hr washes.	
Time.	% hfacac in water wash.	Wash.	% hfacac in water.
5 min.	46%	1.	97%.
10 min.	55%	2.	2.6%
30 min.	88%	3.	0.6%
60 min.	95%		
120 min.	97%		

A practical water wash of 60 minutes duration was observed to remove 95% of the hfacac from a petroleum spirit toluene mixture containing no other acids. The unexpected interference referred to above became apparent when, as the individual stages appeared to be at a suitable development level , a trial run of standards were carried through the full process. Three standards containing radioactive chromium equivalent to 1 µg Cr/L in a 1 mL serum sample were processed using method CDI, except that a 60 minute wash with 4 mL of water was used to remove the excess hfacac. The standard AAS/ETA conditions described in E406 were used with coated profile graphite tubes, and two 25 µL injections were made from each sample. The radiometric recoveries were measured using the usual method (E405). Aliquots of the radioactive chromium solution used were evaporated and dissolved in 100 µL of 0.025M diammonium EDTA solution and used as reference solutions for radiometric recovery checks and AAS measurements. The results are summarised in table 4.11 below.

TABLE 4.11.

AAS/ETA and radiometric recoveries on standards compared.

Tube.	Atomisation peak height mm.			Radiometric recovery.
	i.	ii.	Mean.	
Blank.	13	13	13	
Stds.				
1.	15	16	16	75%
2.	24	36	30	73%
3.	67	13	40	77%
Reference.	67	62	65	100%

The processed standards were seen to show extremely poor reproducibility for both replicate injections from individual tubes and between similarly processed tubes, and severe interference was obviously occurring at the AAS/ETA stage. An experiment to confirm that a 3% retention of hfacac would produce a significant suppression of the chromium signal was carried out. Standard solutions of Cr(III) in 0.005M perchloric acid were prepared together with a duplicate set containing hfacac equivalent to a 3% retention of the usual 250 µL added to each sample in process CDI. The solutions were left for 24

hours at ambient temperature to ensure that hydration of the hfacac, a slow reaction in acid solutions, was complete in the aliquots containing the beta-diketone. Standard AAS/ETA conditions (E406) were used, and duplicate 25 µL injections were made from each solution. Coated profile graphite tubes were fitted. The results are listed in table 4.12 below.

TABLE 4.12.

Comparison of atomisation peak heights, with and without hfacac.

	Atomisation Peak Height mm.					
	Reference soln.			Soln. plus hfacac.		
Cr(III) µg/L.	i.	ii.	Mean.	i.	ii.	Mean.
Zero.	1	1	2	18	19	19
2.5	18	18	18	22	7	15
5.0	32	34	33	8	12	10
10.0	64	68	66	27	26	27
20.0	121	128	125	42	52	47

The above results demonstrated that a 3% retention of hfacac produced severe interference, and also that the hfacac used contained a high concentration of chromium. The conclusion was that if a single wash was to be used then a weakly basic buffer solution was needed, and dipotassium hydrogen phosphate appeared to have the appropriate characteristics.

4.9.3. 1M dipotassium hydrogen phosphate wash.

The suitability of 1M dipotassium hydrogen phosphate as a wash solution was investigated using standards equivalent to 2.8 µg Cr/L in a 1000 µL sample. Standard method CDI was followed except for the wash stage. Aliquots of the standard were washed with water for 60 minutes or with 1M dipotassium phosphate for 15, 30 or 60 minutes. A wash reagent volume of 5 mL was used for all tubes. Radioactive chromium was used for the standards and the distribution of the chromium in the wash solutions, petroleum spirit and ammonia solutions used for the back extractions was determined using the usual conditions (E405). The radioactive chromium left in the tubes used for the evaporation of the ammonia was also measured. The AAS/ETA determination was carried out using standard conditions (E406) with coated profile tubes. Three injections were made from each test solution. The results are

summarised in table 4.13 below.

TABLE 4.13.

1M dipotassium phosphate as a wash solution.

A. Tube identification.

Tube.	Wash solution.	Wash time, minutes.
1.	water.	60
2.	1M phosphate.	15
3.	1M phosphate.	30
4.	1M phosphate.	60

B. Distribution of the radioactive chromium.

Tube.	Wash solution.	Pet. spirit.	Ammonia soln.	Evap. tubes.
1.	0.4%	6.8%	93%	1.2%
2.	7.4%	6.6%	86%	1.5%
3.	9.2%	7.9%	83%	1.5%
4.	15.5%	13.2%	71%	1.5%

C) AAS/ETA chromium measurements.

	Atomisation Peak Height mm.		AAS/ETA	AAS/ETA recovery as
Tube.	Mean.	RSD.	recovery.	% of radiometric.
Blank.	53	1%	/	/
1.	62	43%	24%	26%
2.	137	26%	52%	61%
3.	122	14%	47%	57%
4.	174	0.6%	69%	98%
Reference.	198	0.7%	/	/

Note, The blank value in the table above refers to sample 4. The blank values used for the calculation of the AAS/ETA recoveries for the other samples were derived from this result, assuming similar recoveries and relative responses for blanks and tests, when processed in an identical manner.

The anticipated results of high radiometric recovery but severe interference at the AAS/ETA stage for the water wash sample, and increasing AAS/ETA relative response at the expense of higher losses of chromium at the wash stage were illustrated in the results above. However the failure of the phosphate wash to improve the back extraction efficiency was unexpected. A repeat experiment demonstrated that the introduction of a water wash after the phosphate buffer

treatment was effective, and consistent back extraction efficiencies of greater than 95% were recorded.

4.9.4. 2.2M Dipotassium phosphate wash.

The addition of the acetic acid containing 7.5 % sulphuric acid with the hfacac improved the conversion of chromium to the hexafluoroacetylacetonate, particularly in the total protein-bound chromium series as reported earlier. However a more concentrated phosphate buffer was required to compensate for the extra acidity of the petroleum spirit extracts. The suitability of 2.2M dipotassium hydrogen phosphate was investigated using a solution of radioactive chromium, as the hexafluoroacetylacetonate at a concentration of 1.0 µg Cr/L in petroleum spirit. Two 9 mL aliquots of the petroleum spirit solution were labelled A and B, 250 µL of acetic acid was added to A and 250 µL of hfacac to both. The aliquots were then washed with 4 mL of the 2.2M phosphate solution on a roller mixer for 24 hours. The aliquots were both subsampled (250 µL) at intervals, and the radioactive chromium content of the subsamples measured in the usual way (E405). The final pH values of the wash solutions were measured and compared with 4 mL aliquots of water and phosphate buffer containing 250 µL of hfacac and treated similarly. The results of the experiment are given in table 4.14 below.

TABLE 4.14.

i) Chromium retention during a 2.2M phosphate wash.

	Percentage chromium remaining.	
Wash time.	A (plus acetic acid).	B (no acetic acid).
1 min.	95	85
10 min.	91	79
15 min.	90	80
30 min.	94	75
60 min.	94	70
24 hrs.	90	36

ii) pH values of the wash solutions.

Solution.	pH.
Phosphate buffer (untreated)	9.1
A after 24 hrs.	6.8
B after 24 hrs.	7.9
Phosphate buffer plus hfacac (24 hr. mix).	7.9
Water plus hfacac (24 hr. mix).	2.2

The acetic acid (plus 7.5 % v.v. sulphuric acid) moderated the effects of the phosphate buffer as expected. The chromium lost in a 24 hour wash was reduced from 64% to 10%.

The effectiveness of the 2.2M phosphate wash for removing the excess hfacac was investigated by washing twelve tubes containing 9 mL of petroleum spirit plus 250 µL of hfacac and 250 µL of acetic acid with 4 mL of 2.2M phosphate buffer for 15, 30, 60 minutes and overnight. Tests were carried out in triplicate. The petroleum spirit from all tubes was washed with 4 mL of water for 60 minutes after the phosphate buffer wash. A solution containing 0.1 µg Cr/L in 4.25M ammonia, 0.005M EDTA was prepared and 500 µL of this solution was added to duplicate tubes from each wash series. The third tube was used as a blank and ammonia/EDTA solution containing zero chromium was added to these tubes. All tubes were then placed on a roller mixer and left overnight. The ammonia/EDTA solutions were then removed to small polypropylene tubes and evaporated in a desiccator at 80° C over sulphuric acid and 5 tubes containing 500 µL of the chromium solution were similarly treated. The residues were all dissolved in 100 µL of 0.1M acetic acid at 80° C on a vortex mixer for 5 minutes. The chromium concentrations of the 0.1M acetic acid solutions were then determined using standard AAS/ETA conditions (E406) with uncoated profile graphite tubes. Duplicate 25 µL injections were made from each solution. The results of this experiment are summarised in table 4.15 below.

TABLE 4.15.

AAS/ETA results on processed standards after 2.2M phosphate wash.

Wash time minutes.	Mean peak height test - blank.	Relative std. deviation.	% of reference standard.
15 min.	26	8.3%	88%
30 min.	27	31.4%	93%
60 min.	23	6.1%	80%
16 hr.	30	14.1%	104%
Reference.	29	7.1%	100%

The mean peak heights of the processed standards were compared with the reference mean using Student's t test, and only the value from the samples washed for 60 minutes was found to be significantly different.

The conclusion was that the results confirm that significant signal suppression does not occur after the treatment outlined above, and that even a 15 minute wash with 2.2M phosphate buffer was acceptable.

4.10. THE BACK EXTRACTION PROCESS.

The initial stage of the back extraction process is the removal of the chromium from the petroleum spirit to form a stable solution in the aqueous extraction phase. The chromium must then be concentrated by evaporation and redissolved to form a stable solution suitable for the AAS/ETA stage.

4.10.1. Extraction from the Petroleum Spirit.

The back extraction depends on efficient removal of acids at the earlier wash stage, as discussed earlier. The rate of removal of chromium from a petroleum spirit solution containing hfacac and acetic acid by 4.3 M ammonia solution containing 0.005M EDTA was investigated. Radioactive chromium as the hfacac complex was added to the petroleum spirit to produce a concentration equivalent to that produced by the complete extraction into 9 ml of solvent of the chromium from 4 ml of sample containing 0.1 µg Cr/L. The 9 ml aliquots of petroleum spirit also contained 250 µl of both hfacac and acetic acid. The solutions were washed sequentially with 4 ml of 2.2 M dipotassium hydrogen phosphate and then 4 ml of water, each wash was of 60 minutes duration on a roller mixer. The petroleum spirit aliquots were then extracted with 500 µL of the ammonia/EDTA solution for from 15 minutes to 16 hours. The radioactive chromium in the ammonia solutions was measured using the usual method (E405). The results of the experiment are summarised in table 4.16 below, and the aliquots were processed in duplicate.

TABLE 4.16.

The rate of removal of ^{51}Cr by ammonia soln. from pet. spirit.

	% Cr extracted.			% Cr extracted.	
Time.	i.	ii.	Time.	i.	ii.
15 min.	40	73	120 min.	94	96
30 min.	51	79	150 min.	95	98
60 min.	73	96	16 hours.	>99	>99

The conclusion was that ammonia solution can, under the above conditions give acceptable back extraction recoveries. The extractions should be carried out for at least two hours and ideally overnight.

The stability of trace concentrations of chromium in ammonia solution was demonstrated using radioactive chromium at a concentration of 0.69 µg Cr/L, equivalent to 0.108 µg/L in a serum sample processed using method CDIII, and assuming a recovery of 80%. Four 500 µL aliquots of the ^{51}Cr solution were pipetted into small polypropylene tubes for reference values and the bulk was stored in a screw cap polypropylene tube of the type used for the back extraction stage in the chromium determination process. After 24 hours storage at ambient temperature four 500 µL aliquots were pipetted into small polypropylene tubes without prior mixing. The radioactive concentration of the two sets of tubes were then compared using the usual method (E405). The mean chromium concentration of the test series as a percentage of the mean level in the reference tubes was 101.2%, with a standard deviation of 0.69%. The statistical radioactivity RSD was 0.5%.

The conclusion was that Cr(III) solutions in 4.3M ammonia are stable for at least 24 hours in the concentration range expected in the standard procedure.

4.10.2. Redissolving the chromium after the evaporation step.

The solution of radioactive chromium equivalent to 0.108 µg Cr/L in a specimen referred to above (4.10.1) was used in an experiment to investigate the redissolving of the chromium after the evaporation step. Sixteen 500 µL aliquots were pipetted into polypropylene tubes of the type used for the evaporation stage in method CDIII. The contents of 13 to 16 were evaporated after the addition of 50 µL of 0.025M EDTA. The evaporations were carried out at 80 ^{0} C in a desiccator. The solution of the residues was attempted in 100 µL of various solvents at 80 ^{0} C on a vortex evaporator for 5 minutes, after capping. The tubes were then centrifuged at 3000 rpm at 20 ^{0} C for 10 minutes and then all fluid was removed using a water pump and fine pipette. The empty tubes were then subjected to radioactive chromium determinations in the usual manner (E405). The four remaining aliquots of the original solution were used for reference values. The results are summarised in table 4.17 below.

The results clearly indicated that EDTA must be added to the ammonia before evaporation, for the chromium in the residue to be

readily dissolved.

TABLE 4.17.

Comparison of solvents for redissolving residual chromium.

Tube No.	Solvent.	Percentage ^{51}Cr remaining.		
i) EDTA not added before evaporation.		i.	ii.	Mean.
1 and 2.	0.025M EDTA.	38	32	35
3 and 4.	0.050M EDTA.	32	32	32
5 and 6.	0.025M EDTA in 0.1M acetic acid.	30	25	28
7 and 8.	0.05M hydrochloric acid.	23	20	22
9 and 10.	0.1M hydrochloric acid.	18	17	18
11 and 12.	0.01M perchloric acid.	29	22	26
ii) EDTA added before evaporation.				
13 and 14.	Water.	<0.1	<0.1	<0.1
15 and 16.	0.1M acetic acid.	<0.1	<0.1	<0.1

4.11. INTERFERENCE BY IRON.

The trace metal with the highest probability of producing significant interference in chromium determinations on biological specimens is clearly iron, principally because of the relatively high concentration of iron relative to other trace metals. The ability of both Fe(II) and Fe(III) to form complexes with beta-diketones together with the iron:chromium ratio of about 150:1 in normal human serum indicates that significant competition from iron for hfacac could take place, particularly in samples with high iron concentrations. The presence of a considerable excess of the beta-diketone to drive the complexation reaction in the forward direction in the presence of very low concentrations of chromium is clearly an important factor in obtaining a high recovery. The removal of virtually all the serum iron together with the transferrin-bound chromium at the protein precipitation stage of the alpha-2-globulin-bound chromium determination stream should ensure that interference from this source does not occur with this parameter.

4.11.1. Effect of High Serum Iron Levels.

An experiment was carried out to investigate the effects of high serum iron levels on method CDIII. Aliquots of a human serum pool derived from samples taken with precautions to avoid contamination with

chromium were spiked with radioactive chromium to provide a range of values from 0.086 to 0.69 µg Cr/L. One aliquot was also spiked with Fe(III). The samples were then processed using method CDIII (E403) for both total protein-bound and alpha-2-globulin-bound chromium. Standards equivalent to 0.69 µg Cr/L were also processed, and quarter scale precipitation and wash experiments were performed to measure the fraction of ^{51}Cr precipitated as alpha-2-globulin-bound chromium. The recoveries of ^{51}Cr were determined using the usual technique (E405) and are summarised in table 4.18 below. The table also gives details of the ^{51}Cr and Fe(III) spikes.

TABLE 4.18.

Comparison of the ^{51}Cr recoveries on aliquots of a human serum pool with and without added Fe(III).

Pool.	n.	Spike.		Percentage recovery.			
		^{51}Cr µg/L.	Fe mg/L.	Total Cr.		GTF Cr.	
				Mean.	RSD.	Mean.	RSD.
A.	4	0.69	/	73	2.1	83	3.2
B.	1	0.345	/	73		86	
C.	1	0.173	/	76		85	
D.	1	0.086	/	70		85	
E.	4	0.69	125	59	6.1	85	1.7
Std.	4	0.69	/	75	2.3		

Student's t test confirmed that there was a highly significant difference between the recoveries from pool E compared with those recorded from pool A.

The conclusion was that iron at a concentration about sixty times the normal level does not significantly reduce the recovery of alpha-2-globulin-bound serum chromium (GTF Cr in the table above). However the total protein-bound chromium (Total Cr in the above table) is significantly reduced.

4.11.2. Recovery of Fe(III) using method CDIII.

A second experiment similar to the one described in 4.11.1 above was carried out except that the chromium and iron levels were determined using AAS/ETA and the total protein-bound chromium protocol only was followed. Standard AAS/ETA conditions (E406) with an uncoated "Profile" tube, and no background correction were used. The iron

measurements were carried out under identical conditions except that an iron hollow cathode lamp was used at 248 nm. The spike details and the results are given in table 4.19 below. The chromium in the ferric nitrate used for spiking the serum pool was determined by preparing a solution in 0.1M acetic acid. A solution of chromic nitrate in the same solvent was used as a standard.

TABLE 4.19.

i) Iron recovery on a spiked serum sample using method CDIII.

Pool.	n.	Spike.		AAS/ETA results.	
		^{51}Cr µg/L.	Fe mg/L.	Cr µg/L.	Fe µg/L.
F.	4	/	/	0.074	/
G.	4	0.86	/	0.93	/
H.	4	/	200	37.0	8

ii) Chromium content of ferric nitrate.

35.39 mg Fe/L. 38.4

Cr as a percentage of Fe in the spike solution = 0.109%.

Recovery of chromium in the Fe spiked solution = 18%.

The recovery of the added iron was extremely low (0.004%). The red Fe(III) hfacac complex was observed to be lost from the petroleum spirit at the phosphate wash stage. The relatively high chromium content of the added iron (0.109%) was noted and clearly implied that the interference by iron was more serious than originally concluded from the earlier experiment. The iron/ chromium ratio in the iron spiked serum used in 4.11.1 was, in the light of the high chromium content of the iron, very different from that prevailing in a specimen with a 60 times elevated iron but normal chromium level, as originally envisaged.

4.12. THE EFFECT OF HAEMOLYSIS ON CHROMIUM RECOVERY.

A specimen of blood taken without anticoagulants and with the precautions against contamination with chromium discussed in chapter VII, was lysed by repeated freeze-thawing. The haemoglobin stained serum was then added to unhaemolysed serum taken at the same venepuncture. Two haemolysed specimens were prepared equivalent to 20% and 2% of a whole blood containing 140 g haemoglobin/L. The total iron contaminations resulting from the haemolysis were about 94 and 9.4 mg

Fe/L respectively. Radioactive chromium was added to the haemolysed and to non haemolysed samples to produce a final concentration of 0.18 µg Cr/L. The serum samples were then processed using method CDIII (E403) for both total protein-bound chromium and alpha-2-globulin-bound chromium. A summary of the recovery results is given in table 4.20 below. The radioactive chromium determinations were carried out using the usual method (E405).

TABLE 4.20.

Chromium recoveries on haemolysed serum samples.

A. Total Protein-Bound Chromium.

Sample.	Mean Recovery.	Standard Deviation.	Number.
No haemolyis.	70.5	2.1	4
20% haemolysis.	28.3	4.8	4
2% haemolysis.	69.0	1.4	4

B. Alpha-2-globulin-Bound Chromium.

Sample.	Mean Recovery.	Standard Deviation.	Number.
No haemolysis.	80.1	2.1	4
20% haemolysis.	80.5	2.0	4

Student's t test was applied to the recoveries from the total protein-bound chromium process. The recovery from the specimen with 20% haemolysis showed a highly significant reduction compared with the sample free from haemolysis. However the recovery from the sample with 2% haemolysis was not significantly different from the haemolysis free base serum.

The alpha-2-globulin-bound chromium determination process was clearly much less susceptible to haemolysis and showed no reduction in recovery even with 20% haemolysis.

4.12.1. Conversion of "haemoglobin iron" to the hfacac complex.

The interference only in the total protein-bound chromium series indicates that iron derived from haemoglobin is not responsible. The haematin iron cannot be extracted under the conditions used for precipitating the alpha-2-globulin-bound Cr, and the iron content of the precipitates in the two series must be broadly similar.

The percentages of the total iron content of the 20% haemolysed sample present in the supernatants of the two alternative protein precipitation procedures were compared. The supernatants were diluted

with 0.1M acetic acid 1:20 and the iron concentrations determined as described in E408. The specimen was diluted with 0.1M acetic acid to give solutions corresponding to 12.5, 25, 50 and 100% of the total iron and aliquots of these solutions were used to establish a standard curve. Appropriate blanks were deducted from the readings of the test solutions. The results are summarised in table 4.21 below.

TABLE 4.21.

Percentage of total iron in supernatants.

	n.	Mean.	Standard deviation.
Total protein-bound Cr.	3	3.9	0.2
Alpha-2-globulin-bound Cr.	3	14.4	0.5

The results confirm that although rather less of the haemoglobin derived iron is precipitated in the alpha-2-globulin-bound series, the major fraction is present in the precipitate.

The fraction of the haemoglobin iron that was converted to the hfacac complex in a specimen with 20% haemolysis was determined for both the total protein-bound Cr and the alpha-2-globulin - bound Cr series. A serum sample containing erythrocytes equivalent to a 20% contamination with whole blood was processed in duplicate using method CDIII, except for the modifications described below.

The standard procedure was followed up to and including the extraction with petroleum spirit. The washes with phosphate buffer and water were omitted and the petroleum spirit extracts were subjected to back extraction with 500 µL of a 50:50 mixture of 6M hydrochloric acid and propan-2-ol, overnight on a roller mixer. The aqueous phase was then evaporated at 75 ^{0} C in a small desiccator with the tap open. The extracted hfacac and the hydrated derivative readily evaporate under these acidic conditions, and unlike Cr(III), Fe(III) is extracted into acidified water/propan-2-ol solutions. The residues were dissolved in 500 µL of 0.1M acetic acid, 0.005M EDTA on a vortex mixer at 75 ^{0} C for 30 minutes. The extracts were then diluted 1 in 100 with 0.05M acetic acid. Blanks were put through the procedure and the original "haemolysed serum" sample was diluted 1 in 800, 1 in 1600 and 1 in 3200 in 0.05M acetic acid for comparative purposes. The iron concentrations were then compared as described in E408B. The results are listed in table 4.22 below. The recoveries are of the total iron precipitated,

of which about 99% is derived from haemoglobin.

TABLE 4.22.

"Haemoglobin iron" recoveries from a specimen with 20% haemolysis.

	1.	2.	Mean.
Alpha-2-globulin-bound Cr.	24.4%	24.4%	24.4%
Total Protein-bound Cr.	14.9%	14.8%	14.9%.

The experiment was repeated to confirm that a higher hfacac/iron ratio would result in a higher conversion of haemoglobin derived iron to the beta-diketone complex. A serum sample with added erythrocytes equivalent to 2.5% haemolysis was processed using the total protein bound Cr stream of standard method CDIII. The modifications described above were again used and a 67% recovery of the haemoglobin derived iron was recorded.

4.12.2. Interference by Copper, Iron and Zinc.

The poor recovery recorded for the total protein-bound stream for the 20% haemolysed sample was considered to be a result of the combined competition given by a number of metals present in relatively high concentration in the erythrocyte. The precipitates produced in the alpha-2-globulin stream would contain substantially lower amounts of these metals.

The effects of these metals on the Cr recovery at a concentration about 10 times the upper limit of the normal range were therefore investigated. The recoveries with and without the individual metals was determined on a standard Cr solution containing ^{51}Cr equivalent to a sample containing 0.1 µg Cr/L. The standard method CDIII was used, and the metals were added in 50 µL of acetic acid, except for Fe(II) which was added in hfacac/acetic acid because of the low solubility of Fe(II) sulphate in acetic acid. The bright red Fe(II) complex was observed to change to the orange/red Fe(III) complex on heating. The residues from the back extraction were dissolved in 1000 µL of 0.05M acetic acid and the ^{51}Cr recoveries measured as described in E405. The concentrations of Cu, Fe and Zn in the extracts were determined using the AAS/ETA conditions recommended by the instrument manufacturer, using the original acetic acid solutions diluted 1 in 500 with 0.05M acetic acid as standards. The Fe(III) standard solution was used for both Fe tests. The normal ranges for the metals were taken

from reference (9), and the results are given in table 4.23 below. The recoveries are relative to the unspiked ^{51}Cr solution processed at the same time.

TABLE 4.23.

Effects of Cu, Fe and Zn on ^{51}Cr recovery from a standard.

Metal.	mg/L in a spec.	Recovery ^{51}Cr.	Recovery metal.
Cu(II).	14.3	97.0%	<0.1%
Fe(II).	19.6	100.7%	0.3%
Fe(III).	19.6	103.2%	0.6%
Zn.	10.0	99.4%	<0.1%

n = 2.

S.D. 2.5%. (Stat.RSD. 0.57%).

A series of paired t tests confirmed that there were no significant differences between the recoveries of ^{51}Cr with the above spikes, compared with the Cr standard.

4.12.3. Conclusions, effects of haemolysis.

Haemoglobin iron is accepted as being firmly bound. Ruben et al (10) have shown, using ^{59}Fe, that there is no exchange between haematin iron and ionic iron. Nevertheless experiments in this section have indicated that a high recovery of haemoglobin iron could be achieved with a hfacac/Fe ratio similar to the hfacac/Cr ratio in a "normal" test sample. This observation provides good evidence that the presence of a native Cr(III) compound inert to extraction as the hfacac complex is improbable.

The poor recovery recorded for total protein—bound Cr determinations on samples with 20% haemolysis is puzzling, in view of the resistance to recovery interference shown by the standards spiked individually with Cu, Fe and Zn. However extra Cr was undoubtedly added with the spike solutions, and this may have increased the Cr recoveries. A sample with 20% haemolysis would in any case be normally rejected for the determination of serum components.

The resistance of the alpha-2-globulin—bound Cr series to recovery interferences is noteworthy.

4.13. THE CHARACTERISTICS OF THE TOTAL PROCESS.

The characteristics of method CDIII are discussed below.

4.13.1. Recovery.

The recoveries and the chromium distribution throughout the analytical processes are summarised in table 4.24 below. The data is from three batches processed using method CDIII.

TABLE 4.24.

Recoveries and ^{51}Cr distribution.

A. Recoveries.

i) Standards.

Batch.	Mean.	RSD.	
1.	76%	8.8%	Between batch distribution
2.	73%	2.1%	of means.
3.	75%	2.3%	Mean 74.7%, RSD 2.0%

ii) Total Protein-Bound Chromium.

Batch.	Mean.	RSD.	
1.	74%	3.0%	Between batch distribution
2.	73%	0.8%	of means.
3.	73%	2.1%	Mean 73.3%, RSD 0.8%

iii) Alpha-2-globulin-Bound Chromium.

Batch.	Mean.	RSD.	
1.	79%	5.1%	Between batch distribution
2.	82%	0.6%	of means.
			Mean 80.5%, RSD 2.6%

B. Chromium Distribution in Tests.

	Total Protein-Bound.		Alpha-2-globulin-Bound.	
Solution.	Cr%	SD.	Cr%	SD.
Phosphate buffer.	7.0	2.2	2.4	0.5
Water wash.	2.4	0.3	2.1	0.3
Petroleum spirit.	1.5	1.2	2.5	1.3
Ammonia EDTA.	73.3	2.2	80.5	2.9
Protein mass.	12	6.3	5.3	1.0
Total.	96.2	/	92.8	/
Cr unaccounted for.	3.8	/	7.2	/

The standard pretreatment process CDIII gave recoveries of 73% for the total protein-bound chromium and 81% for the alpha-2-globulin-bound chromium. The major identified location of

unrecovered chromium is the protein mass in both cases. However the chromium left in the less penetrable precipitates from the total protein-bound chromium series at 12% is slightly more than twice the amount left in the alpha-2-globulin-bound precipitates at 5%. The chromium loss in the phosphate buffer wash is also higher in the total protein-bound series, presumably due to the higher acidity of the petroleum spirit extract from the alpha-2-globulin-bound stream. The higher value of the "lost" chromium in the alpha-2-globulin-bound process is due to the less well demarcated petroleum spirit/aqueous phase boundaries at the wash stages leading to less complete transfer of the organic phase, particularly from the wide diameter tuftainers. Haemolysis and high serum iron levels can both reduce the recovery with the total protein-bound chromium process.

4.13.2. AAS/ETA. Relative Response.

The AAS/ETA conditions are discussed in the next chapter,however the relative signals given by chromium in the processed materials compared with unprocessed material are relevant to the pretreatment process, as discussed in the section on excess hfacac.

The AAS/ETA relative response is defined as :-

Peak height x 100 / Expected peak height.

Expected peak height is the peak height given by an equal concentration of chromium in unprocessed ammonia/EDTA/acetic acid solution. The concentrations of chromium in the solution derived from processed samples were calculated from radiometric recoveries. Incremental peak height values were used for the relative responses. The reference values for the increments were reagent blanks and unspiked serum values respectively for determinations on standards and serum samples. Spike values were equivalent to 0.125 µg Cr/L in a specimen.

Standard AAS/ETA conditions as described in E494 were used, with uncoated profile graphite tubes and 25 µL injections. A summary of the results is given in table 4.25 below.

TABLE 4.25.

Relative Responses, Processed and Unprocessed Materials.

i)	Unprocessed standards.	ii)	Processed standards.
	Response 100%, RSD 4.5%, n=4		Response 94%, RSD 3.9%, n=12
iii)	Total Protein-Bound Serum Cr.	iv)	Alpha-2-globulin-Bound Cr.
	Response 99.5%, RSD 3.5%, n=12		Response 82%, RSD 6.5%, n=7

The relative responses of processed material on AAS/ETA compared with unprocessed standards was subjected to Student's t test. The material from the total protein-bound chromium process did not differ significantly from the unprocessed material (95% confidence). However the processed standards and the alpha-2-globulin-bound chromium material both showed significantly different responses from unprocessed material, the confidence limits being 95% and 99% respectively.

4.13.3. Matrix Simplification Achieved.

The weight of the residue from 4 mL of serum processed for total protein-bound chromium using method CDIII was compared with the weight of the total dried solids from 4 mL of the same human serum pool. The mean weight of the processed material was 2.9 mg representing only 0.82% of the solids in the original specimen.

The chromium concentration factors are 24 and 27 for the total protein-bound chromium and alpha-2-globulin-bound chromium processes respectively.

The matrix simplification factor is 3.7 based on 8.8% dried solids in the human pool serum tested compared with 2.4% serum derived solids in the solution for AAS/ETA. However the chromium concentration factor multiplied by the matrix simplification factor is (27 X 3.7) = 100.

Metals other than those present at an oxidation stage capable of forming "inert" complexes will be eliminated at the phosphate wash stage as shown by the recoveries of Cu, Fe and Zn, all well below 1%. Iron and nickel are the only metals forming "inert" complexes which are probably normally present in human serum at levels higher than 1.0 µg/L. However the serum iron is present as Fe(III) (Lemberg et al (8)), and observation has indicated that the possibly inert Fe(II) complex is oxidised to the Fe(III) at the heating stage. The serum nickel is probably less than 5 µg/L and problems due to the presence of metals other than Cr in the test extracts are extremely improbable.

4.13.4. Detection Limit.

The detection limit is clearly governed in this method by the precision of the blank. A gross signal > blank + (4 x blank SD) , can be detected with 95% confidence. Thus a sample chromium level equal to 4 x the blank standard deviation in µg Cr/L equals the detection limit. Table 4.26 below lists the blanks and detection limits of four batches.

TABLE 4.26.

Blank values, and derived Detection Limits.

	Blank µg Cr/L.		Detection Limit.
Batch.	Mean.	Standard Deviation.	µg Cr/L.
I.	0.08	0.0076	0.030
II.	0.08	0.0078	0.031
III.	0.05	0.0086	0.034
IV.	0.09	0.0088	0.035
Means.	0.075	0.0082	0.033

4.13.5. Summary.

A pretreatment has been developed that will extract and concentrate 75% of the total protein—bound chromium, or 80% of the alpha-2-globulin—bound chromium into 120 µL of final solution containing less than 1% of the solids present in the original sample. The final solution appears to be suitable for the determination of chromium by conventional AAS/ETA without background correction. The detection limit using a sample volume of 4 mL is about 0.033 µg Cr/L. However individual specimens need to have a radioactive chromium spiked aliquot processed in parallel to monitor the recovery and the matrix modified AAS/ETA response.

The batch of tests cannot be processed in under three days, and the maximum number of tests in a batch is probably 24, representing duplicate determinations for both serum Cr parameters from a single glucose tolerance test. The radioactive chromium is not expensive, the hfacac is easily the most costly consumable.

E401. Serum chromium determination, standard method CDI.

METHOD.

1. 1000 µL of serum was pipetted into a Tuftainer and 10 mL of protein precipitant added. The serum suspensions were then mixed on a roller mixer for 15 minutes and then centrifuged at 3000 rpm for 15 minutes. The protein precipitants were propan-2-ol for the total protein-bound chromium and 0.6M perchloric acid for the alpha-2-globulin-bound metal.
2. The precipitates were dried after the removal of the supernatants, at 37^0 C for 24 hours.
3. 250 µL of toluene and 250 µL of hfacac were added to the precipitates followed by vigorous mixing on a vortex mixer.

   Standards were set up as follows :-

   i) Zero. (toluene, propan-2-ol, 9 : 1)

   ii) 1.25 µg/L. (5 µg Cr/L, as the chloride, in toluene/propanol).

   iii) 2.50 µg/L. (10 µg Cr/L, as the chloride, in toluene/propanol).

   250 µL of the standard solutions were then taken and 250 µL of hfacac added, followed by vigorous mixing on a vortex mixer.

   Tests and standards were then treated in an identical manner.
4. All Tuftainers were then heated at 100^0 C for 24 hours.
5. The Tuftainers were cooled in a freezer at -20^0 C for at least 30 minutes. 5 mL of cold petroleum spirit (4^0 C) was then added, and the Tuftainers were heated at 75^0 C for 4 hours.
6. 2.5 mL of 1M disodium hydrogen phosphate was then added and the Tuftainer contents mixed on a roller mixer for 15 minutes.
7. The petroleum spirit was removed to a screw cap polypropylene tube containing 2.5 mL of water and the tubes were then placed on a roller mixer for 15 minutes.
8. The petroleum spirit was then extracted with 250 µL of 9M ammonia solution in a similar polypropylene tube on a roller mixer for 24 hours.
9. The ammonia solutions were evaporated on a vortex evaporator at 75^0 C under reduced pressure in 12 x 75 mm polypropylene tubes and the residues dissolved in 100 µL of 0.025M EDTA.

E402. Serum chromium determination, standard method CDII.

METHOD.

1. 5 mL of serum was pipetted into a Tuftainer and 10 mL of protein precipitant added. The serum supensions were then mixed on a roller mixer for 15 minutes and then centrifuged at 3000 rpm for 15 minutes. The protein precipitants were propan-2-ol for the total protein-bound serum chromium and 0.5M hydrochloric acid in propan-2-ol for the alpha-2-globulin-bound chromium.

2. The protein precipitates were then washed x 2 with 10 mL of propan-2-ol using the above mixing and centrifuging procedure, and then with 10 mL of toluene substituting heating at 75^{0} C in a hot air oven for the roller mixing step.

3. 500 µL of toluene and 250 µL of hfacac were added to the precipitates and the Tuftainer contents thoroughly mixed on a vortex mixer.

Standards were set up as follows :-

500 µL of toluene (plus 50 µL acetic acid per 15 mL of toluene).

50 µL of propan-2-ol containing the chromium as the chloride.

250 µL of hfacac.

Thoroughly mixed, and then treated in an identical manner to the tests.

4. All the Tuftainers were then heated at 75^{0} C overnight and then cooled at -20^{0} C for 1 hour.

5. 8 mL of petroleum spirit was then added and the Tuftainers were placed on a roller mixer for 4 hours.

6. 4 mL of 1M dipotassium hydrogen phosphate was then added and the Tuftainers replaced on the roller mixer for a further 30 minutes.

7. The petroleum spirit was then removed and added to screw cap polypropylene tubes containing 4 mL of pure water. The tubes were then roller mixed for a further 2 hours.

8. The petroleum spirit was then extracted with 500 µL of 3.5M ammonia, 0.005M EDTA in similar polypropylene tubes on a roller mixer for 90 minutes.

9. The ammonia solution was removed into 12 x 75 mm polypropylene tubes and evaporated at 75^{0} C under reduced pressure on a vortex evaporator. The residues were dissolved in 100 µL of water at 75^{0} C for 5 minutes on a vortex mixer.

RESULTS.

	Mean radiometric recovery.	Relative standard deviation.
Standards.	74%	3.6%
Serum samples.		
Total protein-bound.	73%	6.0%
Alpha-2-glob.-bound.	84%	4.6%

E403. Serum chromium determination, standard method CDIII.

METHOD.

1. 8 mL of propan-2-ol (resin treated) was added to each Tuftainer. 750 µL of 6M hydrochloric acid (purified by distillation) was added to the propan-2-ol in the Tuftainers for the alpha-2-globulin-bound serum chromium determinations only. The two series were then treated in an identical manner.
2. 4 mL of serum was added slowly to the 8 mL of protein precipitant in the Tuftainer.
3. The Tuftainers were placed on a roller mixer for 15 minutes.
4. Tuftainers centrifuged for 15 minutes at 3000 rpm.
5. The supernatants were carefully removed and discarded.
6. 8 mL of propan-2-ol were added, and the Tuftainers shaken vigorously to resuspend the precipitates.
7. Tuftainers centrifuged for 15 minutes at 3000 rpm.
8. The supernatants were removed and discarded, then 8mL of resin treated toluene was added and the Tuftainers heated at 75 ^{0}C for 15 minutes after a vigorous shake to resuspend the precipitates.
9. Tuftainers were centrifuged at 3000 rpm for 15 minutes.
10. Supernatants were removed and discarded.
11. The treatment with toluene was repeated.
12. 200 µL of acetic acid (purified by ion-exchange resin treatment) containing 7.5% v.v. Aristar sulphuric acid was added.
13. 200 µl of hfacac double distilled from petroleum spirit was added.
14. The Tuftainers were firmly capped and the contents viqorously mixed on a vortex mixer.
15. Standards.

Tuftainers were washed x 2 with 4 mL of propan-2-ol and x 2 with 4 mL of toluene.

1000 µL of toluene and 200 µL of acetic acid containing 7.5% v.v. sulphuric acid and chromium (as the nitrate) at X 20 the nominal value was then added to the Tuftainer, followed by 200 µL of hfacac.

The containers were then firmly capped and vigorously mixed on a vortex mixer.

16. All further treatment refers to both tests and standards.
All Tuftainers were heated at 75 ^{0} C in an oven (fan assisted ovens may give contamination problems) and the container caps were carefully checked for tightness after 15 minutes.

17. The Tuftainers were cooled in a freezer at -20^{0} C for 1 hour.

18. 8 ml of petroleum spirit were added to all Tuftainers.

19. Tuftainer contents thoroughly mixed on a roller mixer for 4 hours after a vigorous shake to detach the precipitates from the container.

20. 4 mL of 2.2M dipotassium hydrogen phosphate, 0.01M EDTA was added to all Tuftainers.

21. Containers placed on a roller mixer for 1 hour.

22. Tuftainers centrifuged at 3000 rpm for 15 minutes and then the petroleum spirit extracts were removed and added to 13 mL screw cap polypropylene tubes containing 4 mL of pure water.

23. The petroleum spirit extracts were then washed for 1 hour with the water on a roller mixer.

24. The petroleum spirit was then removed and extracted with 500 µL of 5.0M ammonia, 0.005M EDTA in a similar polypropylene tube overnight on a roller mixer. The ammonia had been distilled under isothermal conditions and the EDTA was the di-ammonium salt.

25. The ammonia/EDTA solutions were removed and evaporated in 12 x 75 mm polypropylene tubes in a desiccator over concentrated sulphuric acid at 80 ^{0} C.

26. 120 µL of 0.5M ammonia, 0.7M acetic acid was added to the residues. The tubes were capped and then vortexed at 80 ^{0} C for 10 minutes.

27. The tubes were then centrifuged at 3000 rpm for 10 minutes to return any evaporated water which had condensed on the upper walls of the tubes to the base.

E404. Electrophoresis conditions.

Electrophoresis was carried out on cellulose acetate strips at pH 8.6 in a 0.05M barbitone buffer. The proteins were fixed in propan-2-ol and stained with 0.2% bromphenol blue in propan-2-ol.

E405. Radioactive chromium measurements.

A Nuclear Enterprises 1600 gamma counter was used at the cobalt-57 setting. The two diagonally opposing wells 1 and 16 were used to minimise interference. The efficiencies were 2.44 and 2.40% for wells 1 and 16 respectively. The changes in efficiency with respect to the volume of the test material were not significantly different for the two wells and the same correction curve was used for both.

Volume µL.	Relative efficiency.
250	100%
500	99%
1000	94%
2000	76%
4000	47%

E406. AAS/ETA Standard conditions.

A. Electrothermal Atomisation Settings.

Stage.	Time seconds.	Temperature^0 C.
Dry.	20	240
Ash.	40	1400
Delay.	10	/
Atomisation.	5	2900
Delay.	10	/
Tube clean.	5	3000
Delay.	10	/
Tube blank.	5	2900

Water flow 0.5 litres/minute.

Argon flow 3.0 litres/minute.

B. Atomic Absorption Spectrophotometer Settings.

Chromium lamp current 7 mA.

Wavelength 357.9 nm.

Bandpass 0.4 nm.

Direct absorbance signal to recorder. Recorder set at 10mV fsd.

Recorder variab control used to give scale expansion.

Pen deflections corresponding to a change in absorbance of 0.1 were:-

Minimum setting. 26mm.

Mid-point setting. 75mm.

Maximum setting. 100mm.

E407. Total Protein and Albumin Determinations.

The test specimens were the supernatants from the preliminary precipitation stages of the total protein-bound chromium or alpha-2-globulin-bound chromium determination processes. The human serum pool used for these experiments was diluted 1 in 3 with water and 10% increments from zero to 100% prepared from this primary dilution for comparative purposes. A standard human albumin solution was also tested to determine the albumin in the serum pool as a percentage of the total protein.

A. Total protein. (Relative standard deviation 2.83%).

1. 50 µL of sample was diluted to 1000 µL with water.
2. 4 mL of 3% trichloroacetic acid was added and mixed by repeated inversion.
3. The absorbance was measured at 450 nm.

B. Albumin. (Relative standard deviation 2.26%).

1. 35 µL of specimen was added to 3 mL of dye reagent.
2. 35 µL of water, propan-2-ol or 0.5M hydrochloric acid in propan-2-ol was added as appropriate to correct for differences between tests and standards.
3. Mixed by repeated inversions.
4. Allowed to stand at ambient temperature for 10 minutes.
5. The absorbance was measured at 630 nm .

Dye reagent :

Bromcresol green	0.15 mmol/L.
Succinate buffer	0.075 mol/L.

E408. Iron determinations.

A. Spectrophotometric method.

In an acidic medium Fe(III) is released from the transferrin complex. Ascorbic acid reduces the Fe(III) to Fe(II), and the divalent iron then reacts with "ferrozine" to form a coloured complex which is measured spectrophotometrically at 580 nm. Under the conditions of the test the serum proteins remain in solution.

Ferrozine :

3-(2-pyridyl)-5,6-bis(4-phenylsulphonic acid)-1,2,4-triazine.

The serum iron determinations were carried out using a standard method on a Rotochem CFA 2000 centrifugal analyzer using a primary iron standard. The same standard was used for the iron determinations by

AAS/ETA on the supernatents from precipitation with 0.5M hydrochloric acid in propan-2-ol.

B. AAS/ETA.

1. Iron determinations on the supernatant fluids from the preliminary precipitation stage of the alpha-2-globulin-bound chromium determinations were diluted 1 in 11 with 0.1M acetic acid. The diluted supernatants were then analyzed directly.

All injection volumes were 25 µL.

2. AAS/ETA Standard conditions.

i). Electrothermal Atomisation Settings.

Stage.	Time seconds.	Temperature (nominal) ^{0} C.
Dry.	20	240
Ash.	40	1400
Delay.	10	/
Atomisation.	5	2900
Delay.	10	/
Tube clean.	5	3000
Delay.	10	/
Tube blank.	5	2900

Water flow 0.5 litres/minute.

Argon flow 3.0 litres/minute.

ii). Atomic Absorption Spectrophotometer Settings.

Iron lamp current about 15 mA.

Wavelength 248.3 nm.

Bandpass 0.4 nm.

Background correction used. The deuterium lamp emission was reduced using the stepwise variable aperture control to achieve a rough balance.

Fine balance was made by adjusting the iron lamp current.

Direct absorbance signal to recorder. Recorder set at 10mV fsd.

Recorder variab control used to give scale expansion.

Pen deflections corresponding to a change in absorbance of 0.1 were:-

Minimum setting. 26mm.

Mid-point setting. 75mm.

Maximum setting. 100mm.

C. Results.

Twelve specimens of serum with iron concentrations from 4.1 to

40.2 µmol/L were tested.

Method.	Mean µmol/L.	Relative Standard Deviation.
Spectrophotometric.	18.9	2.6%
AAS/ETA.	21.8	9.6%

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CHAPTER V.

THE OPTIMISATION OF AAS/ETA CONDITIONS.

The SP 9-01 digital flameless atomiser has controls only for the voltage applied to the graphite tube and for the duration of time it is applied for. A complete cycle has five heating periods designated :- dry, ash, atomise, tube clean and tube blank. Three delay periods, with time control only, separate the atomise, tube clean and tube blank segments.

5.1. DRY,ASH AND ATOMISATION SETTINGS.

The dry and ash settings are the two phases in the furnace program which must be carefully tailored to optimally suit the analyte and sample type to be investigated. The aim of the ash stage is to remove all matrix components producing chemical interference or background signal at the atomisation step, with no loss of analyte. The drying stage must produce a dry residue of material at the injection point which is in the hottest region of the tube. Spitting, either during the drying stage or at the start of the ash phase will lead to particles of the injected material being redistributed to cooler parts of the tube, and hence to reduced efficiency of both the ash and atomisation processes. A reduction in the efficiency of the ash process may produce increased background signal at the atomisation step, and hence poor analytical precision, which is also an inevitable result of reduced atomisation efficiency.

Presumptive conditions, which were confirmed later using processed materials, were found using chromium in ammonium EDTA solutions as this was anticipated to be the major matrix component in the injection solution derived from the process under development. The role of EDTA in maintaining chromium in a soluble form has been discussed in chapter IV, section 4.10.1., and ammonium salts of low volatility had been found to be useful for controlling the interference by fluorine derived from the excess hfacac, this will be discussed later in this chapter (section 5.3.3.).

5.1.1. Drying Stage.

The drying step was optimised using a small mirror to view the behaviour of the droplet in the graphite tube over the drying and ash segments of the electrothermal atomisation cycle. Illumination by the

neon 352 nm line was found to be useful for this purpose.

A relatively high drying temperature (nominally 240 ^{0} C) was found to be necessary to prevent spitting at the ash stage. A minimal amount of frothing was unavoidable using the steep temperature rise produced by voltage control. A temperature ramp would be an advantage.

5.12. Ash and Atomisation Settings.

The ash and atomisation conditions were selected in the conventional manner using ash/atomisation curves and plots of atomisation peak heights versus notional atomisation temperature. The drying stage settings were confirmed by precision determinations at various drying temperatures using optimum ash and atomisation conditions, and monitoring background absorbance at the neon 352 nm line. The nominal temperatures listed are those quoted by the instrument manufacturer for the settings given. However under voltage control conditions, the only ones available with the equipment used, reproducibility of temperature depends on graphite tube uniformity, as reported by Veillon, et al (1). In practice drying and ash conditions must be checked for each tube. Modest differences from the standard conditions quoted were necessary for some tubes to obtain similar performance characteristics.

The ETA standard conditions listed in table 5.1 below were used in all experiments except where other details are given, or where only slightly different settings were required.

TABLE 5.1.

STANDARD AAS/ETA CONDITIONS.

Electrothermal Atomisation Settings.

Cycle segment.	Time.	Temperature.	
	(seconds).	Setting.	Nominal (^{0} C).
DRY.	20	25	240
ASH.	40	50	1400
DELAY.	10	/	/
ATOMISATION.	5	88	2900
DELAY.	10	/	/
TUBE CLEAN.	5	92	3000
DELAY.	10	/	/
TUBE BLANK.	5	88	2900

Between cycle delay increased from 40 to 55 sec. to prevent partial sample loss due to pressure increase in pipette barrel.

Argon flow 3.0 litres/min. Water flow 0.5 litres/min.

TABLE 5.1. continued.

Atomic Absorption Spectrometer Settings.

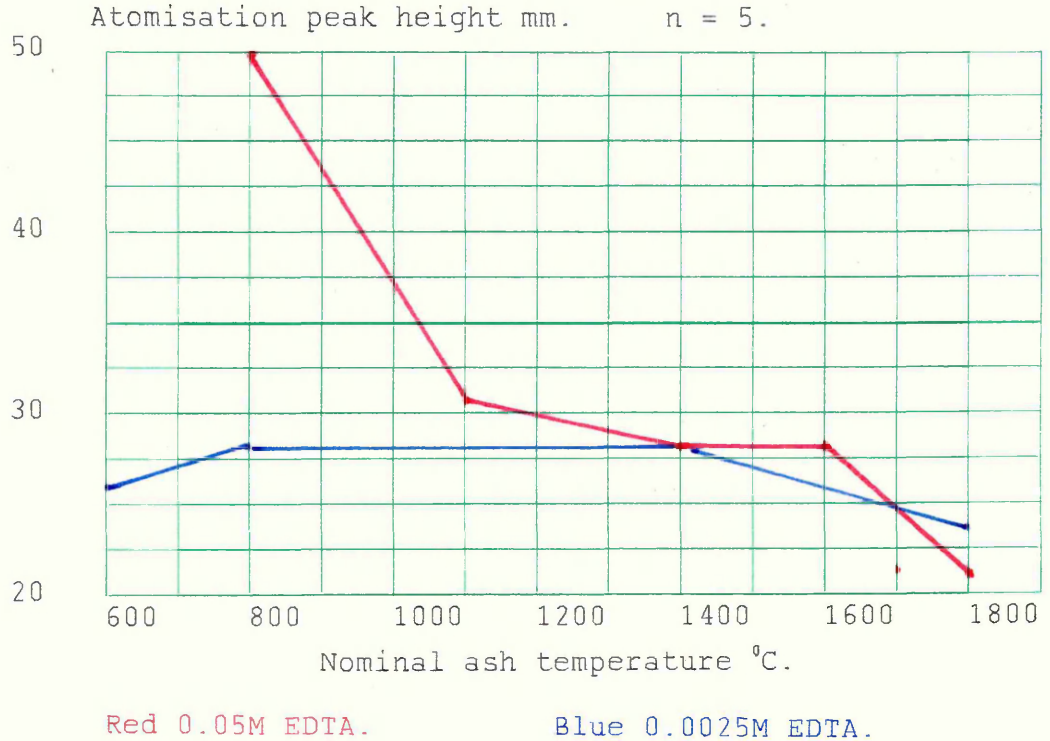
Wavelength 357.9 nm. Bandpass 0.4 nm. Chromium lamp 7 mA.
Direct absorbance signal to recorder (10 mv full scale deflection).
Recorder variab control used to give scale expansion, pen deflections corresponding to a change in absorbance of 0.1 were 26mm to 100mm.

Background correction was not used unless stated, and when used the chromium lamp current was adjusted to balance the unattenuated emission of the deuterium lamp, a reduction to between 5 and 6 mA was satisfactory.

Ash conditions were investigated using a solution of chromium in 0.05M EDTA, about double the concentration used in method CDIII. The higher concentration was used to ensure that settings able to cope with the EDTA, which produces smoke at the atomisation stage with inadequate ash temperatures, were attained. A solution of chromium in 0.0025M EDTA was then injected to confirm that significant losses of chromium did not occur at the 1400 ^{0} C ash temperature selected. The results are illustrated in figure 5.1 below.

FIGURE 5.1.

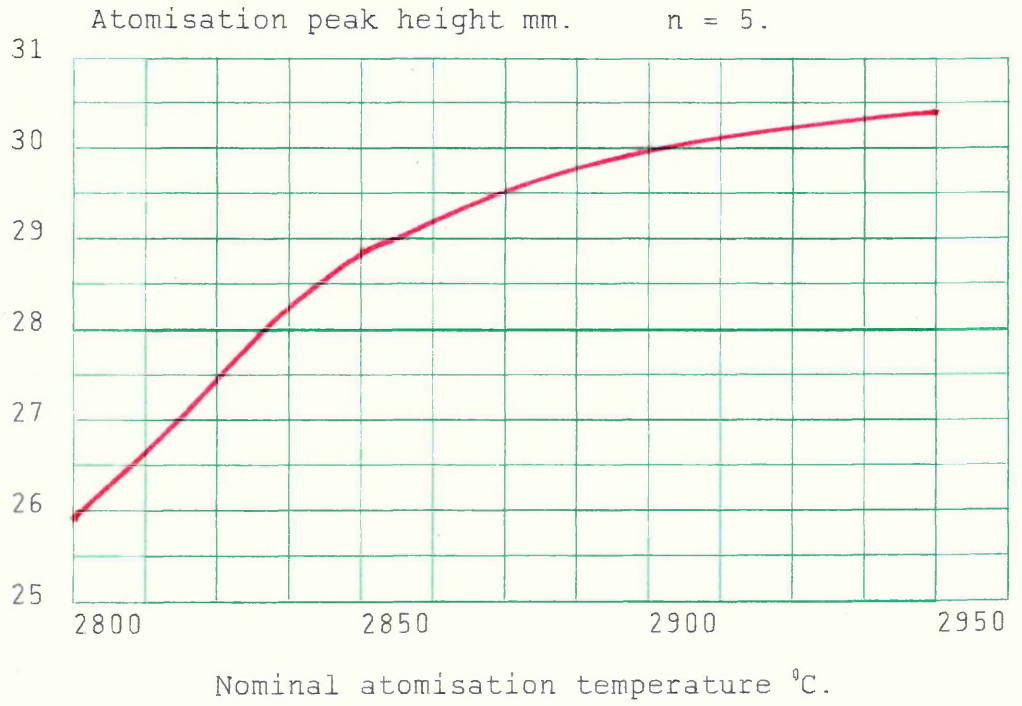
Ash, atomise plot.



The tests were repeated using chromium solutions in 0.1 and 0.5M hydrochloric acid and significant losses of chromium were not recorded using the selected ash temperature. Atomisation temperature was investigated using chromium in 0.025 EDTA solution. The results are given in figure 5.2 below, and show that atomisation peak height does not level off for chromium even up to the maximum temperature.

FIGURE 5.2.

Response relative to temperature.



5.2. INJECTION VOLUME.

Three solutions containing 25, 12.5 and 6.25 µg Cr/L were prepared in 0.05M EDTA. The relatively high chromium concentrations were selected so that good precision was possible from the five replicate injections made from each of the test solutions. The volumes of the aliquots injected were 12.5, 25 and 50 µL respectively, such that 0.313 ng of chromium was injected each time. The results are summarised in table 5.2 below.

The 25 µL injections showed improved precision (95% confidence) over the 50 µL series when calculated in terms of chromium concentration. However there was no significant difference in precision between the 25 µL and 12.5 µL injections. The detection limit is

defined by precision not relative peak height and for this reason the convenient injection volume of 25 µL was selected.

TABLE 5.2.

Comparison of response in relation to injection volume.

µg Cr/L.	6.25	12.5	25
Injection volume µL.	50	25	12.5
	Atomisation peak height mm.		
Mean.	23.8	46	57.5
Standard deviation.	1.7	1.4	2.1
Relative S.D. n = 5.	7.2%	3.1%	3.6%

5.3. COMPARISON OF PYROLYTICALLY COATED AND UNCOATED TUBES.

A series of experiments were carried out to compare the sensitivity, precision, carryover and peak shape characteristics of coated and uncoated profile graphite tubes.

5.3.1. Precision and Carryover characteristics.

The above parameters were compared by injecting three concentrations of chromium: zero, 20 and 5 µg/L in 0.1M acetic acid/0.05M EDTA. Five consecutive 25 µL injections of each solution in the order above were made at three stages in the lives of coated and uncoated profile graphite tubes. The tubes were tested at zero, 100 and 200 firings. Carryover was assessed by measuring the displacement of the first injection of a solution from the mean for that solution series in terms of SD's. A summary of the results is given in table 5.3 below.

TABLE 5.3.

Comparison of coated and uncoated tubes.

	Uncoated tube.		Coated tube.	
Chromium µg/L.	5.	20.	5.	20.
	Atomisation peak height mm.			
Zero firings.				
Mean.	10.4	34.8	27.4	105.4
R.S.D.%.	5.2	13.5	2.0	7.7
1st inj. displacement SD's.	+1.1	-1.2	+1.1	-1.2

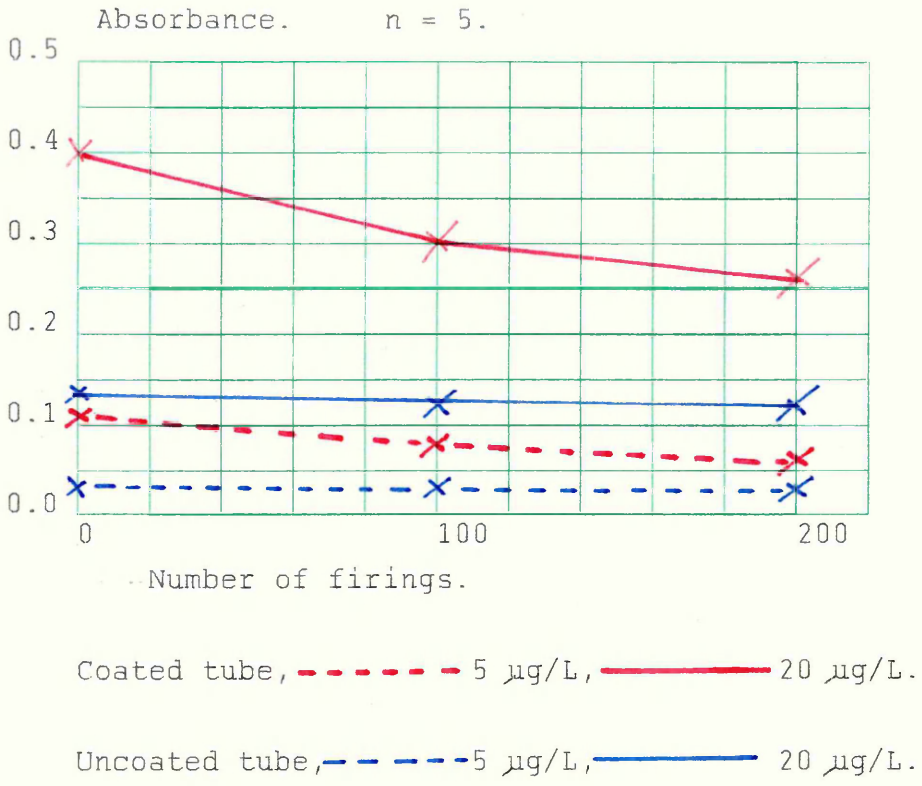
TABLE 5.3. continued.

100 firings.				
Mean.	9.1	31.9	20.6	79
R.S.D.%	6.8	1.3	19.6	10.6
1st inj. displacement SD's.	+5.3	+0.2	+0.3	+0.4
200 firings.				
Mean.	8.6	29.8	17.6	68
R.S.D.%.	15.6	1.9	9.5	2.8
1st inj. displacement SD's.	+11	+1.1	-1.6	0.0

Figure 5.3 below illustrates the difference in sensitivity of the two tube types and the changes with use.

FIGURE 5.3.

Comparison of sensitivity with uncoated and coated graphite tubes, and changes in this parameter over 200 firings.



The linear relationship between atomisation peak height and chromium concentration is shown for new tubes in table 5.4 below.

TABLE 5.4.

Response linearity.

n = 5	Uncoated profile tube.		Coated profile tube.	
µg Cr/L.	Peak absorbance.	Sensitivity.	Peak absorbance.	Sensitivity.
Zero.	<0.0040	/	<0.0040	/
5.	0.0400	0.32	0.1054	0.84
Range.	0.038 to 0.042	0.31 to 0.34	0.103 to 0.108	0.82 to 0.86
20.	0.1338	0.27	0.4054	0.81
Range.	0.116 to 0.152	0.23 to 0.31	0.374 to 0.437	0.75 to 0.87

Sensitivity is defined here as the absorbance change per ng Cr with an injection volume of 25 µL. Range is over 95% confidence limits.

The coated tubes were superior in terms of sensitivity. However although sensitivity decreased with use for both types of tube the fall was much steeper for the coated tubes. The sensitivity ratio fell from 3.03 to 2.28 over 200 firings as the pyrolytic layer became thinner, as illustrated in figure 5.3.

The only significant carryover demonstrated was with the uncoated tube. Positive carryover from the 20 µg/L solution to the 5 µg/L standard was significant after 100 firings, and further deterioration was evident after 200 firings.

The only significant difference in precision was at 100 firings with the 20 µg/L standard. A highly significant advantage in favour of the uncoated tube was recorded.

The linearity of the signal response to the chromium dose was superior for the coated tubes. A response of less than 1mm was recorded for the zero standard prior to injecting aliquots of the 20 µg/L solution in all series for both tubes. The mean value of the 5 µg/L standard was calculated from the appropriate mean peak height of the 20 µg/L solution, assuming a linear response and a zero reading of 1mm. The overall values were 4.99 µg/L for the coated tube and 5.36 for the uncoated. The mean relative standard deviations over the three stages of tube life were 1.2% and 3.3% for coated and uncoated tubes respectively.

The conclusions are that the coated tubes are superior in terms of sensitivity and memory effects. However uncoated tubes appear to show minimal and steady changes in sensitivity over their useful life,

in contrast to the marked changes, particularly over the first one hundred firings recorded for the pyrolytically coated tubes. The poor precision observed at the mid life series for the coated tube was recorded after the injection of processed material and later experiments demonstrated that this was significant.

5.3.2. Comparison of peak area/height ratios.

The above results relate to unprocessed chromium solutions in 0.1M acetic acid/0.05M EDTA. A series of experiments were carried out comparing processed and unprocessed materials in both types of graphite tube. Peak heights and areas were measured, the latter using a DP 101 computing integrator. Ideally peak area/height ratios should be identical for all types of processed materials of similar chromium values, that is for standards, serum samples and urines. The ratio should also be very similar to that recorded for unprocessed chromium solutions in the solvent used for dissolving the processed extracts.

5.3.2.1. Peak area/height ratios in 0.1M acetic acid/0.0125M EDTA.

The peak area/height ratios of processed materials, both standards and serum samples were compared with unprocessed chromium solutions using both pyrolytically coated and uncoated graphite tubes. The standard CDIII process for alpha-2-globulin—bound chromium was used except that the residues were dissolved in 0.1M acetic acid. Three injections of each solution were made. The integrator peak width half-height parameter was evaluated by injecting representative solutions with the recorder chart speed at 300 mm per minute. The default values were used for all other integration parameters.

A. Pyrolytically Coated Tubes.

Table 5.5 below summarises the results obtained with coated tubes.

The absorbance at the 520 nm neon line was monitored, and a very small peak (height < 5 mm) was recorded for all solutions injected, including 0.1M acetic acid/0.0125M EDTA, with and without background correction.

The very poor precision for replicate injections reported in table 5.5 below clearly prohibits the use of coated profile tubes under these conditions, and a discussion of the peak area/height ratios is of little value.

TABLE 5.5.

Peak area/height ratios, coated profile tubes.

n = 3.	Peak Height. mm.		Peak Area. µV sec.		Area/Height. µV sec./mm.	
	Mean.	RSD %.	Mean.	RSD %.	Mean.	RSD %.
i) No bkgr. correction.						
Unprocessed stds.						
5 µg/L.	50	23	1507	25	30.1	2.6
10 µg/L.	09	27	3232	26	30.3	1.6
Processed stds.						
I.	17	13	446	17	27.0	3.9
II.	59	6	2377	12	40.6	5.7
Processed tests.						
I.	73	17	2273	14	31.5	8.5
II.	61	45	2119	34	36.5	10.0
III.	71	33	2503	20	36.4	11.0
IV.	63	20	2150	9	34.4	10.0
ii) Bkgr. correction.						
Unprocessed stds.						
5 µg/L.	59	2	1801	1	30.6	3.5
10 µg/L.	111	27	3316	25	30.0	2.1
Processed tests.						
V.	62	37	1786	33	29.3	4.5
VI.	110	21	3107	21	28.2	0.5
VII.	91	6	2697	6	29.7	1.9

B. Uncoated Tubes.

Table 5.6 below summarises the results obtained with uncoated tubes. Absorbance monitoring at the 520 nm neon line did not produce any peaks therefore no tests were carried out using background correction.

The precisions of the chromium signals from replicate injections were compared for processed standards and tests with coated (no background correction), and uncoated tubes. The differences were significant for four (highly significant for one) of the six pairs tested.

TABLE 5.6.

Peak area/height ratios, uncoated profile tubes.

n = 3.	Peak Height. mm.		Peak Area. µV sec.		Area/Height. µV sec./mm.	
	Mean.	RSD %.	Mean.	RSD %.	Mean.	RSD %.
Unprocessed stds.						
8.6 µg/L.	73	3.2	1830	4.2	25.2	3.5
17.2 µg/L.	139	3.2	3895	5.6	27.9	2.6
Processed stds.						
I.	25	3.1	754	5.4	29.5	3.6
II.	100	3.8	2860	5.2	28.8	1.2
Processed tests.						
I.	63	4.5	1357	3.6	21.6	8.5
II.	135	4.2	3735	3.2	27.4	6.7
III.	46	6.1	984	6.3	21.6	0.3
IV.	33	11	886	16	27.0	6.5
V.	35	36	835	15	24.8	22
VI.	35	0	917	0.5	26.2	0.5

The mean peak area/height ratios of processed standards, test serum samples and unprocessed chromium solutions using both coated and uncoated graphite tubes, the former with and without background correction, are listed in table 5.7 below. The data for processed standards using coated tubes has been omitted because of the large difference beween the values found for the two samples tested, and in view of the very poor precision recorded with this type of tube repeat tests were not carried out.

TABLE 5.7.

Comparison of peak area/height ratios.

	Uncoated tube. µV sec./mm.			Coated tube. µV sec./mm.			Coated tube (BC). µV sec./mm.		
	n.	Mean.	SD.	n.	Mean.	SD.	n.	Mean.	SD.
Unproc. Sol.	9	26.2	1.43	6	29.8	0.57	6	30.3	0.8
Proc. Std.	14	29.2	0.89	/	/	/	/	/	/
Test serum.	18	24.8	3.2	12	34.4	3.5	9	29.1	0.99

A series of t tests were carried out to assess the significance of the differences in peak area/height recorded above. The values reported for uncoated tubes were significantly different (99% confidence) for both processed standards versus unprocessed chromium solutions and processed standards versus serum samples, whilst no significant difference was found between serum samples and unprocessed chromium solutions. In contrast the ratios found for serum samples and unprocessed chromium solutions using coated graphite tubes were significantly different using background correction and highly significant without it.

The conclusions from the above tests are that uncoated tubes appear to be superior under the conditions used. Pyrolytically coated tubes appear to be unsuitable and will not be discussed further.

The material from serum samples has similar peak area/height ratios to the unprocessed chromium solutions but processed standards have higher peak area/height ratios indicating some matrix differences. A tentative explanation is that slightly higher levels of derivatives from the beta-diketone are present in the processed standards compared with serum samples. The extracts from processed standards probably contain higher concentrations of excess beta-diketone because of lower consumption in side reactions compared with serum samples. The serum samples were processed by the alpha-2-globulin-bound Cr method in the experiments reported in this section, because the higher acidity of the protein precipitates produced could result in a less efficient removal of the excess hfacac compared with the total protein-bound Cr process. The addition of EDTA as the di-ammonium salt had been observed to reduce the interference caused by fluoride derived from the diketone, but produced smoke at the atomisation step at other than low concentrations. The removal of fluorine as volatile ammonium fluoride at the ash stage was considered to be a possible mechanism for the beneficial effect, and ammonium acetate was therefore investigated as a matrix modifier.

5.3.2.2. Peak area/height ratios in ammonium acetate solutions.

An excess of acetic acid was added to prevent ammonia vapour from interfering with the pipetting characteristics of the solution. Volatile material can cause an increase in pressure in the pipette barrel as the warmer environment near the graphite furnace is entered, and a partial loss of sample dose can result. The delay between

cycles had been increased to 55 seconds as described in the standard AAS/ETA conditions as a precaution against this, after observing it to occur with a number of solutions. The excess acetic acid also prevents premature loss of the ammonia at the dry stage of the cycle. Table 5.8 below records the results obtained on a series of solutions containing 10 µg Cr/L and a range of ammonium acetate concentrations using a coated profile tube.

TABLE 5.8.

Ammonium acetate solutions in a coated profile graphite tube.

	Atomisation Peak Height mm.	
	Mean	RSD %.
0.1M acetic acid, 0.0025M EDTA.	107	2.9
0.125M ammonia, 0.325M acetic acid.	77	69
1.0M ammonia, 1.8M acetic acid.	34	37

Ammonium acetate solutions are clearly not suitable for use with pyrolytically coated graphite tubes. The above experiment was repeated with an uncoated graphite tube. The results are given in table 5.9 below.

TABLE 5.9.

Ammonium acetate solutions in an uncoated profile graphite tube.

	Atomisation Peak Height mm.	
	Mean.	RSD %.
0.1M acetic acid, 0.0025M EDTA.	57	2.2
0.125M ammonia, 0.325M acetic acid.	59	2.8
0.25M ammonia, 0.60M acetic acid.	58	2.5
0.50M ammonia, 1.4M acetic acid.	58	1.2
1.00M ammonia, 2.8M acetic acid.	40	1.4

It can be seen that uncoated graphite tubes tolerate ammonium acetate/acetic acid up to 0.5M ammonia, 1.4M acetic acid.

An experiment was carried out to check that the standard ash temperature setting (nominally 1400 ^{0} C) of the electrothermal atomisation cycle was suitable for ammonium acetate solutions. Ten aliquots of a serum pool were processed using standard method CDIII. The dried extracts were dissolved in 1.0M ammonia/1.75M acetic acid and

pooled. Five aliquots of the pooled extract were then injected at each setting of the AAS/ETA conditions. The ash temperature was varied from 800 to 1650 ^{0} C, and the absorbance was monitored at the neon 352 nm line and at the 357.9 nm emission from the chromium lamp. The results are given in table 5.10 below.

TABLE 5.10.

Check on ash temperature with ammonium acetate solution.

	Atomisation Peak Height mm. (mean).	
Ash temperature ^{0} C.	Neon 352 nm.	Chromium 357.9 nm.
800	41	/
900	14	/
1000	zero	35
1100	zero	35
1400 (standard)	zero	36
1650	zero	27

The conclusion was that the standard ash setting was suitable for use with ammonium acetate solutions.

The effects of hfacac derivatives on the peak area/height ratio was investigated using aliquots of a solution of the beta diketone converted to the hydrated derivative in ammonia solution. The aliquots were evaporated to dryness over sulphuric acid in a sealed desiccator at 75 ^{0} C. Solutions containing 10 µg Cr/L but with a range of hydrated beta-diketone concentrations corresponding to retentions from 0, through 0.5, 1.0, 2.0 to 4% of the hfacac used in the standard process (CDIII) were prepared. Three sets of these solutions were prepared, one in 0.02M EDTA (diammonium salt), another in 0.02M EDTA plus 0.5M ammonia and the third in 0.02M EDTA with both 0.5M ammonia and 0.7M acetic acid. A summary of the results is given in table 5.11 below. All solutions were injected five times.

The experimental results confirm that traces of hfacac derivatives in 0.02M EDTA produce a highly significant reduction in atomisation peak height at the 0.5 % retention level, and a highly significant deterioration in precision at the 1% hfacac retention level. The reduction in peak area was less pronounced than that recorded for peak height. The result of this was an increase in the peak area/height ratio which was significant at the 2% hfacac retention level.

TABLE 5.10.

Comparison of peak area/height ratios in three solutions.

Solution. n = 5.	hfacac Retention %.	Mean Peak Height. % of reference.	RSD%.	Area/Height ratio. Mean.	RSD%.
1. 0.02M EDTA.					
	Zero	100	4.1	37.9	8.3
	0.5	76	4.2	40.1	8.4
	1.0	79	19	40.4	4.4
	2.0	57	19	43.4	15
2. 0.02M EDTA, 0.5M ammonia.					
	1.0	65	8.2	48.6	7.1
3. 0.02M EDTA, 0.5M ammonia, 0.7M acetic acid.					
	Zero	100	2.8	35.1	6.2
	0.5	98	2.8	38.4	10
	1.0	105	7.5	36.0	7.3
	2.0	100	6.5	37.3	5.2
	4.0	82	8.0	35.6	5.1

The addition of ammonium acetate clearly reduces the interference by hfacac derivatives. The peak area/height ratio was not significantly changed even with 4% hfacac retention, and although a highly significant reduction in peak height was seen at this level no significant changes in this important parameter were recorded up to and including 2% hfacac retention. The precision was however adversely affected by hfacac levels equivalent to 1% retention or more. The addition of ammonia alone did not appear to be effective, presumably because it is volatilised too early in the electrothermal atomisation cycle.

A batch of tests were processed using method CDIII to compare the peak area/height ratios of serum samples and standards as earlier (5.32.1), but this time with ammonium acetate added. A serum pool from samples taken with the necessary precautions was processed in duplicate for both total protein – bound chromium and alpha-2-globulin – bound chromium. A serum sample taken with no special precautions was processed for alpha-2-globulin-bound Cr only. Standards equivalent to

zero and 0.2 µg Cr/L were also processed, the former in triplicate the latter in duplicate. The solutions from each individual source were pooled to obtain volumes of sufficient size for a statistically significant number of injections to be made. The unprocessed standard chromium solutions were prepared in the 0.5M ammonia, 0.7M acetic acid, 0.0125M EDTA used for the processed material, as specified in method CDIII.

The results are listed in table 5.12 below. Pool I was derived from samples taken with special precautions to reduce contamination with exogeneous chromium and pool II was from samples taken in the routine manner.

TABLE 5.12.

Peak area/height ratios for serum samples and standards in ammonium acetate solution.

	Peak Heights. mm.			Peak Areas. µV sec.		Area/Height ratio. µV sec./mm.	
	n.	Mean.	RSD%.	Mean.	RSD%.	Mean.	SD.
Unprocessed Std.							
1. 2.5 µg Cr/L.	5	24	2.9	882	12	36.8	14.5
2. 5.0 µg Cr/L.	5	41	2.8	1461	9.1	35.1	6.2
3. 10.0 µg Cr/L.	5	76	2.7	2582	4.6	33.8	2.9
4. 20.0 µg Cr/L.	5	144	1.0	4530	3.1	31.4	3.0
5. 30.0 µg Cr/L.	5	210	0.86	5885	1.5	28.0	1.6
Processed Std.							
1. "Zero".	9	20.4	13	703	13.3	34.5	2.4
2. "0.2 STD".	6	60.3	0.96	1666	7.7	27.7	1.5
Serum samples.							
A. Alpha-2-globulin-bound Cr.							
1. Pool I.	6	36.0	8.6	1294	7.6	35.9	2.8
2. Pool II.	6	201	4.5	5514	7.0	27.4	1.2
B. Total protein-bound chromium.							
1. Pool I.	6.	39.3	5.3	1422	7.5	36.2	0.87

The peak area/height ratios for the unprocessed standard solutions show a steady decrease with increasing chromium concentration. However the values found for both processed standards and test samples were not significantly different from those of

unprocessed standards of similar peak height. The relative standard deviations of the peak height measurements were smaller than those of the peak areas, significantly so for two standards and highly significant at the 2.5 µg Cr/L level. The linearity of the peak height measurements was also superior as illustrated in figures 5.4 and 5.5. The reproducibility of the transient signal peak heights at the 20 µg Cr/L level (equivalent to about 0.75 µg Cr/L in a sample) is demonstrated by the recorder trace reproduced in figure 5.6.

5.3.2.3. Peak profiles in ammonium acetate solution.

A number of serum samples were processed for total protein-bound chromium and others for alpha-2-globulin-bound chromium using the adopted standard method CDIII. The profiles of the atomisation peaks were recorded under conditions such that 100 mm of pen deflection corresponded to an absorbance change of 0.1, and at a chart speed of 300 mm/second. A selection of peak profiles representing a range of sample chromium concentrations are illustrated in figure 5.7, together with processed standards corresponding to zero, 0.10, 0.35 and 0.50 µg Cr/L. A similar range of profiles from urine samples processed using the standard method adopted for urines, which is described in chapter VI, are also included, plus a number of relevant peak profiles recorded at the neon 352 nm line and "no injection" profiles at both wavebands. The concentrations for comparative profiles from unprocessed Cr standard solutions in ammonium acetate/EDTA range from 0.6125 to 30 µg/L, equivalent to 0.03 and 1.25 µg Cr/L respectively in a test sample. The characteristics of the peak profiles are summarised in table 5.13. W.h/2 is the half height peak width (sec.).

TABLE 5.13.

Peak profile characteristics.

Unproc. stds.		Proc. stds.		Total.P.B.Cr.		Alpha.Glb.Cr.		Urine Cr.	
Abs.	W.h/2.	Abs.	W.h/2.	Abs.	W.h/2.	Abs.	W.h/2.	Abs.	W.h/2.
0.011	3.6	0.012	3.6	0.020	3.6	0.016	3.6	0.021	3.6
0.015	3.6					0.031	3.4		
0.024	3.4	0.036	3.5	0.027	3.4	0.032	3.4		
0.041	3.4	0.063	3.4			0.037	3.4	0.056	3.8
0.076	3.3	0.086	3.4	0.090	3.4			0.104	3.6
0.144	3.3			0.100	3.3			0.138	3.5

FIGURE 5.4.

Plot of peak height versus chromium concentration.

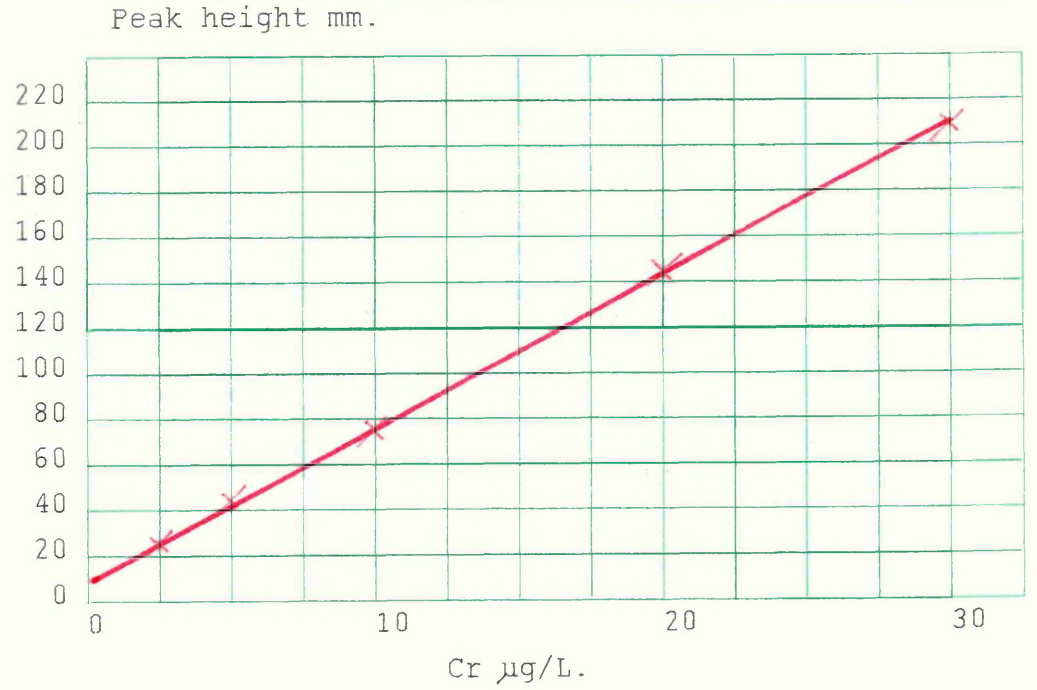
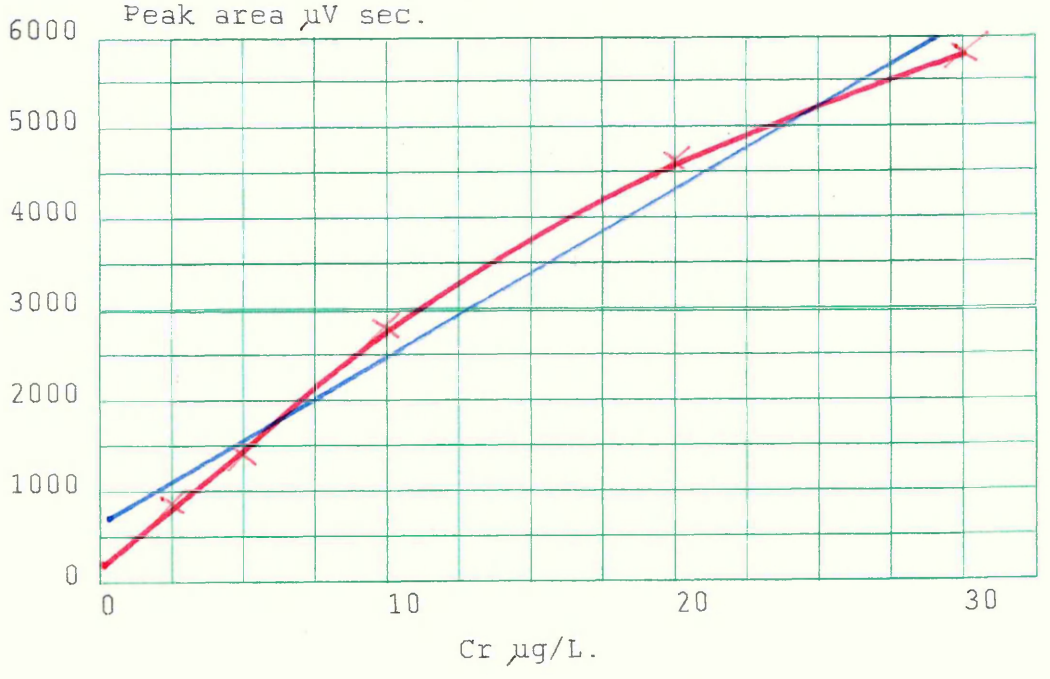


FIGURE 5.5.

Plot of peak area versus chromium concentration.



Blue line = linear regression (least squares).

FIGURE 5.6.

Reproducibility at the 20 µg Cr/L level.

* Chromium peak.

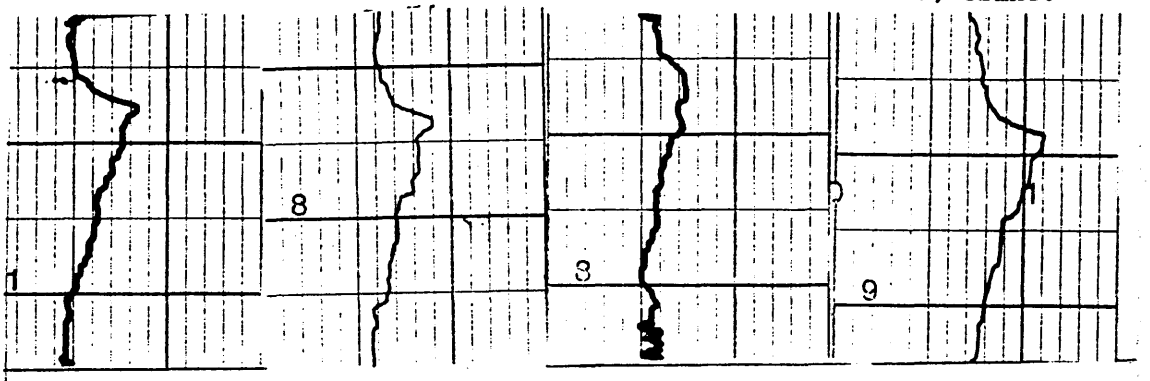
Time scale 10 mm/min.

FIGURE 5.7.

Peak profiles in ammonium acetate.

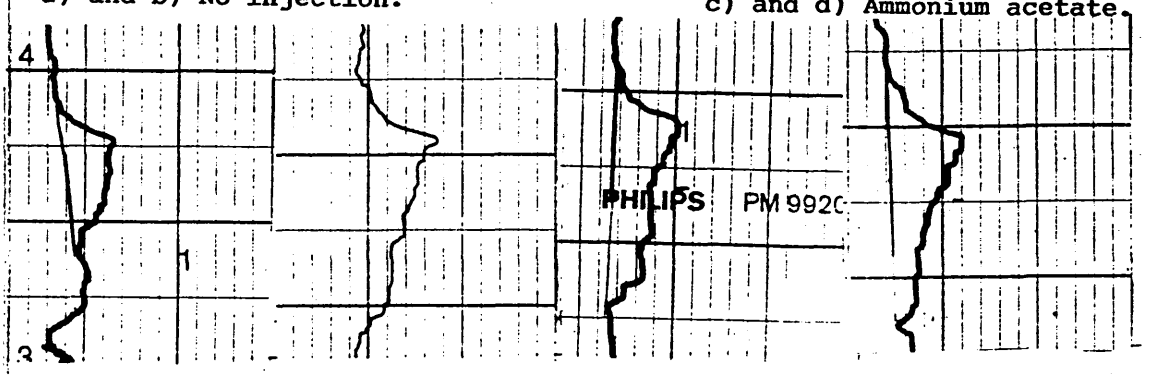
1. At the neon 352 nm line.

a) No injection. b) Ammonium acetate. c) Serum. d) Urine.



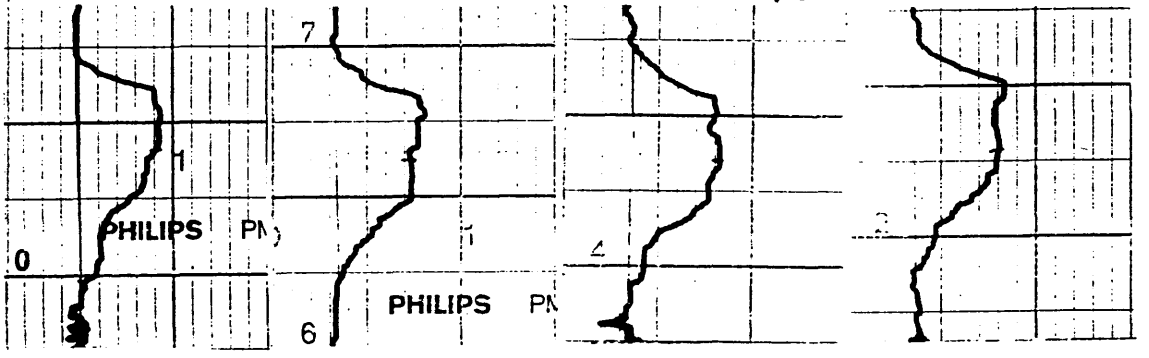
2. At the chromium 357.9 nm line.

a) and b) No injection. c) and d) Ammonium acetate.



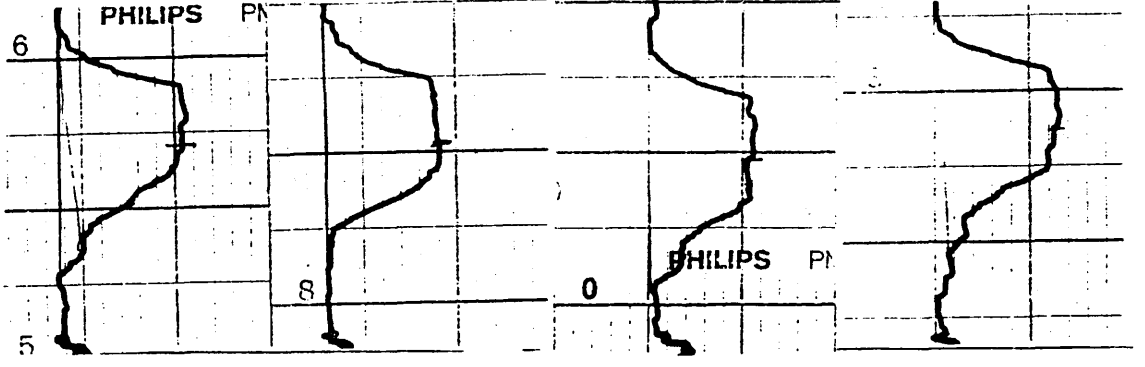
3. At the chromium 357.9 nm line.

a), b), c) and d) Unprocessed standards 0.6125 µg Cr/L.



4. At the chromium 357.9 nm line.

a), b), c) and d) Unprocessed standards 1.25 µg Cr/L.



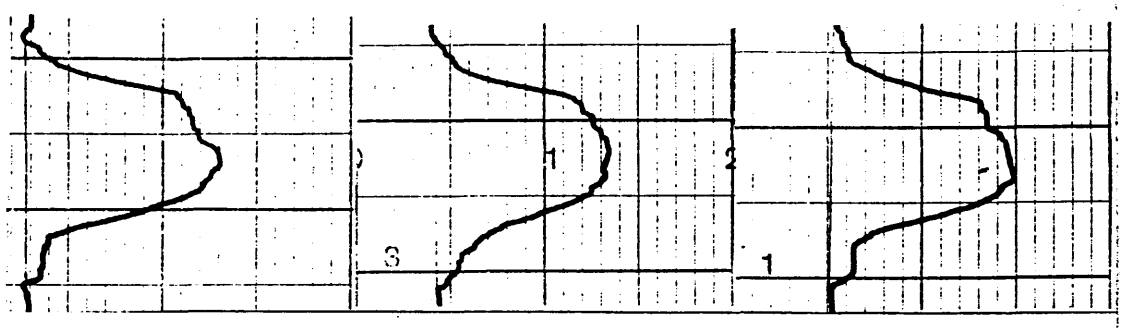
Time scale 300 mm/min. Peak height scale 100 mm/0.1 absorbance.

FIGURE 5.7. continued.

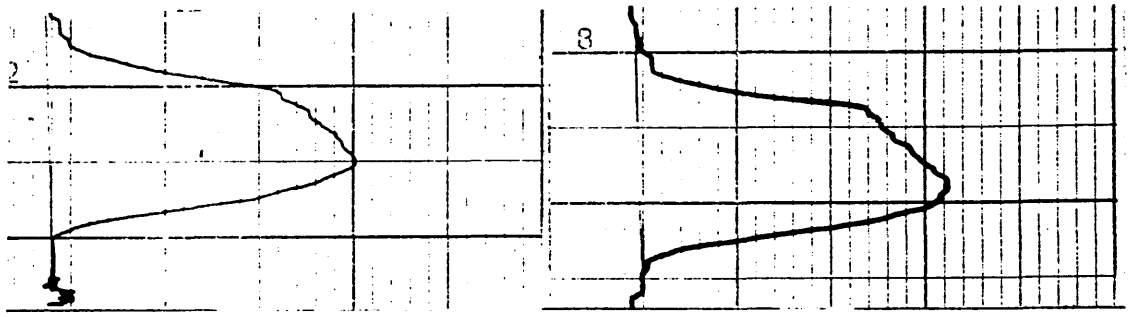
Peak profiles in ammonium acetate.

1, 2, 3, and 4. Unprocessed standards at the 357.9 nm chromium line.

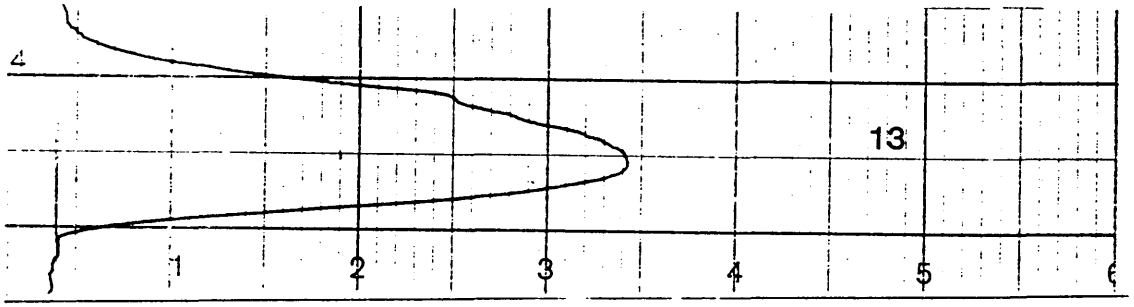
a), b), c) 2.5 µg Cr/L.



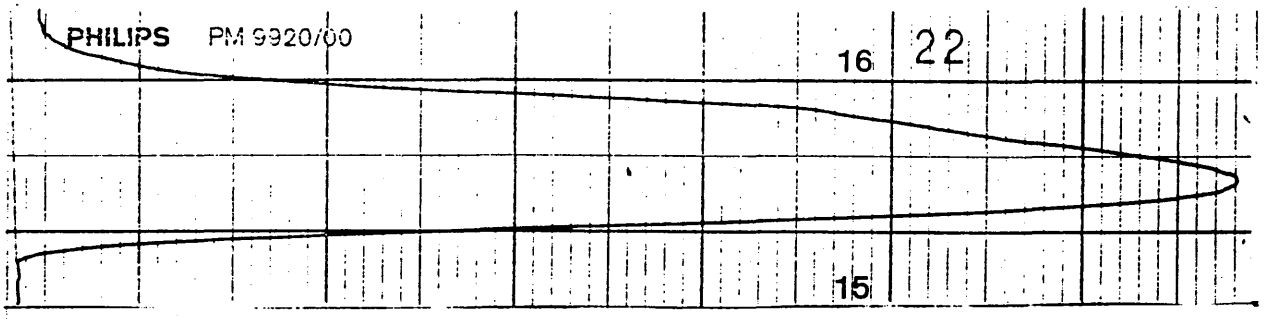
a) and b) 5.0 µg Cr/L.



10.0 µg Cr/L.



20.0 µg Cr/L.



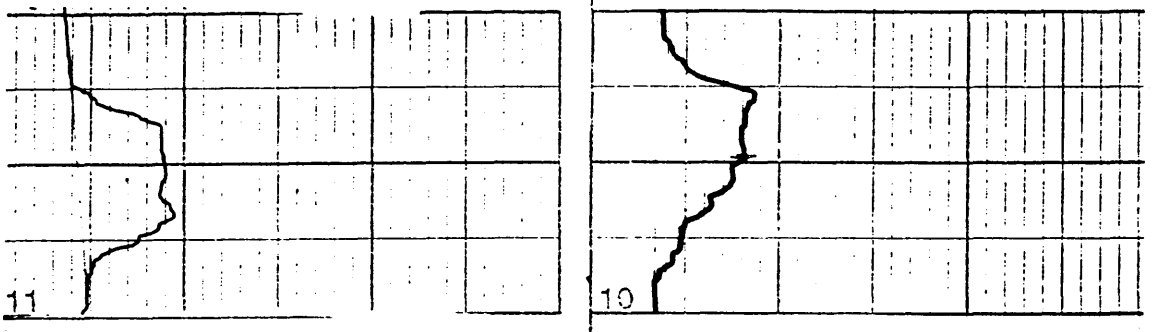
Time scale 300 mm/min. Peak height scale 100 mm/0.1 absorbance.

FIGURE 5.7. continued.

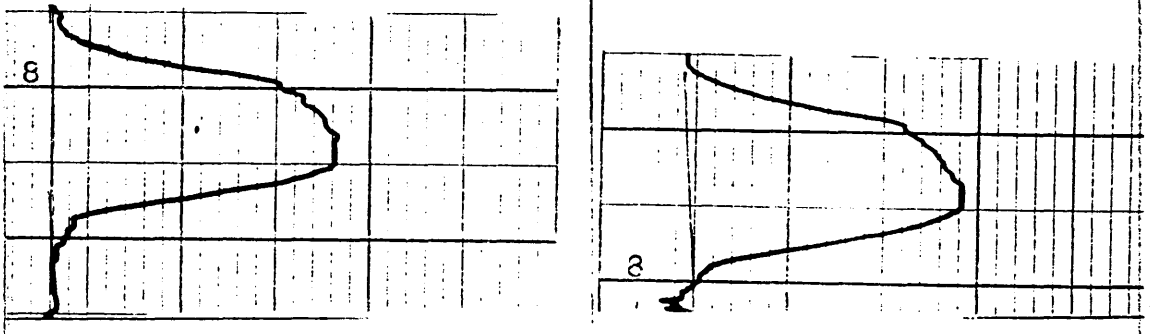
Peak profiles in ammonium acetate.

1, 2, 3, and 4. Processed standards at the 357.9 nm chromium line.

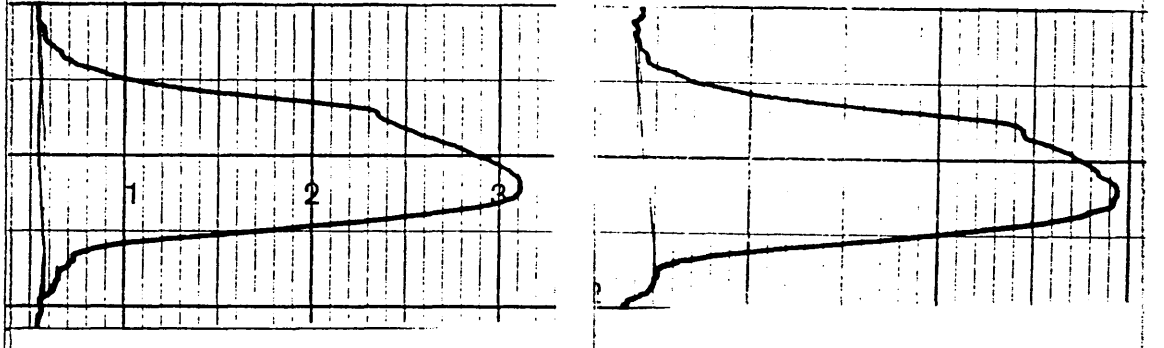
a) and b) Zero standards.



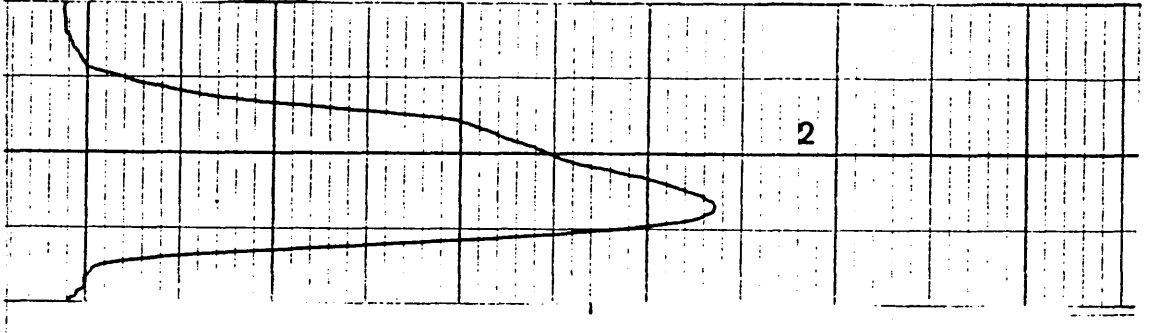
a) and b) Standards equivalent to 0.10 µg Cr/L.



a) and b) Standards equivalent to 0.35 µg Cr/L.



Standard equivalent to 0.50 µg Cr/L.



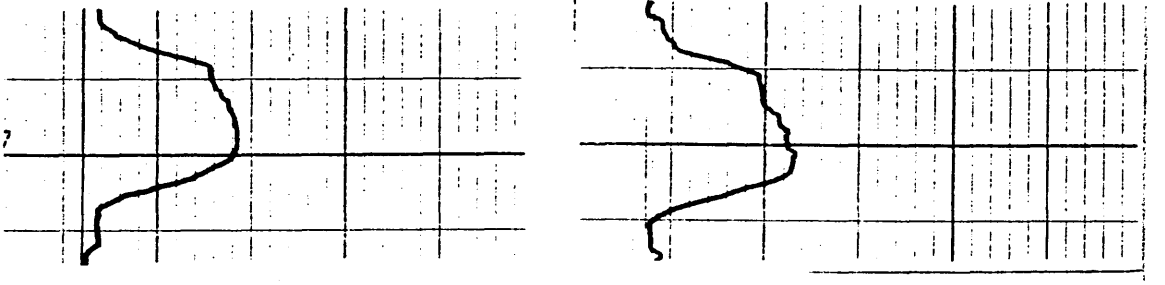
Time scale 300 mm/min. Peak height scale 100 mm/0.1 absorbance.

FIGURE 5.7. continued.

Peak profiles in ammonium acetate.

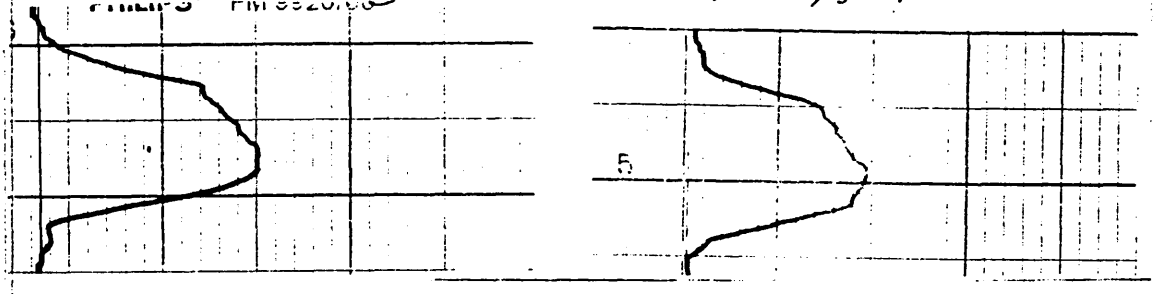
1, 2, 3, and 4. Serum total protein-bound Chromium at the Cr 357.9 nm ~~line.~~

a) and b) 0.05 µg Cr/L.

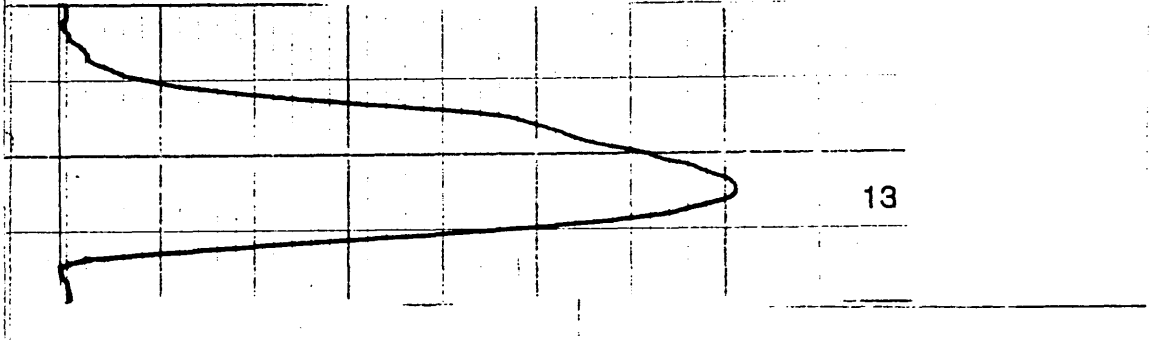


a) 0.10 µg Cr/L.

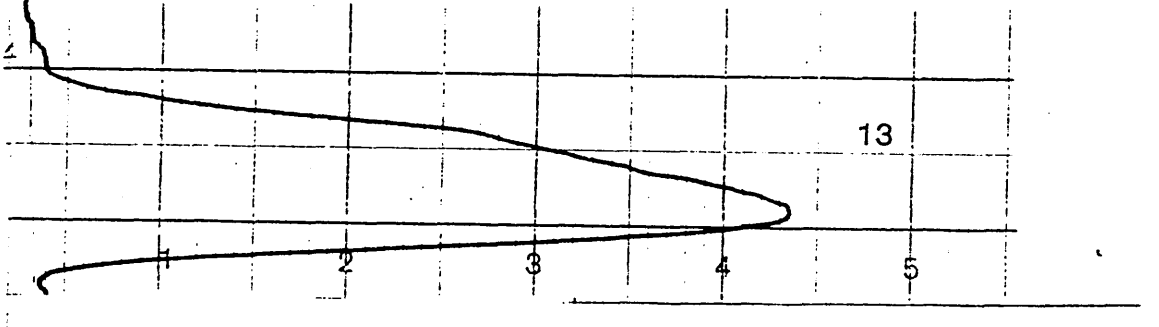
b) 0.07 µg Cr/L.



0.53 µg Cr/L.



0.60 µg Cr/L.



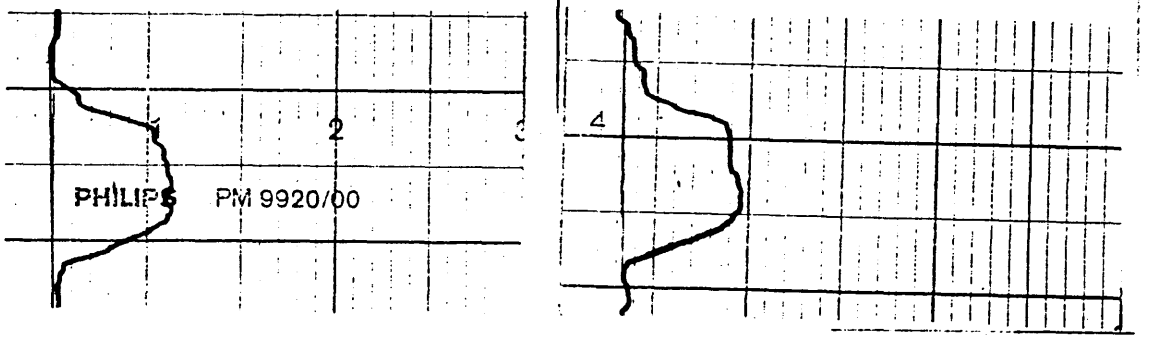
Time scale 300 mm/min. Peak height scale 100 mm/0.1 absorbance.

FIGURE 5.7. continued.

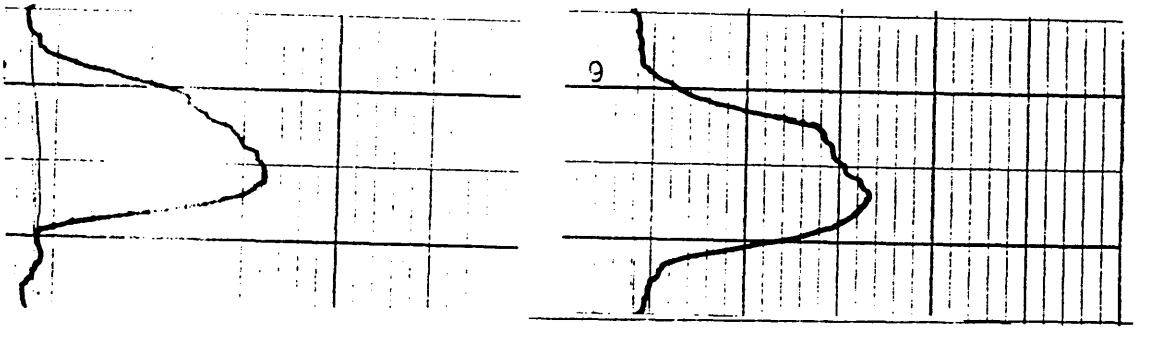
Peak profiles in ammonium acetate.

1, 2, 3, and 4. Serum alpha-2-globulin-bound chromium at the Cr 357.9 nm line.

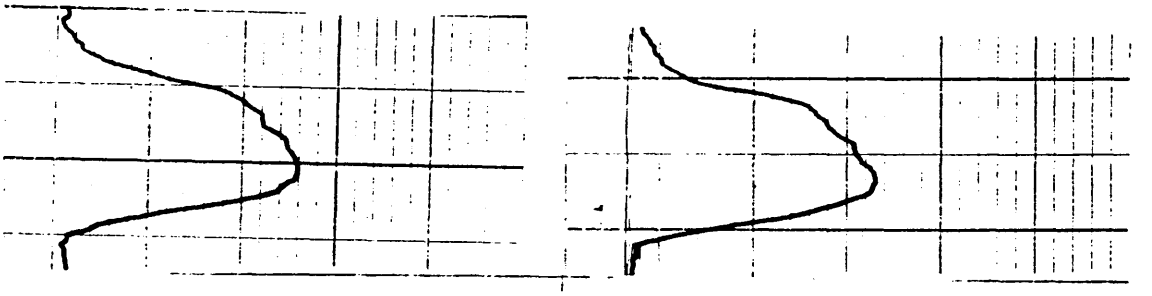
a) and b) below detection limit.



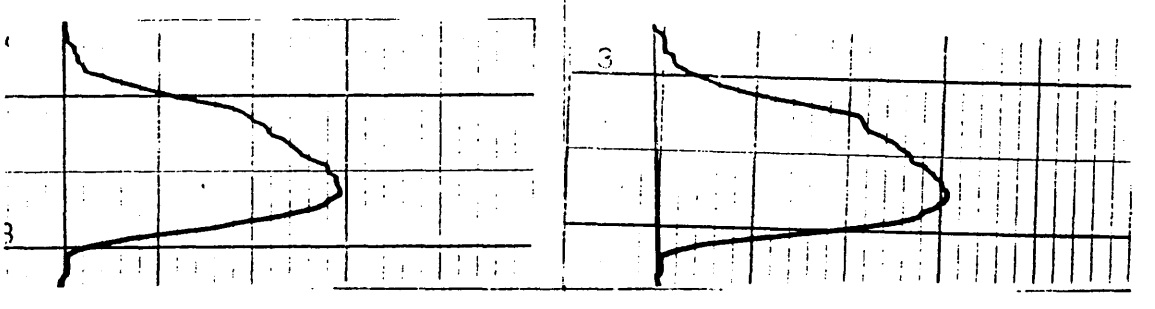
a) 0.12 µg Cr/L. b) 0.11 µg Cr/L.



a) 0.15 µg Cr/L. b) 0.14 µg Cr/L.



a) 0.16 µg Cr/L. b) 0.17 µg Cr/L.



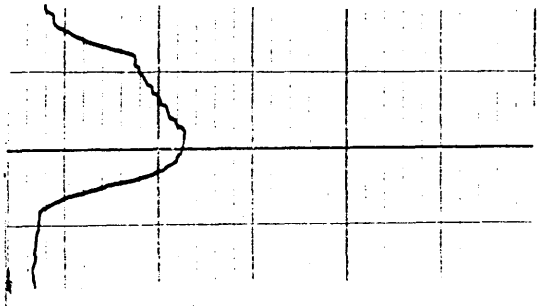
Time scale 300 mm/min. Peak height scale 100 mm/0.1 absorbance.

FIGURE 5.7. continued.

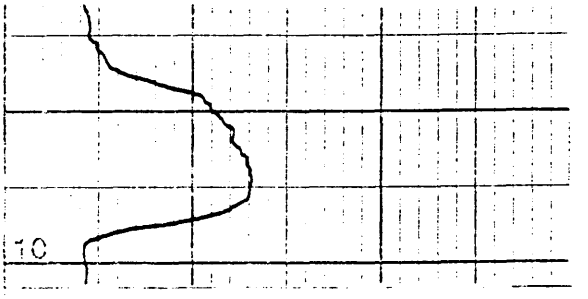
Peak profiles in ammonium acetate.

1, 2, 3, and 4. Urine chromium at the 357.9 nm line.

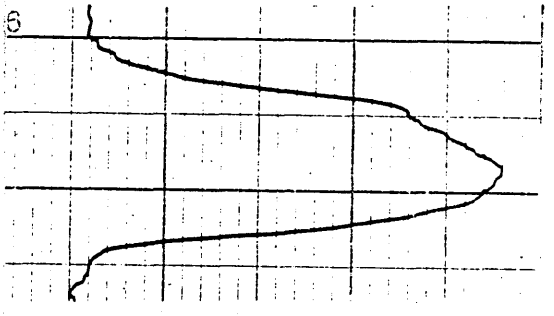
a) below detection limit.



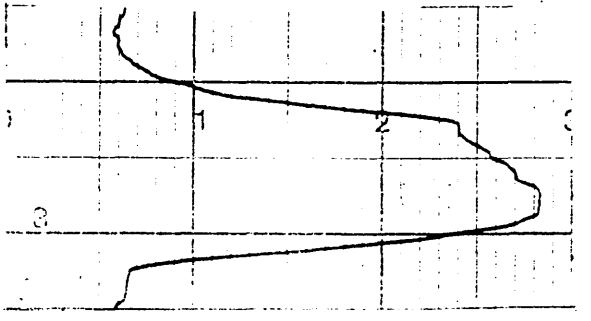
b) 0.05 µg Cr/L.



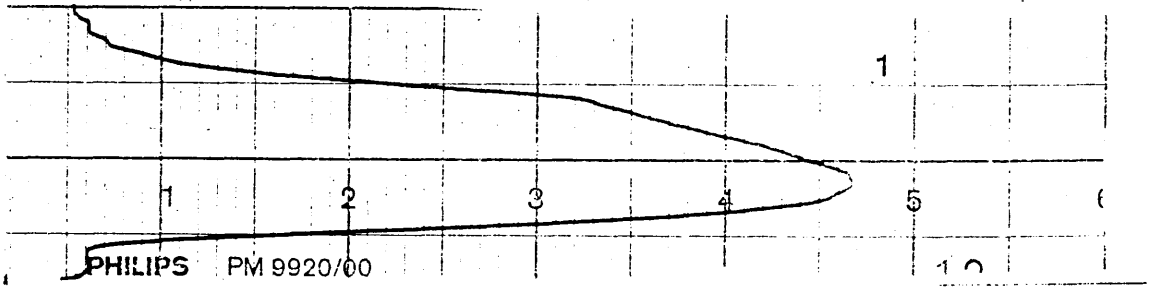
a) 0.31 µg Cr/L.



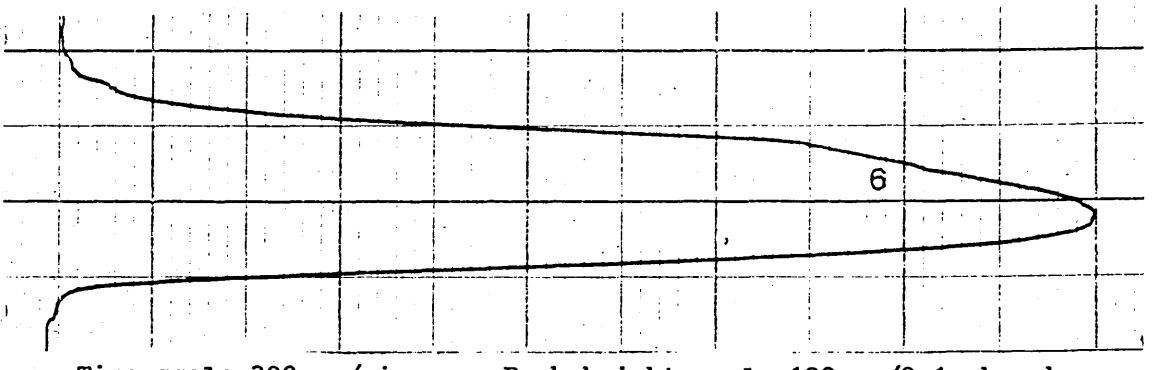
b) 0.31 µg Cr/L.



0.72 µg Cr/L.



0.89 µg Cr/L.



Time scale 300 mm/min. Peak height scale 100 mm/0.1 absorbance.

The peak profiles tend to confirm the conclusions drawn from the peak area/height ratio experiments. The steady decrease with increasing chromium concentration of the peak area/height ratio and the similar profiles seen in processed and unprocessed materials producing similar absorbances can be clearly seen.

The "no injection" peaks have a similar profile to the peaks recorded at the neon 352 nm line, by all classes of material. The peak absorbance in all these traces occurs later in the peak profile than the maximum chromium absorbance and appears to be largely responsible for the increase in peak area/height ratio seen at low absorbances. Background correction does not appear to be required.

5.4. INVESTIGATION OF THE INTERFERENCE MECHANISM.

The mechanism of the hfacac derivative based interference was tentatively investigated by comparing the effects of adding ammonium acetate either before, or after, an ash step. This was to test the hypothesis that: if the interference was caused by the partial loss of chromium as a volatile compound at the ash stage, then the addition of ammonium acetate after this step would be demonstrably less effective. The standard AAS/ETA conditions were used, but the dry and ash stages were duplicated, to allow for the introduction of ammonium acetate between the two dry ash cycles in one series. The base solution contained 10 µg Cr/L in 0.05M EDTA, and for the interference tests beta diketone derivatives equivalent to a retention of 0.5% hfacac, through the wash stages of the standard process, were added. The results of the investigation are summarised below in table 5.14. The injection volumes were 25 µL for both chromium and ammonium acetate solutions.

TABLE 5.14.

Comparison of results with ammonium acetate additions before or after ash stage.

n = 5.	Mean peak height mm.	RSD %.
10 µg Cr/L in 0.05M EDTA.		
A. Single dry and ash steps.		
1. No hfacac.	52.6	3.5
2. Plus hfacac.	27.6	25
B. Double dry and ash steps.		
3. Plus hfacac.	27.3	8.9
4. Ammonium acetate after 1st ash.	44.4	14.4
5. Ammonium acetate after 1st dry.	42.8	24

The ammonium acetate appears to be equally effective, when added either before or after the ash step. The mean peak heights produced by the solution containing the beta-diketone derivatives are virtually identical for the two series with single and double ash steps. These two observations taken together are inconsistent with major losses of chromium through volatilization at the ash stage. The reduction of chromium signal by halides was reported to be expected on theoretical grounds by L'vov (2), and negative interference has been observed in the presence of chloride by several workers and has been attributed to volatile compound formation by Segar et al (3), Aggett et al (4) and Fuller (5). However a vapour phase process was considered to be responsible for the interference by Persson et al (6), Frech et al (7) and Czobik et al (8), whilst analyte occlusion in the matrix was considered to be the cause by Cruz et al (9), Churella et al (10) and Krasowski et al (11). Goodfellow et al (12) worked on the determination of chromium in sodium chloride solutions and found that both EDTA and ammonia enhanced the absorbance of Cr(III) from that recorded in the presence of sodium chloride alone. The EDTA was considered to enhance the chromium signal by preferentially forming the stable transition metal complex so that decomposition to the metal oxide occurred at the ashing stage with minimal formation and subsequent loss of the volatile chloride. Ammonia was found to be more effective than EDTA (1M ammonia cf 0.02M EDTA) but both caused a decrease in precision offsetting any gain in sensitivity. Matsusaki et al (13) investigated the removal of chloride interference in the determination of chromium by AAS/ETA and reported that the presence of cations which form chlorides with high vaporization temperatures such as Ca(II), Sr(II), Ba(II), Cu(II) and Fe(III) caused severe interference, whilst cations forming more volatile chlorides produced less interference. Tetra-ammonium EDTA and acetate ions were both found to be effective in reducing the interference. The acetate ions were assumed to form kinetically inert Cr(III) complexes that are not attacked by chloride, whilst readily volatile ammonium chloride was considered to be produced from the EDTA salt. Matsusaki produced evidence that the interference is not due to loss of chromium at the ash stage, but is due to chromium salts remaining at the atomisation stage. The formation of monochlorochromium (III) ions at the atomisation step was suggested as the source of the reduced signal.

The demonstrated effectiveness of ammonium acetate and of

tetra-ammonium EDTA in reducing the interference produced by the fluoride derived from the hfacac is in agreement with the observations on chloride interference in the references cited above. The failure to correct the poor precision agrees with the observations of Goodfellow et al (12) on the ammonia and EDTA treated materials. The correction of the fluoride based interference by ammonium acetate added after an ash step is consistent with vapour-phase interference by fluoride similar to that suggested by Matsusaki et al (13) for chloride.

5.5. CHARACTERISTICS OF THE AAS/ETA CONDITIONS.

The signal linearity, precision and sensitivity for the AAS/ETA conditions developed are summarized in table 5.15 below. The chromium solutions were in the standard ammonium acetate, EDTA solution and the injection volume was 25 µL. The peak height was taken as the signal.

TABLE 5.15.

Signal linearity, precision and sensitivity using the standard AAS/ETA conditions

Solution. µg Cr/L.	Sample µg Cr/L. (if recovery 75%).	Absorbance.	RSD %.	Value from regression line.
0.6125	0.03	0.011	11	0.66
1.25	0.05	0.015	8.9	1.19
2.5	0.10	0.024	3.8	2.50
5.0	0.21	0.041	2.8	4.99
10.0	0.42	0.076	2.7	10.01
20.0	0.83	0.144	1.0	/
30.0	1.25	0.210	0.86	/

n = 5.

Linear regression analysis using the least squares method produced (y = µg Cr/L and x = peak height in mm) :-

1. y = 0.1469x - 1.00 (r = 0.9999) using all the above values.
2. y = 0.1433x - 0.88 (r = 1.0000) using values up to and including 10 µg Cr/L (0.42 µg/L in a sample). This range is more relevant to serum chromium determinations and was used for the regression line values in table 5.14 above. The standard error of the mean was 0.04 µg Cr/L, which is equivalent to 0.0013 µg/L in a sample giving 95% confidence limits of plus or minus 0.0026 µg Cr/L.

5.6. CONCLUSIONS.

The electrothermal atomisation conditions developed are not entirely satisfactory. The precision of the alpha-2-globulin-bound Cr determinations is significantly worse for pool I and highly significantly worse for pool II compared with that of unprocessed chromium solutions of similar concentrations. The precision of the total protein-bound chromium determination is not significantly different from that of unprocessed material. The AAS/ETA relative response discussed in the last chapter was also found to be less satisfactory for the alpha-2-globulin-bound Cr series (82% compared with 99.5% for the total protein-bound Cr stream). The poor precision and response tend to confirm that the extra acidity of the alpha-2-globulin-bound Cr precipitates is reducing the efficiency of the wash procedure in removing the excess hfacac. However the advantages of a similar wash treatment for the standards and both sample streams are obvious. The three injections for each sample reduce the contribution to the overall precision by the AAS/ETA stage to a level sufficient to produce useful analytical data. However the value of the determinations has certain limitations which will be discussed in chapter VIII.

Traces of hfacac derivatives cause a reduction in both the size of and precision of the chromium signal. The addition of ammonium acetate minimizes the former but does not appear to help with the latter, and is only useful following a process which leaves only minimal traces of the fluorinated diketone. The disadvantages of adding ammonium acetate are the obligatory use of uncoated graphite tubes, and the restriction of the tube life to about 100 firings. The reduced tube life is not a major drawback in the light of the increasing memory effects reported earlier. The initial successful tests with ammonium acetate had been made with a pyrolytically coated tube but it was a much used one, and presumably much of its coating in the critical central zone had been lost.

The failure of coated tubes to cope with ammonium acetate solutions may be due to the less penetrable surface compared with standard electrographite tubes. The failure of the injected material to penetrate the surface may lead to more extensive redistribution at the necessarily high temperature drying stage. However this does not appear to occur on observation with the naked eye using a mirror. The use of

pyrolytically coated tubes with ammonium acetate solution may be possible with a suitably programmed drying ramp.

In general pyrolytically coated graphite tubes were found to be superior in terms of sensitivity and memory effects, although of course, they were not suitable for the methods developed in this study. The only significant carryover demonstrated, was with uncoated tubes after 100 firings, rising to highly significant after 200 cycles. Uncoated tubes do however have some advantages. Sensitivity decreases with use for both types of tube but the fall is steeper for coated ones. The standard electrographite tubes show minimal and steady changes in sensitivity over their useful life.

Manning et al (14) reported that pyrolytically coated tubes showed higher sensitivity than ordinary tubes for chromium in 1976, and this has been confirmed by many workers. Veillon et al (1) reported that pyrolytically coated tubes retained less chromium than uncoated ones. However, Slavin (15) criticised Veillons work on retained chromium. The temperature gradient along a furnace tube exceeds 1000^{0} C and Slavin claimed that much of the radio chromium measured by Veillon had participated in the measurement and then condensed on the cooler edges of the tube. Veillon in a later publication (16) confirmed that the ^{51} Cr had been concentrated near the tube ends. The work by Veillon on the retention of chromium by graphite tubes had involved urine samples, which have a high chloride content, and the apparent lack of retained radio chromium in the central zone suggests that "analyte occlusion" is not a factor in halide interference. The evidence against "volatile chromium" has been discussed earlier and the formation of a non-atomic chromium at the atomisation stage, in the presence of the halide, appears to be the most probable mechanism for interference by chloride and fluoride.

References Chapter V.

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13. Matsusaki,K., et al, Anal. Chim. Acta, 124 (1981) p.163.
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CHAPTER VI.

THE DEVELOPMENT OF A METHOD FOR THE DETERMINATION OF CHROMIUM IN URINE.

The composition of urine is more variable than that of blood. The volume passed per 24 hours depends primarily on the amount of water consumed. The concentrations of analytes are for this reason only useful, in general, for detecting gross changes in an acute situation. The amount of analyte excreted per 24 hours is more meaningful, but is related to the size of the individual concerned. Creatinine excretions are related to the size of the subject, and reporting analytes in relation to a fixed mass of creatinine is probably the most satisfactory parameter for urines. The sample volume taken for analysis can in a similar way be related to the creatinine concentration of the specimen.

6.1. OPTIMISATION OF THE PRETREATMENT PROCESS.

The method developed for the determination of Cr in serum/plasma was adapted for urine. The concurrent protein precipitation and dehydration step used for serum was replaced by evaporation. An acidic solvent miscible with hfacac was necessary to dissolve the dried residue. Acetic acid proved to be satisfactory, and the addition of sulphuric acid, chosen because it is a strong acid free from water, improved the recovery. The method adopted, CDUI is listed in the experimental section at the end of the chapter (E601). Method CDUI was used throughout the development process except for the variations detailed in the particular experimental procedure. The recoveries were monitored using radioactive chromium added as the chloride, before the initial evaporation stage. The radioactive chromium measurements were carried out as outlined in chapter IV (E405).

6.1.1.Medium for the Conversion of Cr to the hfacac Complex.

The results of a number of experiments carried out to find the optimum medium for the conversion of the urinary Cr to the hfacac complex are summarised in table 6.1 below, together with details of the experimental variables.

TABLE 6.1.

Optimisation of the medium for formation of the Cr/hfacac complex.

Evap. temp. ^{0}C.	Volume. acetic acid µL.	% sulphuric acid v.v.	Volume hfacac.	Recovery %. i.	ii.
80	250	zero	250	60	61
80	500	zero	250	62	40
Ambient.	250	zero	250	54	54
80	250*	zero	250	56	59
80	250	1	250	77	76
80	250	5	250	83	77
80	250	7.5	250	82	81
80	250	10	250	80	67

* 2 hrs. at 75^0 C between adding the acetic acid and the hfacac.

Duplicate aliquots of urine corresponding to 10.4 µmol creatinine, and spiked with ^{51}Cr so that each sample contained 3 ng were used in all these investigations. The sample size was chosen to present a reasonable challenge (equivalent to about 1.5 mL of an average urine), so that in these preliminary experiments the analyte recoveries would respond to the conditions in a readily observable manner. The investigations indicated that neither low temperature drying, or pretreatment of the residue with hot acetic acid were beneficial. However the addition of 5 to 7.5 % v.v. sulphuric acid to the acetic acid resulted in significant improvements.

6.1.2. Optimum Temperature.

An experiment was carried out to determine the optimum temperature for the conversion of the Cr to the hfacac complex. The results are listed in table 6.2 below.

TABLE 6.2.

Optimisation of temperature.

Temperature. ^{0}C.	^{51}Cr recovery, %. i.	ii.	Mean.
50	56	63	60
75	76	73	75
100	37	34	36

The spiked urine pool used in 6.1.1. above was used, and the sample volumes used were again equivalent to 10.4 µmol creatinine. The acetic acid used contained 5% sulphuric acid v.v. and 250 µL of this and of hfacac, were added to the residue obtained after evaporation at 75 ^{0} C. The optimum temperature appears to be 75 ^{0} C.

6.1.3. Sample Volume.

The recoveries from sample volumes equivalent to 10.4 and 20.8 µmol creatinine were compared using the conditions and urine pool described in section 6.1.2. above, but only at 75 ^{0} C. The tests were carried out in duplicate and the results are listed in table 6.3. below.

TABLE 6.3.

Effect of sample size on recovery.

Sample volume = µmol creatinine.	^{51} Cr recovery, %. i.	ii.	Mean.
10.4	76	73	75
20.8	47	56	52

The recovery is clearly sensitive to the quantity of dried solids. The effect of increasing the volumes of acetic acid/sulphuric acid and hfacac to 500 µL was investigated, using urine volumes equivalent to a range of creatinines from 12 to 72 µmol. All the samples were spiked with 2.0 ng ^{51}Cr. The results are summarised in table 6.4 below.

TABLE 6.4.

Effect of urine "concentration" on ^{51}Cr recoveries.

Urine. n=1.	Sample creatinine, µmol.	^{51} Cr recovery, %.
1.	12.0	74
2.	15.6	75
3.	21.6	64
4.	27.6	64
5.	28.4	71
6.	72.4	54

The experiment was repeated on two urines, the volumes of the aliquots taken corresponding to about 20 µmol creatinine. The ^{51} Cr

spike was again 2.0 ng per sample. The results are listed in table 6.5 below.

TABLE 6.5.

^{51}Cr recovery using standard sample volume.

Urine. n=2.	Sample creatinine, µmol.	^{51}Cr recoveries, %. i.	ii.	Mean.
1.	21.8	76	73	75
2.	18.6	83	81	82

The conclusions from the above experiments were that the volume of urine taken for Cr determination should be : 20/x mL, where x = the creatinine concentration in mmol/L, and that 500 µL of acetic acid (containing 7.5% v.v. sulphuric acid) and 500 µL of hfacac should be used for each sample. The above urine volume corresponds to about 3 mL of an average urine sample. The increase in acetic acid and hfacac to double that used for serum samples increases the blank value, but does not double it, and allows the urine sample size to be increased by a factor of two. The recoveries from urine samples must be similar to the standard recoveries for reliable calibrations to be made, as the fraction of the high blank which is susceptible to the recovery differences is very difficult to measure.

6.2. CHARACTERISTICS OF THE METHOD.

The characteristics of method CDUI are discussed below.

6.2.1. Recovery.

Table 6.6. below lists the recovery and distribution of 2.0 ng ^{51}Cr added to a sample of urine equivalent to 20 µmol of creatinine.

The high value for the "lost" ^{51}Cr is difficult to explain. the values quoted for the residue and phosphate buffer wash solution are not strictly true, as the petroleum spirit extract must be removed from the Tuftainer before washing with buffer, to obtain these as separate values. However, even when the standard procedure was followed, and the residue and phosphate buffer wash ^{51}Cr are not separated, a similar high value was found for the lost ^{51}Cr. A possible explanation is that the ^{51}Cr in the residue is difficult to measure with accuracy, because of the insoluble nature of the charred mass, and the low efficiency for ^{51}Cr of the gamma counter used.

TABLE.6.6.

Recoveries and ^{51}Cr distribution.

n=4.	^{51}Cr %.		
Solution.	Mean.	S.D.	
Phosphate buffer.	6.8	1.7	
Water wash.	<1	/	
Petroleum spirit.	<1	/	
Ammonia,EDTA.	74.5	1.9	(cf Standard mean 75%).
Residue.	8.4	2.5	
Total.	89.7	/	
^{51}Cr unaccounted for.	10.3	/	

6.2.2. AAS/ETA Relative Response.

The relative signals given by Cr in the processed materials compared with unprocessed materials was measured as described in chapter IV, section 4.13.2. Incremental peak heights using spikes equivalent to 0.5 µg Cr/L were used on pools of processed materials. A summary of the relative responses are given in table 6.7 below.

TABLE 6.7.

Relative responses, processed and unprocessed materials.

Material.	Mean response %.	R.S.D.	n.
Unprocessed standard.	100	4.5	5
Processed standard.	93	4.9	5
Urine.	95	5.1	8

The relative responses of urines and processed standards were compared using Student's t test. The responses did not differ significantly.

6.2.3. Detection Limit.

The detection limit is governed in this method by the precision of the relatively high blank. A gross signal > blank + (4 X blank SD) can be detected with 95% confidence, providing that the Cr recoveries of tests and standards are not significantly different. Thus a sample Cr level equal to (4 X blank SD) in µg Cr/ 10 mmol creatinine equals the detection limit. Table 6.8 below lists the detection limits of the four best batches, together with a selection of the best detection

limits in the literature. The published values are in µg Cr/L, however in our laboratory the mean adult creatinine is 13 mmol/L, and 24 hr. urine volumes are usually between 1 and 2 litres, therefore the figures are broadly comparable.

TABLE 6.8.

Blank values, and derived detection limits.

µg Cr/ 10 mmol creatinine.

Batch.	Blank.	S.D.	Detection limit.
1. n=4	0.11	0.017	0.07
2. n=4	0.11	0.012	0.05
3. n=4	0.13	0.012	0.05
4. n=3	0.07	0.010	0.04
Mean.	0.105	0.013	0.05

Reference.	Detection limit µg Cr/L.
Veillion et al, (1).	0.03
Brodie et al, (2).	0.05
Halls et al, (3).	0.08
Kumpulainen et al, (4).	0.05
Nomiyama et al, (5).	0.1
Vanderlinde et al, (6).	0.055

The detection limit is broadly similar to the best ones published to date.

6.3. SUMMARY.

A pretreatment process has been developed that will extract and concentrate about 75% of the Cr in a urine sample corresponding to 20 µmol of creatinine into 120 µL of solution. The final solution appears to be suitable for conventional AAS/ETA without background correction. However individual samples need to have a radioactive Cr spiked aliquot processed in parallel to monitor the recovery. The detection limit is about 0.05 µg Cr/ 10 mmol creatinine.

E601. Urine chromium determination, standard method CDUI.

METHOD.

The determinations were carried out in quadruplicate, and two of the four were spiked with radioactive chromium , as the chloride, at a concentration equivalent to 0.5 µg Cr/ 10 mmol creatinine.

1. y mls of urine were added to a Tuftainer, where y = 20/x, and x = the creatinine concentration of the urine in mmol/L.
2. The urine samples were evaporated to dryness in a desiccator containing an open vessel of sulphuric acid at 75 ^{0} C.
3. The sealed Tuftainers were allowed to cool to ambient temperature.
4. 500 µL of acetic acid (purified by ion-exchange resin treatment) containing 7.5% v.v. Aristar sulphuric acid was added, and the Tuftainers contents were mixed on an orbital mixer for two hours, and then thoroughly vortexed. The orbital mixer "swirls" the solvent over the residue encouraging solution.
5. 500 µl of hfacac double distilled from petroleum spirit was added.
6. The Tuftainers were firmly capped and the contents vigorously mixed on a vortex mixer.
7. Standards.

Tuftainers were washed x 2 with 4 mL of propan-2-ol and x 2 with 4 mL of purified acetic acid. 500 µL of purified acetic acid containing 7.5% v.v. sulphuric acid and chromium (as the nitrate) at x 8 the nominal value was then added to the Tuftainer, followed by 500 µL of hfacac. The containers were then firmly capped and vigorously mixed on a vortex mixer.

The nominal values of the standards in µg Cr/ 10 mmol creatinine were:

i) Zero.
ii) 0.25
iii) 0.5
iv) 1.0

The zero standard was set up in quadruplicate, the others in duplicate.

8. All further treatment refers to both tests and standards.

All Tuftainers were heated at 75 ^{0} C in an oven (fan assisted ovens may give contamination problems) and the container caps were carefully checked for tightness after 15 minutes.

9. The Tuftainers were cooled in a freezer at -20 ^{0} C for 1 hour.

10. 8 ml of petroleum spirit were added to all Tuftainers.

11. Tuftainer were heated at 75 ^{0} C for 4 hours. Then cooled to ambient temperature on a roller mixer.

12. 4 mL of 2.2M dipotassium hydrogen phosphate,0.01M EDTA was added to all Tuftainers.

13. Containers placed on a roller mixer for 1 hour.

14. Tuftainers centrifuged at 3000 rpm for 15 minutes and then the petroleum spirit extracts were removed and added to 13 mL screw cap polypropylene tubes containing 4 mL of pure water.

15. The petroleum spirit extracts were then washed for 1 hour with the water on a roller mixer.

16. The petroleum spirit was then removed and extracted with 500 µL of 4.25M ammonia, 0.005M EDTA in a similar polypropylene tube overnight on a roller mixer. The ammonia had been distilled under isothermal conditions and the EDTA was the di-ammonium salt.

17. The ammonia/EDTA solutions were removed and evaporated in 12 x 75 mm polypropylene tubes in a desiccator over concentrated sulphuric acid at 75 ^{0} C.

18. 120 µL of 0.5M ammonia, 0.7M acetic acid was added to the residues. The tubes were capped and then vortexed at 80^{0} C for 10 minutes.

19. The tubes were then centrifuged at 3000 rpm for 10 minutes to return any evaporated water which had condensed on the upper walls of the tubes to the base.

20. The chromium concentrations in the solutions were determined using atomic absorption spectrometry under the standard conditions described in chapter IV (E406).

References Chapter VI.

1. Veillon,C., et al, Anal. Chim. Acta, 136 (1982) p.233.
2. Brodie,K.G., et al, Clin. Biochem., 17 (1984) p.19.
3. Halls,D.J., et al, in Trace Elements-Analytical Chemistry in Medicine and Biology, Vol. 2, Ed. P.Bratter and P.Schramel, (1983) p.667, Walter de Gruyter and Co., Berlin.
4. Kumpulainen,J., et al, in Trace Element-Analytical Chemistry in Medicine and Biology, Vol. 2, Ed. P.Bratter and P.Schramel, (1983) p.951, Walter dr Gruyter and Co., Berlin.
5. Nomiyamam,H., et al, Am. Ind. Hyg. Assoc., 41 (1980) p.98.
6. Vanderlinde,R.E., et al, in Chromium in Nutrition and Metabolism, Ed. D.Shapcott and J.Hubert, (1979) p.49, Elsiever, North Holland.

CHAPTER VII.

CONTAMINATION CONTROL.

Nieboer and Jusys (1) stated that interlaboratory comparisons and the use of standard reference materials clearly show contamination to be the limiting factor in trace and ultratrace analyses.

The difficulties encountered in this work were consistent with the above statement. The reduction and control of the relatively high and variable blank was a major problem, and one that was not entirely overcome.

7.1. ENVIRONMENT.

The steps taken to minimise contamination from the immediate environment in the present investigation are detailed below.

The time containers were open was reduced to the minimum, and all dispensing and transfer operations were carried out in a fume cupboard reserved for this purpose. The work was carried out on a frequently changed sheet of Benchkote, and the fume cupboard fan was switched on only when potentially harmful hfacac was being dispensed. The interior of the fume cupboard, which was constructed of uPVC was regularly washed down with 10% Decon. The enclosure had no stainless steel or chromium plated surfaces or fittings.

Evaporation stages, the only ones in which vessels were open for longer than a minute were carried out in small desiccators, with not more than four tubes or two tuftainers per desiccator. The evaporations were all carried out at 75 ^{0} C in a hot air oven. The desiccators were sealed (PTFE taps) for procedures related to serum or urine chromium determinations, and contained an open vessel of concentrated sulphuric acid to remove ammonia / and or water vapour, so encouraging evaporation, and preventing drips from the inner surface of the lid.

Talc free latex gloves were put on, and rinsed on the exterior with pure water and dried with paper towels prior to all experimental procedures.

The data reported by Sansoni et al (2) on the composition of the dust in the unfiltered air from a laboratory atmosphere are reproduced in table 7.1 below.

TABLE 7.1.

Trace element levels in laboratory dust.

Element.	mg/kg dust.	Element.	mg/kg dust.
Al	3000	Mg	2390
As	55	Mn	116
Br	23	Na	2950
Ca	2690	Ni	70
Cd	3	P	1150
Cl	2	Pb	2150
Co	9	S	20000
Cr	39	Sb	15
Cu	213	Sn	10
F	1	Sr	14
Fe	3230	Ti	258
I	3	V	259
K	7920	Zn	1640

The data in the above table indicates that although chromium represented a trace element in laboratory dust, to increase the value of a typical reagent blank by 100% would have required less than 1 µg of dust.

7.2. CONTAINERS.

Cleaning should remove "less strongly bound" material from the container walls, but active adsorption sites should not be created. The contamination tests, and hence the cleaning methods used for particular pieces of equipment must be related to the uses to which they are to be put: clearly "less strongly bound" is a relative term.

Containers made from relatively hydrophobic materials used for aqueous solutions at ambient temperature were less critically tested than ones exposed to acidic organic solvents at elevated temperatures. Equipment and reagents were tested under the conditions of use whenever practicable.

Three cleaning protocols were compared on the polypropylene tubes. Two treatments involved a 24 hour soak at ambient temperature in either acetic acid plus 5% sulphuric acid or 10% nitric acid, followed by 10 rinses in pure water. The third set of tubes were merely rinsed

10 times in pure water. The tubes were tested in their respective roles in method CDIII. all the tubes produced results equivalent to a contribution of less than 0.005 µg Cr/L to a 4 ml sample, and this included the reagent blank. However with both types of polypropylene tubes random tests on unrinsed specimens showed some high chromium values and rinsing is clearly necessary.

7.2.1. Universal Containers.

These are 30 mL polystyrene containers with a screw cap, and were used for sample collection, and storage of some aqueous reagents at ambient temperatures or less. Universal containers were used new, untreated: there was no evidence of contamination from these bottles under the conditions under which they were used.

7.2.2. 12 X 75 mm polypropylene tubes.

These tubes were used for the evaporation of the ammonia /EDTA back extraction solutions prior to AAS/ETA. The ten rinses in pure water was adopted as the standard treatment and the push-on polyethylene caps were similarly treated.

7.2.3. 13 mL screw cap polypropylene tubes.

The screw cap polypropylene tubes were used at the petroleum spirit extract wash stage of the chromium determination procedure. New tubes from sealed packs were used after 10 rinses with pure water immediately before use, both tubes and tops were so treated.

The screw cap polypropylene tubes were also used for the storage of purified acetic acid and hfacac. The tubes used for reagent storage were soaked in 5% v.v. sulphuric acid in acetic acid for 48 hours, and then rinsed at least 15 times with pure water. The final rinse was with purified acetic acid for the storage of this reagent and with Aristar grade sulphuric acid for the distilled hfacac. The distilled hfacac was stored over Aristar grade sulphuric acid as detailed in the section on the treatment and storage of the diketone.

7.2.4. Tuftainers.

The reactions carried out in the tuftainers, particularly the conversion of chromium to the hfacac complex at 75 ^{0} C make this the most critical container in terms of contamination. The investigation of various protocols for cleaning new tuftainers was investigated using method CDI. Reagent blanks were processed in the tuftainer and "cold" blanks in screw cap polypropylene tubes were used as reference reagent blanks to assess the relative contribution from the container.

The details of the cleaning protocols and results are summarised in table 7.2 below.

TABLE 7.2.

Comparison of various cleaning protocols for new Tuftainers.

Protocols.

1. All soaked in 10% decon for 24 hours at ambient temperature.
2. Rinsed in tap water.
3. 3/4 filled with :-

A. 10% nitric acid, ambient temperature.

B. tfacac, 125 ^{0} C.

C. Acetylacetone 125 ^{0} C.

D. 5% v.v. sulphuric acid in acetic acid, 125 ^{0} C.

All tuftainers 24 hours upright, and then 24 hours inverted.

After cooling :-

4. B and C rinsed 3 times with propan-2-ol.
5. All rinsed thoroughly with tap water then pure water.
6. Rinsed with propan-2-ol, then toluene.

Results.

Cr µg/L (equivalent values in a processed sample).

Protocol.	i.	ii.	iii.	iv.	Mean.
A.	1.2	3.4	2.3	lost.	2.3
B.	All greater than 10 µg/L.				
C.	2.9	1.9	2.4	1.1	2.1
D.	0.05	0.07	0.06	0.06	0.06

Protocol D appeared to be very effective in cleaning new tuftainers. This treatment was on new Tuftainers before they were introduced to the stock in use. The procedure removed that Cr present in the new container which was available for contamination of the tests. The acetic acid/sulphuric acid solution is a good solvent for many Cr species, and the organic acid presumably makes better contact with, and penetrates, the surface micro-irregularities better, than an inorganic acid, or aqueous solution can.

Routine cleaning of Tuftainers.

The routine cleaning protocol listed in table 7.3 below was used to return the containers to the same condition after each batch of tests.

TABLE 7.3.

Protocol for the routine cleaning of Tuftainers.

Stage.	Treatment.	Purpose.
1.	24 hr. soak at 75 ^{0} C in 10% Decon solution, tops and bases in separate 2L flasks, then thorough rinse in tap water.	Removes organic material which chars if left.
2.	Immerse the tuftainers in 5% v.v. sulphuric acid in acetic acid at ambient temperature, then thorough rinse in a) tap water, and b) pure water. Remove from flasks.	Removes any Cr from surface, particularly screw threads.
3.	3/4 fill individual Tuftainers with conc. nitric acid (AR) and heat at 75 ^{0} C in an upright position, 24 hrs., then invert and repeat heat treatment.Thoroughly rinse in pure water individually.	Oxidises and dissolves any organic material left.
4.	3/4 fill with 10% v.v. hydrochloric acid (Aristar) in propan-2-ol (AR), and repeat heating, inversion cycle of stage 3.	Removes traces of oxidant, wets container, and removes residual Cr.

Note. If the hot nitric acid stage is omitted a gradual build up of charred organic material takes place. The Tuftainers were examined when cool, after the nitric acid treatment, and containers showing a relatively deeper colour were subjected to repeated stage 3 treatments until no difference from the other Tuftainers could be seen.

7.2.5. Polypropylene bottles.

Polypropylene bottles of 250, 500 and 1000 mL capacity were used for reagent storage and for treating reagents with ion-exchange resins. The new bottles were soaked in 5% v.v. sulphuric acid in acetic acid (AR grades) for 24 hours, and then thoroughly rinsed in tap water followed by at least 15 rinses in pure water. The bottles were then rinsed in the fluids which they were to contain. Pre-rinses in propan-2-ol were used before introducing solvents not miscible with water.

7.2.6. Finnpipette tips.

Tips were stored in sealed plastic containers and carefully

removed wearing washed latex gloves. The tips were rinsed three times in the solution to be dispensed, except when dispensing serum, three rinses in pure water were then used.

7.2.7. Polythene transfer pipettes.

These were stored in sealed plastic containers and rinsed three times in the pure solvent corresponding to the major component of the solution to be transferred, inverting to rinse all the internal surface. A minimal immersion in the solution to be transferred was carefully made when aspirating.

7.3. REAGENTS.

Two techniques, distillation and treatment with ion-exchange resins were used for the purification of reagents. The purification processes used together with the initial and final chromium concentrations are summarised in table 7.4 below. The methods used for the determinations of the chromium concentrations are outlined in the experimental section at the end of the chapter.

TABLE 7.4.

Comparison of purification treatments and initial and final chromium concentrations.

Reagent.	Treatment.		Cr µg/L.
1.Acetic acid.	Untreated.		4.8
(Aristar).	Distillation using magnetic stirrer and teflon condenser.		1.3
	Double distillation.	Mean.	0.2
		Best.	0.05
	Resin treatment.		
	i) 'Dowex' 50W-X8		0.14
	ii) 'Amberlite' IR-120		1.9
2.Ammonia.	Untreated.		1.14
(Aristar).	Boiling water bath, teflon condenser.		0.14
Cr conc. in	Resin treatment, 'Amberlite' IRA 400.		0.64
16M soln.	Isothermal distillation.		0.15
3.hfacac.	Untreated.		9.7
i) BDH batch	Distillation at b.pt. in borosilicate.		
5720210B.	Distillation X 1.		2.6
	Distillation X 2.		1.4
	Distillation X 3.		1.1

TABLE 7.4. continued.

Reagent.	Treatment.	Cr µg/L.
hfacac.	Distillation X 4.	0.6
BDH. continued.	Distillation at 56 ^{0} C in borosilicate glass.	0.4
	Distillation from an equal volume of toluene, boiling water bath, teflon condenser. Distillation X 2.	<0.02
ii)Koch-Light. 99560.	Untreated.	0.32
	Distilled from an equal volume of petroleum spirit, boiling water bath, teflon condenser. Distilled X 2.	<0.02
4. Hydrochloric acid. (Aristar.) Cr conc. corrected to 12M soln.	Untreated.	1.8
	Distillation, boiling water bath, teflon condenser.	0.2
	Isothermal distillation.	0.3
5. Petroleum spirit. 120-160 ^{0} C, b.pt. (AR).	Untreated.	0.007
	Treated with 'Amberlite' monobed ion-exchange resin MB-1.	<0.002
6. Toluene. (Aristar).	Treated with 'Amberlite' monobed ion exchange resin MB-1.	<0.002
7. Propan-2-ol. (Aristar).	Untreated.	0.03
	Distilled in borosilicate glass.	0.09
	Treated with 'Amberlite' monobed ion-exchange resin MB-1.	<0.002

7.3.1. Ion-exchange Resin Treatment.

A mixed bed ion-exchange resin can be expected to remove chromium present as ionic species, either anionic or cationic. However ion-exchange resins can be effective in both base and acid catalysed reactions. Experiments confirmed that a mixed bed resin can remove chromium present as complexes which are susceptible to either acidic or basic conditions, in both propan-2-ol and petroleum spirit. The resin treatments were carried by mixing solvent and resin in about 10:1 v.v. proportions on a roller mixer. Petroleum spirit was treated in a similar manner with sulphuric acid and aluminium oxide in two other

experiments. The purification of propan-2-ol and petroleum spirit was investigated using radioactive chromium labelled complexes with the ligands listed below. The procedures used in the preparation of the complexes are outlined in E704.

i) Benzoylacetone. (benzoylac).

ii) 4,4,4-trifluoro-1-(2-thienyl)butane-1,3-dione. (thenoylac).

iii) 1.1.1,5,5,5-hexafluoropenta-2,4-dione. (hfacac).

The results of the treatment are given in table 7.5 below.

In conclusion, ion-exchange resin treatment is an attractive purification process as it can be readily carried out in the period preceding use, and contamination from the storage container should be minimal. Thorough washing with pure water appears to be necessary before using the resins on reagents. A wash of at least 48 hours duration, gently mixing the resin using 1:10 resin:water volumes is satisfactory. The wash presumably redistributes the chromium present in the resin skeleton onto the true ion exchange sites. Contamination of the reagents by microparticulate material derived from the resin could be a problem but can be avoided in practice by decanting the "fines" at the water wash stage.

7.3.2. Distillation.

The ideal equipment for producing high quality reagents for trace metal determinations by distillation is the vitreous silica sub-boiling apparatus. This appliance uses quiescent evaporation of the liquid via infrared heating at the surface to prevent violent boiling. The efficiency of the process is due to the prevention of the formation of fine particles of spray, and to the high purity and inert character of the vitreous silica.

A simple piece of equipment with similar characteristics was therefore designed, this appliance is illustrated in figure 7.1 and described below.

A long necked 100 mL polypropylene flask was used as the boiler, and a glass beaker water bath on a magnetic stirrer hotplate was used to heat the boiler. The water level in the water bath was maintained above the level of fluid in the boiler, and stirred vigorously with a teflon coated spinbar. The high water level and vigorous stirring minimise spray formation, and the long necked flask is effective in reducing spray transfer to the distillate. Direct heating using a borosilicate flask with the teflon coated spinbar inside was used for

TABLE 7.5.

A) Radioactive chromium content of the treated propan-2-ol.

	% ^{51}Cr remaining.		
	24 hrs.	48 hrs.	60 hrs.
Chromium chloride.	<0.1	<0.1	<0.1
benzoylac.	0.6	0.6	0.6
thenoylac.	0.3	<0.1	<0.1
hfacac.	<0.1	<0.1	<0.1

B) Radioactive chromium content of the treated petroleum spirit.

i) 'Amberlyst' resin treatment.

	% ^{51}Cr remaining.	
	24 hr.	48 hr.
benzoylac.	34	30
thenoylac.	63	60
hfacac.	89	82

ii) 'Amberlite' monobed resin treatment.

	% ^{51}Cr remaining.		
	24 hr.	48 hr.	60 hr.
benzoylac.	3.9	3.9	3.9
thenoylac.	0.01	<0.01	<0.01
hfacac.	0.07	0.02	0.02

iii)Sulphuric acid treatment.

	% ^{51}Cr remaining.	
	24 hr.	48 hr.
benzoylac.	2.0	1.4
thenoylac.	2.0	0.5
hfacac.	102	104

iv) Aluminium oxide treatment.

	% ^{51}Cr remaining.	
	24 hr.	48 hr.
benzoylac.	31	30
thenoylac.	56	57
hfacac.	3	<0.01

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distillation above 100 ^{0} C. A teflon tube connected between two teflon "tube to joint" connectors was used to to produce a chemically inert condensing surface. A wide bore PVC tube between two wash bottle heads

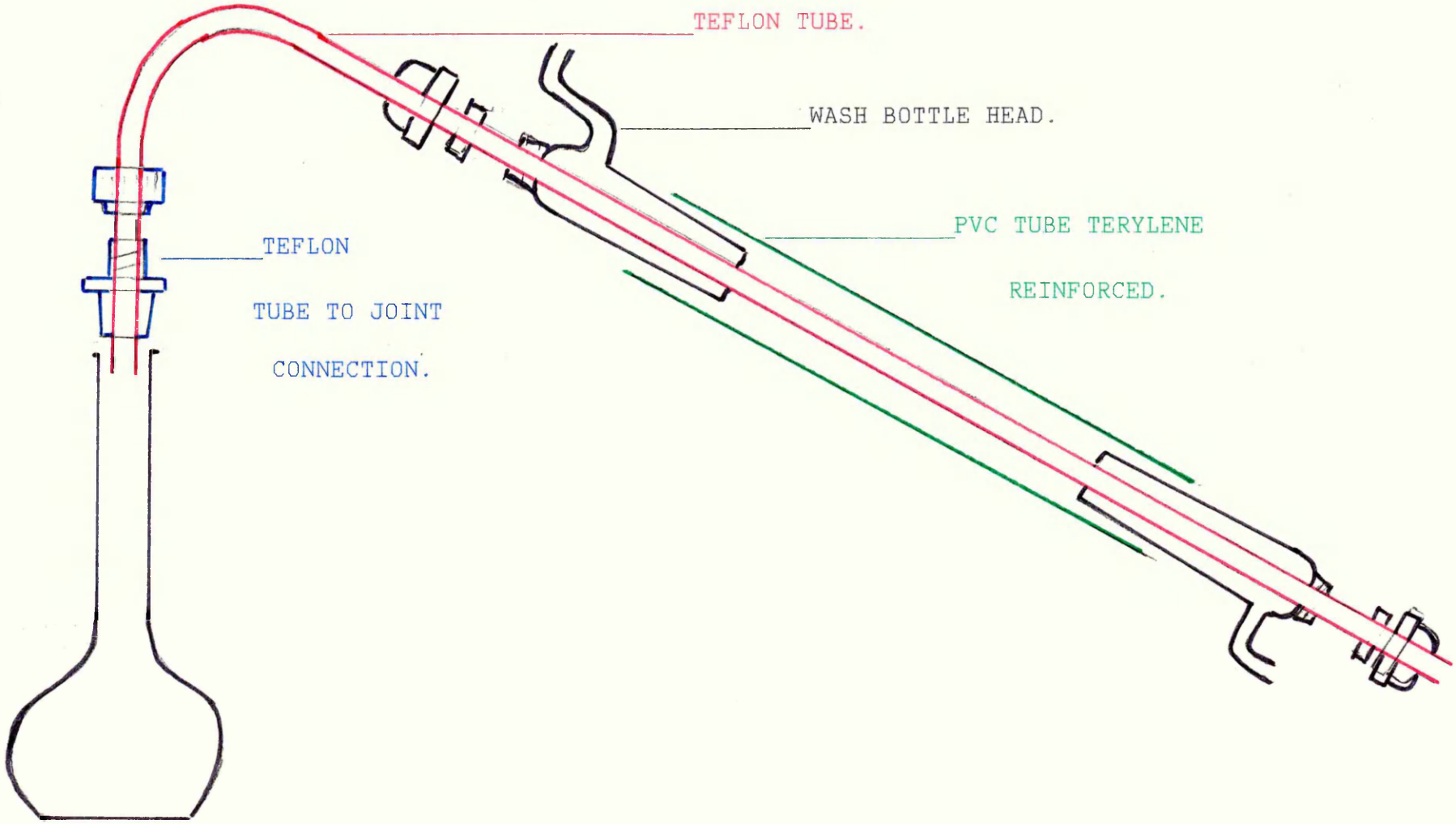

FIGURE 7.1 **TEFLON TUBE CONDENSER DISTILLATION EQUIPMENT.**

which sealed round the teflon tube formed a water jacket.

Mitchell (3) reported sub-boiling distilled hydrochloric acid to have a chromium content of 0.3 ng/g. The simple equipment described above produced hydrochloric acid of 0.5 ng/g after one distillation.

The most successful method found for the purification of hfacac was distillation from an equal volume of petroleum spirit (120-160 ^{0} C b.pt.) using the boiling water bath variation outlined above. A sample of hfacac containing 13.8 µg ^{51}Cr/L was treated and a distillate containing only 0.28% of the added ^{51}Cr was produced.

The data on other materials produced using this equipment can be found in table 7.4.

7.3.3. Isothermal distillation.

Hildebrand et al (4) stated that from thermodymamic principles a purification process based on vapourisation and condensation of the vapour is more efficient the lower the temperature at which the process is conducted. Isothermal distillation can produce a high purity but medium strength product from volatile reagents, and this technique was used to purify both ammonia and hydrochloric acid.

The isothermal distillations were carried out using a large desiccator, and the data on the distillates can be found in table 7.4.

7.4. STORAGE OF PURIFIED REAGENTS.

Ammonia as a 5M solution, and hydrochloric acid as a 6M solution were stored in 30 mL polystyrene containers at minus 20 ^{0} C.

The acetic acid was stored in screw cap polypropylene tubes at minus 20 ^{0} C and thawed out at ambient temperature immediately prior to use.

The hfacac was stored in similar polypropylene tubes in 10 mL aliquots, with 1 mL of sulphuric acid (Aristar grade) in each tube. The sulphuric acid reconverted any hydrated hfacac produced during distillation and tests indicated that sulphuric acid removed simple Cr(III) ions irreversibly from the diketone.

The storage at low temperature minimises extraction of chromium from the containers and the dissolving of any particulate matter present. A very small amount of petroleum spirit distills over with the hfacac in the distillation process and during the low temperature storage when miscibility is reduced some separates from the diketone and can be removed from the surface. All fluids were stored in an

upright position.

Propan-2-ol and petroleum spirit were resin treated over the 24 hours prior to commencing a batch of tests. Centrifugation at 3000 rpm, for 30 minutes to minimise contamination by particulate matter was carried out, in the treatment bottles on the day of use.

The phosphate buffer used was made by dissolving 500g of dipotassium hydrogen orthophosphate (tri-hydrate) in pure water in the original container. EDTA as the diammonium salt (3.62g, Spectrosol grade) was added and the solution made up to 1 litre in a graduated polypropylene bottle.

7.5. SAMPLE COLLECTION.

The controversy about the effects of stainless steel needles on sample collection was mentioned in chapter III. Kumpulainen et al (5) and Versieck et al (6) both produced evidence that blood samples collected using stainless steel needles were contaminated with Cr from the needle. The results of the investigations by Kumpulainen and Versieck together with data from 8 samples taken for routine laboratory tests and subjected for total protein-bound Cr as a part of this work are summarized in table 7.6.

TABLE 7.6.

Effect of stainless steel needles on serum Cr levels.

	Serum Cr µg/L.	
Ref.	Plastic catheter samples.	Steel needle samples.
	Individual samples tested.	Pool from 20 samples.
(5).	Mean 0.12 SD 0.05 n=6	Mean 0.43 SD 0.05
CDIII.	Mean 0.11 SD 0.04 n=43	Sample. Cr. Sample. Cr.
(This work).		1. 0.13 5. 0.10
		2. 0.07 6. 0.10
		3. 0.07 7. 1.10
		4. 0.16 8. 0.20
		Mean 0.25
(6).	Mean 0.16 SD 0.083 n=20	4 successive 20 mL
	Range 0.038 to 0.352	samples.
Note. The 4 successive 20 ml samples were		Sample. Cr. Sample. Cr.
collected using an irradiated needle on a		1. 89.9 3. 10.4
plastic tube, simulating a venepuncture.		2. 12.7 4. 15.9

Versieck's "irradiated needle" may have undergone some structural changes during the exposure to the neutron flux, and the results reported on the samples taken in the simulated venepuncture experiment are therefore possibly invalid. Kumpulainen may have found only a few of the 20 samples were contaminated if the samples had been tested individually. Vanderlinde (8) reported a mean serum Cr level of 0.075 µg/L on samples taken using stainless steel needles. The conclusion is that although blood samples taken through stainless steel needles are not always contaminated with Cr the possibity of this occurring must be rigorously excluded.

7.5.1. Serum and Plasma.

Samples were taken during oral glucose tolerance tests. Blood was taken from an anterior cubital vein without stasis. A "Medicut" polyethylene intravenous cannula was used. The needle was removed after piercing the vein and a plastic tap was substituted for the syringe. The first 10 mL of blood flowing from the indwelling plastic cannula were discarded and 4 fasting samples of about 25 mL were collected into polystyrene universal containers, labelled 1 to 4, in order. A 5 mL sample was collected into a tube containing potassium fluoride/oxalate mixture for glucose determinations. The tap and cannula were flushed with "Hepsal" sterile heparinised saline (10 units heparin/mL) to prevent clotting after this and all other sampling sessions. On one occasion blood was collected into universal bottles containing 100 µL of heparin. The specimens were stored at 4^{0} C and the plasma separated after centrifugation at 4^{0} C. Total protein-bound Cr and alpha-2-globulin-bound Cr determinations were commenced within 2 hours of sample collection.

A solution containing 50 g glucose was given orally after the collection of the fasting samples, samples of blood were then taken every 30 minutes for three hours. The first 10 mL of blood were discarded and the next 5 mL taken into a fluoride/oxalate tube for glucose measurement. Two 25 mL samples were then collected for chromium and insulin determinations. The blood collected for chromium and insulin determinations was allowed to clot at 4^{0} C, and the separated serum was stored at minus 20^{0} C.

7.5.2. Urine.

Urine samples were collected from males only directly into universal containers, after discarding the first 20 mL. Samples of

urine were collected fasting and at 1, 2 and 3 hours after imbibing the glucose solution. Samples of urine were also collected from a number of young males, before and after vigorous exercise. Males only were used for urine collection because of the contamination problem with female donors.

7.6. CONCLUSIONS.

The contamination control procedures outlined in this chapter were not entirely successful. This is indicated by comparison of the blank values recorded with those of other workers who reported similar serum chromium levels, see table 7.7 below. The control procedures were accepted however under the dual constraints of time and monetary resources.

The theoretical contribution to the blank was relatively easy to deduce for many reagents, and these values are listed below in table 7.7 together with the blanks reported by some workers and referred to above.

TABLE 7.7.

Contributions to a theoretical reagent blank.

Reagent.	Cr µg/L.	Contribution to blank, µg Cr/L.
Acetic acid.	0.2	0.01
Ammonia.	0.14	0.007
hfacac.	0.02	0.001
Theoretical blank.		0.02
Experimental blank.		
All batch mean.		0.08
Lowest batch mean.		0.04
Blanks by other workers.		
Veillon, et al,(7).		0.02
Vanderlinde, et al,(8).		0.03
Offenbacher, et al,(9). Serum.		0.02
Citrated plasma.		0.03
Heparinised plasma.		0.05

The teflon condenser, magnetic stirrer distillation appliance was very successful for purifying acetic acid, ammonia, hfacac and hydrochloric acid.

The contribution to a serum sample processed by the alpha-2-globulin-bound chromium procedure by hydrochloric acid was computed to be <0.002 µg Cr/L, as an experiment indicated that less than 10% of the chromium present in this reagent was present in the precipitated protein, and some of this probably represents exchanged rather than additive chromium.

The contributions by propan-2-ol and petroleum spirit are more difficult to deduce as no detectable chromium was found in the purified reagents. However the mean serum total protein-bound chromium was found to be 0.10 µg/L compared with a mean alpha-2-globulin-bound chromium of 0.07 µg/L. Therefore, as less than 10% of the chromium in the acidified propan-2-ol used for the latter fraction is precipitated, the propan-2-ol probably makes a very modest contribution. The treatment with ion-exchange resin was convenient and appeared to be effective for reducing the chromium concentrations in the propan-2-ol and petroleum spirit, both of which are used in relatively large volumes. The mixed bed resin Amberlite MB1 was demonstrated to remove chromium present as the simple solvated Cr(III) ion, and to break down complexes susceptible to either base catalysed or acid catalysed degradation.

In summary, the experimental blank values recorded are not entirely satisfactory relative to the very low values found for serum chromium levels in normal subjects. However the purification methods used did reduce reagent chromium levels to ultra low levels. The cleaning procedure adopted for the containers identified as the major chromium contamination source was effective in significantly reducing the chromium elution. A sampling procedure was used for blood collection which avoided the obvious potential contamination from stainless steel needles.

E701. AAS/ETA conditions.

The standard conditions listed in chapter IV (E406) were used. Uncoated profile graphite tubes were used at all times.

E702. Radioactive chromium determinations.

All measurements of radioactive chromium were carried out as described in chapter IV (E405).

E703. Methods used in reagent chromium determinations.

1. Acetic acid.

i) 500 µL of acetic acid was evaporated in 12 X 75 mm polypropylene tubes at 100° C in a desiccator with the tap open.

ii) Residue dissolved in 100 µL of 0.1M acetic acid, 0.0125M EDTA at 80° C in a vortex heater.

iii) Chromium concentration measured using AAS/ETA.

2. Ammonia.

i) 25 µL of 0.05M EDTA was added to 500 µL of ammonia in a 12 X 75 mm polypropylene tube prior to evaporation at 100° C in a desiccator containing an open vessel of sulphuric acid.

ii) Residue dissolved in 100 µL of 0.1M acetic acid at 80° C in a vortex heater.

iii) Chromium concentration measured using AAS/ETA.

3. Hydrochloric acid.

i) 500 µL of hydrochloric acid was evaporated at 80° C in 12 X 75 mm polypropylene tubes in a desiccator containing an open vessel of sulphuric acid.

ii) Residue dissolved in 100 µL of 0.1M acetic acid, 0.0125M EDTA at 80° C for 10 minutes on a vortex heater.

iii) Chromium concentration measured using AAS/ETA.

4. Propan-2-ol.

i) 10 mL of propan-2-ol plus 100 µL of 0.025M EDTA were evaporated in Tuftainers standing in the updraught zone against the rear wall of a fume cupboard, at ambient temperature overnight.

ii) Residue dissolved in 100 µL of 0.1M acetic acid at 80° C for 30 minutes on a vortex heater.

iii) Chromium concentrations measured using AAS/ETA.

5. Petroleum spirit.

i) 10 mL of petroleum spirit plus 50 µL of acetic acid were evaporated in Tuftainers standing in the updraught zone against the rear wall of a fume cupboard, at ambient temperature overnight.

A blank of 50 µL of acetic acid was also evaporated.

ii) Residue dissolved in 100 µL of 0.1M acetic acid, 0.0125M EDTA at 80^0 C for 30 minutes on a vortex heater.

iii) Chromium concentration measured using AAS/ETA.

6. Toluene.

The chromium determinations on toluene were carried out in an identical manner to the determinations on petroleum spirit above.

7. hfacac.

Treated as "blank" in standard method CDIII, but no heating at 75 ^{0} C. Blank similar but without hfacac.

E704. Preparation of complexes.

The complexes listed below were used for petroleum spirit and propan-2-ol purification investigations.

i) Benzoylacetone. (benzoylac).

ii) 4,4,4-trifluoro-1-(2-thienyl)butane-1,3-dione. (thenoylac).

iii) 1.1.1,5,5,5-hexafluoropenta-2,4-dione. (hfacac).

Method.

0.1g of benzoylac and thenoylac were each dissolved in 1000 µL of acetone. Acetic acid (10 µL) and 25 µL of radioactive chromium as the chloride (6.9 mg Cr/L) were added to the two solutions above and to 1000 µL of hfacac. The solutions were then incubated at 50^0 C for three hours. Aliquots (20 mL) of both propan-2-ol and petroleum spirit were then individually spiked with 50 µL of the three radioactive chromium solutions. Aliquots of the two solvents were also directly spiked with an equivalent amount of radioactive chromium as the chloride.

The solutions were thoroughly mixed on a roller mixer for 30 minutes. The solutions were then centrifuged at 3000 rpm for 10 minutes and 500 µL aliquots removed for use as reference solutions. The radioactive chromium concentrations in the reference solutions are listed below in table 7.8. Chromium chloride is clearly virtually completely insoluble in petroleum spirit, and ^{51}Cr concentrations in this solvent can therefore be taken as the true concentrations of the

particular chromium complexes in the solvents. The excess ^{51}Cr in the propan-2-ol being present as chromium chloride.

TABLE 7.8.

Radioactive chromium in reference solutions.

^{51}Cr µg/L.

	Petroleum spirit.	Propan-2-ol.	
		Total ^{51}Cr.	^{51}Cr as complex.
Chromium chloride.	<0.001	0.345	/
benzoylac	0.100	0.223	0.100
thenoylac	0.219	0.387	0.219
hfacac	0.046	0.057	0.046

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CHAPTER VIII.

CHROMIUM LEVELS IN SERUM AND URINE.

The serum chromium levels found using method CDIII, and the urinary chromium excretion as determined using method CDUI, are discussed, and compared with other values reported in the literature, in this chapter.

8.1. SERUM CHROMIUM.

The total protein-bound chromium was found to represent at least 95% of the serum chromium (Chapter IV, section 4.2.), in agreement with Lim et al (1). The values found for this parameter should therefore be similar to the total serum chromium levels reported by other workers.

The serum chromium concentrations reported over the last decade (table 3.1, Chapter III) appear to fall into three main groups, as summarised in table 8.1 below. The members of each group tend to support their own results by citing the other members in the group.

The mean levels reported by the group I members form the "tightest" grouping, the overall mean is 0.125 and the relative standard deviation is a modest 23%. A similar tight group including 7 reported mean values cannot be assembled at any other part of the serum chromium "spectrum". The samples analysed by 5 of the members of group I were, as reported and discussed in Chapter III, section 3.1.1.1. and Chapter VII, section, 7.5., taken with stringent precautions against sample contamination. Members of group I have attributed the higher values reported by other workers to contamination from stainless steel needles. The most popular argument put forward by workers reporting higher values, is that a volatile chromium fraction present in biological fluids has been wholly, or partly lost, by those reporting lower values. The volatile chromium controversy has been discussed in chapter III, section 3.4.1., and there appears to be good evidence that the volatile biological chromium fraction is mythical, and that workers reporting low values have taken adequate precautions against losses by volatilisation.

The mean value for total protein-bound chromium using method CDIII for 43 samples taken with stringent precautions against sample

contamination from 11 subjects was 0.11 µg/L, with a standard deviation of 0.04 µg/L. This value clearly supports group I, and if included gives a group mean of 0.123 µg Cr/L, with a relative standard deviation of 22%.

TABLE 8.1.

Serum chromium levels over the last decade.

Reference.	Mean Cr, µg/L.	Year.
Group I (group mean 0.125, RSD 23%, n=7).		
Versieck et al,(2).	0.16	1979.
Kayne et al,(3).	0.14	1978.
Vanderlinde et al,(4).	0.075	1979.
Kumpulainen et al,(5).	0.12	1983.
Anderson et al,(6).	0.12	1984.
Veillon et al,(7).	0.11	1984.
Offenbacher et al,(8).	0.15 (plasma 0.27)	1986.
This study (CDIII).	0.11	
Group II (group mean 1.14, RSD 54%, n=7).		
Liu et al,(9).	1.7	1978.
Kasperek et al,(10).	0.45	1979.
Seeling et al,(11).	0.70 (plasma 1.25)	1979.
Thompson,(12).	2.0	1980.
Abraham et al,(13).	1.6	1981.
Liu et al,(14).	1.0	1982.
Morris et al,(15).	0.56	1985.
Group III (group mean 6.8, RSD 27%, n=4).		
Newman et al,(16).	6.0	1978.
Salvadeo et al,(17).	8.2	1979.
Gedik et al,(18).	4.6	1980.
Simonoff et al,(19).	8.5	1984.

RSD = relative standard deviation.

The relatively low values found for serum chromium using method CDIII are clearly not false due to the loss of a volatile Cr fraction derived from the biological sample. The specimens are never heated above 75 ^{0} C, and are in sealed containers at all temperatures above ambient. The loss of Cr as the volatile hfacac complex is very unlikely as shown in Chapter IV, section 4.6.1. (<1% lost in 48 hrs. at ambient

temps. open vessel), and is of equal opportunity for both tests and standards. The compositions of processed standards and samples are almost identical, and reasonable proof has been given that the AAS/ETA measurements are valid (chapter V).

An equally confident case cannot be made for the validity of the radioactive Cr spike as a meaningful guide to recovery of the native Cr, even after equilibration. The experiments reported in chapter IV, section 4.2. clearly indicate that the added ^{51}Cr exchanged with the native element. The recovery tests carried out in this study are suspect if lower levels than the ones found are postulated, as the percentage of the added ^{51}Cr present as native molecular species of which Cr is a normal component (excluding transferrin) would then be very low, reducing their contribution to the recovery. The reverse is true if the supposition is that the values found using method CDIII are falsely low, and under these circumstances the recovery tests appear to be more reliable, The recovery of haemoglobin-derived iron reported in chapter IV, section 4.12.1. gives some support to the validity of the pretreatment used. The administration of radioactive Cr to a human subject, the only completely satisfactory route to recovery confirmation, was considered to be unjustified on ethical grounds.

The pretreatment used in method CDIII has virtually nothing in common with the pretreatments used in any of the methods cited in group I, which include: 2 using dry ash, 2 wet oxidative digestion, 1 partial digestion with bacterial protease, 1 direct injection, and 1 (6) with no information. The agreement shown by 8 methods with such diverse methodology tends to support the overall mean of 0.123 µg Cr/L, as the best guide to the true value.

8.1.1. Analytical Characteristics.

The major analytical characteristics of 7 of the 8 methods cited in group I, table 8.1, are summarised in table 8.2 below, in comparison with method CDIII.

A comparison of the precisions of the 7 methods is difficult to make, as this parameter has been determined on specimens with inappropriately high Cr concentrations in many reports. The level of 19.51 µg Cr/g quoted in reference (2) is probably a misprint, but 19.91 ng/g would be an unsuitable concentration. The high value of 13% for a specimen with a mean level of 0.06 µg Cr/L for method CDIII must be considered in this light. The high blank value of 0.08 µg Cr/L is

TABLE 8.2.

Analytical characteristics of serum Cr methods.

Reference.	Sample µL.	Pretreatment and measurement.	Detection Blank.	Detection limit.	Mean.	Precision. SD.	Precision. RSD.
(2).	1000	Dry ash, NAA.	0.0478 (0.0262-0.0704)	19.51(a) n=6.		1.39	7.1
(3).	500	Digestion, nitric acid, peroxide. AAS/ETA.BC.WI.	/	/	/		/
(4).	500	Digestion, nitric acid, peroxide. AAS/ETA.BC.WI.	/	/	/		/
(5).	100	Direct injection, AAS/ETA.BC.WI. Std.Add.		0.05	0.43(b) n=4	0.048	11.1
(7).	1000-2000	Dry ash. AAS/ETA.BC.WI.	0.02	0.03	0.27(b) n=26	0.050	18.5
					0.27(c) n=8	0.020	7.4
(8).	500	Partial digestion, bacterial protease, AAS/ETA.BC.Zeeman. Std.Add. Auto-sampler.	0.02	0.03	0.29(b) n=12	0.030	10.3
					0.50(c)	0.014	2.8
					0.10(c)	0.006	6.3
CDIII.	4000	Solvent extraction as hfacac complex. AAS/ETA.	0.08	0.03	0.13(d) n=6	0.009	7.0
					0.06(c)	0.008	13.0

Note:

"Mean" in above table refers to the sample used for precision tests, not to reference range mean.

BC= background correction, WI= tungsten iodide.

Std.Add.=calibration by standard additions.

Cr concentrations are in µg/L, except for (a) where µg/g are quoted!

SD = standard deviation in the same units as relevant mean.

RSD = relative standard deviation %.

(b) between batch precision. (c) within batch precision.

(d) between batch precision, but on mean of quadruplicate tests.

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the principal disadvantage with method CDIII, and is clearly significantly inferior to the value of 0.02 reported by both (7) and

(8). The method of Offenbacher et al (8) appears to be very impressive, with a conveniently small sample requirement, simple pretreatment, and very good precision with a low blank value. However a high quality AAS instrument is required with high sensitivity, as the sample was diluted 1 in 2, and the background correction system used reduces sensitivity, as reported in chapter III, section 3.3.1.1.1. The auto-sampler used may have made significant contributions to the precision. Background correction is required and the expensive Zeeman system may be obligatory,

8.2. ALPHA-2-GLOBULIN-BOUND SERUM CHROMIUM.

The alpha-2-globulin-bound serum chromium cannot be compared with others in the literature, as no other worker has attempted to fractionate serum chromium. The hypothesis was that alpha-2-globulin-bound Cr would show a greater relative response than total serum Cr to a glucose challenge. This paramater was considered to be less prone to modest contamination at the sampling stage, as added Cr(III) is initially bound predominantly by the beta-globulin transferrin, and to a lesser extent by albumin.

The mean alpha-2-globulin-bound serum Cr was 0.07 µg/L, compared with 0.10 µg/L for the total protein-bound Cr on the same samples (36 specimens from 4 subjects). The determination is less prone to recovery problems than the total protein-bound Cr stream. The within batch precision on a pool of routine laboratory specimens produced a relative standard deviation of 1.6%, with a mean value of 0.92 µg Cr/L (standard deviation 0.015 µg Cr/L), demonstrating that the method would be excellent, if the analyte levels were ten times higher.

8.3. URINARY CHROMIUM.

The urinary Cr levels reported over the past decade can be divided into three groups in a similar manner to the serum Cr values, and are listed in table 8.3 below. All the methods listed use AAS/ETA.

The three methods in group I are all direct injection techniques with tungsten iodide background correction, and standard additions calibration. Veillon et al (20) and Anderson et al (21) probably used identical techniques, both reported that their urine method had been verified by stable isotope gas chromatography/mass spectrometry, and also by a method using the wavelength modulated echelle monochromator AAS developed by Harnley et al (30). However, Guthrie et al (22) in

group II used the wavelength modulated echelle monochromator AAS system and reported a level of 0.5 µg Cr/L, also confirmed by stable isotope dilution gas chromatography/mass spectrometry. Veillon et al (20) argued that as Cr absorption had been proven to be between 0.5 and 1.0% by radiotracer experiments, and as diets in the U.S.A. supplied only about 100 µg Cr/day, excretions must be less than 1 µg/day. Egila et al (31) using tube wall, platform and probe atomisations with Zeeman correction reported good agreement by all three techniques with the method of Halls et al (28) in group III. However the samples used were all from subjects on Cr supplementation and were reported to be in the range 1.6 to 7.4 µg Cr/L.

TABLE.8.3.

Urine chromium levels reported over the last decade.

Reference.	Mean Cr, µg/L.	Year.
Group I (group mean 0.13, RSD 15%, n=3).		
Veillon et al,(20).	0.15(i)	1981.
Kumpulainen et al,(5).	0.13	1983.
Anderson et al,(21).	0.11(i)	1983.
Group II (group mean 0.44, RSD 19%, n=5).		
Guthrie et al,(22).	0.50(i)	1978.
Vanderlinde et al,(4).	0.40	1979.
Nomiyama et al,(23).	0.48	1980.
Brodie et al,(24).	0.50	1984.
Morris et al,(25).	0.31	1985.
This study (CDUI).	0.33	
Group III (group mean 0.85, RSD 25%, n=5).		
Kayne et al,(3).	1.10	1978.
Routh et al,(26).	0.79	1980.
Ping et al,(27).	0.72 (20 to 35 yrs.).	1982.
	0.39 (47 to 69 yrs.).	
Halls et al,(28).	0.80	1983.
Mianzhi et al,(29).	1.00	1983.

(i) reported to be confirmed by stable isotope dilution, gas chromatography/mass spectrometry.

The mean urinary Cr obtained using method CDUI was 0.57 µg

Cr/day (SD 0.3) based on a creatinine excretion of 13 mmol/day, and this gives a mean Cr concentration of 0.33 µg/L if a mean daily urinary excretion of 1.75 litres is assumed. The value is in agreement with group II in table 8.3, but is not compatible with group I.

8.3.1. Analytical Characteristics.

The major analytical characteristics of some of the methods in table 8.3 above are listed in table 8.4 below. The methods selected were those from the first two groups which provided suitable information in the report. Method CDUI is included for comparative purposes.

TABLE 8.4.

Analytical characteristics of urinary Cr methods.

Reference.	Methodology.	limit.	Precision Mean	SD.	RSD.
(20).	Direct injection.	0.03	0.14(a)	0.012	8.6%
	BC.WI. Std.Add.		n=40		
(5).	Direct injection.		0.13(a)	0.011	8.7%
	BC.WI. Std Add.		n=10		
(23).	Direct injection.	0.10			
	No BC.				
(24).	Direct injection.	0.05	0.5	0.05	10%
	BC.D2. Auto-sampler Std.Add.				
(28).	Direct injection dil 1/2.	0.08	0.58	0.104	18%
	BC.D2.		0.79	0.103	13%
CDUI	Solvent Ext., hfacac complex.	0.05	0.43(b)	0.056	13%

Note:

"Mean" (µg Cr/L) in above table refers to the sample used for precision tests, not to reference range mean.

SD = standard deviation in µg Cr/L.

BC= background correction, WI= tungsten iodide, D2= deuterium.

(a) between batch precision.

(b) between batch precision but on mean of duplicate tests.

The conclusion from table 8.4 is that direct injection techniques are suitable for urinary Cr determinations, particularly if tungsten iodide background correction is available. The atomisation, and particularly the ashing temperatures must be very carefully

controlled to obtain useful results. The ability of Nomiyama et al (23) to obtain apparently satisfactory results without background correction using a direct injection of undiluted urine, is in contradiction to the findings of other workers in this field.

8.4. SERUM CHROMIUM RESPONSE TO A GLUCOSE CHALLENGE.

A number of workers have investigated the serum/plasma Cr profile through a glucose tolerance test, but the reported results have been contradictory.

8.4.1. CDIII Cr Levels in Response to a Glucose Challenge.

In the present study oral glucose tolerance tests were carried out on 4 normal subjects, and samples were collected for serum Cr, serum insulin and plasma glucose as detailed in chapter VII.

The overall mean serum Cr results for the glucose tolerance test samples are given in table 8.5 below.

TABLE 8.5.

Serum chromium levels during an oral glucose tolerance test.

	Total protein-bound Cr.		Alpha-2-globulin-bound Cr.	
Sample.	Mean.	SD.	Mean.	SD.
Fasting.	0.11	0.02	0.06	0.02
30 min.	0.12	0.02	0.05	0.005
60 min.	0.12	0.02	0.07	0.01
90 min.	0.12	0.04	0.07	0.03
120 min.	0.09	0.03	0.05	0.01
150 min.	/	/	0.08	0.03
180 min.	0.12	0.02	0.07	0.02
	n=3		n=4, fasting to 90 min.	
			n=3, 120 to 180 min.	

The specimens taken during the oral glucose tolerance tests were also subjected to glucose determinations, and insulin determinations were carried out on two series of samples. The methods used for the glucose and insulin determinations are given in appendix B. The data from the individual glucose tolerance tests are listed in table 8.6 below.

TABLE 8.6.

Chromium, glucose and insulin levels on normal subjects during an oral glucose tolerance test.

Sample.	Total Cr. µg/L.	GTF Cr. µg/L.	Glucose. mmol/L.	Insulin. mU/L.
Subject A, female, 18 yrs.				
Fasting.	0.11	0.07	4.4	<10
30 min.	0.12	0.06	10.1	51
60 min.	0.14	0.06	11.2	47
90 min.	0.14	0.04	8.6	30
120 min.	0.12	0.04	4.5	<10
150 min.	/	0.05	3.5	<10
180 min.	0.14	0.05	4.0	<10
Subject B, male, 19 yrs.				
Fasting.	0.13	0.07	4.5	<10
30 min.	0.10	0.05	8.1	34
60 min.	0.12	0.08	7.7	34
90 min.	0.08	0.09	6.5	51
120 min.	0.06	0.06	5.1	21
150 min.	0.12	0.10	4.2	12
180 min.	0.10	0.07	4.0	10
Subject C, male, 53 yrs.				
Fasting.	0.10	0.07	5.3	
30 min.	0.14	0.05	7.5	
60 min.	0.10	0.19	3.8	
90 min.	0.15	0.10	4.5	
120 min.	0.09	0.06	4.4	
150 min.	/	0.08	4.1	
180 min.	0.11	0.08	3.6	
Subject D, female, 46 yrs.				
Fasting.		0.04	4.8	
30 min.		0.05	5.3	
60 min.		0.08	4.5	
90 min.		0.05	6.8	

Note:

Total Cr = total protein-bound Cr., GTF Cr = alpha-2-globulin-bound Cr.

The oral glucose tolerance test was repeated on subject C, but 11 sequential fasting samples were taken, and 4 specimens 60 minutes after the glucose ingestion. The large number of fasting samples were taken to confirm that the fasting values recorded were not falsely elevated, through a failure to wash out traces of Cr from the initial venepuncture. The total protein-bound Cr and alpha-2-globulin-bound Cr levels were determined, in duplicate on the specimens. The results are summarised in table 8.7 below.

TABLE 8.7.

Fasting and 60 minute post glucose serum Cr values.

i) Sequential fasting samples, total protein-bound Cr.

Sample No.	Cr µg/L (Mean of duplicate determinations).
1.	0.10
2.	0.08
3.	/
4.	0.14
5.	0.08
6.	0.12
7.	0.07
8.	2.95 rejected.
9.	0.10
10.	0.09
11.	0.07

ii) Mean values, total protein-bound Cr and alpha-2-globulin-bound Cr.

Cr µg/L.

	Total protein-bound Cr.			Alpha-2-globulin-bound Cr.		
Sample.	Mean.	SD.	n.	Mean.	SD.	n.
Fasting.	0.094	0.024	9	0.051	0.008	6
60 minute.	0.090	0.051	6	0.056	0.016	6

Student's t tests confirmed that there were no significant differences between the fasting and 60 minute post glucose samples, for either of the two serum chromium parameters in the table above.

8.4.2. Fall in Plasma Chromium.

Davidson et al (32), reported that an intravenous glucose load produced a rapid fall in plasma Cr. An oral glucose challenge was found to produce a less well marked decline. The reported results are

characteristic of group III (high) methodology (table 8.1) and are summarised in table 8.8 below.

TABLE 8.8.

Plasma Cr response to a glucose load, Davidson et al (32).

	Plasma Cr µg/L.			Plasma Cr µg/L.	
Sample.	Mean.	SD.	Sample.	Mean.	SD.
Fasting.	4.7	0.15	40 min.	3.12	0.21
10 min.	3.89	0.29	50 min.	3.24	0.23
20 min.	3.48	0.17	60 min.	3.16	0.19
30 min.	3.31	0.19			

The means are from 10 normal subjects. SD= standard deviation.

8.4.3. Rise in Serum Cr.

Gedik et al (18), reported significant increases in serum Cr for normal subjects, and for those with adult, or juvenile onset diabetes mellitus. Chromium levels before and during the oral glucose tolerance tests were found to be not significantly different in the three groups. The results are summarised in table 8.9 below, and again the results are "high" (group III of table 8.1).

TABLE 8.9.

Serum Cr responses to a glucose load, Gedik et al (18).

	Serum Cr µg/L.					
Sample.	Normal (n=10).		Adult onset DM (n=9).		Juvenile (n=8).	
	Mean.	SD.	Mean.	SD.	Mean.	SD.
Fasting.	4.6	0.6	4.7	0.9	4.4	0.9
30 min.	7.0	0.7	7.6	0.9	5.8	0.8
60 min.	6.4	0.7	7.2	0.6	6.5	0.9
90 min.	6.0	0.5	5.9	0.7	6.5	0.9
120 min.	5.5	0.7	3.7	0.5	5.3	0.8

SD= standard deviation.

The reference range was reported to be 4.4 to 6.1 µg Cr/L. The method used was that of Pekarek et al (33). However Pekarek reported the mean normal serum Cr to be 1.62 µg/L.

8.4.4. Rise in normal status, fall in deficient subjects.

Liu et al (9) investigated the "relative Cr response" (RCR) as

an indicator of Cr status. The RCR was defined as :

100 x (1 hr. serum Cr)/(fasting serum Cr).

The 27 subjects tested were re-examined after receiving 5 g of brewer's yeast daily for 3 months. The results are summarised in table 8.10 below. The Cr concentrations are characteristic of group II methodology (table 8.1).

TABLE 8.10.

Relative Cr response, Liu et al (35).

	Serum Cr µg/L. Fasting.	60 min.	RCR %.
A. Normal. (n=15).			
Baseline.	1.67	1.43	107
Post yeast.	1.46	1.86	140
B. Hyperglycaemic. (n=12).			
Baseline.	1.89	1.03	81
Post yeast.	0.94	1.03	149

Note. The RCRs reported do not appear to refer to the mean Cr levels of the groups, but to the 73% of normals and the 75% of hyperglycaemics who showed an improved response.

8.4.5. Response to Glucose Challenge, Conclusions.

The serum Cr responses reported by other workers and referred to above are all based on serum Cr levels which appear to be at least ten times higher than the values indicated by method CDIII, and they do not agree on Cr levels, or on the direction of the response. A method with a low recovery could give a reproducible profile, a technique producing grossly elevated values would not be expected to do so. The higher levels, if due to contamination, or measuring signals from, or unduly influenced by, matrix components independent of chromium, should be random in distribution. The ability of a method to demonstrate a reproducible, characteristic pattern of serum Cr levels, in response to a glucose challenge, must be taken as evidence that the values found are not falsely elevated. This argument would be reinforced if different, characteristic reproducible patterns were given by classes of subjects with abnormalities of carbohydrate metabolism.

A glucose tolerance profile using one of the group I serum Cr techniques (table 8.1) has not been reported. Method CDIII failed to

demonstrate a significant change in either total protein-bound serum Cr, or alpha-2-globulin-bound Cr. However a simpler technique with superior precision such as the one developed by Offenbacher et al (8) would be more sensitive in this respect. The insulin level in human serum is about 0.15 nmol/L, assuming that the total Cr concentration is about 0.12 µg/L (2 nmol/L), then as little as 10% of the total serum Cr could be true GTF Cr, if it is assumed that the concentrations of insulin and GTF in moles per litre are similar. The changes in total serum Cr would then be a relatively modest 50%, even if the GTF levels changed in parallel to the insulin levels.

In summary, the developed method for measuring total protein-bound Cr, and alpha-2-globulin-bound Cr has failed to demonstrate a response of either of these parameters to a glucose challenge in normal subjects. Saner (34) stated in 1981 that there was general agreement that an increase in plasma Cr levels occured after glucose administration, but this still awaits indisputable proof, as evidence based on acceptable Cr levels has yet to be produced.

8.5. DIAGNOSTIC VALUE OF URINE CHROMIUM DETERMINATIONS.

The diagnostic value of urinary Cr determinations is a matter of some controversy, and this is to be expected in the light of the uncertainty about the validity of the analytical methods used.

The urinary Cr should reflect the level of non-protein-bound Cr, and therefore, possibly "free GTF" levels. The concept that the alpha-2-globulin-bound Cr represents a reserve of GTF, in equilibrium with a low level of the free factor , is a reasonable one by analogy with the thyroid hormones. The free GTF and its degradation products would be excreted in the urine. Anderson et al investigated the effects of Cr supplementation and a glucose load on urinary excretion (21). The baseline Cr levels in these investigations were in group I of table 8.3, and well below the 1 µg Cr/day maximum, derived from radiotracer absorption studies and dietary considerations, discussed earlier (section 8.3).

8.5.1. Urinary Chromium Response to a Glucose Challenge.

Anderson et al (21) investigated the urinary Cr response following a glucose load on a large number of subjects, both before and after Cr supplementation. The mean daily Cr excretion increased from 0.22 µg/day to 0.99 µg/day on a schedule of 200 µg Cr, as the chloride,

per day. The urinary Cr concentration was found to correlate significantly with the creatinine concentration for urine samples taken after a morning void, and 90 minutes after glucose ingestion, both before and during Cr supplementation. However there was no correlation between Cr concentration and 90 minute glucose levels, or fasting or 90 minute insulin levels.

8.5.1.1. Urinary chromium response to a glucose challenge, using method CDU1.

In the present study two subjects were investigated and the results are summarised in table 8.11 below. Duplicate tests plus duplicate recovery spikes were set up for each sample.

TABLE 8.11.

Urinary Cr before and after a glucose load.

µg Cr/10 mmol creatinine.

	Subject B.		Subject C.	
n=1.	Mean.	SD.	Mean.	SD.
Fasting.	0.58	0.26	0.16	0.04
60 min. post glucose.	0.94	0.14	0.22	0.06

SD= standard deviation.

There is insufficient data for reliable statistical analysis in the above table. The method for urine was developed very late in the project and there was insufficient time to carry out further investigations. A low priority had been allocated to this field, as Anderson et al had found no significant response. The results justify the analysis of more samples from a larger number of subjects.

8.5.2. Effects of Exercise on Urinary Chromium Excretion.

Vigorous exercise induces increases in glucose utilization and consequent changes in urinary Cr excretion could occur.

8.5.2.1. Effect of exercise on urinary Cr, investigation using method CDU1.

Urine specimens were collected immediately prior to, and immediately after runs of 5 to 10 miles duration. Two male subjects B and D, aged 19 and 24 years respectively, provided a total of 16 pairs of pre, and post run urines. The results are summarised in 8.12 below. The urine determinations were carried out in duplicate, plus two spiked

aliquots to monitor the recovery.

TABLE 8.12.

Urinary Cr, before and after vigorous exercise.

µg Cr/10 mmol creatinine.

	Pre exercise.		Post exercise.	
	Mean.	SD.	Mean.	SD.
Subject B. (n=6)	0.59	0.15	0.60	0.32
Subject D. (n=10)	0.49	0.12	0.42	0.25

SD= standard deviation.

Student's t test confirmed that there was no significant difference between the pre and post exercise urine Cr levels.

8.5.2.2. Effects of Exercise on Urinary Chromium Excretion, Anderson et al.

Anderson et al (35) investigated the effects of vigorous exercise on urinary Cr excretion. Nine male runners were asked to run a 6 mile course at or near their maximal capacity, and urine samples were collected immediately prior to, immediately following and 2 hours after running. The mean urinary Cr/creatinine ratio was significantly increased in the 2 hour post exercise urine, relative to the pre-run sample. The mean factor was 4.7, with a range of 1.5 to 8.6.

8.5.2.3. Effect of exercise in urinary Cr, conclusions.

In the light of the report by Anderson et al the tests should be repeated on specimens collected 2 hours after the termination of the vigorous exercise.

8.5.3. Value of Urinary Chromium Determinations Discussed.

The value of urinary Cr determinations appears to be limited to toxicological studies, and confirmation of compliance in Cr supplementation studies.

The determinations on specimens collected before and after a glucose load should be repeated in the light of the results recorded in table 8.11., and if a significant change is demonstrated for normal subjects, then this test may possibly be of value.

8.6. CONCLUSIONS.

The value of this study is clearly very limited. The method developed for the determination of serum Cr has the disadvantages of a

large sample requirement and modest throughput. Contamination is difficult to control and about 1 in 12 determinations must be rejected because of obvious failure in this field. The total protein-bound Cr and urine Cr determinations are prone to recovery problems, and this parameter must be strictly monitored, and results on samples with recoveries below the minus 2 standard deviation limit rejected. However the radioactive spike provides a recovery check independent of the AAS/ETA values, and hence four independent results are available if tests and spikes are set up in duplicate.

The alpha-2-globulin-bound Cr procedure is more robust, but failed to demonstrate a response to a glucose challenge. The high blanks, and consequent high detection limits of the developed methods have prevented a confident definition of the lower limits of the normal range for the measured parameters. However all other methods developed to date appear to have similar failings.

The data from this work has made very modest contributions to the highly controversial serum and urinary Cr fields. The data from serum Cr determinations have given added weight to the argument that the mean serum Cr level in normal humans is about 0.12 µg/L. A second contribution is that about one third of the Cr in human serum is normally bound to transferrin, the other two thirds being mainly bound by proteins of the alpha-2-globulin fraction.

The data from this project indicates that neither serum total protein-bound Cr or alpha-2-globulin-bound Cr levels, show changes of similar proportions to those demonstrated for either insulin or glucose values, in response to a glucose load. However, more modest responses of these parameters may occur.

The value of the urinary Cr data is limited to supporting the urinary excretion level at less than 1 µg Cr/day in normal subjects.

Contributions to analytical chemistry outside the field discussed above are necessarily very small. The simple cheap distillation equipment described in chapter VII, section 7.3.2., can produce distillates of extremely high purity. The analytical methods developed can possibly be adapted for other trace metals in serum/plasma and urine, as beta-diketones form complexes with the majority of trace metals which are of interest to the clinical chemist. The metal beta-diketonates are generally suitable for solvent extraction and some may be produced under similar conditions, a certain

amount of multi-element analysis may thus be possible. The following trace metals which are of current interest in clinical chemistry, as reported in chapter I, table 1.3, are reported by Moshier et al (36) to form complexes with hfacac: aluminium, nickel(II) and manganese(II). The concentration of aluminium in serum is probably higher than that of chromium by a factor of at least one hundred, and the factor for the other two metals is possibly about five (see chapter I, table 1.1). The methods may be more satisfactory for analytes present at higher levels, as the precision recorded on a pool with elevated Cr values was good, as described in section 8.2. The simplified back extraction described in chapter IV, section 4.12.1. is suitable for metals which form labile complexes with hfacac.

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APPENDIX A.

EQUIPMENT AND REAGENTS USED.

1. Milli-RQ Water Purifier.

   Quality 1 megohm-cm resistivity.

   Supplied by :

   Millipore UK Ltd.,
   Millipore House,
   Abbey Road,
   London NW10 7SP.

2. NE 1600 Gamma Counter.

   Used in the ^{57}Co mode.

   Supplied by :

   Nuclear Enterprises Ltd.,
   Sighthill,
   Edinburgh EH11 4EY.

3. MX10 10 Roller Mixer.

   Supplied by :

   Coulter Electronics Ltd.,
   Coldharbour Lane,
   Harpenden,
   Hertfordshire.

4. Beckman TJ-6R Refrigerated Centrifuge.

   RCF 1500 g at 3000 rpm.

   Supplied by :

   Beckman - RIIC Ltd.,
   Progress Road,
   Sands Industrial Estate,
   High Wycombe,
   Buckinghamshire HP12 4JL.

5. SP 2900 Atomic Absorption Spetrometer.

   Equiped with SP9-01 Digital Flameless Atomiser.

   DP 101 Computing Integrator.

   Supplied by :

   Pye Unicam Ltd.,
   York Street,
   Cambridge CB1 2PX.

6.a) Finnpipettes.

Continously adjustable micropipettes.

Code.	Range µL.	Set volume.	Mean. n=10	RSD%.
H40/25	5 to 50	25	24.9	1.94
		50	49.8	1.64
H40/26	50 to 250	125	121	0.32
		250	246	0.26
H40/27	200 to 1000	250	252	0.25
		500	502	0.32
		1000	1006	0.16
H40/29	1000 to 5000	4000	3960	0.73

Used with polypropylene tips.

b) Desiccators.

Jencons Dry - Seal Desiccators.

4" and 12".

c) PTFE tubing.

Cat. No. H66/7 normal wall.

0.25" id, 0.025" wall.

d) PVC high pressure terylene reinforced hose.

Cat. No. TR 24.

0.75" id, 1.125" od.

Supplied by :

Jencons Scientific Ltd.,
Mark Road,
Hemel Hempstead,
Hertfordshire.

7. Teflon connector.

Tube to joint connector 1206.

Supplied by :

Chemplast Inc.,
150 Dey Road,
Wayne,
New Jersey 07470,
U.S.A.

8. Quickfit Wash Bottle Head,

Dreschsel/ADJ.

Code MF 27/3/13

Supplied by:

J.Bibby Science Products Ltd.,
Stone,
Staffordshire ST15 0SA.

9. Tuftainers.

Teflon PFA vials.

15 mL capacity, Cat. No. 14045.

Manufacturers state:

Teflon -PFA-main chain carbon fluorine backbone contains perfluoroalkoxy (PFA) side chains connected through flexible oxygen links. Stable -196 ^{0} C to 260 ^{0} C.

All Tuftainer products are moulded from Teflon-PFA, no plasticisers or other organics are added , making Tuftainers the product of choice for handling severe corrosive liquids, and for trace metal analysis. Knurled closure, 4 ribbed areas on body. Inner secondary seal. Seamless. Radiused inner corners. Marking area in centre of cap.

Supplied by :

Pierce and Warriner (UK) ltd.,
44 Upper Northgate Street,
Chester,
Cheshire CH1 4EF.

10.a) Universal Containers.

30 mL polystyrene screw cap bottle.

Code 33621.

b) Polythene Transfer Pipettes.

Code 28928.

Supplied by :

L.I.P. (Equipment and Services) Ltd.,
111, Dockfield Road,
Shipley,
West Yorkshire BD17 7AS.

11.a) 12 X 75 mm polypropylene tube.

Code 55/526.

Cap (skirted).

Code 65/719.

b) 13 mL polypropylene screw cap tube plus cap.

Code 60/541.

Supplied by :

Sarstedt Ltd.,
68 Boston Road,
Beaumont Leys,
Leicester LE4 1AW.

12. Chemicals.

Supplied by :

A. BDH Chemicals Ltd.,
Broom Road,
Parkstone,
Poole BH12 4NN.

Ammonia Aristar.	Product 45200.
Acetic acid Aristar.	Product 45001.
Acetic acid AR.	Product 10001.
Benzoyl acetone.	Product 27347.
Chromium III nitrate, standard solution, "Spectrosol" for atomic spectroscopy. 1 mL = 1 mg Cr.	Product 14137.
Decon 90. General purpose surfactant, especially suitable for biological and radioactive work.	Product 56022.
EDTA (diammonium salt). "Spectrosol" grade.	Product 14111.
1,1,1,5,5,5-Hexafluoropenta-2,4-dione.	Product 28623.
Hydrochloric acid Aristar.	Product 45002.
Nitric acid AR.	Product 10168.
Perchloric acid Aristar (70%).	Product 45008.
Petroleum spirit AR (120 to 160 ^{0} C b.pt.).	Product 10182.
di-Potasium hydrogen orthophosphate AR.	Product 10349.
Propan-2-ol Aristar.	Product 45103.
Propan-2-ol AR.	Product 10224.
Sulphuric acid Aristar.	Product 45006.
2-Thenoyltrifluoroacetone.	Product 13128.
Toluene Aristar.	Product 45109.

Ion-exchange resins.

Amberlite IRA 400 AR grade. Product 55005.

Amberlite IR 120 (H) AR grade. Product 55001.

Amberlite monobed MB1 AR grade. Product 55007.

Amberlyst 15 macroreticular strong acid. Product 55137.

Amberlyst 27 macroreticular strong base. Product 55137.

Dowex 50WX8 (H) standard grade. Product 55162.

B. Koch-Light Ltd.,
37 Hollands Road,
Haverhill,
Suffolk,
CB9 8PU.

1,1,1,5,5,5-Hexafluoropenta-2,4-dione. Product 3055-55.

C. Amersham International PLC.,
Amersham,
Buckinghamshire.

^{51}Cr. Chromium Chloride in 0.1M hydrochloric acid.

Code CJS 2.

13. Materials used in blood collection.

1. Medicut cannula, size 1.7 mm.

Supplied by :

Sherwood Medical Industries Ltd.,
London Road,
County Oak,
Crawley,
West Sussex RH10 2TL.

2. Hepsal sterile heparinised saline, 10 units heparin per mL.

Supplied by :

Weddel Pharmaceuticals Ltd.,
Wrexham,
CLWYD LL13 9PX.

3. 3-way Stopcock, VYGON VG1.

Supplied by :

Vygon UK Ltd.,
Bridge Road,
Cirencester,
Gloucestershire C17 1PT.

APPENDIX B.

ANALYTICAL METHODS.

1. Creatinine Determination in Urine.

Technicon AA1 system, no dialysis, using the reaction between creatinine and 3,5-dinitrobenzoic acid.

Adapted from the method of Parekh,A.C., and Sims,C., Clin. Chem., 23 (1977) p.2066.

Technicon AA1 system supplied by :

Technicon Instruments Co. Ltd.,
Hamilton Close,
Basingstoke,
Hampshire RG21 2YE.

2. Glucose Determinations in Blood.

Beckman Glucose Analyser II.

Glucose oxidase method, partial pressure of oxygen measured.

Supplied by :

Beckman-RIIC Ltd.,
Progress Road,
Sands Industrial Estate,
High Wycombe,
Buckinghamshire HP12 4JL.

3. Insulin Determinations in Serum.

Insulin RIA kit, code 1M78.

Supplied by :

Amersham International PLC.,
Amersham,
Buckinghamshire.

4. Serum Iron Determinations.

CFA 2000 Rotochem Centrifugal Analyser.

Ferrozine reagent.

Supplied by :

Travenol Laboratories Inc.,
9299 Washington Boulevard,
Savage,
Maryland 20863.
U.S.A.

APPENDIX C.

STATISTICAL METHODS.

Student's t test was used to compare the means of two series of results as a test for significant difference. The formulae used were taken from :

Statistics for Analytical Chemistry. (1984) p.54-55.

by J.C.Miller and J.N.Miller.

Published by John Wiley and Sons, New York.

1. Applied when the standard deviations of the two series were not significantly different.

The "pooled" estimate of standard deviation was calculated from the individual standard deviations s_1 and s_2 by using the equation:

$$s^2 = \left\{(n_1 - 1)s_1^2 + (n_2 - 1)s_2^2\right\}/(n_1 + n_2 - 2)$$

Then t is given by:

$$t = (\bar{x}_1 - \bar{x}_2)/s\sqrt{(1/n_1 + 1/n_2)}$$

where t has (n + n -2) degrees of freedom.

2. Applied when the standard deviations of the two series were significantly different.

$$t = (\bar{x}_1 - \bar{x}_2)/\sqrt{(s_1^2/n_1 + s_2^2/n_2)}$$

and the number of degrees of freedom was calculated from:

$$\text{degrees of freedom} = \left\{\frac{(s_1^2/n_1 + s_2^2/n_2)}{\frac{(s_1^2/n_1)}{n_1 + 1} + \frac{(s_2^2/n_2)}{n_2 + 1}}\right\} - 2$$

the result being rounded to the nearest whole number.

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