NOVEL METHODS IN TRACE METAL DETOXIFICATION

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ABSTRACT

Two putative methods to deal with the problems caused by toxic trace metals were examined. Firstly, Escherichia coli cells into which had been cloned a gene thought to encode a metal-binding protein were examined. It is hoped that by such methods it may be possible to clone metal resistance into bacteria. Secondly, an approach was made to determine the feasability of using immobilised plant metal binding peptides for the clearance of toxic metals from waste streams. Using this second approach, previously unavailable information on the metal-binding characteristics of (gammaEC)$_n$G has been obtained. These data have implications for the physiology and biochemistry of (gammaEC)$_n$G, in addition to their possible use in bioreclamation.

Patent application.

It is noted that a patent application has been filed by University of California attorneys on certain biotechnological applications of (gammaEC)$_n$G and related molecules. (DOE case number : S-16, 864 ; W-7405-ENG-36).

The patent is filed in the names of :

Dr N. J. Robinson, Department of Biological Sciences, University of Durham, England.

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Some of the work described in this report may relate to the above named patent application.

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I. GENERAL INTRODUCTION

The careless disposal of wastes contaminated with toxic trace metals is a problem which governments are having to recognise as a serious threat to the environment. Recently, there has been an agreed commitment to dramatically reduce the legal limits for levels of Cd deposited into the North Sea (Pearce 1988). The following report is an examination of two possible approaches which could be applied to dealing with toxic trace metals from industrial wastes.

The first part of the project involves the analysis of bacteria into which a synthetic gene, designed to encode a putative metal-binding protein, has been cloned. It is hoped that by cloning such genes into non-resistant organisms that resistance may be conferred on them. This could lead to the production of bacteria or yeasts with increased metal resistance for detoxification of various industrial effluents. The second approach involves the attachment of metal-binding polypeptides from plant cells onto cyanogen bromide-activated Sepharose, with the aim of subsequently producing metal-affinity matrices which are capable of removing metals from contaminated waste streams.

Toxicity of trace metals

The toxicity of heavy metals, particularly Pb and Cd, has been widely researched in recent years. Many reviews exist on the detrimental effect that exposure to toxic trace metals, particularly chronic exposure, can have on human health (Brockhaus *et al* 1988 ; Davison *et al*

1988).

Sources of Cd

Cd is one of the most toxic of the trace metals, and will be focused on in this report. It is a silver white ductile metal, with melting point 321°C, which emits highly toxic fumes of Cd and CdO_2 at sufficiently high temperatures. The industrial uses of Cd include its application as a protective coating to other metals; its use as an alloy with Cu to which it imparts strength without impairing electrical conductivity; as a neutron absorber in nuclear reactors; and as a constituent of low melting point alloys used in such items as the tips of fire sprinklers, safety plugs and electrical fuses. In addition, Cd compounds are often used as pigments in the production of paints, plastics, rubber, inks and glass. Mining and smelting of various other metals constitutes a further environmental source of Cd.

A major non-occupational source to humans is cigarette smoking (via agricultural application of Cd-containing agrochemicals to tobacco fields). A small amount (up to 50 ug day^{-1}) may be attributed to dietary intake. Legal limits for occupational Cd exposure reflect its high toxicity and are set extremely low (0.05 mg m^{-3}, 8 h time-weighted average [Health & Safety Executive 1986]).

Effects of Cd on human health

The main acute effects of Cd exposure are respiratory (after inhalation) or gastro-intestinal (after ingestion). Fumes of Cd cause

irritation of the eyes, nose and throat, followed by coughing, headache, chills, fever and breathlessness. Pulmonary damage may be delayed for several days, and may be accompanied by damage to liver and kidneys. Several fatalities have resulted from short term exposure to high Cd concentrations (Beton et al 1966). Chronic exposure to Cd is associated with slow accumulation in the renal cortex causing abnormality at a critical level thought to be about 200 to 400 ppm. (Health and Safety Executive 1986). Long term exposure can also result in malformation of bone tissue, leading to "Itai Itai" disease (Christopherson et al 1988) where the bones become extemely brittle resulting in multiple fractures to the arms and legs. There is also some evidence that chronic exposure may be associated with increased incidence of cancer of the prostate (Lemen et al 1976). Studies on the mutagenicity of Cd have shown that it will bring about cell transformation in vitro at concentrations of <1% in cultures of Syrian hamster embryo cells (Di Paolo & Casto 1979).

It has been demonstrated that Cd will directly damage DNA, by use of the Rec$^-$ assay in Bacillus subtilis (Nishioka 1975). In vitro studies on Cd administration to mammalian cells have shown increased occurrence of chromosomal aberrations, though only at very high concentrations. In vivo studies in mice and rats have revealed some evidence of the induction of cancers, particularly testicular, when Cd is administered parenterally (Gunn et al 1963). Extensive investigation of possible teratogenic effects of Cd has produced only slight evidence that Cd is fetotoxic when administered orally (Webster 1978). Parenteral administration, however, has been shown to cause major defects to

developing rat and mouse foetuses (Samarawickrama & Webb 1979).

Many of the toxic effects brought about by Cd are the result of an effect on Zn metabolism, and in some cases administration of Zn can ameliorate the toxic effects of Cd (Gunn et al 1963). Zn, which is known to be a co-factor to over two hundred enzymes, is important in many diverse cellular processes. Of those elements encountered at the active site of enzymes, Zn is the only one which participates with representatives of all six classes of enzyme (oxidoreductases, transferases, hydrolases, lyases, isomerases and ligases - IUB classification). The key role of Zn in enzyme activity is considered to be in the formation and stabilisation of protein secondary structure, often through the formation of "Zn fingers" (Miller et al 1985). Therefore, Zn has a key role in the metabolic and proliferative status of the cell, via actions on DNA replication, RNA transcription, and protein synthesis and degradation (Vallee 1987). Displacement of Zn by Cd can inactivate Zn-requiring enzymes, and therefore can have a dramatic effect on cell metabolism.

II. ATTEMPTED CLONING FOR METAL RESISTANCE IN BACTERIA.

Introduction.

The toxic metals contained in various industrial wastes can become the limiting factor for bacterial growth, and thus inhibit the degradative organisms which would otherwise be instrumental in detoxifying and breaking down other toxic molecules (Omberek et al 1985). Disposal of such heavy metal contaminated wastes into the sewage system can also result in decreased bacterial activity at water treatment works (Randall 1984), therefore slower breakdown of domestic wastes.

Several approaches have been made in an attempt solve this problem (Macasckie et al 1986, Kiff & Little 1985). One proposed method, that of removal of the metal ion prior to disposal, will be dealt with later. The approach outlined here is an attempt to genetically modify bacteria to induce metal tolerance.

The test organism used in these experiments was the bacterium Escherichia coli, since this organism represents the simplest system for cloning and manipulation of genes. The means to attempt to bring about heavy metal resistance was to clone into E. coli a synthetic gene encoding a metal-binding protein. The structure of the protein product of the synthetic gene mimics that of metal-binding polypeptides $(gammaEC)_nG$, produced by higher plants in response to challenge with heavy metals. Its structure is shown in Figure 1.

Met-Glu-Cys-Glu-Cys-Glu-Cys-Gly

Figure 1. The synthetic protein.

This protein differs from $(gammaEC)_nG$ in that whereas in the plant product, glutamate and cysteine residues are linked via gamma-glutamyl carboxamide bonds, the product of the synthetic gene results from the translation of mRNA, and therefore contains exclusively alpha-carboxamide linkages. Details of construction of the cloning vector (pHI-28) can be found in Appendix 1. The gene was cloned in front of the pho A promoter and signal sequences. Pho A, part of the pho regulon of E. coli, encodes a periplasmic alkaline phosphatase under the control of the pho A promoter. The primary gene product has a 21 amino acid leader sequence (Michaelis & Beckwith 1982: 436 fig.2), which has been shown to direct the peptide to the periplasmic space, where it is then cleaved (Inouye et al 1982 ; Michaelis et al 1983). Expression of the pho regulon is under control of external phosphate levels, being switched on in conditions of phosphate deprivation. Expression has been shown to be maximal 15 to 20 min after the onset of phosphate deprivation (Case et al 1986). It was thus hoped that induction of translation of the synthetic protein product (by growth in a low phosphate medium) would give rise to a fusion protein which would be directed to the periplasmic space. Metal ions could therefore be chelated in the periplasmic space by the synthetic gene product, allowing growth in high concentrations of heavy metals, and furthermore, removal of metals from solution. If the cell produces the synthetic

product, there may also be the possibility of extraction of the protein for use in the construction of metal affinity matrices, which will be discussed in Section II.

Toxic metal resistance in bacteria.

Naturally occurring mechanisms which bacteria have evolved in order to grow in toxic metal-contaminated environments are generally plasmid-encoded (Miller & Harmon 1967). However, bacteria are also able to adapt physiologically to increasing concentrations of Cd as well as other metals, via changes in cell wall transport mechanisms, and the induction of repair enzymes (Mitra & Bernstein 1977). Production of metal-chelating molecules by prokaryotic cells is found in species of cyanobacteria, some of which have been shown to produce class II metallothioneins (Olafson et al 1988). Higham et al (1984) have demonstrated the existence of a Cd associated protein in Pseudomonas putida, although this differs a great deal from all of the metal-chelating proteins so far isolated.

Plasmid-encoded metal resistance in bacteria is more often associated with modification of ion transport systems. For example, the Staphylococcus aureus energy-dependant Mn transport system (by which Cd enters the cell) is modulated by the plasmid-encoded cad A and cad B genes in such a way that Cd ions are expelled from the cell and do not accumulate (Smith & Novick 1972). E.coli has not been demonstrated to produce a metal-chelating protein, therfore, it is necessary for successful cloning of the synthetic gene that the cell is able to deal

with the synthetic protein. This is dependant on several factors: The gene must be properly transcribed and translated (This has been demonstrated for other protein fusions using the same cloning vector ; P. Lee unpublished results), and the protein produced must not interfere with other cellular processes. If transport to the periplasm is facilitated there is greater chance of metal ion chelation and detoxification without interferance of the toxic metal on other cellular processes.

Materials and methods.

Media and buffers.

1. 2XL medium (for 1 litre).

   20 g trypticase

   10 g yeast extract

   1 g NaCl pH 7 with NaOH

   (autoclaved)

   10 ml 20 % (w/v) glucose.

2. Low phosphate medium.

   (Gerdes and Rosenberg 1973)

   50 mM tris, pH 7

   10 mM KCl 0.4 mM $MgSO_4$

   10 mM $(NH_4)_2SO_4$

   0.1% (w/v) yeast extract

   20 mM glucose

   1 mM methionine

   E. coli RR1 Requirements:

   added as 5 ml of

   0.2 g proline

   0.1 g leucine

   0.5 mg thiamine

3. S.T.E. buffer.

   10 mM tris, pH 8

   100 mM NaCl

   1 mM EDTA, pH 8

4. 20X SSC (for 1 litre).

   175.3 g NaCl

   88.2 g citric acid

   pH 7.0 with 10 N NaOH

5. Restriction buffer (medium)

50 mM NaCl

10 mM tris Cl, pH 7.5

10 mM $MgCl_2$

1 mM Dithiothreitol

6. T.E.buffer, pH 8

10 mM tris Cl, pH 8

1 mM EDTA, pH 8

Preparation of frozen protoplasts of E. coli (competent cells).

E. coli strain RR1 was made competent for transformation using the protocol of D. Denny (Unpublished results). An aliquot (2 ml) of an overnight culture of E coli was added to 100 ml of pre-warmed 2XL medium (30 °C). This was incubated at 30 °C until the OD_{600} was approximately 0.2 then 2 M $MgCl_2$ added to give a final concentration of 20 mM. Incubation was continued until OD_{600} was 0.5, and the cells were cooled on ice for 2 h.

Aliquots (50 ml) of the cells were then centrifuged at 3000 g for 10 min (M.S.E. High Speed 18 centrifuge), the supernatant was removed, and the cells resuspended in one half the original volume of ice-cold 100 mM $CaCl_2$, 70 mM $MnCl_2$, 40 mM NaAc, pH 5.5. The suspension was then incubated on ice for 40 min prior to being centrifuged at 1400 g (M.S.E. High Speed 18). The cells were then gently resuspended in 5 ml of the same buffer containing 15% (v/v) glycerol. Aliquots (0.2 ml) were transferred to 1.5 ml Eppendorfs and frozen in liquid nitrogen (5 min).

The competent cells were stored at -80 °C.

Transformation of competent cells.

Prior to transformation of cells with the pHI-28-plasmids, the transformation efficiency of the cells was tested using unmodified pBR 322, encoding ampicillin resistance. Ampicillin-containing agar plates were made using 2XL agar with 2 ml litre^{-1} of a 50 mg ml^{-1} solution of ampicillin. The competent cells prepared as above were transformed with 12.6 ng pBR 322 (in a volume of 100 µl) using the following procedure (D. Denny, unpublished results) :

1. Cells were thawed on ice and used immediately on thawing.
2. pBR 322 DNA (12.6 ng in a volume of 100 µl) was added to the cells and the mixture left on ice for 30 min.
3. The mixture was heat-shocked at 37°C for 5 min and pre-warmed (37 °C) 2XL medium added to give a final volume of 4 ml.
4. The cells were shaken for 100 min at 37 °C, and then plated on YT amp$^+$ plates.

As a control, normal E. coli cells and untransformed competent cells on 2XL amp$^+$ plates. Transformation of E. coli with the synthetic gene-containing plasmids was done using the same protocol as above. About 10 ng (precise amount not known) of plasmid DNA was added to the competent cells in a volume of 100 µl and transformed cells selected on 2XL plates containing kanamycin.

Isolation of plasmid DNA (mini-preps).

Plasmid mini-preps were made for each of the pHI-28-SG clones using the alkaline lysis method of Maniatis et al (1982) :

1. A 10 ml overnight culture of each clone was grown up in 2XL medium containing kanamycin.
2. Cells from 1.5 ml of the culture were transferred to an Eppendorf and harvested by centrifugation in an MSE Microcentaur centrifuge.
3. The pellet was resuspended in 100 µl of 50 mM glucose, 10 mM EDTA and 25 mM Tris (pH 8).
4. After 5 min incubation at room temperature, 200 µl of 0.2 N NaOH containing 1% (v/v) sodium dodecyl sulphate was added and the tube stored on ice for 5 min.
5. An aliquot (150 µl) of potassium acetate solution (made up as shown above) was added, and the tube vortexed gently in an inverted position for 10 s. This was then stored on ice for 5 min before centrifugation in an MSE Microcentaur centrifuge. The supernatant was removed and transferred to a fresh tube.
6. An equal volume of phenol/chloroform was added and, after centrifugation for 2 min, the supernatant was transferred to a fresh tube.
7. Two volumes of ethanol were added and the tube left at room temperature for 2 min.
8. After centrifugation for 5 min, the supernatant was removed and 1 ml 70 % (v/v) ethanol added. The pellet was resuspended by vortexing for 2 min and re-pelleted by centrifugation. This

was repeated and the pellet dried under vacuum in a desiccator.

Digestion of plasmid DNA with restriction endonuclease.

Plasmid DNA from mini-preps prepared as above were restricted according to the method of Maniatis et al (1982). T.E. buffer (50 µl, pH 8) containing 20 µg ml^{-1} DNase-free pancreatic RNase was added and the tube vortexed briefly. To 10 µl of this solution, 1.2 µl of restriction buffer and 1 unit of restriction enzyme was added. This was then incubated at 37 °C for 2 h.

Gel electrophoresis of restricted DNA.

Restricted DNA was analysed by agarose gel electrophoresis using the method of Maniatis et al (1982). Gels were prepared using 1.5 % (w/v) agarose dissolved in T.B.E. plus 10 ul ethidium bromide and DNA was separated by electrophoresis in tanks with volumes of 400 ml (mini-gels) or 2 litres (full-sized gels) containing T.B.E plus 100 µl ethidium bromide. A voltage of 120 v or 50 v was applied, and the gel run for 5 h or 12 h respectively. Gels were photographed under ultraviolet light using an aperture of 1.8 and a 1 s. exposure.

Testing clones for Cd sensitivity

Clones were tested for sensitivity to Cd by growth in micro-titre plates, using the following method :

Cells to be tested were grown overnight in 10 ml cultures of 2XL medium, and an aliquot of the overnight culture (500 µl) used to initiate 10 ml

log phase cultures in the same medium. After 4 h growth, 1.5 ml of the log-phase cells was centrifuged at high speed in an M.S.E. Micro-Centaur microfuge. The cells were washed twice in sterile S.T.E. buffer, resuspended in low phosphate medium and incubated at 37 °C for 10 min After 10 min incubation, 10 µl (or 5 µl for smaller inocula) of the cell suspension was added to 990 ul aliquots of low phosphate medium (with kanamycin) containing various concentrations of Cd. Immediately on inoculation with the cells the Cd-containing medium was vortex-mixed, and 300 µl of the cell suspension was pipetted into a well of a microtitre plate. Plates were then incubated at 37 °C for 24 h and cell growth measured as a function of cell density by measuring OD_{600} using a Titretek plate reader (Flow Laboratories)

In initial tests no log phase culture was set up, the cells being treated as above thereafter.

Preparation of bacterial extracts and HPLC analylis.

Extracts were made of several of the SG clones (numbers 1, 3, 7, 11, 22 and 23) to test for production of the gene product. Overnight cultures of each clone were prepared by growth in low phosphate medium containing kanamycin and the cells harvested by centrifugation (3000 g, 10 min. MSE High Speed 18 centrifuge). The cells were resuspended in 100 µl of extraction buffer containing 36 ul of 2 mercapto-ethanol (final molarity 10.18 M) and 50 µl 3 M HCl. After sonication for 1 min

on ice (MSE Soniprep 150), cell debris was removed by centrifugation (4 min. high speed in MSE Microcentaur Eppendorf centrifuge). The supernatant was filtered though a Centricon 30 Microconcentrator filtration unit (cut off 30000 Da.) and analysed by HPLC. HPLC analysis was performed using the method described for plant cells in the following section.

Isolation of total RNA.

RNA was isolated from E. coli using the method of Kornblum et al (1987). Cells were grown in 10 ml of 2XL containing kanamycin for 14 h, 1.5 ml removed to an Eppendorf, and harvested by centrifugation (3 min high speed in MSE Microcentaur). After removal of the supernatant, 100 µl of lysis buffer was added and the cells incubated on ice for 10 min. Lysis of the cells was then completed by the addition of 100 µl of SDS. Proteinase K (10 µl of a 5 mg ml^{-1} solution) was added and the mixture incubated at room temperature for 15 min. The lysate was then frozen and thawed twice using liquid nitrogen and water at a temperature of 45 °C. After addition of 50 µl of the loading dyes, the integrity of the RNA in the lysate was checked (see below), prior to electrophoresis.

Formaldehyde gel electrophoresis of RNA.

The integrity of the RNA lysates prepared as above was tested by electrophoresis of a 6 µl aliquot on a 1 % agarose mini-gel containing 10 µl ethidium bromide. Electrophoresis was carried out at 80 V until the dye had migrated two thirds the distance from the origin of the gel.

Two distinct ribosomal RNA bands were visible on all the lysates prepared (4 were prepared), meaning that they were considered suitable for use in the rest of the experiment.

RNA species were separated on an agarose / formaldehyde gel by the method of Kornblum et al (1987). A photograph of one of these gels is shown in Appendix II.

Northern blotting of RNA gels.

Northern blots were made of four RNA gels prepared as above. The procedures used were as in Maniatis et al (1982). The gel was soaked first in several changes of water, then in 50 mM NaOH and 10 mM NaCl for 45 min. Next, the gel was transferred to 20X SSC for 1 h, prior to RNA transfer by established procedures. After transfer, the gel was washed in 3X SSC, dried in air for 2 h, and baked for 4 h at 80 °C under vacuum.

Attempted hybridisation of probe DNA complementary to the putative mRNA product of the synthetic gene was performed by workers at Los Alamos National Laboratory.

Results and discussion.

E.coli was successfully transformed with the seven plasmids which had been provided. This, added to four clones obtained as previously transformed bacteria, gave a total of eleven pHI-28-SG clones to be tested. These were labelled as; pHI-28-SG 1, 2, 3, 7, 9, 11, 14, 17, 19, 22 & 23 (Labelling was adopted from original engineering and transformation).

Restriction analysis of SG plasmids.

DNA restriction analysis of the eleven plasmids using the restriction enzyme Bam H1 is shown in Photograph 2 (Appendix II). All of the plasmids (except number 1 and also number 2, which is known to contain no insert), show the typical migration of covalently closed circular DNA. This indicates that these plasmids do indeed contain an insert which has destroyed the Bam H1 restriction site (see Appendix 1). The restriction pattern shown by clone 1 is slightly different from the others, indicating that some form of rearrangement has taken place. No further investigation of this was made.

Cd tolerance testing.

Transformed cells were analysed to determine any enhanced resistance to Cd. The results presented in Table 1 were obtained by growth of cells in the wells of microtitre plates. Growth was initiated by the addition of an inoculum which had entered the lag phase of growth (12 h

Time (h)	Clone	Cd Concentration (μM)					
		0	1	2	5	10	20
t=0 h	1	0.038 3.3×10^{-3}	0.038 2.3×10^{-3}	0.036 2.7×10^{-3}	0.037 3.4×10^{-3}	0.038 3.8×10^{-3}	0.031 2.4×10^{-3}
	2	0.033 1.6×10^{-3}	0.033 2.4×10^{-3}	.0355 2.4×10^{-3}	.034 2.7×10^{-3}	0.036 3.8×10^{-3}	0.038 3.5×10^{-3}
	11	0.032 4.5×10^{-3}	0.034 5.8×10^{-3}	0.034 7.7×10^{-3}	0.032 5.1×10^{-3}	0.032 6.8×10^{-3}	0.031 3.7×10^{-3}
	23	0.032 2.8×10^{-3}	0.032 3.2×10^{-3}	0.031 2.8×10^{-3}	0.034 6.8×10^{-3}	0.03 2.8×10^{-3}	0.031 3.5×10^{-3}
t=5 h	1	0.217 0.015	0.218 0.013	0.21 0.023	0.179 0.013	0.171 0.018	0.09 0.013
	2	0.19 8.2^{-3}	0.186 0.013	0.167 0.015	0.134 0.02	0.09 0.014	0.048 4.15^{-3}
	11	0.216 0.032	0.225 0.01	0.21 0.016	0.176 0.043	0.134 0.025	0.106 0.023
	23	0.208 0.013	0.212 8.5×20^{-3}	0.205 9×10^{-3}	0.177 0.01	0.129 0.013	0.096 0.017
t=20 h	1	0.197 0.011	0.208 8.2×10^{-3}	0.208 0.013	0.213 9.3×10^{-3}	0.208 3.9×10^{-3}	0.203 0.014
	2	0.185 0.013	0.196 7.7×10^{-3}	0.2 0.012	0.202 0.011	0.184 0.012	0.16 0.012
	11	0.198 0.03	0.21 0.02	0.212 0.012	0.212 0.014	0.202 0.025	0.22 0.015
	23	0.197 0.013	0.21 0.013	0.208 9.3×10^{-3}	0.203 7.8×10^{-3}	0.204 8.3×10^{-3}	0.209 0.02

Table 1. Growth of clones pHI-SG-28 in Cd concentrations of 0, 1, 2, 5, 10 and 20 μM. The experiment was done using lag phase cells, with methods as outlined previously.

incubation of a 10 ml culture). Clones pHI-28-SG 1, 11 and 23 were tested, using pHI-28-SG 2, which has no insert, as a control. For this test triplicate plating was done for each of three identically set up tests. The resulting small standard deviations demonstrate the validity of this method for tolerance testing. In further experiments plating was not done in triplicate.

The data shown in table 1 shows an apparent increased resistance to >10 uM Cd for the three clones tested (pHI-28-SG 1, 11 and 23, as measured against the control, pHI-28-SG 2). However, it was noted that pHI-28-SG 2 showed slightly reduced growth even in the absence of Cd. A possible expla nation of this is that pHI-28-SG 2, which has no insert (and therefore no signal to stop translation of the alkaline phosphatase gene - see Appendix I.), is producing excess alkaline phosphatase. This would be a drain on the cells recources and so result in reduced growth. Furthermore, when growth curves were constructed for several of the clones, including pHI-28-SG 11, apparent enhanced resistance to Cd of synthetic gene-containing clones was not confirmed (Table 2). To further investigate this, experiments were carried out with decreased inoculum size (see Materials and methods), and increased Cd concentrations. This was done in order to emphasise any effect that the synthetic gene may have on growth. The results, given in Appendix III, show that deceased inoculum size has a detrimental effect on cell growth in Cd. This may be due to each cell being exposed to a higher amount of Cd. The results showed, however, that there was no significant difference in response to Cd for clones pHI-28-SG 1, 2, 11, and 23.

Time (h)	Clone	Cd concentration (μM).					
		0	1	2	5	10	20
t=0 h	2	0.054 0.004	0.055 0.003	0.047 0.001	0.054 0.002	0.055 0.002	0.058 0.001
	9	0.055 0.5×10^{-3}	0.054 0.001	0.058 0.005	0.058 0.004	0.063 0.002	0.058 0.003
	11	0.052 0.6×10^{-3}	0.059 0.004	0.053 0.005	0.053 0	0.058 0.005	0.056 0.002
	19	0.049 0.001	0.056 0.002	0.054 0.001	0.055 0.003	0.056 0.001	0.051 0.006
t=3 h	2	0.102 0.001	0.108 0.003	0.079 0.002	0.086 0.009	0.077 0.001	0.042 0.007
	9	0.099 0.006	0.098 0.033	0.099 0.01	0.088 0.002	0.086 0.002	0.072 0.004
	11	0.095 0.006	0.102 0.004	0.087 0.01	0.083 0.004	0.08 0.004	0.07 0.001
	19	0.101 0.002	0.106 0.003	0.093 0.004	0.092 0.003	0.083 0.003	0.064 0.7×10^{-3}
t=10 h	2	0.134 0.001	0.144 0.009	0.12 0.003	0.115 0.005	0.092 0.002	0.088 0.001
	9	0.135 0.007	0.136 0.01	0.136 0.016	0.109 0.008	0.1 0.004	0.088 0.009
	11	0.13 0.003	0.136 0.008	0.118 0.015	0.104 0.013	0.092 0.008	0.081 0.5×10^{-3}
	19	0.13 0.002	0.131 0.007	0.119 0.007	0.115 0.01	0.095 0.003	0.068 0.001

Table 2. Data from growth of clones pHI-28-SG 2, 9, 11 and 19 in Cd concentrations of 1, 2, 5, 10 and 20 μM. Data was obtained using an inoculum of cells which had entered the log-phase. Cell density at times 1, 3 and 10 h were measured as described in the text. Apparent differences in growth of clone 11, as seen

A change in the experimental protocol (inoculation using log-phase cells), again failed to detect increased resistance to Cd for any of the SG clones. In order to establish any enhanced tolerance to Cd, a total of three different sets of experiments were carried out using slight modifications to the experimental protocol. Representative results obtained for several of the pHI-SG clones tested are presented in Figure 2. Full results for tests done on log-phase cells for all clones are given in Appendix III. Log-phase cells are less resistant to Cd (Appendix III), either due to increased metablolic activity in the log- as opposed to lag-phase cells, or because the cell wall is more permeable at this stage of the cell cycle. However, this presents greater opportunity for the SG product to have a beneficial effect if produced. The results presented indicate that cells transformed with the synthetic gene are not showing increased tolerance to Cd compared to cells transformed with pHI-28 without the SG insert.

HPLC analysis of cell extracts.

Although no detectable change in response to Cd was seen in the transformed bacteria, the possibility remained that the gene was being expressed, though producing no change in growth characteristics. This may occur if the protein is degraded by proteolytic enzymes before it can reach the periplasm and bind metals. It is also possible that the protein is not capable of binding Cd. This would be the case if the protein is oxidised after production to give disulphide linkages, which

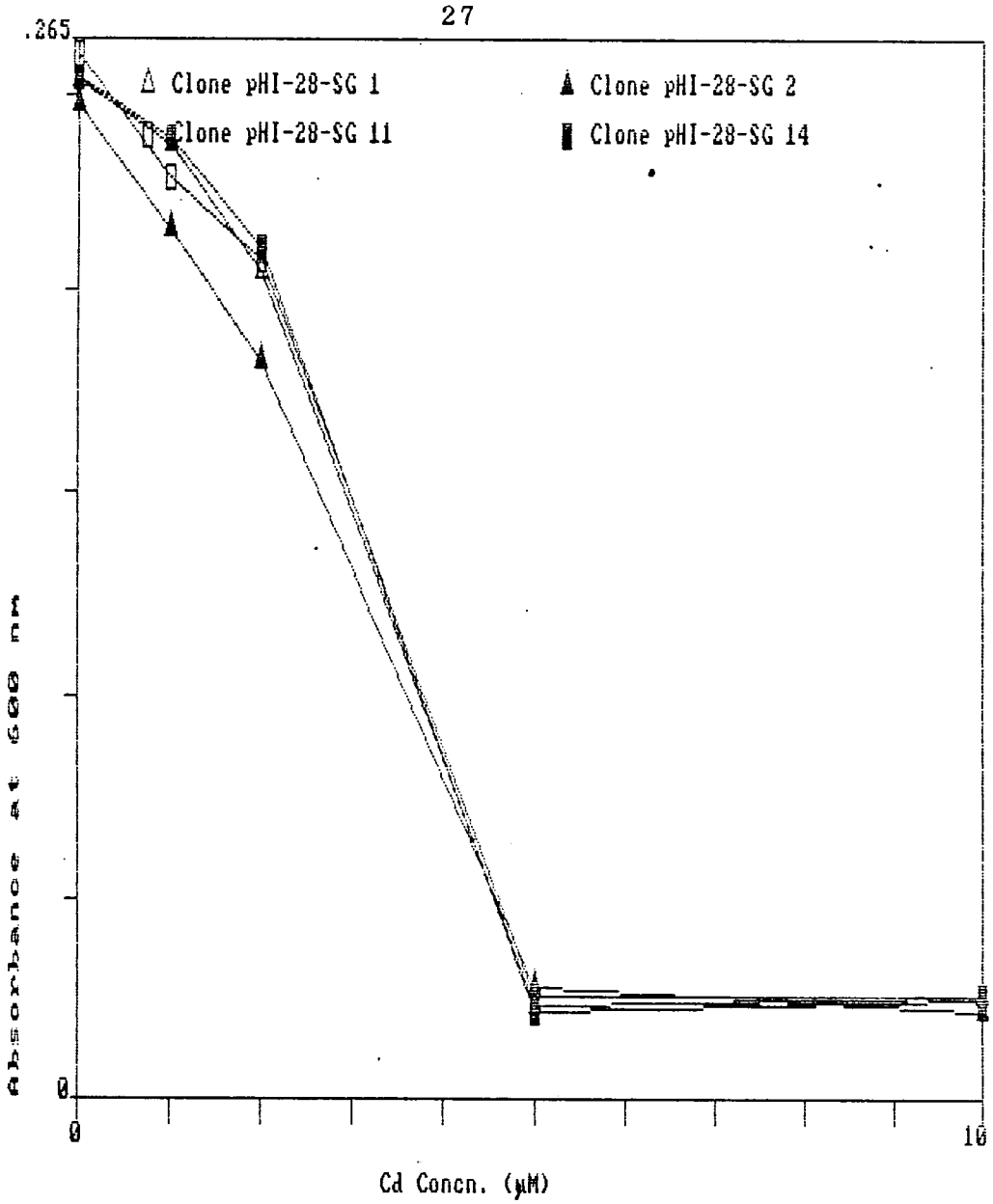

Figure 2. The response of four of the pHI-28-SG clones to Cd. Experimental procedure was as given in the text and in materials and methods, using log-phase cells. Clones 1, 2, 11 and 14 are shown, which are representative of the results obtained. Complete results are given in Appendix III.

would not allow the thiol groups to co-ordinate Cd. Cells were examined for production of the SG protein by HPLC analysis of cell extracts. Extracts were prepared and run as indicated in materials and methods. A typical elution profile of one of the extracts is shown in Figure 3. This shows no significant thiol-rich fraction on the acetonitrile gradient 0-20 %.(The thiol rich peptide, if produced could be expected to elute from the column at around 15 % acetonitrile - fraction 30 on the profile shown).

Hybridization to northern blots.

A complimentary DNA which would hybridise to RNA produced by the synthetic gene was used to attempt hybridization to Northern blots prepared from total RNA from each of the eleven clones. Hybridization was not performed by the author and the results of this are not shown. No hybridization to the probe DNA was recorded, suggesting that the gene was not being expressed. This may indicate that the gene is in the incorrect orientation.

Conclusions

The results indicate that the gene encoding the synthetic protein may have been in an incorrect orientation in each case. This would suggest that there has been selective pressure against those clones in which the gene is inserted correctly. Since the phoA promoter should not be active under normal (non-phosphate limited) conditions there is no

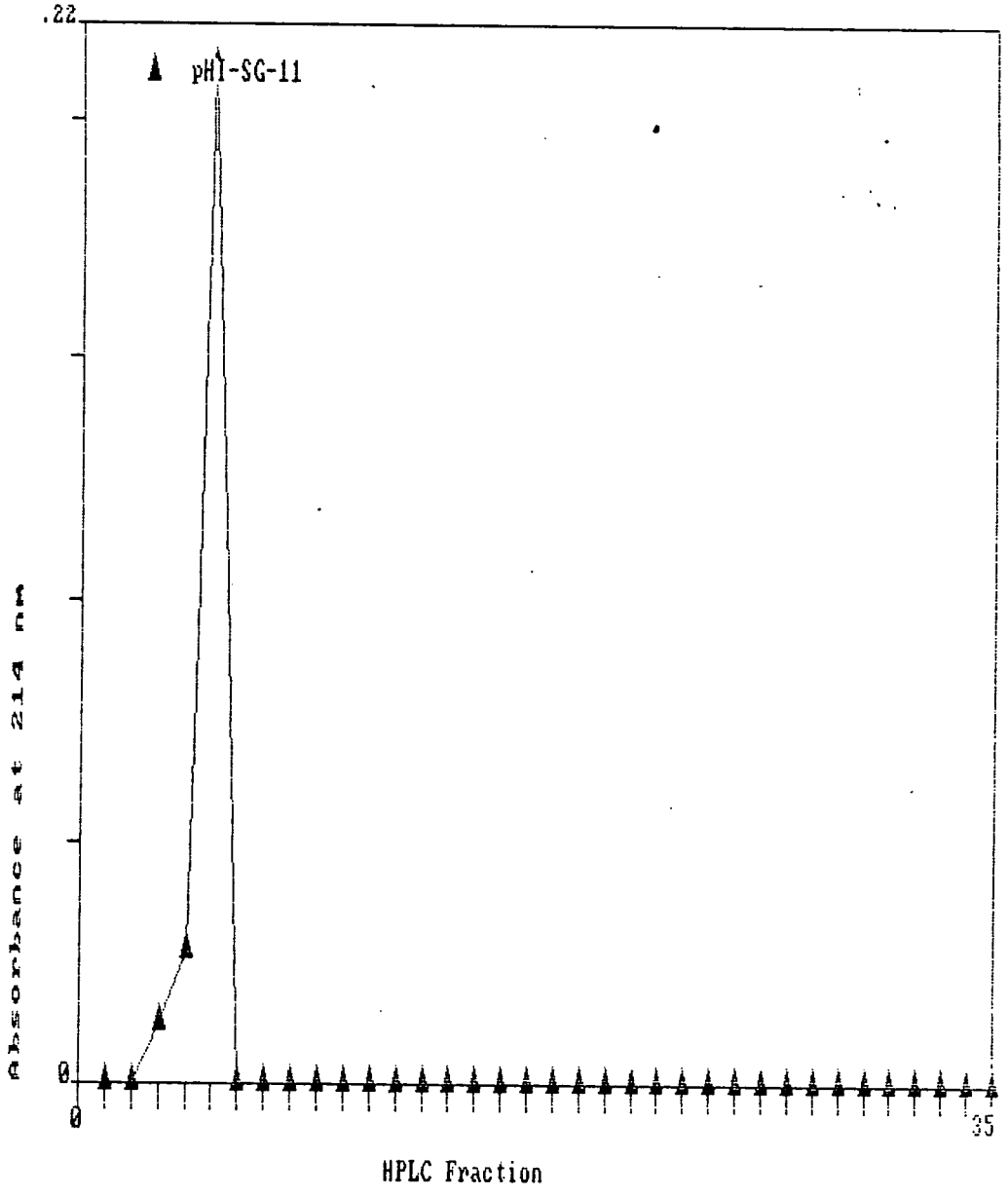

Figure 3. HPLC analysis of an extract of clone pHI-28-SG 11. HPLC analysis and Ellman's testing of fractions was performed as outlined in materials and methods. Five of the clones were tested, pHI-28-SG 1, 11, 7, 22 and 23. All gave results similar to above.

apparent reason why this should occur. One possible explanation of this effect is that the phoA promoter is allowing "leaky" expression of the gene in non-phosphate limited conditions. In these circumstances production of a metal-chelating protein may sequester essential trace elements, and therefore kill the cell.

Further experiments are planned to test the possibilities of cloning metal-resistance genes in microorganisms. Attempts to clone a metal-chelating protein in E coli have not yet proved successful. In a future series of experiments it is hoped to use a different host organism. Yeast cells of the species Schizosaccharomyces pombe are known to produce metal-binding peptides identical in structure to those produced by plants. Mutants, which are unable to produce these peptides, and are therefore hypersensitive to Cd, have been obtained at Durham (A gift from Y. Hayashi, Institute of Developmental Research, Japan - see Mutoh and Hayashi 1983). Cloning of the synthetic gene into one of these mutants could be performed in an attempt to re-establish metal tolerance. Given that the cell is known to produce a structural analogue of the protein which is implicated in metal detoxification, the synthetic product may be dealt with successfully. This will lead to a greater understanding of the tolerance mechanism in S.pombe and should lead to more successful means of cloning for metal resistance in other organisms. Vectors are available for cloning in yeasts which place genes under control of the metal regulatory elements of the CUP 1 locus of Saccharomyces cerevisiae (Theile et al 1986). These may be useful in engineering appropriate expression of the synthetic gene in S. pombe.

In addition it is hoped to initiate work into the isolation of metal-regulatory elements (mre's) from prokaryotic cells. Cyanobacteria (Synechococcus spp.) have recently been shown to produce a metal-binding protein, classified as class II metallothionein. This protein has recently been sequenced (Olafson et al 1988). This should allow the production of synthetic oligonucleotides which may be used to isolate the corresponding gene. Since these genes are known to be transcriptionally regulated by Zn and Cu (Olafson et al 1988), it may be possible in the long term to isolate metal-regulated sequences which will be active in prokaryotic cells. These would be invaluable in cloning for metal resistance in bacteria, and may possibly be useful tools for the manipulation of other genes in prokaryotes.

III. BIOTECHNOLOGICAL APPLICATIONS OF (gammaEC)$_n$G.

Introduction.

A second approach to dealing with metal-contaminated wastes is to employ some means to remove the toxic heavy metals prior to disposal. Currently, toxic metals are not dealt with specifically and this often results in large amounts of Cd, Zn and Cu being dumped either at sea or in land-fill sites.

Current investigations are underway to allow an increase in the amount of sewage sludge which can be dumped at a given site. It is also desireable to use the organic content of sewage sludge via application to agricultural land (Vermes 1985) and to mining and waste-disposal sites prior to reclaimation (Sopper 1985). Strict limits are now in force to restrict the amounts of toxic metals which can be applied in such cases, making toxic metal content a critical factor in how much waste can be applied. With the British government urging that the "best available technology not entailing exessive costs" should be employed for the removal of Cd, new methods of extraction are being investigated. Methods under investigation include biological techniques such as polyacrylamide-immobilised organisms to accumulate Cd (Macaskie et al 1986), as well as chemical and engineering techniques (Matis and Zouboulis 1985).

This current investigation applies to the use of a novel system for the removal of Cd, Cu and Zn from waste streams. The aim is to immobilise metal-binding peptides, isolated from over-producing plant

cells. These peptides can then be used to strip metals from contaminated wastes.

Structure and function of $(gammaEC)_nG$.

Metal-binding peptides isolated from higher plants (Robinson and Jackson 1986), some algae (Gekeler et al 1988) and the fission yeast S. pombe (Murasugi et al 1981) have the structure $(gammaEC)_nG$ (where n can = 2-5). Gamma- linkages between the glutamate and cysteine residues distinguishes these molecules from primary gene products and suggests that they are probably products of a biosynthetic pathway. This has been confirmed using synthetic oligonucleotides to identify any mRNA sequences which could encode protein precursors of $(gammaEC)_nG$ (Robinson et al 1988). $(gammaEC)_nG$ were first isolated from S.pombe cells and subsequently from metal-tolerant plant cells, where they have been demonstrated to bind more than 80 % of cellular Cd (Jackson et al 1987). They are rapidly induced by Cd and Cu exposure, and may also be involved in the detoxification of Zn, Pb and Hg.

Although a role in metal detoxificaton has been established, these molecules are produced constitutively in both sensitive and resistant cells indicating that they may have another function in the cell. In addition, toxic trace metals have not been at high enough concentrations in the environment, for sufficient time, to bring about the evolution of specialised detoxification systems (Karin 1985). There has been some speculaton that $(gammaEC)_nG$ may be involved in sulphur (S) metabolism (Robinson 1988). This is based on several observations of the APS

sulphotransferase system for assimilatory sulphate reduction. A carrier molecule, which may be $(gammaEC)_nG$ has been isolated from Chlorella spp. (Tsang and Schiff 1978). Also, in higher plants a sulpho-accepter molecule has been identified which is structurally similar to glutathione, but of a higher molecular weight (Schiff and Fankhauser 1981). Cd-peptide complexes isolated from both S. pombe and higher plants have been shown to include acid-labile S (Reese et al 1988, Murasugi 1983). Other postulated constitutive functions for $(gammaEC)_nG$ include metal ion homeostasis and a role as a functional analogue of glutathione (Robinson 1988).

Biotechnological applications of $(gammaEC)_nG$.

Whatever other constitutive functions $(gammaEC)_nG$ may have in plant cells, their role in metal detoxification is now well established. It is this property which this investigation seeks to exploit. Peptides were isolated from plant cells and used to construct metal-affinity matrices. This was done by immobilising the peptides onto cyanogen bromide-activated agarose beads. It is hoped that under immobilised conditions the metal-chelating ability of the peptides will be retained. Therefore, the peptides will be capable of stripping metals from solutions passed through the matrix. This would have direct application in water treatment as mentioned previously, although alternative support matrices may be more useful. Comparative efficiency of chelation of various metal ions can be quantified by determination of K_d and Q_{max}, as extrapolated from the kinetics of immobilized enzymes.

Other potential applications exist. It has been postulated that immobilised peptides may be useful in the construction of biosensors. If binding of metals alters the conformation of the peptides, this interaction may be used in creating the impulse required in the manufacture of biosensors. No attempt was made in the present study to assess the feasibility of this application.

As well as the biotechnological considerations of the work, the data produced should be useful in determining some of the physiological characteristics of the peptides. Such information is necessary to understand the interactions of (gammaEC)$_n$G in the plant cell.

Materials and methods.

1. Maintenance of plant suspension cultures.

The Cd-resistant cell line used in these experiments was kindly provided by workers at Los Alamos National Laboratory, New Mexico, where the original in vitro selection and isolation of resistant cells had been performed (Jackson et al 1983). Suspension cultures of Cd-resistant Datura innoxia (Cd-300) were maintained in the dark in 100 ml batch suspension cultures as described by Jackson et al (1983). Cells were diluted every 48 h. to maintain a concentration of cells in the logarithmic growth phase between 2×10^5 and 2×10^6 cells ml^{-1}.

2. Exposure of cells to Cd and radiolabelling.

Cells from which $(gammaEC)_nG$ was to be extracted were exposed to 300 uM Cd at the time of dilution and extraction done 48 h after exposure. Labelling of $(gammaEC)_nG$ was performed at the time of exposure by adding 0.1 μCi ml^{-1} carrier-free ($^{109}CdCl_2$) (E.I. du Pont de Nemours and Co., Inc., NEN Products, Boston.),to the growth medium.

3. Cell extraction and isolation of Cd-binding polypeptides.

Cells were removed from the medium by centrifugation at 800 g for 3 min prior to being resuspended and washed in ice-cold buffer containing 10 mM tris, pH 7.4, 10 mM KCl, 1.5 mM $MgCl_2$, and 20 mM 2-mercaptoethanol. The pellet was then suspended in 5 ml of this buffer and disrupted by homogenisation in an Elvehjem Tissue Grinder with a teflon pestle. The resulting homogenate was centrifuged for 20 min at 7000 g

and 4°C. The supernatant was collected and subjected to either gel filtration on columns (2.2 x 100 cm) of Sephadex G-50 (fine), or to heat-denaturation at 60°C followed by centrifugation at 10 000 g for final purification of peptides. Eluate from G-50 columns was collected in 10 ml fractions. These samples were assayed for the Cd-containing fraction by atomic absorption spectrophotometry or by liquid scintillation counting (in cases where radioactive ^{109}Cd had been added to the growth medium).

Atomic absorption was read using a Perkin Elmer HGA-500 model. Cd was detected at a wavelength of 228.8 nm. Detection of radioactive ^{109}Cd was by liquid scintillation spectrometry after addition of 3 ml of Formula 963 Aqueous Counting Cocktail (New England Nuclear, Boston, MA) to 100 µl of each 10 ml fraction. Counting was done using a Packard Instruments liquid scintillation counter. The Cd-containing portion of the collected fractions was pooled, the volume measured and this material concentrated using an Amicon Model 52 Ultrafiltration unit (Amicon Corporation Danvers MA) with a YM 2 filtration membrane (cut-off 1000 Da), to give a final volume of 15 ml.

Purified Cd-binding peptides obtained in this way were finally perpared for coupling to activated Sepharose by changing the buffer to $NaHCO_3$ using Pharamacia PD10 desalting columns (Pharmacia Fine Chemicals, Uppsala, Sweden). These are pre-packed G-25 Sephadex gel-permeation separation columns (1 x 5 cm). Equilibration of the column with the appropriate buffer was followed by loading of the sample in a volume of 2.5 ml. Elution of the void volume, which contained the

peptide/Cd aggregate, was performed by the addition of a further 3.5 ml of buffer, thus leaving the purified peptides in the new buffer, free of cysteine or glutathione contamination.

An aliquot of the purified preparation (200 µl) was removed and acidified for reverse phase HPLC as described below, in order to give an indication of the amount of peptide in the preparation as well as its composition in terms of the various forms (n=2, n=3, et.c.).

4. Activation of Sepharose.

Sepharose 4B (Pharmacia Fine Chemicals) was activated using cyanogen bromide, as devised by Porath (1972). The following method was used to prepare duplicate control and peptide matrices:

1. Sepharose 4B slurry (10 ml) was washed using 1 500 ml of distilled water though a scintered glass filtration unit.
2. Washed Sepharose (30 g) was suspended in 30 ml of 5 M KPO_4 and the slurry chilled on ice for 10 min.
3. An aliquot (12 ml) of a 100 mg/ml solution of cyanogen bromide was added dropwise, with gentle stirring (using a magnetic stirrer), for 4 min, and then allowed to react a further 8 minutes on ice.
4. The reacted Sepharose was washed with 300 ml of 250 mM $NaHCO_3$, then 10 g of this material added to each of two 50 ml screw-capped Falcon tubes.
5. Either purified peptide preparation (10 ml) or 10 ml of $NaHCO_3$ buffer for control matrices was added to the activated Sepharose.

The mixture was incubated with gentle agitation (on a rotary mixer) for 12 h at 25 °C.

5. Column preparation.

Matrices were set up in 20 ml Bio-rad Econo-columns (Biorad Laboratories, Ltd., Watford, England.)

1. Reacted Sepharose slurries were poured into the prepared columns and loading eluates collected. An aliquot (40 ml) of 250 mM $NaHCO_3$, followed by 50 ml $NaHCO_3$ /1 M NaCl was then passed though the column and the eluate collected and combined with the loading eluate.

2. Ethanolamine (50 ml of a 1 M solution, at pH 9) was loaded into the column, and allowed to react for 14 h. (This should react with any free groups which have not reacted with CNBr)

3. Ethanolamine was washed out of the column using 50 ml $NaHCO_3$ (pH 9), followed by 50 ml of water.

4. The column outflow was directed to a fraction collector and 3ml samples collected. Na oxalate (200 mM/pH 2) was then passed through the column and the resulting fractions assayed for Cd using dithizone, details of which are given below. Cd-containing fractions were pooled and added to the initial buffer washes. The pooled washes were retained in order that total eluted peptide could be estimated (by assaying for thiol groups - see below) and subtracted from that obtained for the peptide sample loaded.

6. Construction of glutathione matrix

An affinity matrix was made using glutathione adopting the same procedures as outlined above for peptide matrices. Glutathione (Sigma Chemical Co.) was dissolved in 10 ml $NaHCO_3$ to give a 40 mg ml^{-1} solution. Cd was added to this to give a concentration of 35 mM, and this added to 10 g of activated Sepharose. A control matrix was made at the same time using 10 ml $NaHCO_3$. The amount binding to Sepharose was calculated from the amounts contained in the washes, as for the peptide matrix.

7. Ellman's test for thiol groups.

The thiol test used in these experiments, in order to quantify the amount of $(gammaEC)_nG$ in a given sample, was devised by Ellman (1959). The assay was scaled down for use on micro-titre plates, 150 µl of reagent being added to 150 µl of test solution in the wells of the plate. Samples were read for absorbance at 214 nm after 20 min. Glutathione standards, for calibration, were assayed using the same procedure.

8.Estimation of peptide content of columns.

Estimation of the amount of peptide loaded onto each column was done by determination of the total number of thiol groups present, as

outlined above. The assay was calibrated with glutathione (0 to 100 μg ml^{-1}) reacted in the same buffer (tris-Cl, pH 9) at the same pH (pH 1.5) as the reduced peptides. The values thus obtained could then be used to calculate the relative amounts of peptide present in mg-equivalents of glutathione. The buffers of each sample were changed to tris-Cl, pH 9 using Pharmacia PD-10 columns, which also served to remove any residual glutathione and free cysteine.

9. Determining the pH of Cd dissociation from immobilised peptides.

To investigate the characteristics of pH dissociation of immobilized peptides, an aliquot of unstripped matrix (i.e. the matrix preparation at stage 3. of the preparation procedure, prior to running water through the column) was used. Na_2HPO_4-Citric acid buffer was prepared at a range of pH's by mixing various amounts of 0.2 M Na_2HPO_4 and 0.1 M citric acid in a total volume of 20 ml (Dawson 1968). The pH was recorded, an equal volume of tris pH 9 was added, and the pH re-recorded. This was done to determine what the pH of the buffer would be after the addition of an equal volume of unstripped matrix in tris pH 9. To test the pH dissociation characteristics of the immobilised peptides, 200 μl of buffer solution was added to 200 μl of matrix suspended in tris pH 9 in an Eppendorf tube. The mixture was vortexed gently for 30 s, and the matrix was removed by centrifugation (1 min MSE Microcentaur). An aliquot (150 μl)of the resulting supernatant was removed and the Cd concentration measured using dithizone (see below). A series of calibration curves made at the various pH's used showed no

significant effect on the dithizone assay. Cd dissociation was measured at pH 2.30, 2.80, 5.27, 6.17, 7.27 and 7.87. By assuming complete dissociation at pH 2.30 and complete association at pH 7.87, values were calculated as percentage of Cd bound.

10. Reverse phase HPLC analysis of isolated peptides.

Reverse phase high performance liquid chromatography was used to separate the various forms of $(gammaEC)_nG$. An aliquot (1 ml) of each of the extracts to be analysed was reduced by the addition of 1/4 volume of 1 M HCl, and filtered though a Centricon 30 micro-concentrator (Amicon, cut off 1000 Da). Reverse phase HPLC analysis (Beckman Instruments HPLC system) was then performed using 250 µl of this material. Samples were applied to a 250 x 4.6 mm column of nucleosil C-18 (BioRad). Samples were eluted with a 20 ml linear gradient of 0.1% (v/v) triflouroacetic acid (TFA) to 0.1% (v/v) TFA containing 20% (v/v) acetonitrile, at a flow rate of 2 ml/min. Fractions of 1 ml were collected and 500 µl of each reacted with 150 µl of Ellman's reagent. After 20 min absorbance was measured at 210 nm and these readings calibrated using glutathione. Results of HPLC analyses are presented in mg equivalents of glutathione.

11. Dithizone assay for Cd, Zn, and Cu.

Since characterization of the affinity matrices required repeated analysis of column eluate for free metals, it was considered worthwhile to adapt the simple chemical assay utilising dithizone (diphenyl-

thiocarbazone) for use with a microtitre plate reader. The results of this work are presented in Appendix IV. Cd was assayed by the addition of 200 µl of test solution to 100 µl of 50 µg ml^{-1} dithizone dissolved in 1 M NaOH. The resulting colour was detected using a Titertek Multiskan plate-reader (Flow Laboratories). Similarly Zn was detected using 50 µg ml^{-1} dithizone and monitoring absorbance at 540 nm, and Cu using 100 µg ml^{-1} and monitoring absorbance at 620 nm. In the case of both Cu and Zn, dithizone was dissolved in acetonitrile.

12. Determination of binding characteristics of $(GammaEC)_nG$.

Two methods were used to determine binding affinities of the isolated peptides. Data was first generated using the Sepharose-bound peptides in the columns in which they had been originally prepared. In this case, the amount of metal ion binding was monitored using the "breakthrough" method. Data was generated by flowing solutions containing different metal ion concentrations through the matrix until the point of breakthrough was detected, by analysis of the eluent with dithizone. The second method of analysis was to use aliquots of the matrix in suspensions of various concentrations of metal ion, data being generated by determining the amount of metal removed from solution. This was achieved by spinning down the Sepharose and analysing (by atomic absorption spectrophotometry) the amount of metal remaining in solution. In each case, since Sepharose will bind some metal, the amount bound to the peptides was determined by comparison of control- and peptide-matrices. A complete treatment of data is given in Appendix

13. Summary of methods used.

In summary, three metal affinity matrices were made. The first, prepared using glutathione, used 10 g of Sepharose. The second (using 6 g of Sepharose) was prepared using $(gammaEC)_nG$ isolated from Datura innoxia. This was the extract from 80 ml of cells, purified by G-50 gel filtration. A third matrix (5 g of Sepharose) was made using the extract from 200 ml of cells, purified by heat denaturation.

For column tests the total weight of Sepharose was used in each case. For suspension tests 0.8 g aliquots of matrix were suspended in 4 ml of distilled water, and 200 µl of this suspension added to each concentration of metal. Data was then corrected for the amount of metal binding per g of matrix, and the amount of metal bound per mg of peptide subsequently estimated.

Apparent Q_{max} and K_D values were found for Cd, both using data generated in columns and in suspension. For column tests, solutions of 1, 5, 10, 15 and 25 µM Cd were passed run through the column. Preliminary data for Cu and Zn were also generated in this way. For suspension tests, metal ion concentrations of 1, 3, 5, 10, 15 and 25 µM were used, and readings taken at 15 min, 1 h and 2 h. Experiments were performed in 10 mM tris (pH 9). Incubation of suspensions was at 37 °C unless otherwise stated.

Results.

Quantification of the amount of peptide and GSH bound to Sepharose.

Amounts of peptide and GSH immobilised onto both the denaturation-prepared peptide matrix and the GSH matrix were calculated as follows :

A known amount of GSH (400 mg) was allowed to react with 10 ml of 35 mM Cd in a volume of 10 ml. This amount of GSH was chosen since it was hoped that the amount of GSH immobilized would compare with the published maximal values for immobilization of proteins such as trypsin, which are around 20 mg per g of Sepharose. Cd was added since it was hoped that immobilisation of GSH in such a conformation as enabled maximal metal ion chelation could be achieved in this way. The amount of the added GSH which actually bound to the Sepharose on activation was quantified by analysis of the amount of GSH eluted in the various washes performed at the time that the matrix was set up. The amounts eluted from the matrix were quantified by testing for thiols using Ellman's reagent. Calibration curves were set up in each of the buffers for which elution of GSH was to be tested.

The results produced were as follows :

Amount loaded : 400 mg

Amount detected in initial eluate and $NaHCO_3$ wash : 306.4 mg
(volume 15.8 ml)

Amount detected in NaCl wash and Oxalate strip : 35.28 mg

(volume 98 ml)

The amount bound to 10 g of matrix was therefore 58.32 mg

For the peptide matrix, anestimate of the total peptide was first made using Ellman's reagent. The amount present was calibrated using standard curves made up using GSH. This gave an estimate of peptide in "milligram equivalents" of GSH. As for the GSH matrix the total amount immobilized onto Sepharose was calculated by subtraction of the amount eluted from the matrix at the time of setting up the column. The amounts calculated in each case were :

Amount of peptide in 10 ml of the initial loading buffer : 10.5 mg

Total amount eluted (pooled 107 ml) 2.1 mg

Estimated total amount bound (in mg equivalents of GSH) 8.396 mg

No estimate was made of the amount of G-50 purified peptide immobilized to Sepharose. An amino acid analysis of the loaded sample and collected eluent is required before this can be quantified. Amino acid analysis of the samples should allow a more accurate estimate of the amount bound

The results of all of the experiments which used the three matrices prepared are presented in Figures 1 to 15.

Reverse phase HPLC profiles : determination of molar ratios for Cd and Zn binding to $(gammaEC)_nG$

The reverse phase HPLC separation shown in Figure 3 was of an aliquot (150 µl) of the crude extract which was subsequently heat denatured and used to form the second affinity matrix. This reveals a ratio of the different forms of peptide to be 3 : 3 : 3 : 1, for the forms n = 2, 3, 4 and 5 respectively. Given that these 4 forms have molecular weights of 579, 771, 1003 and 1235, the "average" M_r for the preparation as a whole can be calculated as around 817. The molecular weight of GSH is 306.

The data show that the quantities of GSH and $(gammaEC)_nG$ immobilised were 16×10^{-6} M and 2.05×10^{-6} M, respectively.

Figures 11 and 12 show the binding characteristics of the matrix made using the preparation described above. This shows that at maximal binding, 1 mg of peptide co-ordinates 260 nM of Cd (Figure 11) and 315 nM of Zn (Figure 12). Maximal binding of Cd was taken to be 260 nM, as at this amount the graph levels out. Subsequent apparent steady increase in binding may reflect inaccuracy of the method used to detect Cd (atomic absorbance). Inaccuracy is encountered in the measurement of high Cd concentrations (see Figure 14). Subsequent data analysis (see Appendix V) will have the effect of emphasizing any error. The molar ratios as determined from the figures above are 4.5 : 1 and 3.8 : 1 for $(gammaEC)_nG$: Cd and $(gammaEC)_nG$: Zn, respectively.

Binding constants for Cd and Zn binding to (gammaEC)$_n$G.

The constants K_D and Q_{max} can be used to quantify metal-binding to (gammaEC)$_n$G. Apparent K_D and Q_{max} values were extrapolated from the graphs presented in Figures 6, 7, 8 and 9 (for data obtained by breakthrough) and Figures 11 and 12 (for data obtained from suspension tests). Q_{max} values were taken as the maximal amount of metal binding per mg of immobilised material. K_D values were obtained by reading from the x-axis of the graph the metal concentration which gives half maximal binding.

The apparent values obtained were :

(A) By breakthrough analysis :

1. GSH Q_{max} = 150 nM Cd / mg
   K_D = 5 uM Cd
2. G-50 Purified (gammaEC)$_n$G Q_{max} = 70 nM Cd (per quantity of peptide on 1 g matrix)
   K_D = 4.5 µM Cd
3. Heat denaturation purified (gammaEC)$_n$G Q_{max} = 168 nM Cd / mg
   K_D = 1.3 µM Cd
4. " " " Q_{max} = 190 nM Zn / mg
   K_D = 5 µM Zn

(B) By suspension method :

Heat denaturation purified (gammaEC)$_n$G only :

Q_{max} = 315 nM Zn / mg
K_D = 2.8 µM Zn

Q_{max} = 255 nM Cd / mg
K_D = 0.5 µM Cd

In addition, a preliminary test was done using the breakthrough method to determine whether the immobilised peptides were capable of binding Cu. This showed that at a feedstream concentration of 25 μM Cd, 187.5 nM of Cu was bound to the peptides. This result was not confirmed by suspension testing.

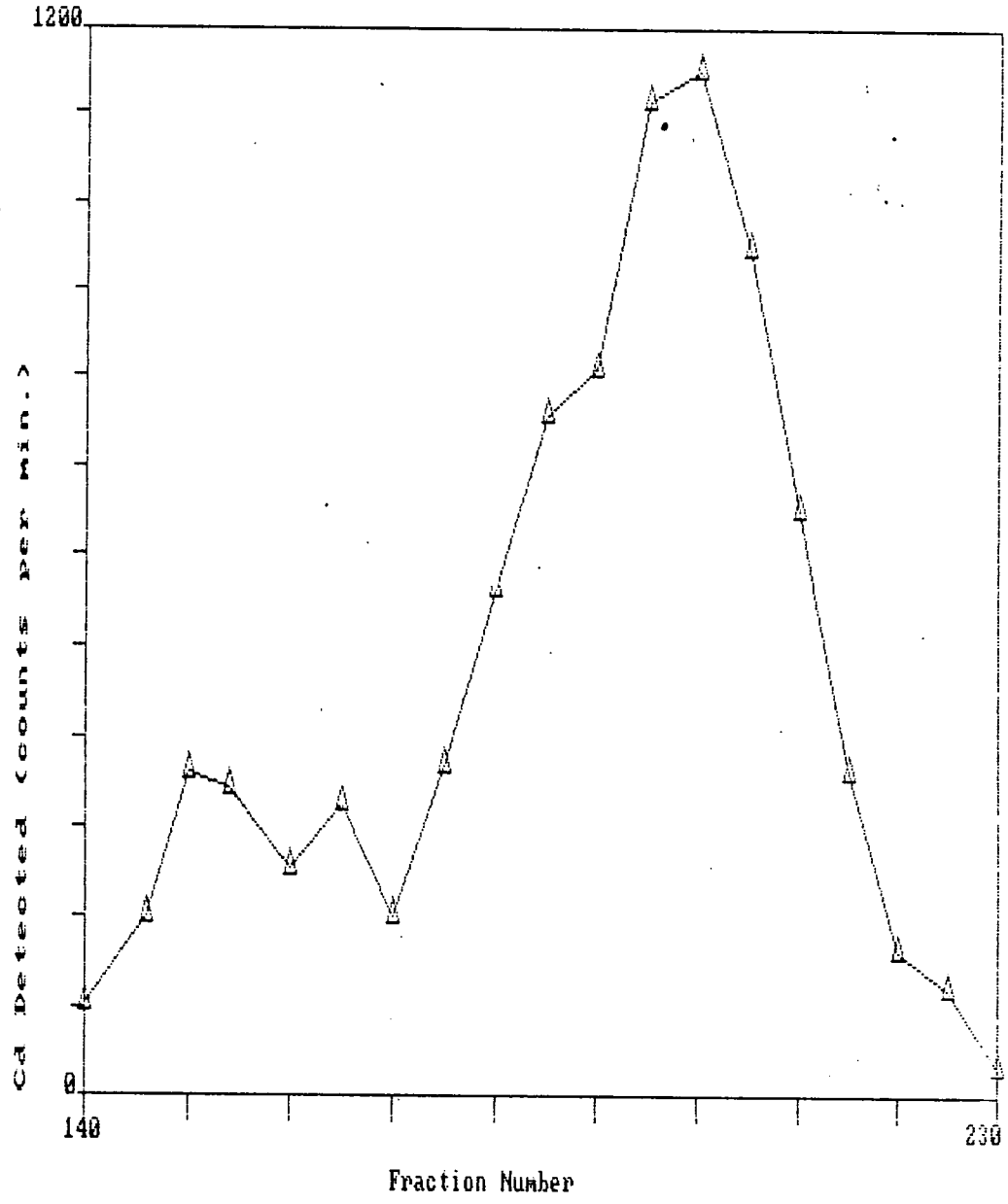

Figure 1 Profile of (gammaEC)$_n$G complexes eluting from the Sephadex G-50 column. Volumes of 8 ml were collected and measured for radioactivity as described in materials and methods. The peak shown, from fractions 148 to 220 (a volume of 570 ml from 1184 ml to 1760 ml) were pooled and concentrated as previously described. The peak shown eluted midway between the void and total volumes of the column.

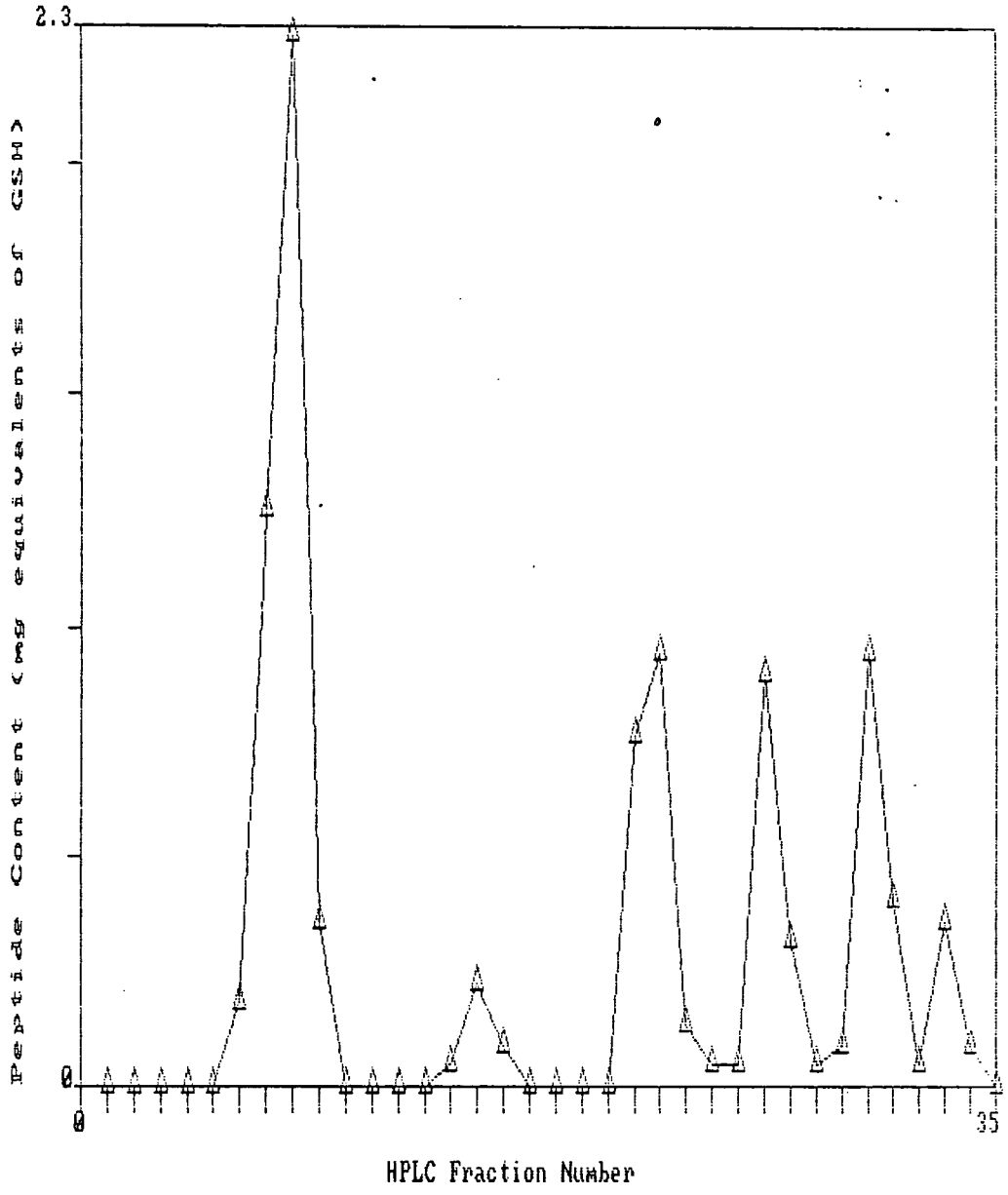

Figure 2. Reverse phase HPLC separation of (gammaEC)$_n$G purified by gel filtration on Sephadex G-50. An aliquot of the purified material was reduced, separated by HPLC and the fractions analysed for thiols by testing with Ellman's reagent. The large peak to the left represents free cysteine, 2 mercaptoethanol (from the extraction buffer), and GSH. This is followed by a small peak representing oxidised GSH. The four peaks to the right represent (gammaEC)$_n$G molecules where n = 2, 3, 4 and 5, respectively.

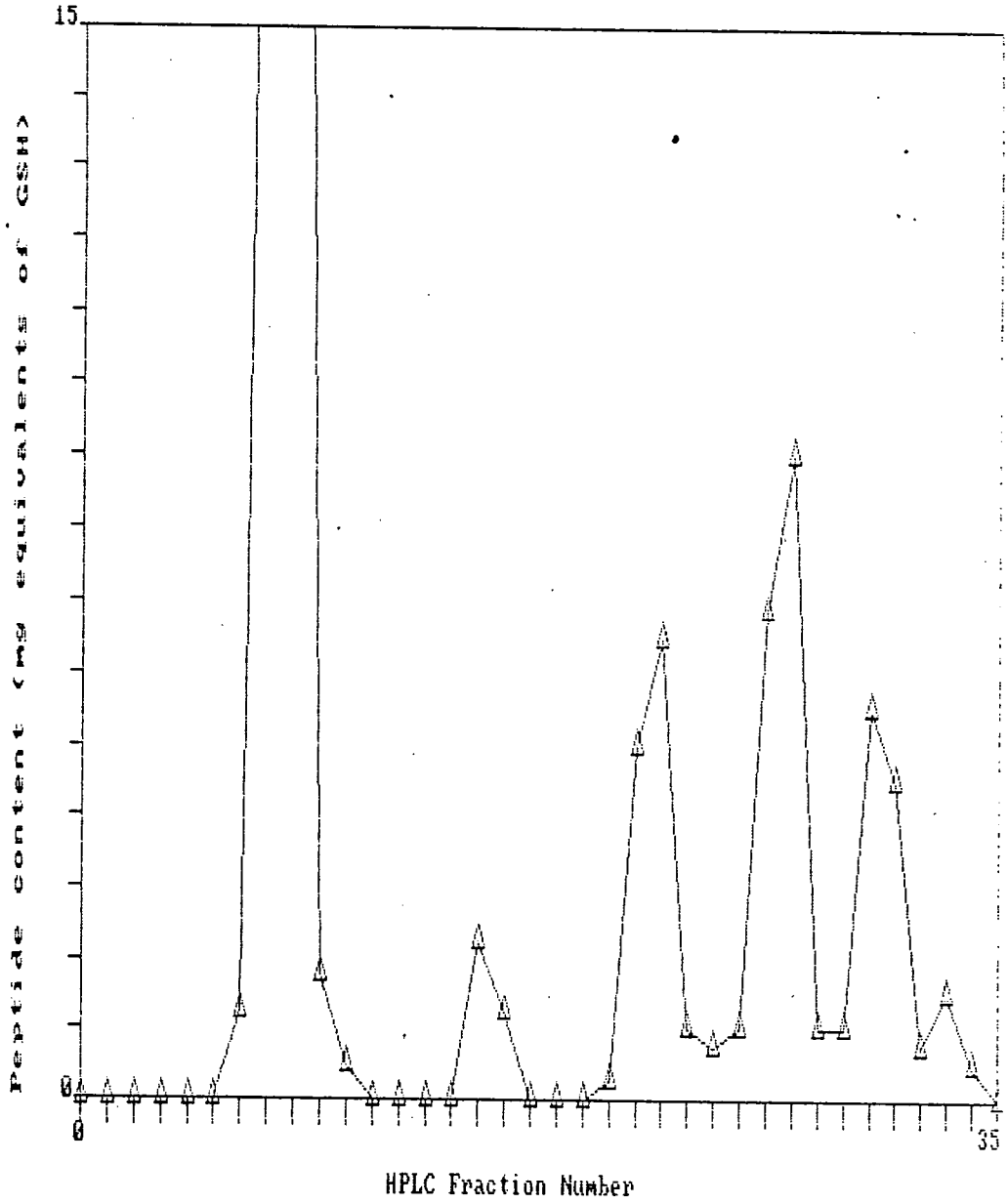

Figure 3. HPLC Analysis of a crude extract of Cd-exposed Cd-300 cells. The extract was subsequently purified by heat denaturation prior to immobilisation onto cyanogen bromide-activated Sepharose 4B. The profile shows the same peaks as described for Figure 2.

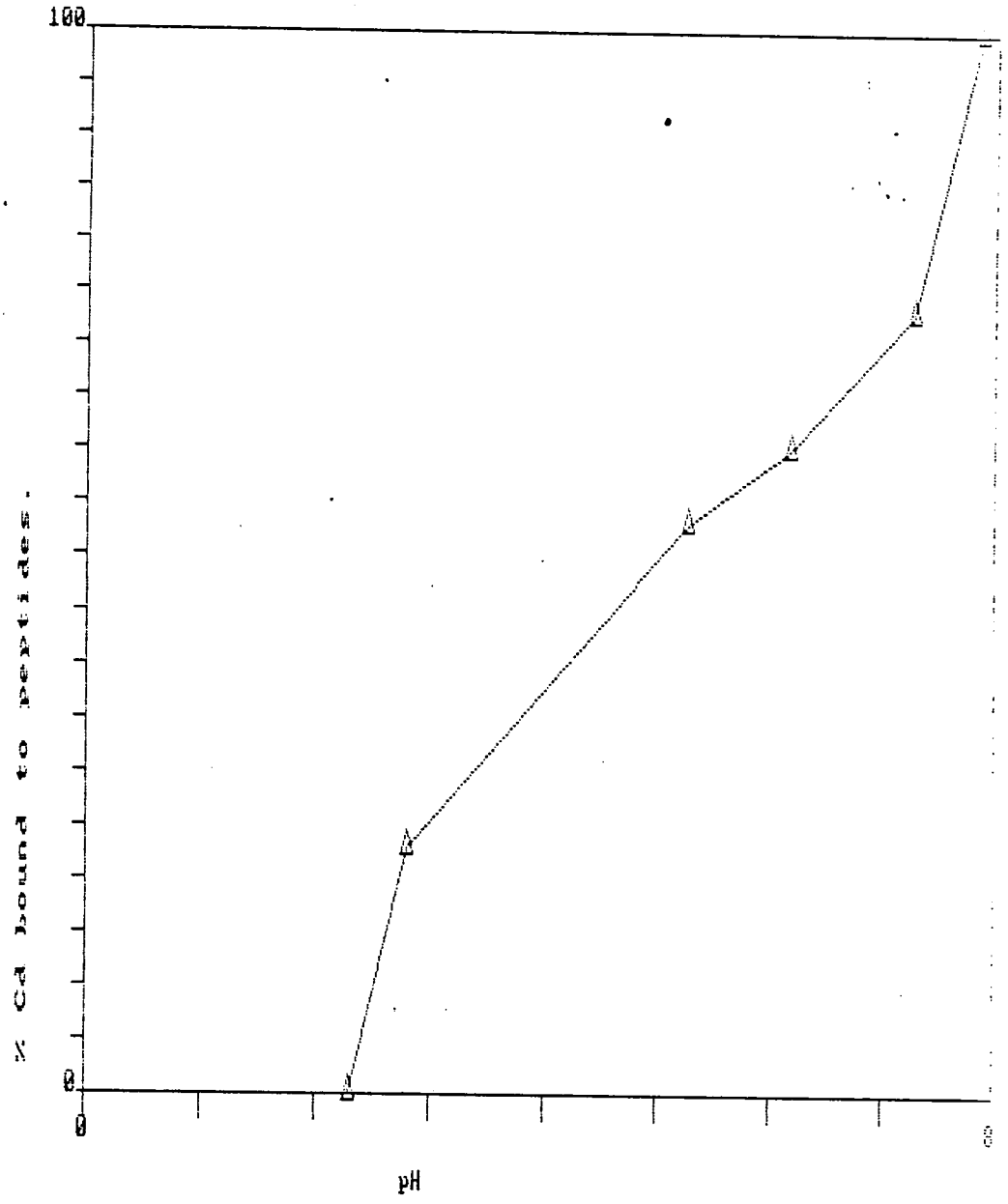

Figure 4. pH Dissociation of Cd from Sepharose-immobilised heat-denatured peptide preparation. Cd was stripped from the matrix using buffers of varying pH and the released Cd assayed using dithizone as outlined in Materials and Methods. It was assumed that Cd would be 100 % bound at pH 8, and 100 % dissociated at pH 2.3.

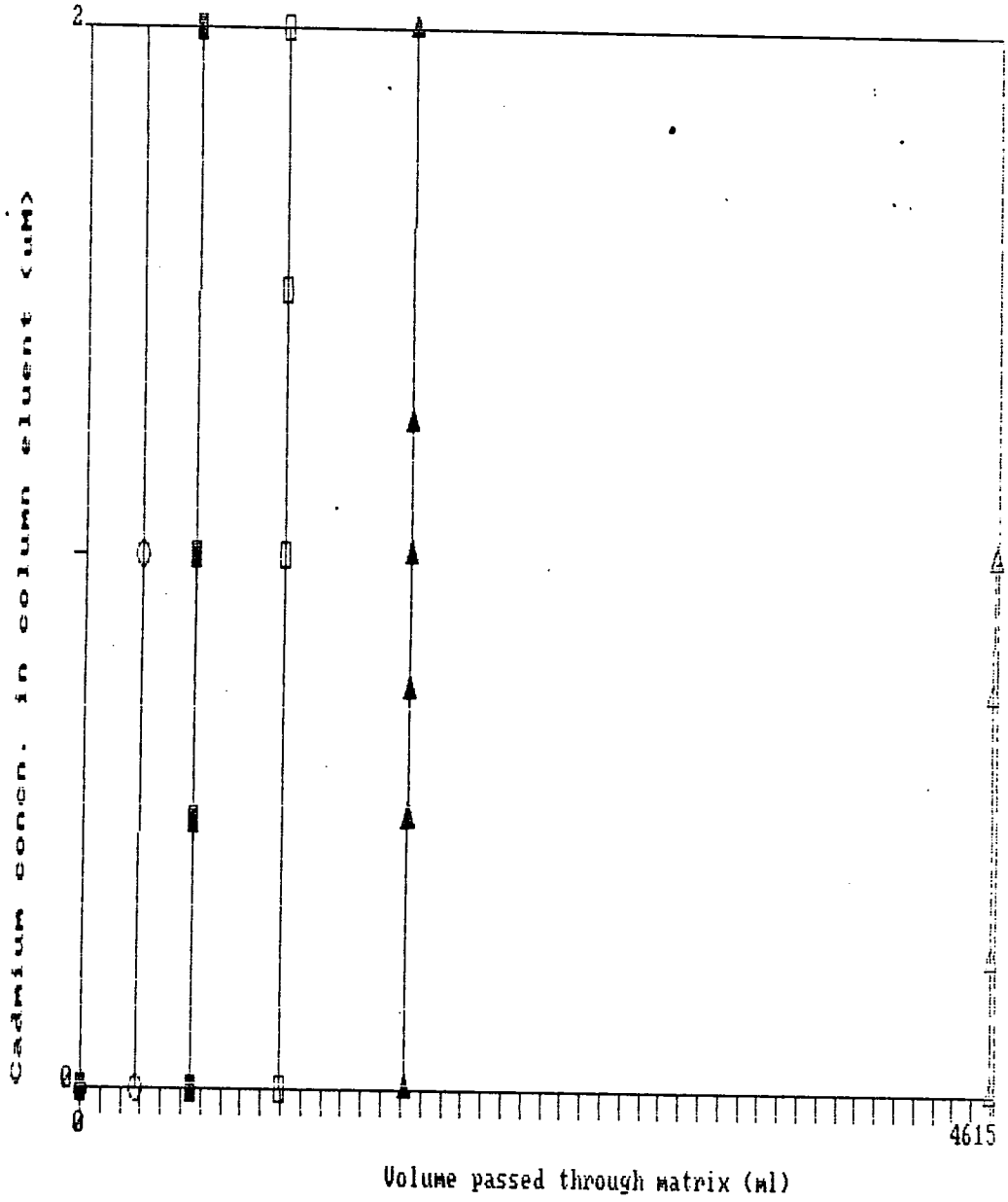

Figure 5. This shows the breakthrough volumes for various concentrations of Cd passing through the GSH matrix. Cd concentrations of 1 (Δ), 5 (▲), 10 (□), 25 (■) and 50 (◊) μM were passed through the matrix and the point of breakthrough recorded as described previously. Breakthrough volumes were similarly recorded for a control matrix, and calculation made of the amount of Cd binding to GSH molecules.

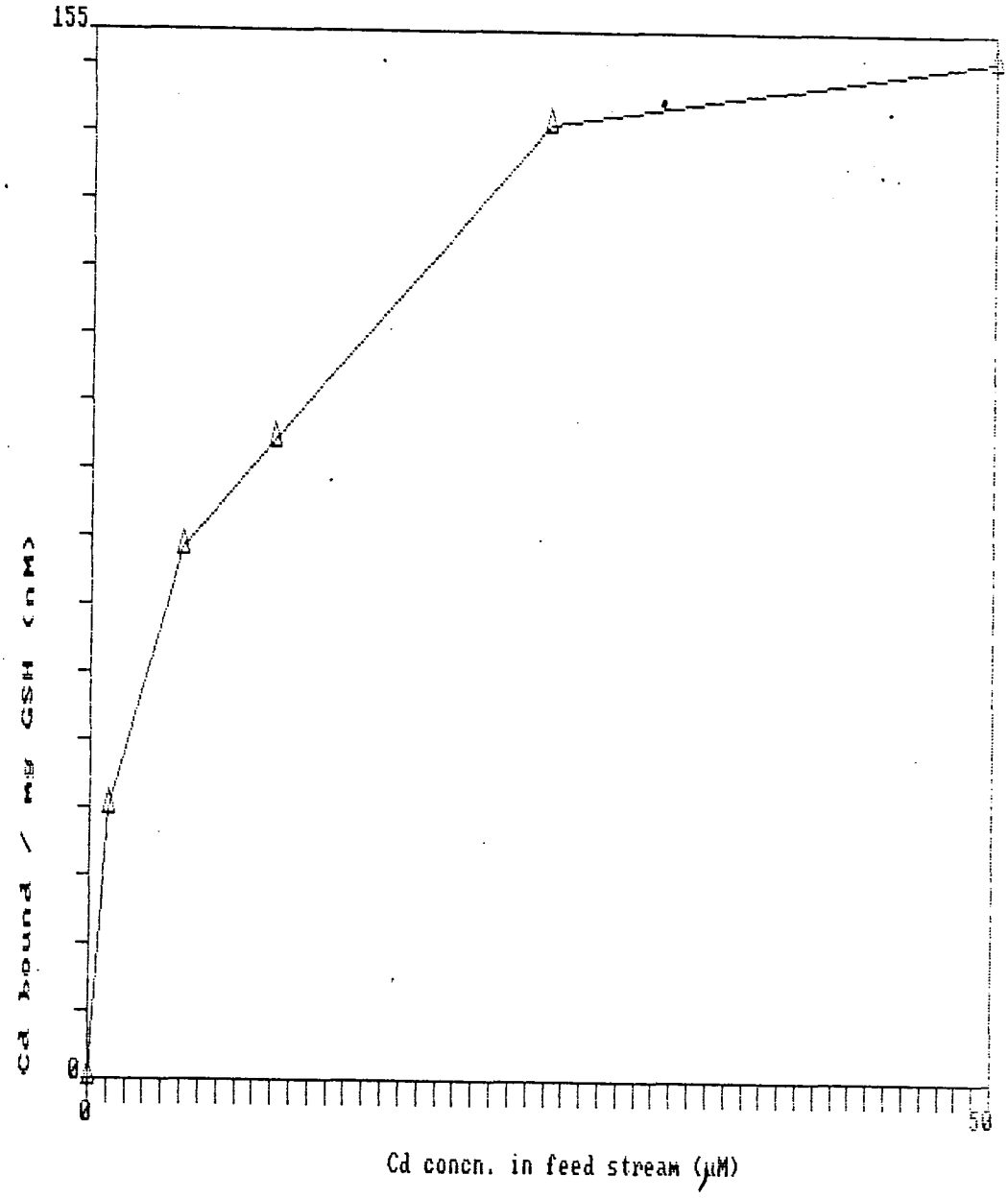

Figure 6. Analysis of Cd binding to immobilised GSH using the breakthrough method of analysis. Data was obtained as described in Figure 5, and in materials and methods.

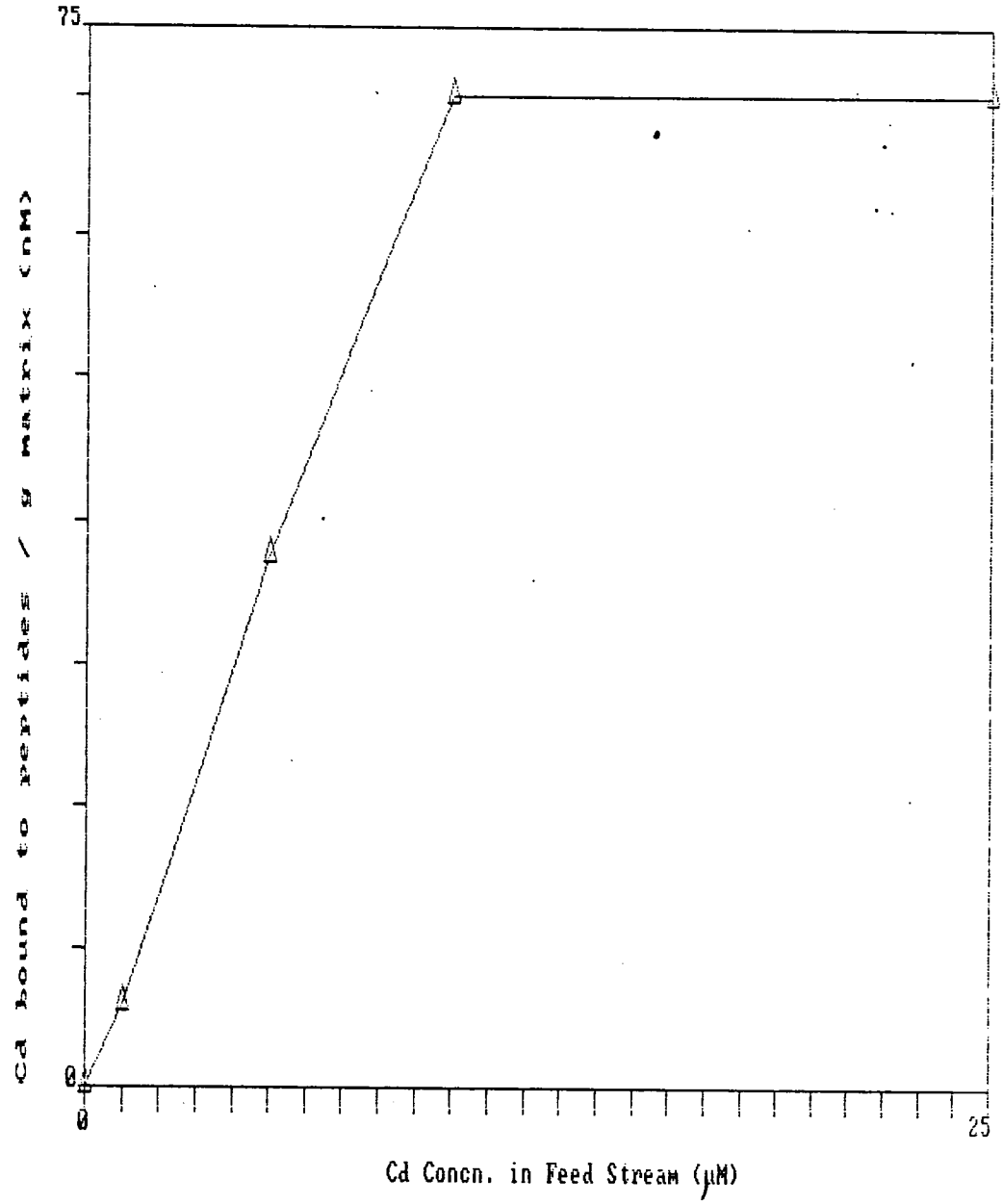

Figure 7. Cd binding to immobilised G-50 purified (gammaEC)$_n$G. Values are given as the amount of Cd binding to immobilised (gammaEC)$_n$G per g of matrix as no data was available for the total amount of (gammaEC)$_n$G immobilised. The results were obtained using the breakthrough method as previously described for the GSH matrix.

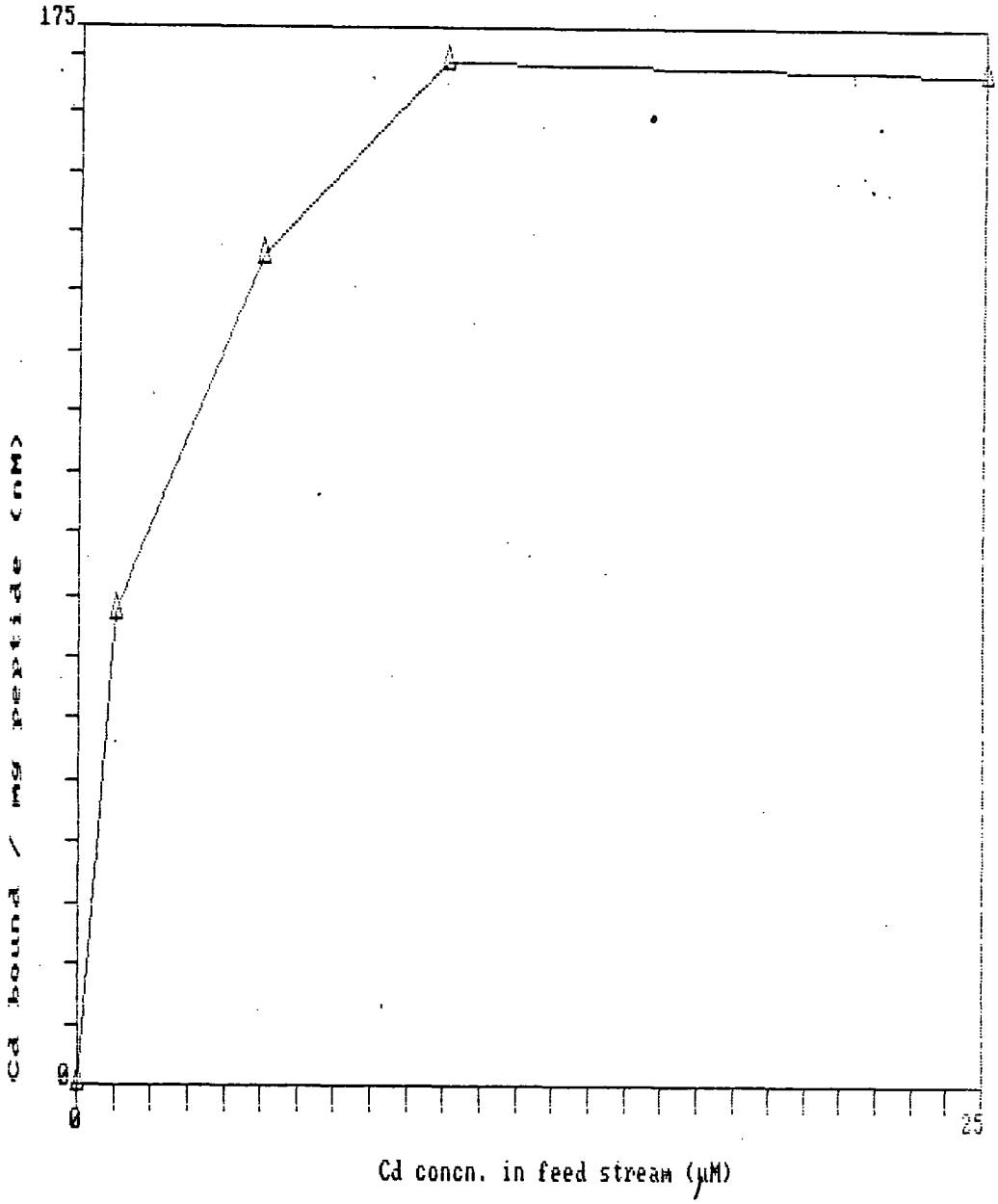

Figure 8. Analysis of Cd binding to immobilised heat denatured peptide preparation. The data was obtained using the breakthrough method as for the GSH matrix. Values for the amount of Cd bound refer to values per mg of $(gammaEC)_nG$ in equivalents of GSH.

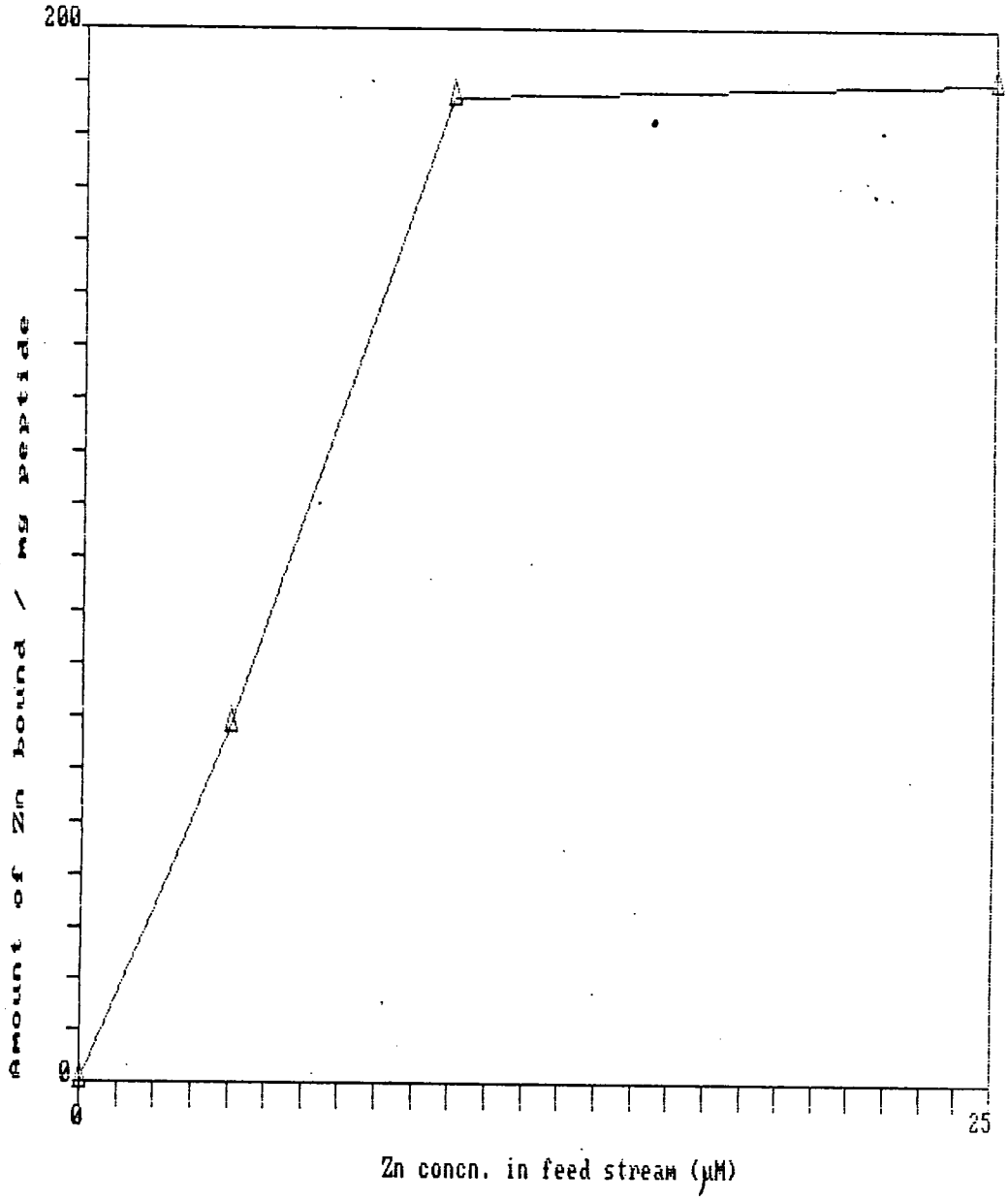

Figure 9 Analysis of Zn binding to immobilised heat-denaturation purified $(gammaEC)_nG$. This data was generated using the breakthrough method as described for Cd-binding analyses.

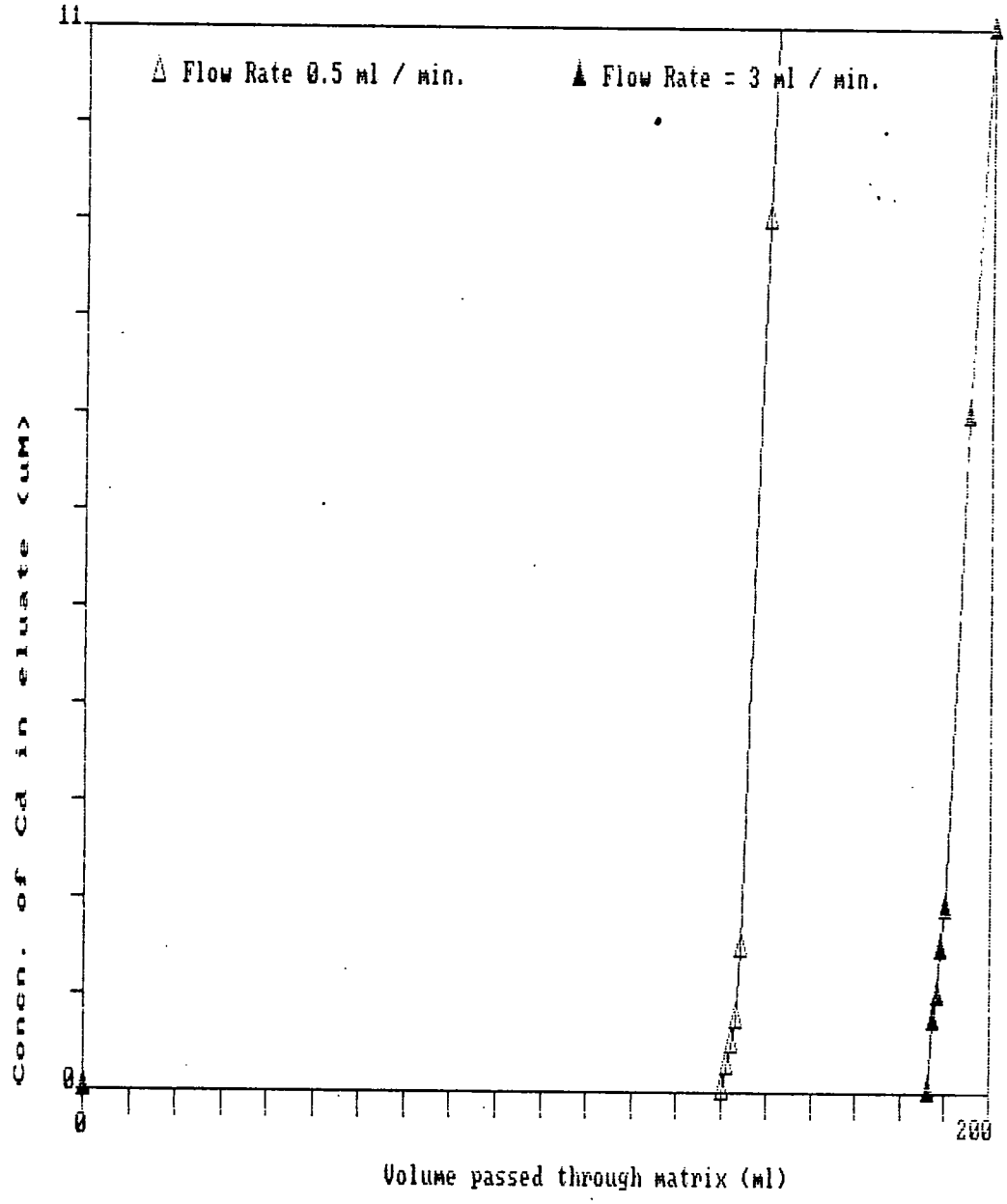

Figure 10. The effect of the flow rate through the column on the amount of metal ion binding to both Sepharose and immobilised $(gammaEC)_nG$. A Cd solution of 25 μM was passed through the matrix firstly at 0.5 ml min^{-1}, then at 3 ml $min.^{-1}$, and the volume to breakthrough recorded in each case. The data shows that flow rate clearly has an effect on the amount of Cd binding.

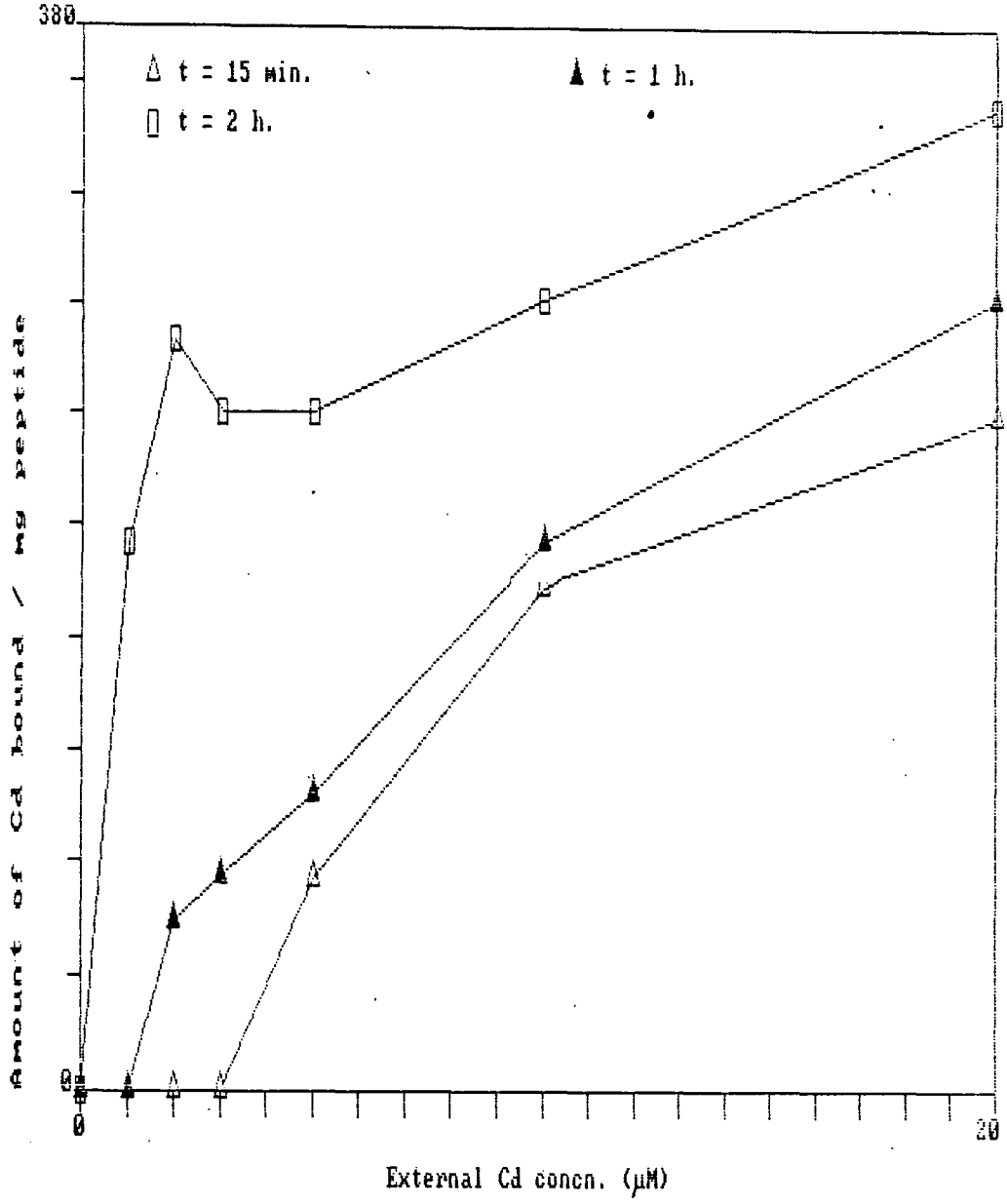

Figure 11. Time taken for Cd - (gammaEC)$_n$G complexes to form at pH 9 in suspension. Data was obtained as outlined in Appendix 2, using figures A7, A8 and A9. The difference between the amounts bound at 1 h and 2 h indicate that even after 2 h the system may not have reached complete equilibrium.

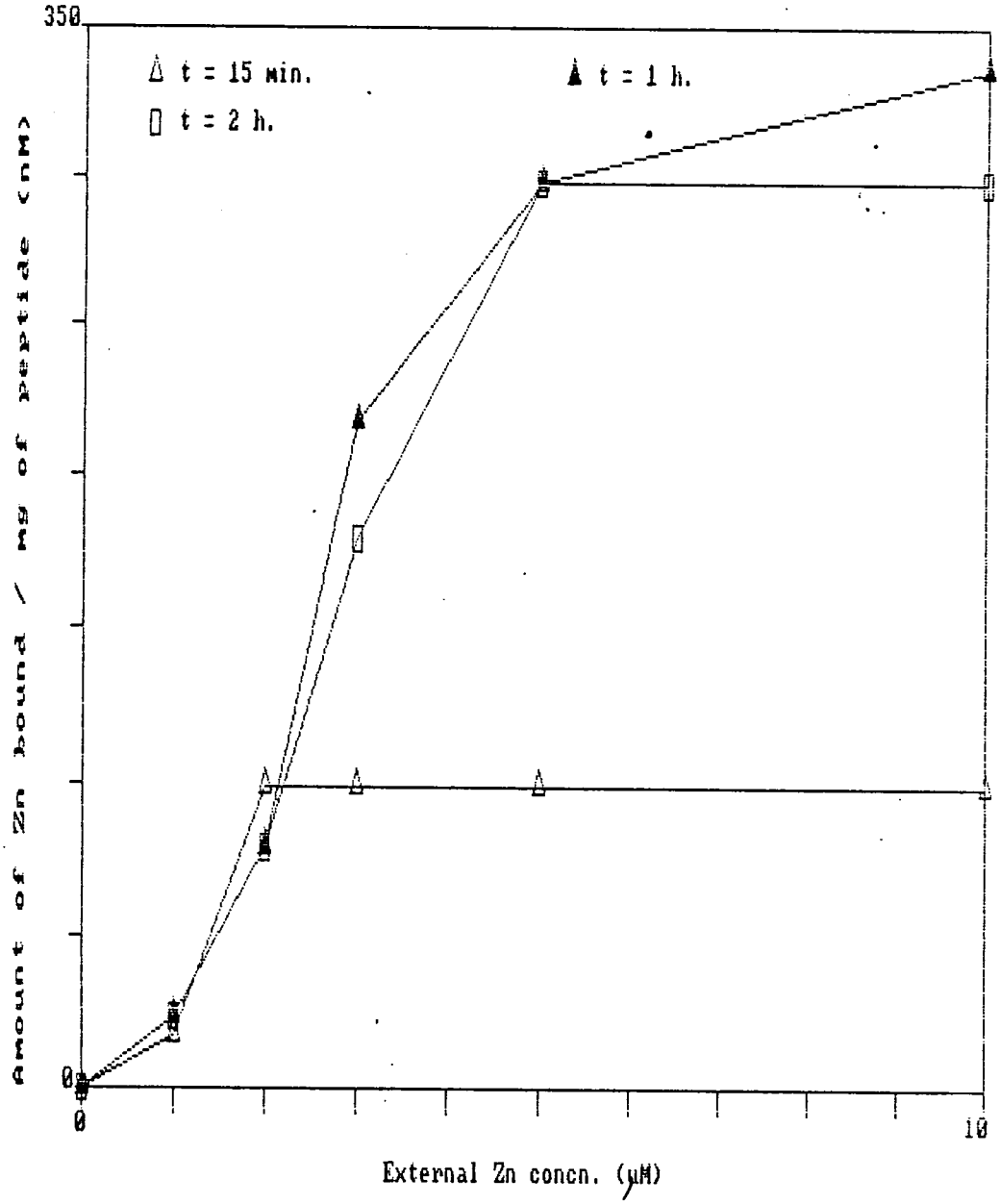

Figure 12. Time of formation of Zn - (gammaEC)$_n$G complexes. Data was obtained as for Cd binding in suspension. The similarity of the curves at 1 h and 2 h indicates that equilibrium of the complexes was reached within 1 h.

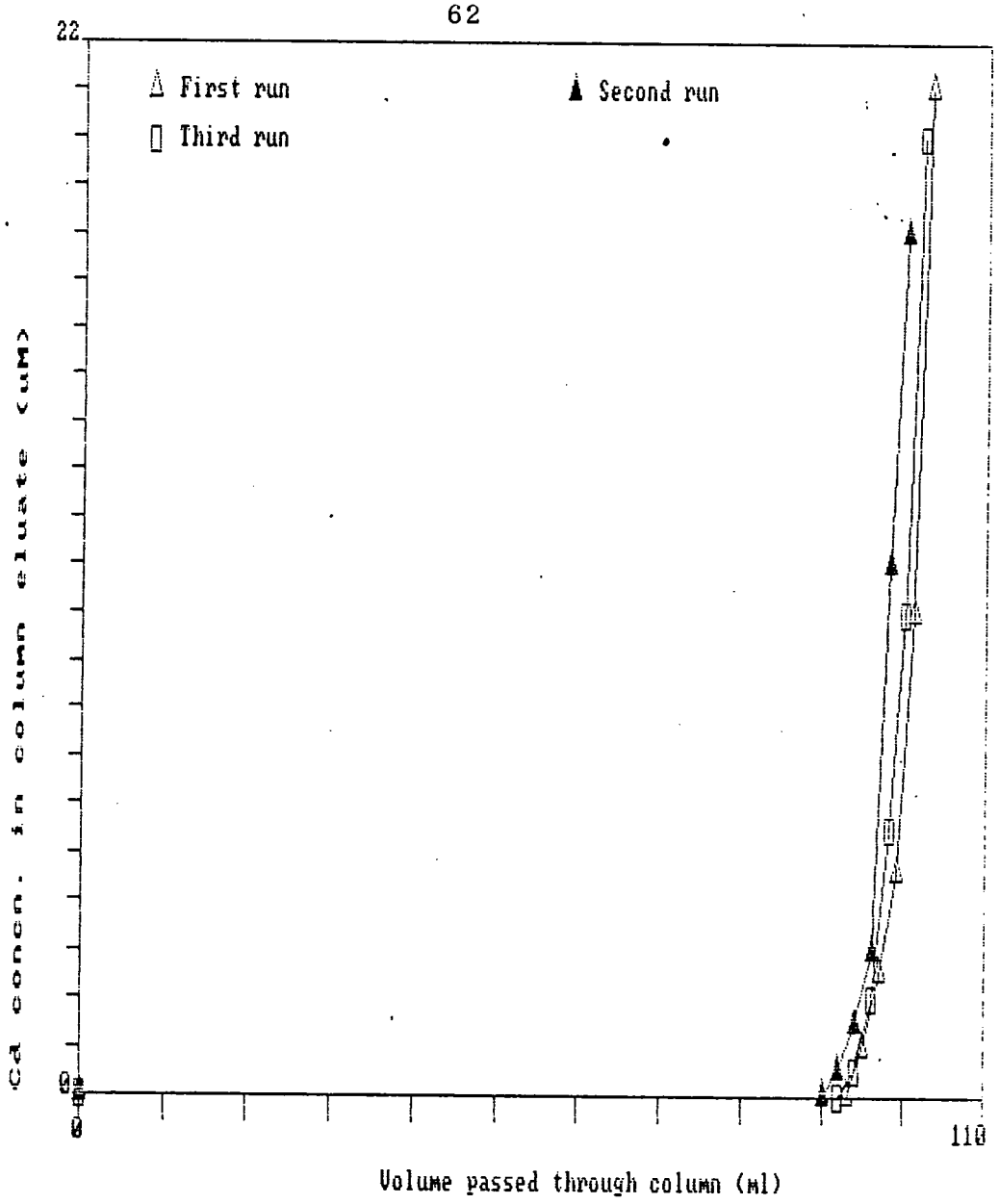

Figure 13. Re-cycling of matrix after Cd binding. The peptide matrix made using heat denaturation-purified peptides was used to test for stability of the immobilized peptides. The volume to breakthrough of 25 μM Cd was recorded, the matrix stripped using Na oxalate (pH 2) and the process repeated.

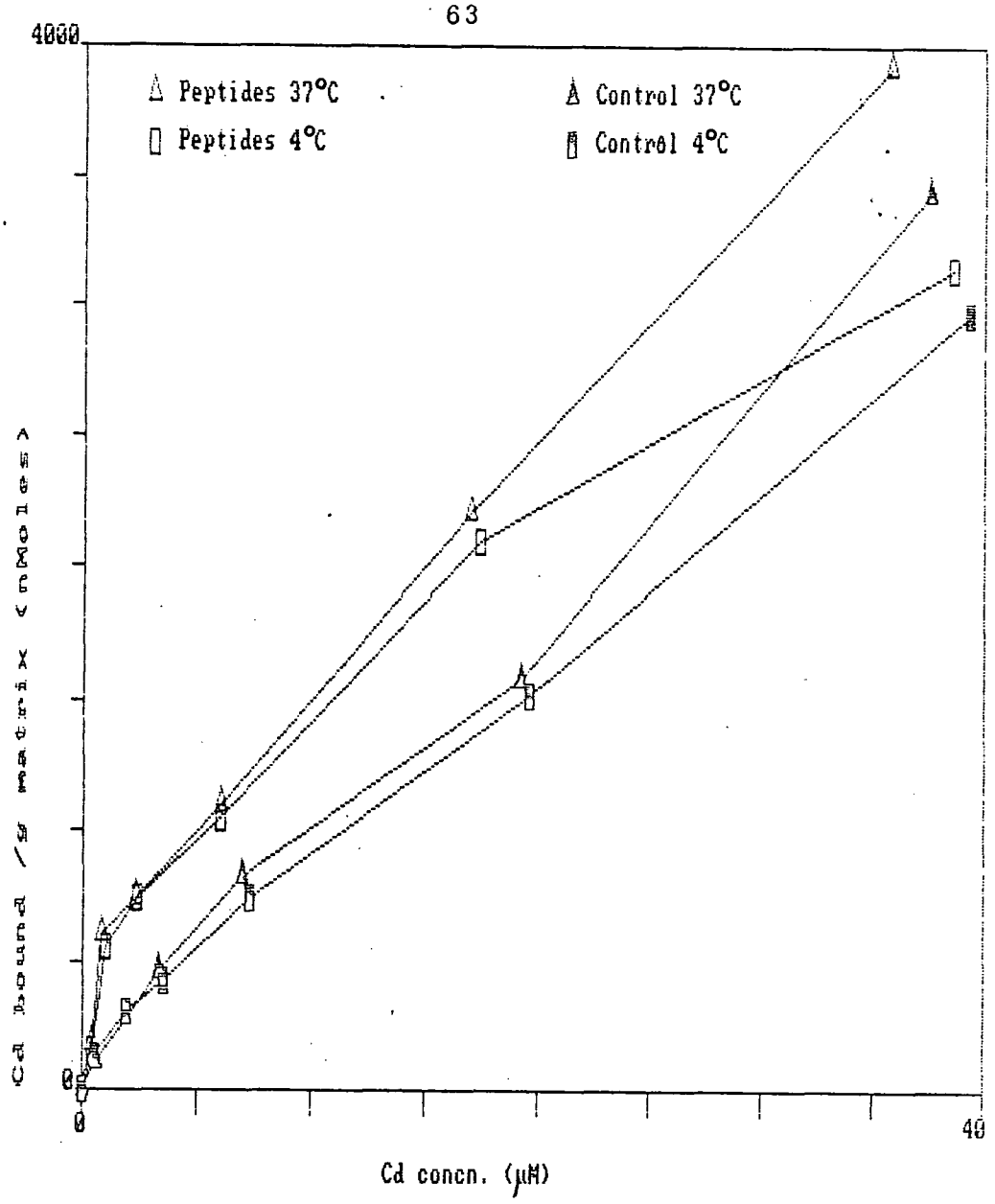

Figure 14. The effect of temperature on Cd binding to (gammaEC)$_n$G and Sepharose binding Cd. Analysis was done using suspensions which were incubated at the appropriate temperature for 2 h. Calculation of the amount of Cd bound was as for other tests. The data shows that temperature does affect Cd chelation. It is also demonstrated that there is some degree of inaccuracy in the analysis using the atomic absorbance spectrophotometer at high Cd concentrations.

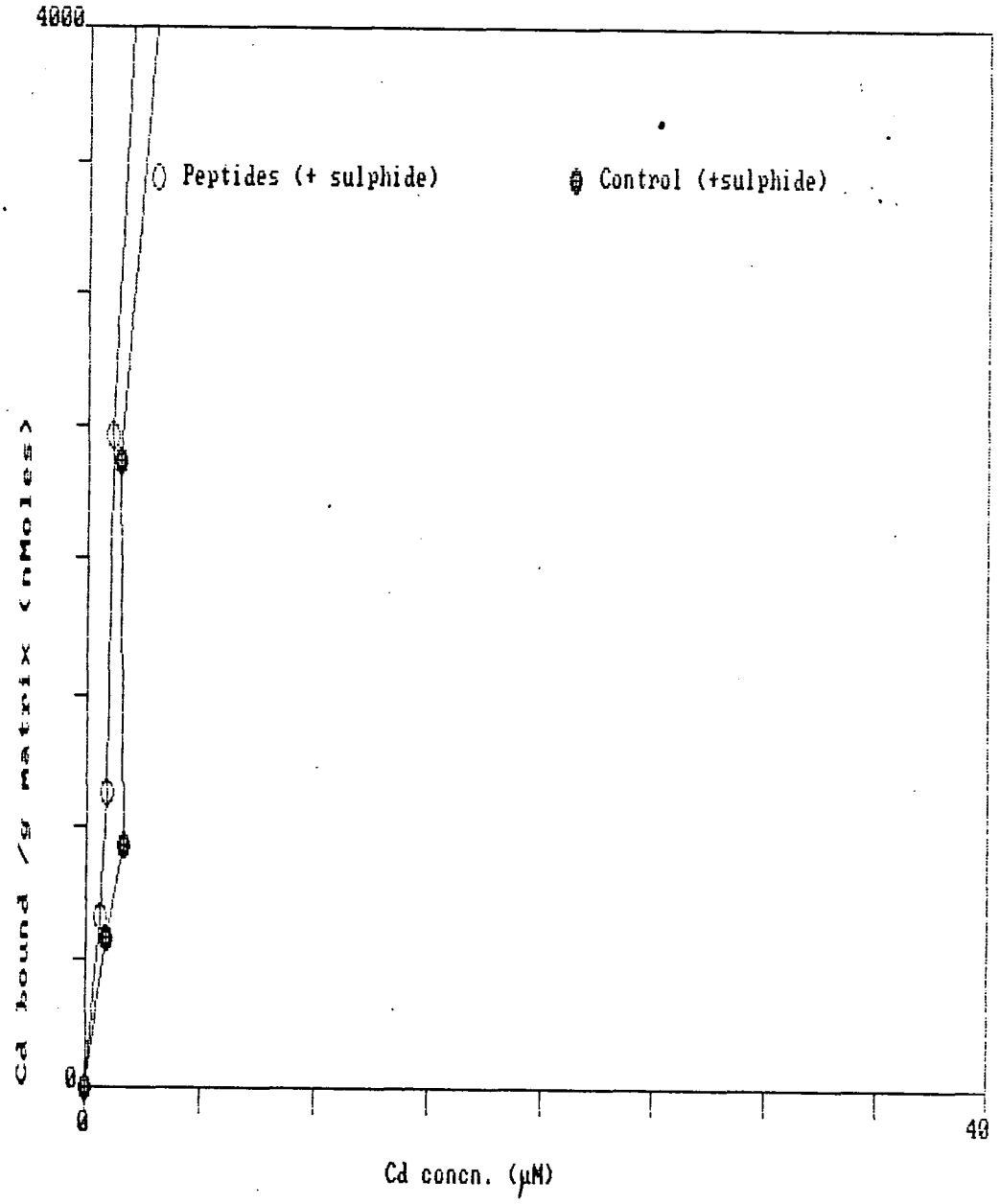

Figure 15. The effect of the addition of sulphide to both the control- and $(gammaEC)_nG$ matrices. The results reveal that sulphur is very effective in co-ordinating the binding of Cd, not only to the immobilised molecules, but also to Sepharose.

Discussion

(a) Efficiency of GSH and $(gammaEC)_nG$ immobilization.

Although in terms of weight only relatively small amounts of GSH and $(gammaEC)_nG$ were immobilised onto the Sepharose support, it is apparent that in terms of molarity, in both cases, the molecules were reasonably efficiently immobilised. The quoted maximal value for the immobilisation of trypsin is 20 mg g^{-1} compared to values of 5 mg g^{-1} for GSH and 1.68 mg g^{-1} for $(gammaEC)_nG$. However, this represents only 8.96×10^{-7} M of trypsin immobilised per g of Sepharose, as against 16×10^{-6} M, and 2.05×10^{-6} M for GSH and $(gammaEC)_nG$, respectively.

GSH and $(gammaEC)_nG$ are similar in size. The results obtained for the immobilization of GSH indicates that there is scope for increasing the molarity of $(gammaEC)_nG$ making up the matrix. The amount of GSH immobilised probably represents a maximal figure, due to the vast excess provided at the activation step. Therefore seems likely that by simply increasing the amount of$(gammaEC)_nG$ available at activation, a more efficient matrix could be prepared. However, complication exists due to loss of peptide when the matrix is initially stripped using oxalate. The first oxalate strip of the matrix brings about the removal of a number of molecules which were not bound directly to Sepharose, but were possibly immobilised as part of a Cd - $(gammaEC)_nG$ complex. Stripping of the Cd destroys the complex, therefore leads to the removal of a

significant amount of polypeptide. Increasing the overall amount of complex immobilised could increase the size of this component, and so could theoretically decrease the eventual amount of $(gammaEC)_nG$ on the matrix. It is not known how effective an increased amount of $(gammaEC)_nG$ at the immobilisation step will be.

Loss of peptides from the matrix, as described above, is illustrated in the figures obtained for the relative amount of peptide molecules involved in the co-ordination of Cd and Zn. The ratios produced (4.5 : 1 and 3.8 : 1 for Cd and Zn, respectively) are unlikely to reflect the situation *in vivo*. This also shows the importance of optimising the immobilisation procedure with respect to the ratio of peptide to Sepharose. At a ratio where the $(gammaEC)_nG$ complexes are least disrupted and all the $(gammaEC)_nG$ molecules in a complex are immobilized, the amounts of metal which can bind per molecule of peptide should be dramatically increased.

It has been demonstrated that the longer peptide forms have a higher affinity for Cd than do the shorter forms (Murasugi *et al* 1988). Therefore, manipulation of the cell cultures which over-produce the polypeptides to produce more of the longer forms, may be beneficial. *In vivo* manipulation of the $(gammaEC)_nG$ complex in cultured *D. innoxia* cells has not been demonstrated. However, it would be expected that changes in exposure to Cd could affect the peptides involved in complex formation. It has been shown that variation of the concentration of Cd to which whole tobacco plants are exposed can alter the binding characteristics of the peptides produced (Reese and Wagner 1987). It

has also been shown that supplementing the medium with the amino acids making up the polypeptides will increase their production (Berger et al 1988).

(b) Reverse phase HPLC analysis of $(gammaEC)_nG$.

The reverse phase HPLC profiles presented in Figures 2 and 3, give details of the peptide constituent of the G-50 and heat-denatured preparations, respectively. It is shown from the HPLC profiles that the $(gammaEC)_nG$ content of the two preparations are quite distinct from one another, especially in terms of the relative amounts of $(gammaEC)_2G$ and $(gammaEC)_3G$. It has been demonstrated by Murasugi et al, that in S. pombe cells, there is a difference in the relative amounts of the different peptide forms involved in Cd chelation as compared to the total cellular constituent. The G-50 purified material represents only those components which are combined into complexes with Cd. In contrast, the extract from the crude preparation represents total cellular $(gammaEC)_nG$. The differences seen may, therefore, reflect the difference between those polypeptides involved in Cd chelation and the total cellular complement. The two samples were, however, drawn from separate cell cultures. The difference seen may alternatively be accounted for by differences in $(gammaEC)_nG$ production in the two samples.

(c) Relative stability of immobilized $(gammaEC)_nG$ complexes.

Having immobilised $(gammaEC)_nG$, it was important that the behaviour

of the immobilised complexes could be related to their behaviour *in vivo*. To demonstrate the binding capabilities of metal-chelating molecules, it is often useful to demonstrate the pH of half dissociation. This measurement is often quoted as a measure of the binding affinity between molecules and has been demonstrated for $(gammaEC)_nG$ to be around pH 5 (see Table 1).

Research Group	Weber *et al* 1987	Reese *et al* 1987		Hayashi *et al* 1987		Reese & Wagner 1987		
Quoted pH of half dissociation	5.7[1]	5.4[2]	4[3]	4.8[4]	5.2[5]	5[6]	5.4[7]	5.7[8]

Table 1. Quoted pH's of half dissociation of Cd from $(gammaEC)_nG$ complexes. Examples presented : (1) Cd-binding peptide II isolated from *Euglena gracilis*. (these have a sulphide component). (2) Cd-binding peptide I (non S-containing) from *S. pombe*. (3) Cd-binding peptide II (S-containing) from *S. pombe*. (4) *In vitro*-synthesised $(gammaEC)_3G$. (5) *In vitro*-synthesised $(gammaEC)_2G$. (6) Tobacco Cd-binding protein isolated from plants exposed to 90 μM Cd. (7) Cd-binding protein isolated from cultured tobacco cells. (8) Tobacco Cd-binding protein isolated from plants exposed to 3 μM Cd.

The results for half dissociation of Cd from immobilised (gamma EC)$_n$G obtained in this study, at pH 4.9 (see Figure 4), agree reasonably well with published results for half dissociation of Cd from peptide complexes in free solution. The finding that around 20 % of the original Cd remains bound at pH 2.8 would represent a slight increase in pH stability of the complex relative to results obtained in free solution. Since behaviour of the immobilized complexes appears similar to the results obtained for the unmodified peptides, it is reasonable to extrapolate the results obtained in this study to characteristics of immobilized (gammaEC)$_n$G in vivo. As previously mentioned, however, the metal-chelating ability of the initial complex may change considerably on primary stripping using oxalate. In subsequent work, it is hoped to confirm that the peptides behave in a similar manner after oxalate stripping as before, by determining the pH of half dissociation of the reconstiuted complex.

(d) Initial characterization of metal binding to GSH and (gammaEC)$_n$G matrices.

Characterisation of both the GSH and (gammaEC)$_n$G matrices was first attempted using the breakthrough method as described in Appendix V. This allowed the partial characterisation of the matrices as illustrated in Figures 4, 5 and 6.

To rationalise the binding characteristics of the immobilised molecules, apparent binding constants were determined (see Results Section). The equilibrium constants Q_{max} and K_D, which are applied to

the kinetics of enzymic reactions, can be applied to the equilibrium which develops between two molecules which have an affinity for one another (Godfrey & Reichelt 1983). In terms of enzymic reactions, Q_{max} represents the maximal rate of reaction which is induced in conditions of excess substrate. At low substrate concentrations, the reaction rate reduces as the equilibrium moves away from product formation, as a consequence of less interaction between enzyme and substrate. The value K_D (defined as the substrate concentration giving half Q_{max}) is used as a measure of the affinity of a given enzyme for a given substrate. The lower the K_D the higher the affinity of enzyme for substrate. By analogy, it is possible to define a term K_D as the concentration of metal ion ("substrate") which will allow half maximal binding of metal ion to the available binding sites on the peptides. This is because the surrounding concentration of metal ions has a similar effect on the amounts bound to $(gammaEC)_nG$ as the substrate concentration has on the interaction between enzyme and substrate. The amount of Cd completely saturating all of the metal-binding sites of the peptides can similarly be referred to as Q_{max}

Although it proved possible to obtain binding constants as shown in the results section using the breakthrough method, several factors led to the conclusion that there was a certain amount of inaccuracy in the method. It was noted that the concentration at breakthrough was not the same as that in the feedstream, as is clearly noticeable in Figure 13. In practice, the concentration of the feedstream was generally not achieved until the passage of up to 20 ml of the metal ion solution in

excess of breakthrough, dependant on the concentration entering the matrix. This suggested that at the time of breakthrough, the system had not achieved equilibrium, and that the time taken to come to an equilibrium would differ from one loading concentration to another. In addition to this, an experiment was designed to determine whether the flow rate could affect the amount of Cd binding (see Section h), which suggested that variation in flow rate could introduce inaccuracies to the test.

In addition to the problem of the flow rate affecting the calculation of K_D and Q_{max} values, it was noted that another factor, which could not be attributed to the methods used in the experiment, was bringing about slight errors in some of the results obtained (see Figure 14 for subsequent testing of this hypothesis). It was considered possible that changing temperature may have an effect in determining how much metal would bind and that this may lead to slight errors in the initial calculations made. Thus, although the results obtained were useful in describing the ability of immobilized $(gammaEC)_nG$ to remove metals from solution, the data obtained were not considered an accurate reflection of the binding characteristics of the peptides *in vivo*.

(e) The use of matrix suspensions for characterization of $(gammaEC)_nG$.

For the reasons outlined above, it was decided to adopt an alternative approach to calculation of K_D and Q_{max} values. The second approach, outlined in Appendix V, was to use suspensions of matrix in

varying metal concentrations. This allowed amelioration of any flow rate effect by allowing equilibria to develop in a constant environment. The perceived problem of changing temperature was avoided by incubation of the suspensions at a constant temperature of 37 °C. Only the heat denatured peptide matrix was used in these tests.

The results presented from this data show that the peptides isolated from D. innoxia cells have an apparent K_D and Q_{max} for Zn of 2.8 μM and 315 nM mg^{-1} respectively. It is clearly shown that the affinity of the peptides for Cd is very much higher for Cd than for Zn, with an apparent K_D value of less than 0.5 μM. The value K_D for Cd may be lower than 0.5 μM for two reasons. Firstly, examination of the graph showing formation of the complex (Figure 10) demonstrates that after 2 h. it cannot be certain that complete equilibrium has been reached, due to the disparity between the curves at 1 h. and 2 h. Secondly, the lower limit of accurate detection of Cd using atomic absorption spectrophotometry is around 0.5 μM. Since small errors in detection can be inflated by subsequent treatment of data, it is essential that the method used is accurate. Final confirmation of the K_D value for Cd will therefore depend on the use of a more accurate method of analysis. The use of isotope labelling and subsequent detection by liquid scintillation counting is the most sensitive method for trace metal analysis, and may be useful in subsequent experiments if the K_D value is to be accurately measured.

The Q_{max} value for Cd is lower than that for Zn. This suggests that some sites are available on the $(gammaEC)_nG$ molecule for Zn

sequestration than are available to Cd. The major site for metal ion binding to these molecules is well established as the thiol groups of the cysteine residues. It has been shown, however, that metal ions will bind to other sites on GSH (Perrin & Watt 1971). It is possible, therefore, that Zn has access to such sites on the (gammaEC)$_n$G molecule, from which Cd is excluded. Affinity for these sites would be considerably less than for the thiol groups.

Given the very high affinity of (gammaEC)$_n$G for Cd, it is difficult to explain the slowness of complex formation. This may be related to the accessibility of the binding site, though repitition of the experiment is required to confirm this result, possibly utilizing more accurate methods of analysis.

(f) Implications of Zn binding data.

The finding that these peptides are capable of binding Zn is of interest in determining a possible constitutive role for (gammaEC)$_n$G in plant cells. As mentioned in the introduction, it has been postulated that (gammaEC)$_n$G may be involved in Zn homeostasis by acting as a storage pool for the metal. Zn, however, has not been demonstrated to be associated with the peptides *in vivo*. Although this experiment shows that binding to Zn does occur, the high K_D value would suggest that this role in Zn homeostasis may not be likely. The chelated Zn would be too readily given up by the peptides in response to moderately low Zn concentrations or to the many molecules in the cell with higher

affinities.

(g) Investigation of the effect of sulphide on Cd chelation.

Several of the Cd - (gammaEC)$_n$G complexes isolated have been shown to have a sulphide component, which, when compared to a non sulphide-containing complex from the same organism, is found to bind Cd more strongly. This prompted the experiment to investigate whether sulphide could be incorporated into the immobilised complex *in vitro*. Reduced S, in the form of Na sulphide was added to the suspensions. This was added at a concentration twice that of the Cd in each suspension since the ratio of sulpide to Cd in the *S. pombe* cells was shown to be 2.4 : 1. The results presented in Figure 15 show clearly that sulphide is extremely effective in increasing chelation of Cd, not only by the immobilised peptides, but also by the Sepharose itself. This indicates that sulphide, as a component of a complex would increase the affinity of the complex for Cd. However, due to interference caused by binding to Sepharose, in this experiment it was not possible to provide evidence of reconstitution of such complexes.

(h) Metal detoxification using immobilised (gammaEC)$_n$G.

The results presented have implications for the possible use of immobilised (gammaEC)$_n$G in detoxification of water polluted with toxic trace metals. Firsty, it is shown that the immobilized polypeptides will remove metal ions from solutions containing dissolved metal salts. The preliminary results produced by running metal-containing solutions

through the affinity matrices shows that both Cd and Zn are effectively removed from solution. Cu-binding was also demonstrated by these tests. The more detailed analysis of the binding affinities of the immobilised polypeptides using suspensions of matrix, demonstrated that at low concentrations of metal ions, Cd will be more effectively removed than Zn. The results indicate, however, that the matrices have a higher capacity for Zn, and that Zn complexes will tend to form more rapidly than Cd complexes.

The fact that increasing flow rate has the effect of increasing the amount of metal binding to immobilized $(gammaEC)_nG$ (Figure 8), is of interest if polluted waste streams were to be cleared by passing through matrix containing $(gammaEC)_nG$. The result of this experiment was surprising since it was expected that decreased residence time in the matrix would lead to a decrease in binding as has been noted for immobilised enzymes and bacteria (Macaskie et al 1988). It is likely that the increased flow rate has the effect of "pushing" a greater amount of metal ions into the system, increasing the concentration of metal surrounding the peptides, therefore leading to increased binding. This finding is of importance in terms of using the matrix as a metal detoxification system since the higher the flow rates achieved, the more efficiently the system can be operated. This will obviously be related to the flow rates which can be maintained by the supporting medium. In this case, Sepharose, which is relatively delicate cannot support flow rates which are likely to be required on an industrial scale. However, other supporting media are available and it is hoped that these will be

tested in the future for their effectiveness in allowing metal ion chelation by immobilized $(gammaEC)_n$

The immobilised $(gammaEC)_nG$ are capable of chelating Cd and Zn from contaminated feedstreams. Therefore it was necessary to find out whether the matrices would remain stable through a number of cycles of binding metals and acid stripping. Figure 13 is a demonstration that the matrix remains stable over a series of loading and stripping. In practice the matrix was loaded and stripped for both Zn and Cd many times with no detectable loss in capacity

IV SUMMARY AND FUTURE WORK

1. Synthetic metal resistance genes.

The work involving synthetic metal resistance genes indicated that the system used did not confer metal resistance on the host cell. Further experiments are planned in attempting to engineer for metal resistance :

1. The plasmids containing the synthetic gene will be sequenced in order to determine whether the insert is in the correct orientation.
2. pho regulon products will be radiolabelled with ^{35}S, and the periplasmic shock proteins isolated and screened for the SG product.
3. A new host organism will be used in further experimentstoclone for metal resistance. S. pombe mutants which are more likely to be able to deal with the synthetic gene product may be used.
4. It is possible that prokaryotic metal regulatory elements will be isolated in the future. If so, these will be utilised to attempt to engineer for metal resistance in bacteria.

2. Metal affinity matrices.

The results obtained in this work indicate that immobilization of $(gammaEC)_nG$ for the clearance of metal contaminated wastes may be feasible. Furthermore, the method used allows the determination of binding characteristics of the immobilized molecules. This data showed that the immobilized peptides have a very high affinity for Cd (K_D < 0.5) and also that $(gammaEC)_nG$ can co-ordinate Zn, with a K_D of 2.8.

More work is planned to better characterize the metal affinities of $(gammaEC)_nG$, and to improve metal removing ability by the bound peptides. .This may include :

1. The use of more sensitive methods to determine precise binding constants for metals which bind to $(gammaEC)_nG$.
2. Maximizing the amount of peptide bound to the support.
3. Attempting to establish whether the peptides can be made ion-specific by retaining a particular conformation on immobilization.
4. The use of different types of support to maximize the amount of $(gammaEC)_nG$ immobilized, and to improve the applicability of the system to waste treatment.
5. Determining the resistance of the molecules to degradation by proteolysis.
6. Analysis of the pH of half dissociation of reconstituted complexes.

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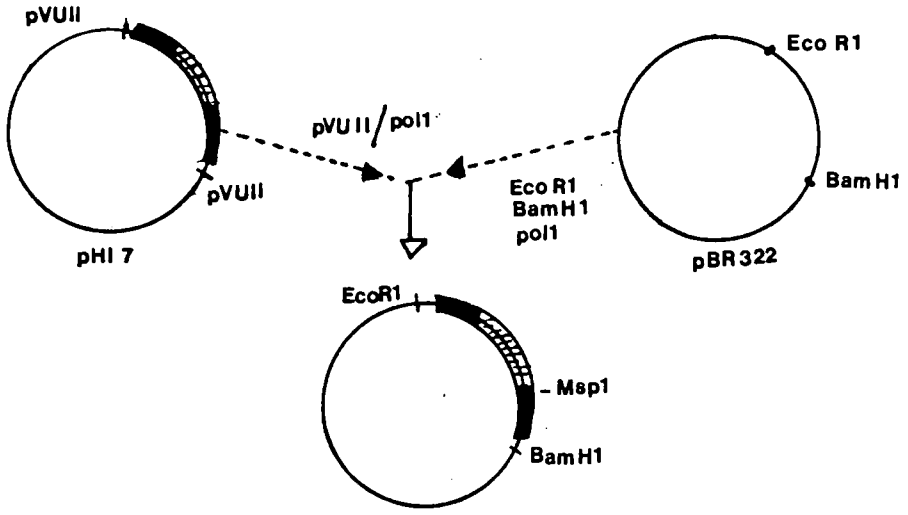
APPENDIX I

Construction of the plasmid pHI-28-SG

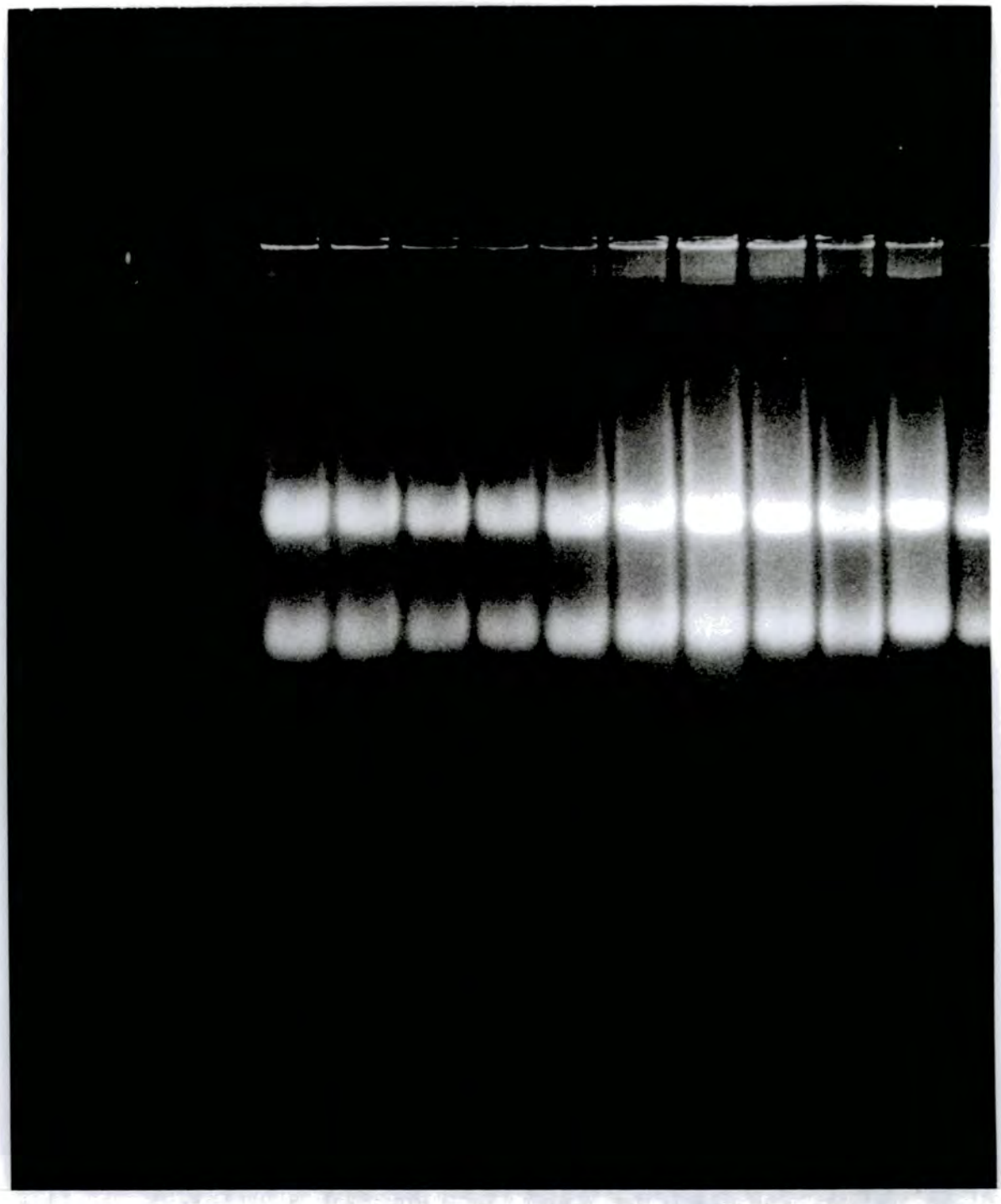
Plasmid pHI-28-SG was constructed by P.Lee (Harvard, unpublished results). The Pvu II restriction fragment from pHI 7 (containing the phoA promoter, signal sequence and 185 amino acids of the structural gene) was removed and ligated into pBR 322, as shown below.



This intermediate was then digested with Msp I, which has a site just inside the phoA structural gene, and the digest was "filled in" using DNA polymerase I. This generates a population of fragments, all of which are blunt-ended. Eco RI digestion of this group of fragments generates a phoA / phoAss fragment which has one blunt end and one Eco RI-generated single-stranded end. A vector was created for the selective ligation of these fragments by digestion of pBR322 with Bam

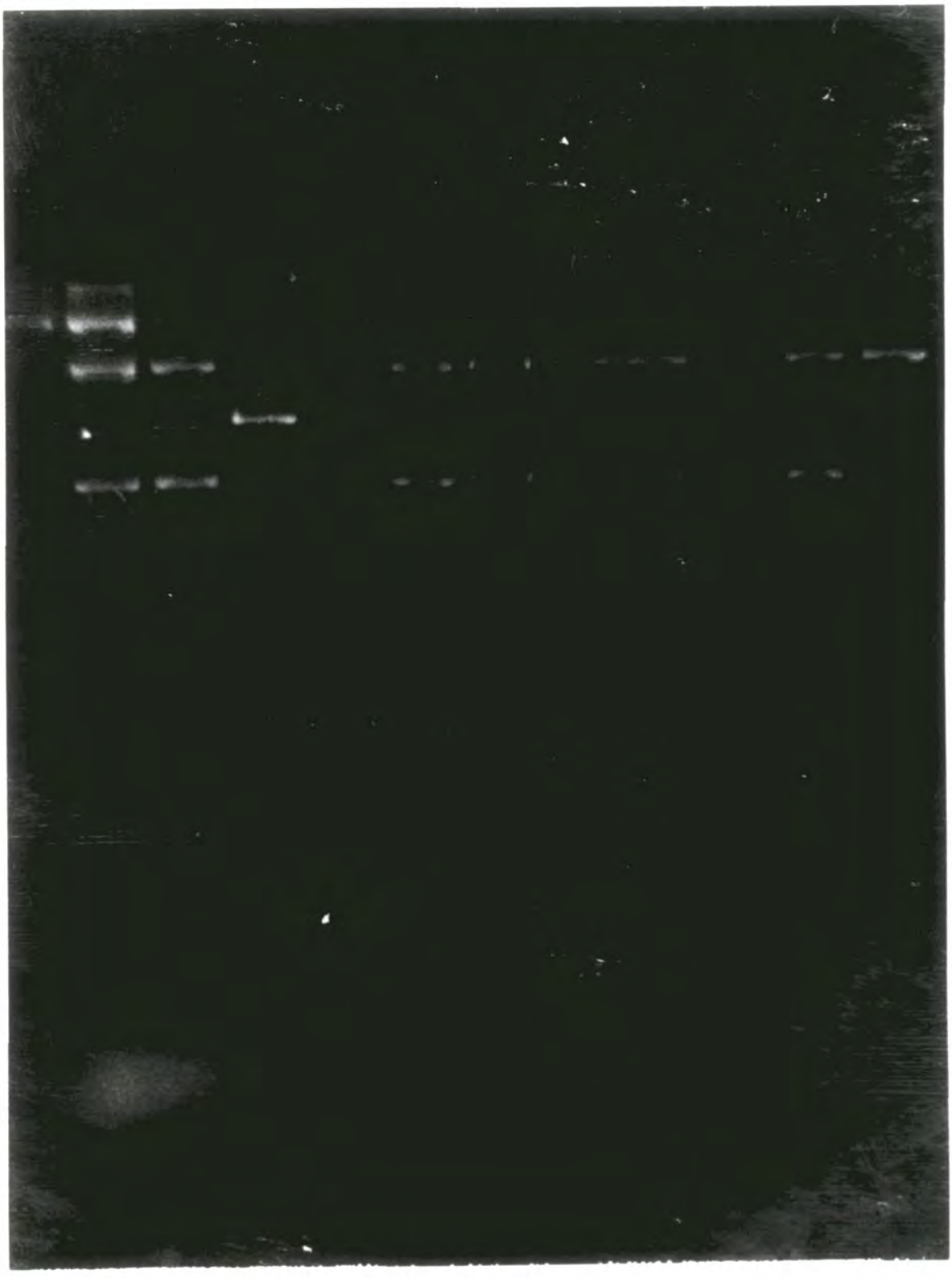
HI, followed by filling using DNA pol I, and re-digestion using Eco RI. The fragment was ligated in to give plasmid pHI-25. The kanamycin resistance gene from transposon Tn 5 was cloned into pHI 25, and further manipulation done to generate a Bam HI restriction site adjacent to the phoAss region.

Subsequent cloning of the synthetic gene into this site destroys the Bam HI site and puts the gene under control of the phoA promoter. The product has the phoA signal sequence at its amino terminus



APPENDIX II.

Photograph I. RNA formaldehyde gel. Two distinct ribosomal RNA bands are visible. Four gels such as this were used for northern blotting, and the blots probed as explained in the text. The running order of the gel is : (left to right) pHI-28-SG 17, 19, 22, 7, 23, 11, 1, 2, 3 and 9.



APPENDIX II (cont.)

Photograph 11. pHI-28-SG clones, restricted using Bam HI endonuclease. All clones except 2 (control) and 1 (possible DNA rearrangement) were restricted. The running order of the gel is : (right to left) pHI-28-SG 17, 19, 22, 7, 14, 23, 11, 1, 2, 3 and 9.

APPENDIX III.

Clone	Cd Concentration (μM)		
	0	5	10
1	7×10^{-3}	6×10^{-3}	7.6×10^{-3}
	2×10^{-3}	1.4×10^{-3}	2.8×10^{-3}
2	8.5×10^{-3}	5.6×10^{-3}	8×10^{-3}
	7.1×10^{-4}	3.2×10^{-3}	3.5×10^{-3}
11	9×10^{-3}	7.6×10^{-3}	6×10^{-3}
	1×10^{-3}	1.5×10^{-3}	1.7×10^{-3}
23	8×10^{-3}	8.6×10^{-3}	9.3×10^{-3}
	2×10^{-3}	2.1×10^{-3}	5.7×10^{-3}
1	0.146	0.039	0
	8.1×10^{-3}	7×10^{-3}	0
2	0.156	0.053	0
	5.6×10^{-3}	8.1×10^{-3}	0
11	0.150	0.049	0
	3×10^{-3}	8.6×10^{-3}	0
23	0.151	0.059	0
	6.6×10^{-3}	7×10^{-3}	0

Table A1. Growth of clones pHI-28-SG 1, 2, 11 and 23 in 0, 5 and 10 μM Cd. Smaller inocula were used in this experiment to initiate growth (see materials and methods for details). Data given above refer to A_{600} at 0 h (above), and at 12 h (below). The results show that decreasing the inoculum has a detrimental effect on the ability of the cells to survive in Cd. No difference was observed in Cd tolerance of the cells tested.

APPENDIX III (cont.)

Clone	Cd concentration (μM)			
	0	1	2	5
1	0.026	0.028	0.029	0.028
	0.5×10^{-3}	0.001	0.001	0.001
2	0.036	0.036	0.04	0.035
	0.002	0.003	0.005	0.002
11	0.032	0.031	0.033	0.035
	0.001	0.001	0.002	0.002
14	0.032	0.033	0.036	0.035
	0.003	0.005	0.002	0.002
17	0.03	0.032	0.026	0.028
	0.003	0.001	0.006	0.001
22	0.031	0.026	0.025	0.028
	0.014	0.008	0.008	0.008
23	0.033	0.028	0.031	0.032
	0.002	0.002	0.002	0.01
3	0.036	0.033	0.037	0.034
	0.001	0.006	0.006	0
7	0.037	0.037	0.036	0.037
	0.003	0	0.001	0.005
19	0.041	0.034	0.049	0.038
	0.002	0	0.002	0
9	0.038	0.04	0.043	0.037
	0	0	0.001	0.005

Table AII. Growth of all pHI-28-SG clones in 0, 1, 2 and 5 μM Cd. Growth was initiated from log-phase cells after 4 h. growth in 2XL medium. Measurements shown are at 0 h (above) and 12 h (opposite). Experiments were performed as outlined in materials and methods.

Clone	Cd Concentration (μM).			
	0	1	2	5
1	0.245	0.238	0.205	0.023
	0.013	0.019	0.023	0.003
2	0.248	0.216	0.182	0.028
	0.005	0.003	0.044	0.003
11	0.260	0.229	0.208	0.026
	0.024	0.022	0.025	0.005
14	0.255	0.239	0.211	0.022
	0.013	0.01	0.022	0.009
17	0.273	0.248	0.216	0.024
	0.014	0.017	0.027	0.003
22	0.263	0.232	0.216	0.022
	0.013	0.011	0.031	0.002
23	0.259	0.246	0.212	0.028
	0.001	0.003	0.025	0.002
3	0.214	0.146	0.078	0.056
	0.017	0.041	0.003	0.021
7	0.244	0.193	0.09	0.038
	0.009	0.009	0.03	0.016
19	0.115	0.067	0.034	0.026
	0.009	0.008	0.004	0.002
9	0.116	0.068	0.03	0.024
	0.015	0.024	0.002	0.004

APPENDIX IV.

The Dithizone Assay.

Dithizone (Diphenylthiocarbazone) was first prepared in 1878 (Fischer 1878), and has been used extensively in various situations where detection of trace amounts of metal is required. It is obtained as a violet-black solid (Sigma Chemical Co.) which, is soluble in most organic solvents but is almost completely insoluble in neutral or acidic aqueous solutions. Two solvents, chloroform and carbon tetrachloride, have been used almost exclusively in the preparation of dithizone solutions for the detection of trace amounts of metals.

Detection of trace metals using dithizone is dependant on the colour change brought about by complexing of the molecule with metal ions in solution. In basic aqueous solutions, dithizone dissolves to give a yellow/orange colour whereas dilute organic solutions tend to be dichroic (red in transmitted light, green in reflected light). Interference by a wide range of metals (e.g. Mn, Fe, Co, Ni, Cu, Zn, Pb, Ag, Cd, Au and Hg) will, under appropriate conditions, alter the conformation of the molecule and so produce a colorimetric reaction.

The structure of dithizone is shown in Figure 1. When complexed with a particular metal ion, it can exist in two tautomeric forms, keto and enol. The enol form of the Zn-dithizonate complex is shown as an example

in Figure 2.

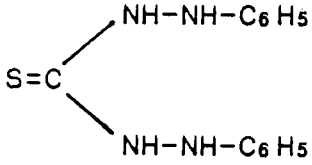


Figure 1.

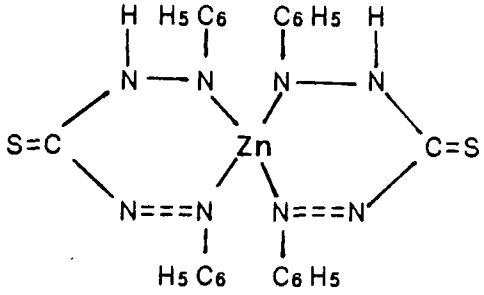


Figure 2.

A wide range of methods have been developed for the use of dithizone in trace metal analysis (Sandell 1959). Although these can allow detection of minute traces of various metals, they were considered to be too complex and time consuming for the present work. The use of dithizone was therefore approached from the standpoint of simplifying its use and developing it for use with the available microtitre plate reader (The plate reader was from Flow Laboratories, the plates from Becton Dickinson Labware). The two solvents most often used with dithizone, chloroform and carbon tetrachloride, were not suitable for use in these tests because they dissolved the polystyrene of the microtitre plates. The fist step was, therefore, to choose a suitable solvent which would allow reaction with the metals to be tested (Cd, Cu and Zn). Acetonitrile was selected as this did not damage the plates

and allowed adequate dissolution of dithizone. In addition dithizone was found to dissolve adequately in 1 M NaOH and this was also tested.

Solutions of each of the metals to be tested (200 μM) were added to what was considered a reasonable concentration of dithizone (500 μg ml^{-1}) and the solutions monitored for maximal colour change. Dithizone was found to react strongly with Cd when dissolved in NaOH but did not react with Cu or Zn. Similarly, there was a strong colour reaction between dithizone dissolved in acetonitrile and both Cu and Zn but not with Cd. Initial tests with lower molarities of the three metals indicated that a 2:1 mix of test solution to dithizone produced best results at this concentration of dithizone and this convention was adopted throughout the rest of the experiments (results not shown).

In order to determine the maximal absorbance wavelength of each of the metal dithizonates, 1 ml of the reacted solution was placed in a cuvette and the absorbance spectrum read in comparison with a distilled water/dithizone blank. The spectra obtained are presented as Figures A1, A2 and A3. Based on these profiles (and the wavelengths available on the plate-reader), Cd and Zn were read at 540 nm, and Cu at 620 nm.

The next stage was to determine a concentration of dithizone which allowed maximal detection levels and produced a linear plot over a reasonable range of metal concentrations. This was done simply by creating a series of calibration curves using concentrations from 10 μg ml^{-1} to 500 ug ml^{-1} dithizone. These are presented as Figures A4, A5 and A6.

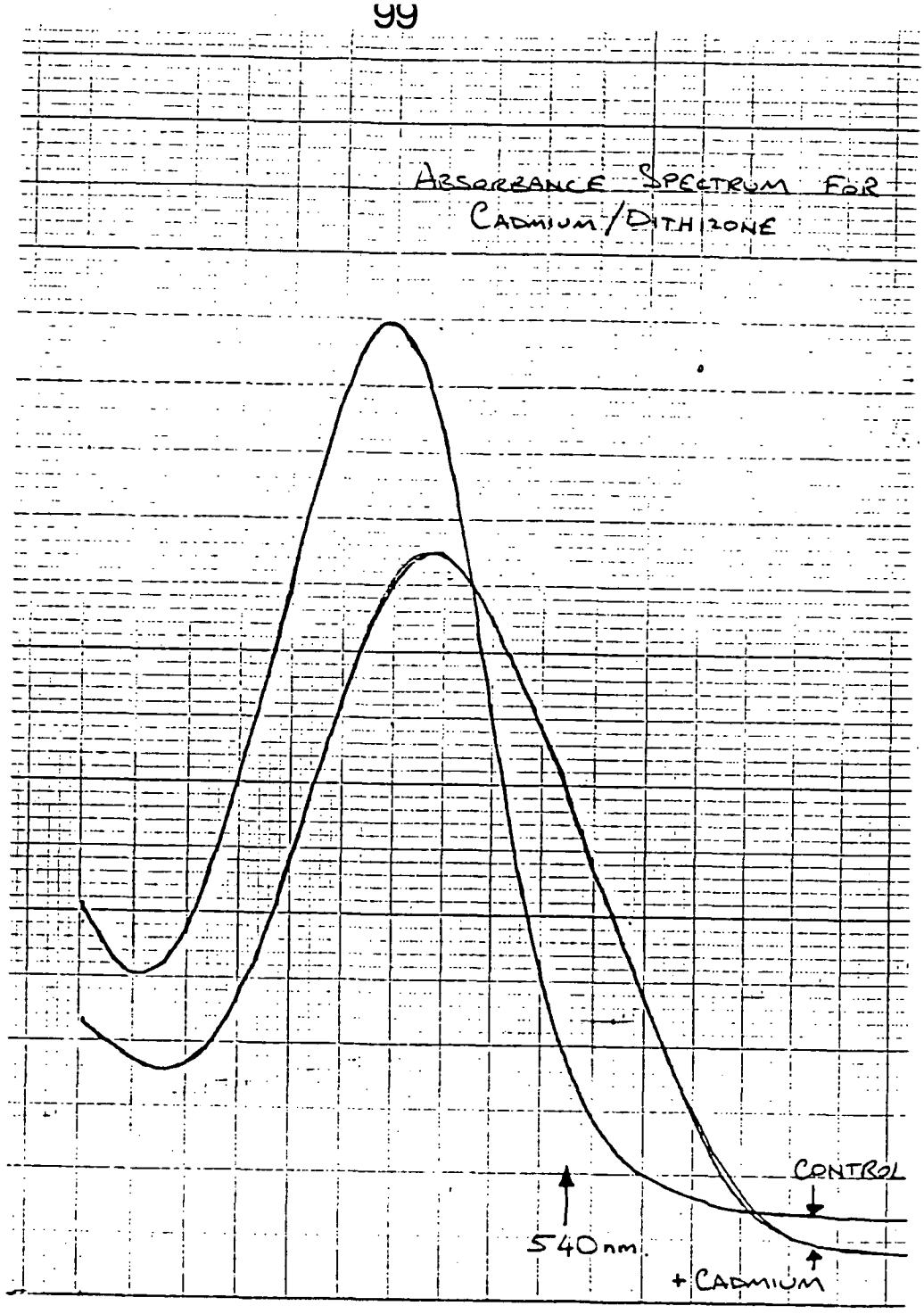


Figure A1.

Figures A1, A2 and A3. The absorbance spectra for Cd (A1), Zn (A2) and Cu (A3) reacting with dithizone. Measurements were made as explained in the text.

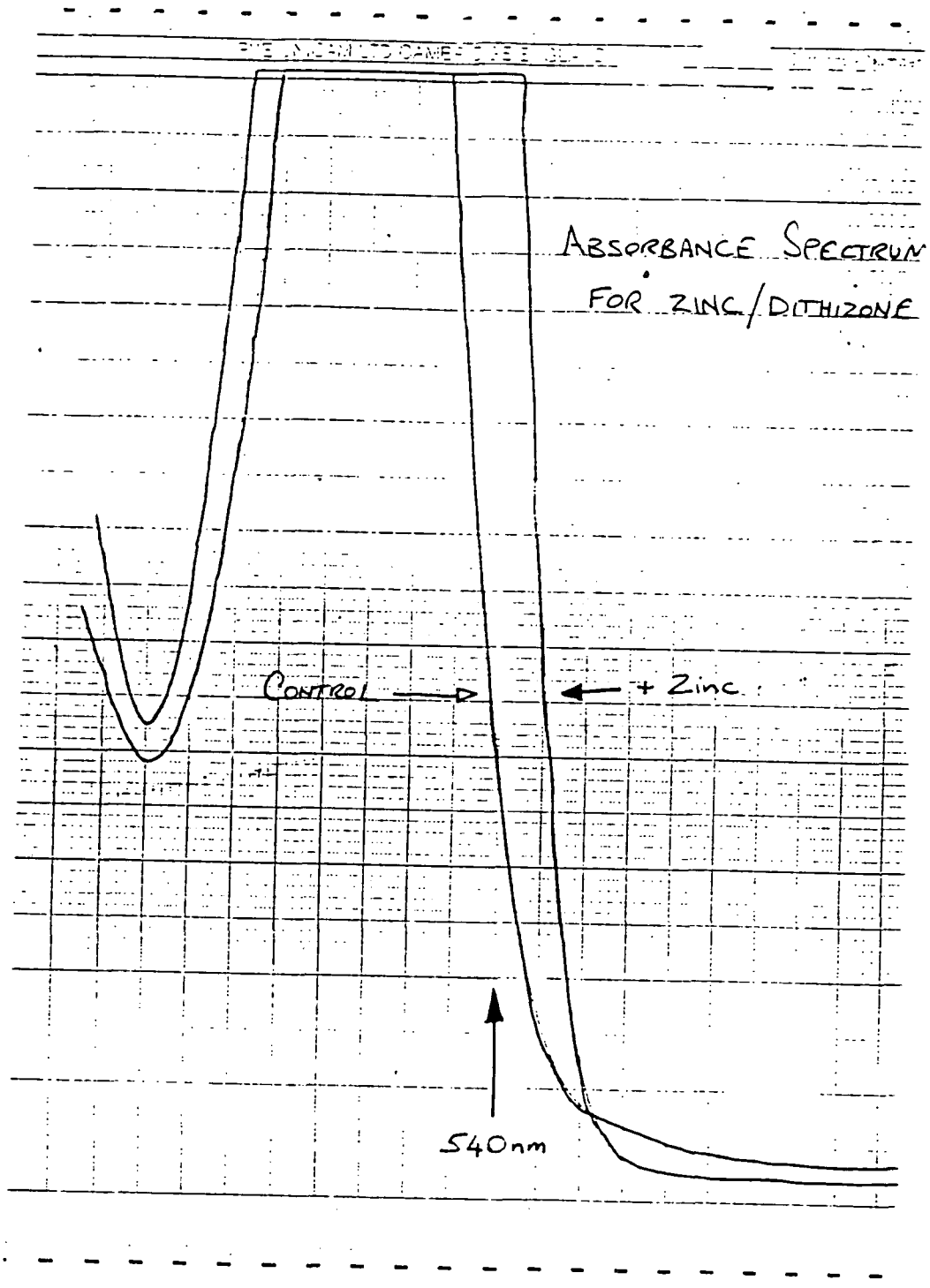

ABSORBANCE SPECTRUM
FOR ZINC/DITHIZONE
CONTROL
+ ZINC
540nm

Figure A2.

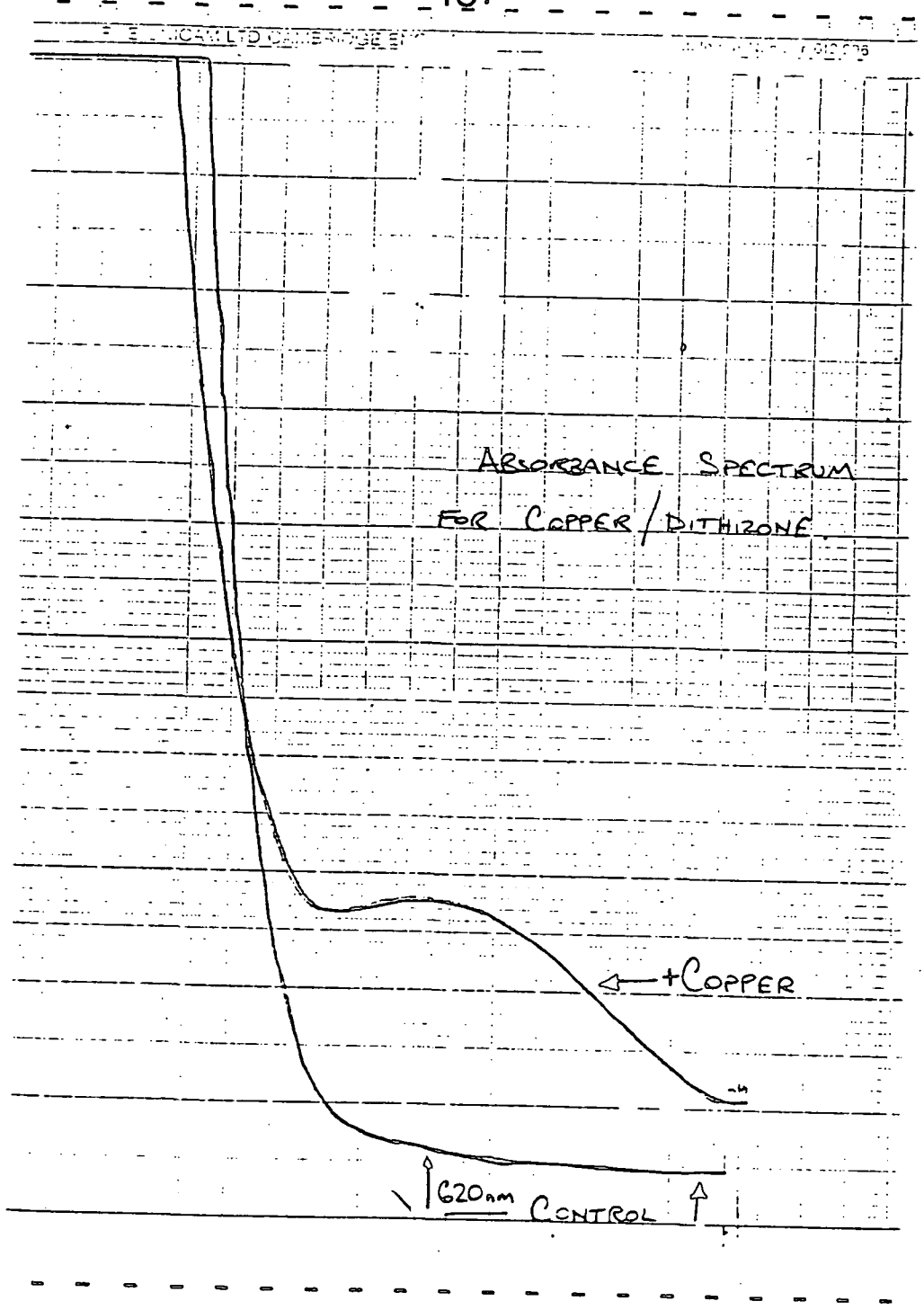
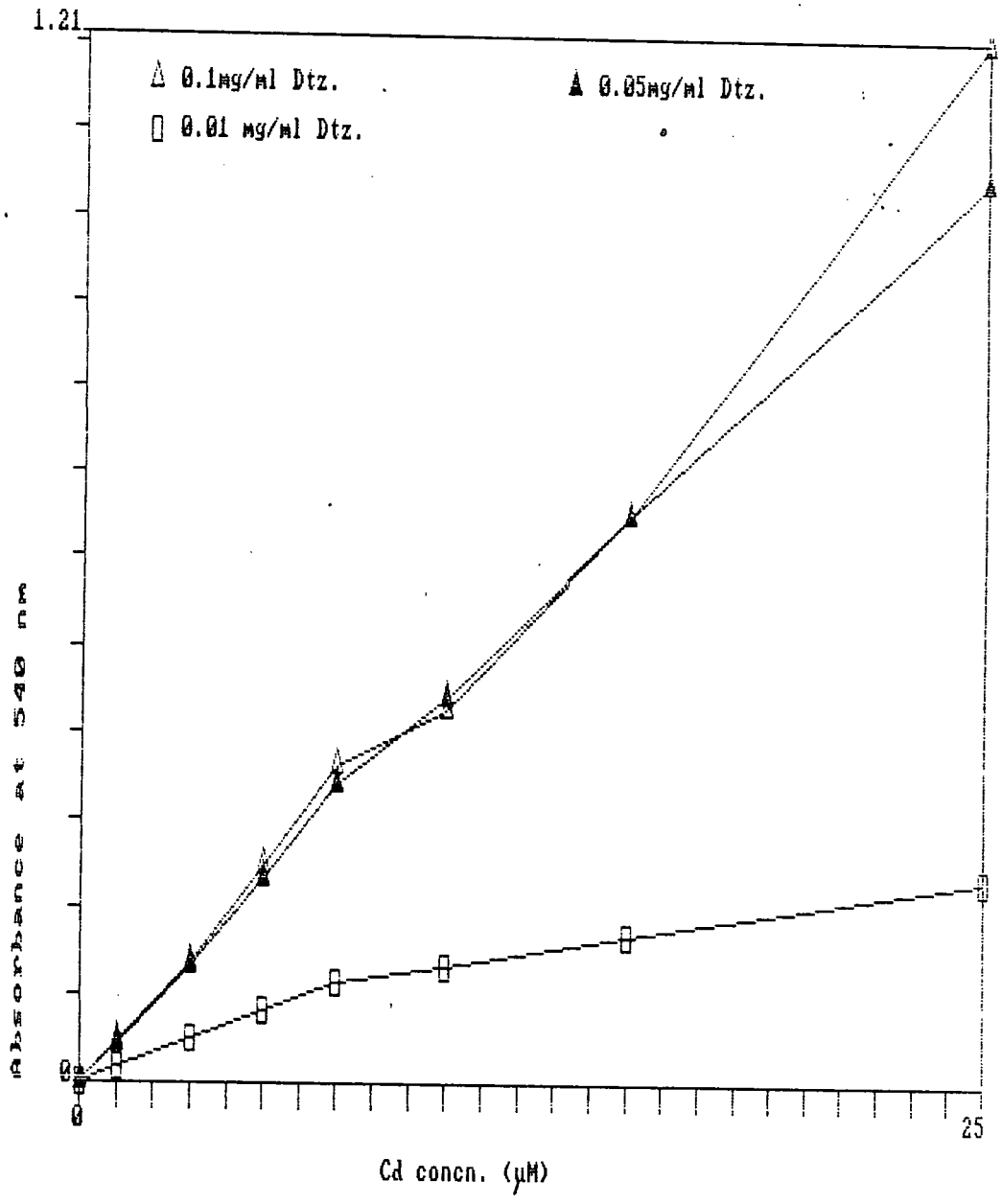


Figure A3.



Figures A4, A5 and A6. Calibration curves for the colour reaction of dithizone with Cd, Zn and Cu respectively. The graphs show the reaction of dithizone at a variety of concentrations with a series of standards of the three metals. Analysis was performed as discussed in the text.

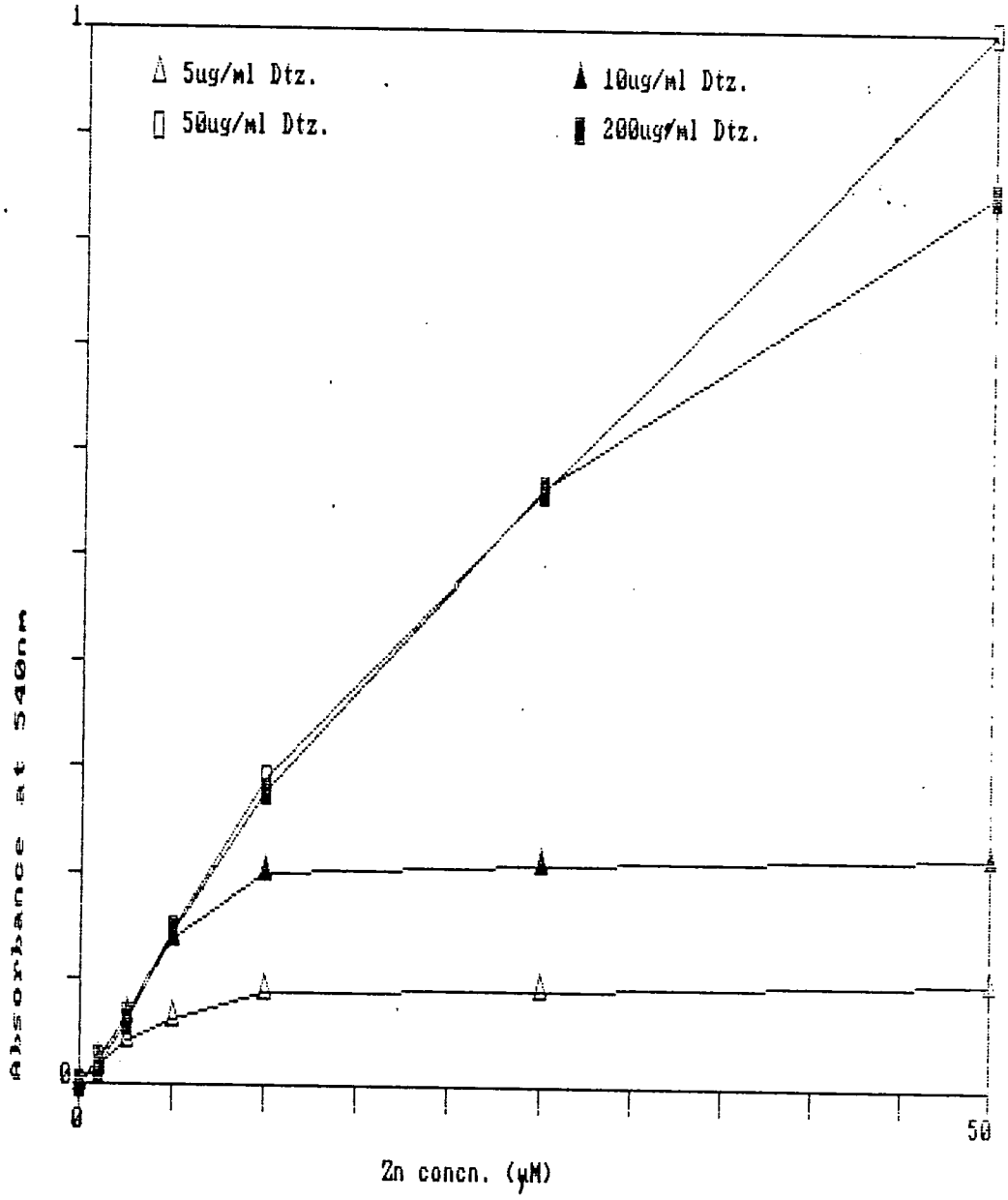

1
5ug/ml Dtz.
10ug/ml Dtz.
50ug/ml Dtz.
200ug/ml Dtz.
Absorbance at 540nm
0
0
50
Zn concn. (μM)

Figure A5.

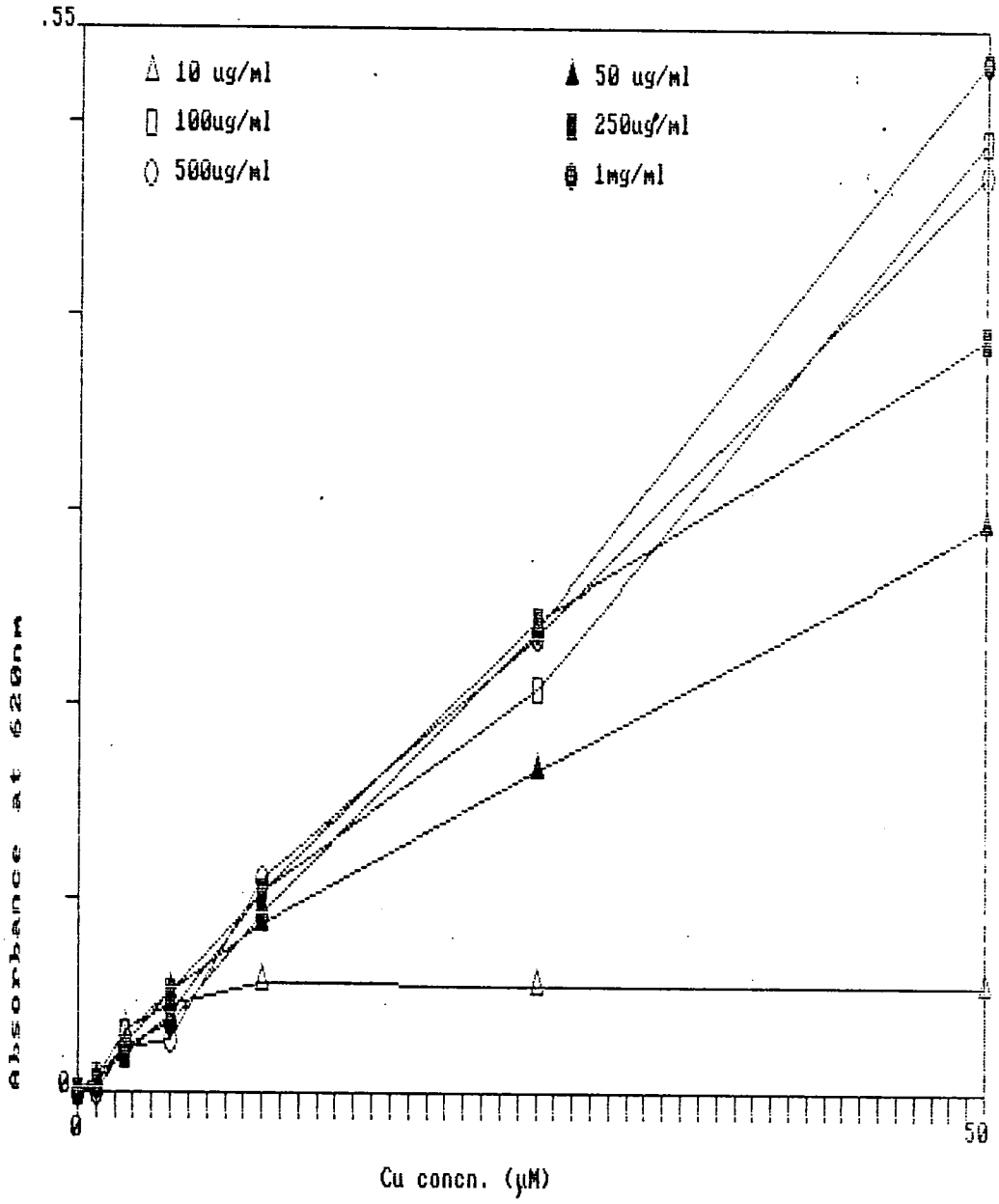

.55
10 ug/ml
50 ug/ml
100ug/ml
250ug/ml
500ug/ml
1mg/ml
Absorbance at 620nm
0
0
50
Cu concn. (µM)

Figure A6

APPENDIX V.

Treatment of data from affinity matrices.

1.Column data.

Data from metal-affinity columns was generated by recording the volume of a given metal-containing solution passing through a column before breakthrough of the metal ion occurred. Knowing the concentration of the feed solution, it was then possible to calculate the amount binding to the matrix. For example, if a feed solution of 10 uM Cd breaks though a matrix after the passage of 100 ml, the amount bound to the matrix must be 1 μMole. Subtraction of the values obtained for the control matrix from those obtained for the peptide/glutathione matrix gave the amounts bound to peptides/glutathione. If this is performed for a series of concentrations of metal ion, the amounts bound can be graphed as in the results section. Values were adjusted to give amounts bound per g of matrix. Also, knowing the amounts of peptide or glutathione bound per g, this value was used to calculate the amounts of metal bound per mg of peptide or glutathione.

Values calculated for Cd-binding to the heat-denaturation-purified peptides were as shown below.

Cd conc. in feed (ml)	volume to breakthrough control (ml)	peptide (ml)	Difference (ml)	Amount bound*/g matrix (nM)	Amt.Bound/mg Peptide+ (nM)
25	116	172	56	280	167.6
10	218	360	142	284	170
5	374	602	228	228	136.5
1	1214	1862	698	130	77.8

(* Amount bound to peptides; + in mg equivalents of GSH)

Suspension tests.

Data from suspension tests was treated as follows. The amount bound to the aliquot of matrix was determined by centrifuging the matrix from suspension and measuring the amount of the metal ion remaining in solution. Since 200 µl of a total volume of 4.4 ml containing 0.8 g of matrix was added to each test, the value obtained was multiplied by 27.5 to give the amount bound per g of matrix.

The results obtained were plotted as nMoles bound per g of matrix against external Cd concentration (the amount remaining in solution after the removal of the matrix). An example of this plot is shown in Figures A1, A2 and A3. In practice a large-scale graph was made and this used to measure the difference between the control and peptide matrices. The value obtained, representing the amount bound to peptides per g of matrix, were multiplied by 1.67 as above to give the amount of metal bound per mg of peptide.

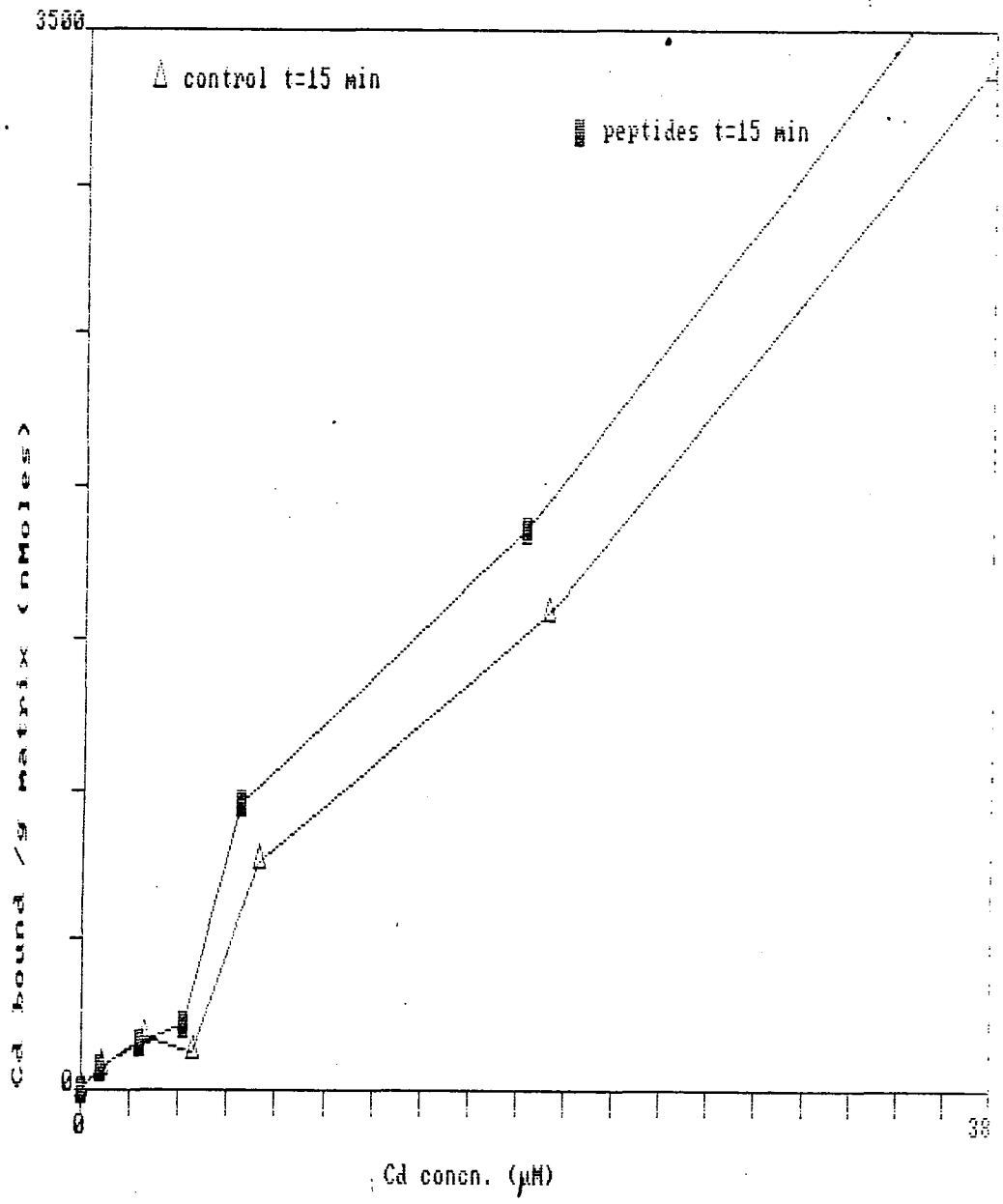


Figure A7

Figures A7, A8 and A9. Cd removal by control- and $(gammaEC)_nG$-matrices at times 15 min (A7), 1 h (A8) and 2 h (A9). Subtraction of the amount binding to the control matrix from that binding to the $(gammaEC)_nG$ matrix gives the total amount bound to $(gammaEC)_nG$ molecules. Graphs like these were used to construct Figures 11 and 12.

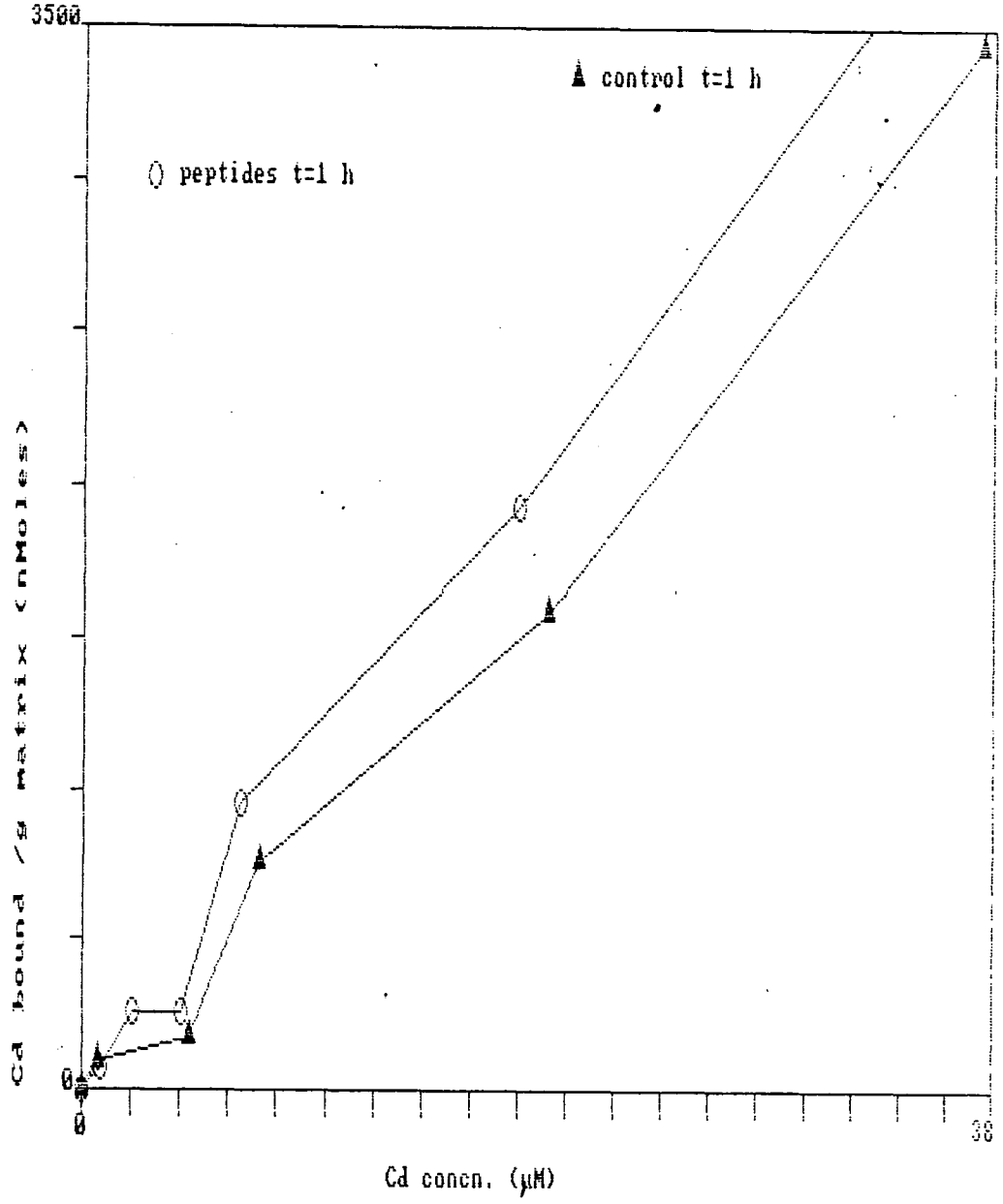

3500
control t=1 h
() peptides t=1 h
Cd bound / g matrix (nMoles)
0
0
30
Cd concn. (µM)

Figure A8.

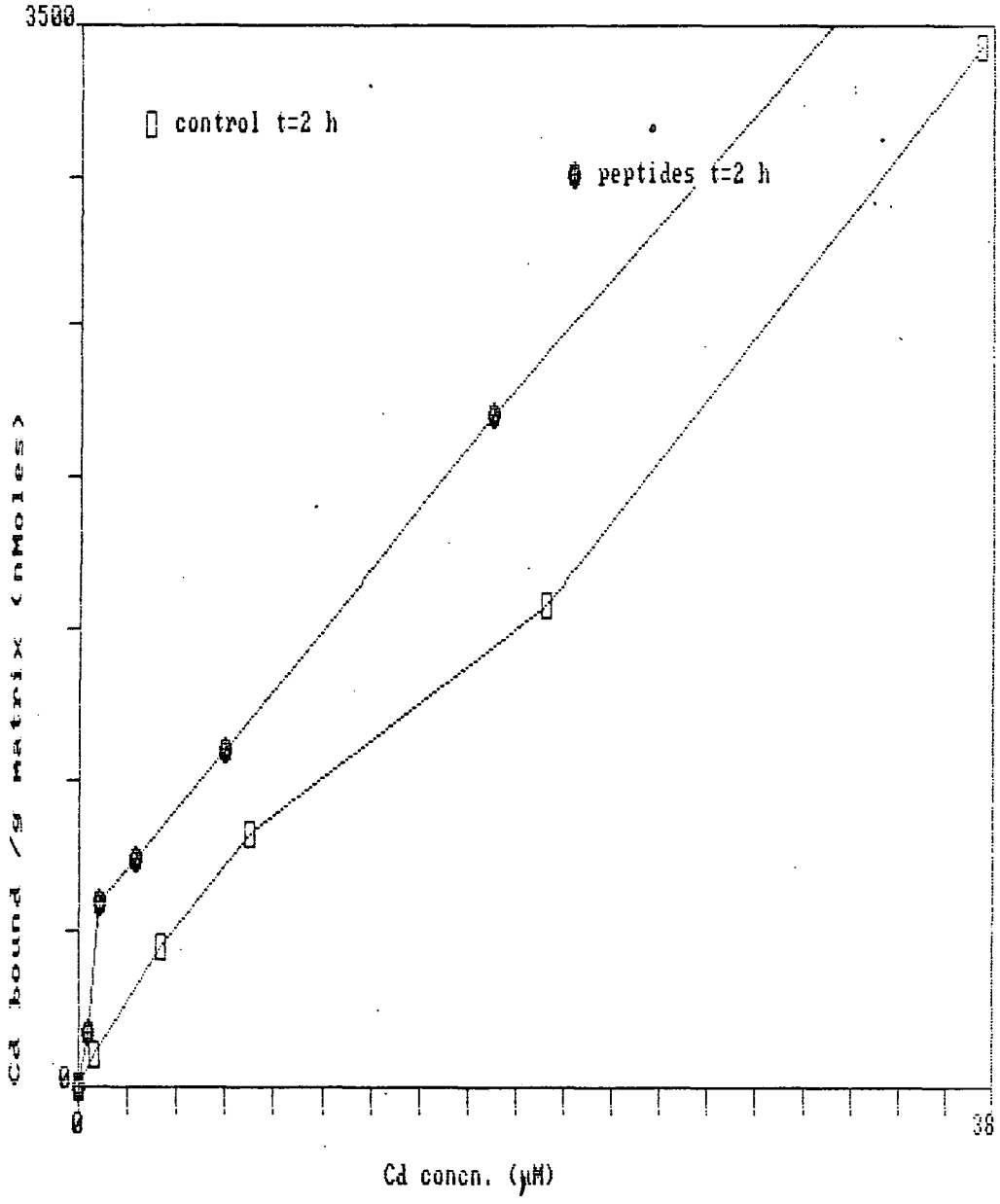


Figure A9.